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A COMPARISON BETWEEN THE HEMAGGLUTINATION TEST WITH FORMALIN TREATED ERYTHROCYTES AND THE IMMUNOFLUORESCENCE TEST WITH WORM PARTICLES, FOR SCHISTOSOMIASIS

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SUMMARY

Results obtained with a hemagglutination test and a fluorescence test in serum from 105 patients with Manson's schistosomiasis and from 175 individuals having no schistosomiasis, were compared. In both, adult *S. mansoni* antigens were employed, in the first as formalin-treated erythrocytes coated with worm extracts, in the second as worm particles fixed to microscope slides. The two tests are easily performed even with blood samples from finger puncture and could be useful for populational studies. Although titers were always higher for the hemagglutination test, a narrow concordance was observed between both tests. Reproducibility studies of the tests indicate four-fold differences in titers as significant. In the parasitized group, with chronic forms of the disease, positive fluorescence tests were seen in every case and positive hemagglutination tests in 96% of the cases. In the non-parasitized group, 2.6% of positive results were obtained for both techniques although occurring in different serums in each test. In treated cases, serologic curves with similar patterns were obtained in both tests.

INTRODUCTION

The search for new foci of schistosomiasis, as well as the evaluation of therapeutic and sanitary measures could find a great help in serologic techniques for the diagnosis of this widespread parasitosis. Such techniques should be sensitive, specific and practical enough for extensive populational studies.

In our laboratory, two techniques have been developed for this purpose, the immunofluorescence test with particles of adult worms [4], and the hemagglutination test with formalin-treated human erythrocytes sensitized with worm extracts [7]. This paper reports a comparative evaluation of their results in a group of schistosomiasis patients and in individuals having no schistosomiasis. Serologic curves obtained after specific therapy were also compared in a few cases.

MATERIAL AND METHODS

Blood samples — Were obtained from parasitologically proven cases of Manson's schistosomiasis, from patients with other diseases, parasitic or not, and from clinically normal individuals. Venous blood samples were divided in small amounts and kept at -20°C. For use, one sample was thawed and diluted at 1:20 and on, in saline solution (0.15 M NaCl) containing 1% normal rabbit serum. Thawed samples were not kept for further use. Blood samples obtained by finger puncture

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were collected on thin filter paper *, dried and kept at room temperature for no longer than two weeks. For use, with the help of a punch, 4 cm^2 paper discs were cut from the blood impregnated areas and soaked for one hour in 0.5 ml amounts of 1% rabbit normal serum in saline solution. Eluates so obtained, corresponding approximately to 1:10 dilutions of serum, were further diluted for use and then discarded.

Positive and negative standards — Large amounts of 10 positive serums obtained from cases of schistosomiasis, were pooled, distributed in 0.2 ml amounts and kept at -20°C. Four different pools were so prepared, as well as a negative standard with serum from clinically normal individuals.

Immunofluorescence tests — Antigens were prepared as described by CAMARGO et al. [4] with minor modifications. About 50 or 60 worms, washed in saline solution and vacuum-dried at -15°C over calcium chloride, were ground in an ice-cool mortar. Twenty milliliters of absolute ethyl alcohol at -20°C were added and the suspension centrifuged in the cold at 3,000 rpm for 5 minutes. The sediment was washed in two changes of 20 ml anhydrous ether at -20°C and vacuum-dried. Delipidized worm particles so obtained were suspended in about 5 ml of saline solution and the same volume of 3% formaldehyde in PBS (0.15 M NaCl 0.01 M phosphates, pH 7.2) was added to the suspension. After incubating at 37°C for 18 hours, the particles were centrifuged, washed 3 times in saline solution and suspended in about 3 ml of this solution. A few minutes were sufficient for large particles to settle down, and drops of the remaining supernatant suspension were distributed on small areas drawn with nail polish on microscope slides. Twenty such areas could be traced on each slide and so, a large number of tests could be run on a few slides. Drying at 37°C for about 60 minutes was sufficient to fix worm particles on slides which could be kept for several months at -20°C without any decrease in antigenic activity. About 1,000 reactions could be done with the antigen obtained from 50 worms.

Anti-human globulin conjugates were prepared from serum samples of immunized rabbits showing antiglobulin titers of 1:64 or more, as determined by gel diffusion [2]. Labeling with fluorescein isothiocyanate * was performed by a fluorochrome slow adding dialysis technique [5], to a fluorescein-protein weight ratio (g per mg) of 10 to 15. Free fluorochrome was removed through gel-filtration in Sephadex G-25. In the test, dilutions of conjugates were used according to titer, to ensure maximum reactivity [3]. Such dilutions were made in PBS containing Evans blue at 1 mg per 100 ml. Tests were done at 37°C in a wet chamber with serum samples at 1:20 and on, in a doubling dilution ratio. Each serum dilution was alloted to a different antigenic area on the slide. After 30 minutes incubation at 37°C, the slides were washed in two changes of PBS, 10 minutes each, and dried for a few minutes under a ventilator, blotting with paper being avoided. The conjugate dilution was then pipetted on each area, and the slides were incubated again for 30 minutes, twice washed in PBS for 10 minutes each and mounted with coverslip and buffered glycerine, pH8. Reactions were judged under a 40 x immersion objective in a binocular microscope provided with dark-field, HBO-200 as the light source, BG 12 as exciter filter and 50 (Zeiss) as barrier filter. In positive tests, worm particles were seen as entirely fluorescent irregular structures. In weakly positive tests fluorescence was limited to the outline of the particles, which, as in negative tests, showed a red hue determined by the Evans blue counterstaining. Titers were given as the highest dilution of serum still producing any degree of fluorescence.

Hemagglutination tests — Were performed as described by HOSHINO et al. [7]. Formalin-treated human erythrocytes were tanned by mixing a 1.5% suspension of cells, with an equal volume of tannic acid dilution at 1:15,000 in PBS and incubating for 30 minutes at 37°C. After washing for twice in

* Whatman no. 1 or Klabin 80

* Crystalline, chromatographically pure, Isomer I, Baltimore Biological Laboratories, Maryland, U.S.A.

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saline solution, tanned cells were sensitized with *S. manson's* antigens by incubating at 37°C for 45 minutes in adult worm extracts diluted at 200 μg of protein per milliliter of saline solution buffered with phosphates to pH 6.4. Sensitized cells washed for three times in saline solution, were suspended at 1.5 x 10^8 per milliliter of this solution and could be kept at 4°C or -20°C for many months with no diminished antigenic activity. Worm extracts for coating cells were obtained by grinding adult worms in saline solution, at 4°C, with the help of a Potter-Evelyn tissue grinder. After centrifuging (3,000 rpm, 10 minutes) to remove insoluble particles, extracts were distributed in small volumes, frozen and kept at -20°C.

Tests were performed in perspex plates * with V-shaped wells of 0.15 ml capacity. Doubling dilutions of serum specimens or filter paper blood eluates, from 1:40 on in saline solution containing 1% rabbit serum or 0.35% Haemaccel **, were distributed in the wells in 0.1 ml volumes (4 drops). One drop of the coated cell suspension was then added to each well, the mixture homogeneized and the plates incubated overnight at room temperature. Readings were performed as referred [7].

RESULTS

A) *Comparison of results in patients with and without schistosomiasis*

A hundred patients with chronic forms of Manson's schistosomiasis were tested for antibodies by both techniques. In every case a positive fluorescence test was seen and in 96 patients, positive hemagglutination tests were also observed (Table I). In four other cases, not included in this series, with very recent parasitisms, three of which presented well documented acute forms of schistosomiasis, hemagglutination tests were negative but, in one a fluorescence titer of 1:20 was observed.

TABLE I

Results of hemagglutination and immunofluorescence tests in 100 cases with chronic forms of Manson's schistosomiasis

Hemagglutination titers	Immunofluorescence titers								Total
	< 20	20	40	80	160	320	640	1,280	
< 40		4							4
40		8	3						11
80		4	4	2					10
160			1	6	1				8
320			4	7	4	1	1		17
640			2	1	6	2			11
1,280				2	4	3	1		10
2,560			1	6	1	1	1		10
5,120				1	4	1	2		8
10,240					1	1	2	1	5
20,480					2		1	1	4
40,960						1			1
81,920						1			1
Total	0	16	15	25	23	11	8	2	100

* Microtitrator Takatsy, Labor, Budapest, Hungary
** Haemaccel, plasma expansor, Behringwerke AG, Bundesrepublik Germany

Serums from 175 individuals were also tested, who did not present clinical, laboratory and epidemiologic evidences of schistosomiasis. Table II includes results obtained by the fluorescence and hemagglutination tests in 152 such cases for whom a diagnosis had been established. Only in one case of Chagas' disease and in three cases of *Pemphigus foliaceus*, positive hemagglutination tests of low titers (1:80 or less) were obtained. Positive fluorescence tests were seen in other four patients, one with Kala-azar and three with malaria. Titers were also low (1:40 or less). In this group, presence of intestinal parasites was common, as for example *Ascaris lumbricoides, Trichuris trichiura, Strongyloides stercoralis, Giardia lamblia* and *Entamoeba*, many times a large number of cists, eggs or larvae being found by fecal examinations.

The remaining 23 cases in this group showed positive or doubtful positive intradermal tests with *S. mansoni* antigens, and had been selected from a tested group of 589 individuals studied by PELLEGRINO et al.[15] in the

TABLE II

Hemagglutination and immunofluorescence tests with *S. mansoni* antigens in patients having no Manson's schistosomiasis

Diagnosis	no. of cases	Hemagglutination test *		Immunofluorescence test **	
		Positive	Negative	Positive	Negative
Chagas' disease	7	1	6	—	7
Mucocutaneous leishmaniasis	5	—	5	—	5
Kala-azar	6	—	6	1	5
Toxoplasmosis	9	—	9	—	9
Malaria	5	—	5	3	2
Hidatidosis	10	—	10	—	10
Cisticercosis	4	—	4	—	4
Tuberculosis	2	—	2	—	2
Leprosy	5	—	5	—	5
Syphilis	9	—	9	—	9
South American blastomycosis	1	—	1	—	1
Rheumatic fever	5	—	5	—	5
Infectious diseases	2	—	2	—	2
Systemic Lupus Erythematosus	5	—	5	—	5
Pemphigus foliaceus	5	3	2	—	5
Infectious hepatitis	2	—	2	—	2
Hepatic cirrhosis	3	—	3	—	3
Atrophic gastritis	4	—	4	—	4
Pernicious anemia	3	—	3	—	3
Gastric carcinoma	2	—	2	—	2
Polycistic kidney	1	—	1	—	1
Infectious mononucleosis	1	—	1	—	1
Hodgkin disease	1	—	1	—	1
Clinically normal	55	—	55	—	55
Total	152	4(2.63%)	148	4(2.63%)	148

* Serums diluted at 1:40
** Serums diluted at 1:20

Provinces of Corrientes and Misiones, Argentina, where no cases of schistosomiasis were found. Hemagglutination tests were negative in all 23 sera, but in 3, immunofluorescence tests were positive at 1:10 and doubtful in 6, which showed only very weak fluorescences at this dilution.

Five spinal fluids from cases of cerebral cysticercosis, which strongly reacted in complement fixation tests with antigens of *Cysticercus cellulosae,* did not react in the hemagglutination and immunofluorescence tests with *S. mansoni* antigens.

B) *Evaluation of the reproducibility of the immunofluorescence and hemagglutination titers*

Sixty positive serums, previously titrated by the immunofluorescence and hemagglutination tests at different periods and with different batches of antigens, were submitted to two further titrations. In this way, frozen aliquots of such serums were thawed and twice titrated by both tests on successive days. Titers (x) were normalized (n) through the equation $x = 2^n \times 5$ and medium titers and standard errors calculated. The values obtained, of 5.02 ± 1.04, indicate as significant for the fluorescence tests, a difference in titers of two dilutions, since a unit corresponds to successive doubling dilutions. The same values, obtained for the corresponding hemagglutination titers were 8.5 ± 1.22, the differences between their respective variances being highly significant at a 5% probability level.

Pearson's correlation coefficient, $r = 0.66$, ($t = 10.4$; d.f. $= 177$; $p < 0.001$), indicates an association between the results of the hemagglutination and of the immunofluorescence tests. A regression equation could be established between fluorescence titers (Y) and hemagglutination titers (X): (Fig. 1).

$$Y = 5.02 + 0.4031 (x - 8.51)$$

C) *Serologic curves in schistosomiasis patients submitted to specific therapy*

Eight patients were submitted to treatment with nitrothiamidazol (Ciba 32.644 — Ba) and tests were performed before and after treatment, every one or two weeks, for three months. Figure 2 shows medium titers and ranges of titers for these 8 patients, in both tests. As a rule, an evident increase in titers was observed about 30 days after the treatment, followed by a slow fall to lower levels.

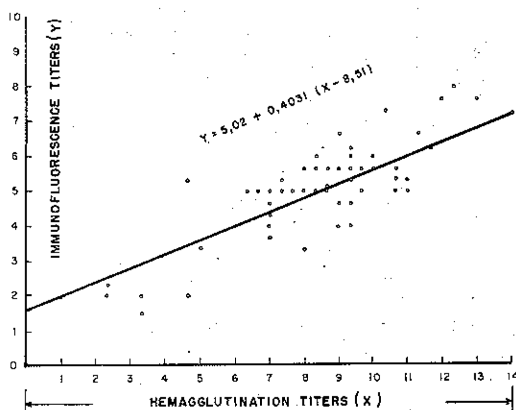

Fig. 1 — Regression equation between immunofluorescence and hemagglutination titers obtained in 60 patients with Manson's schistosomiasis. Titers expressed as $2^n \times 5$ (see text)

D) *Comparative results between serum specimens and eluates of blood samples collected on filter paper*

Blood samples were obtained on filter paper by finger puncture from 17 patients with schistosomiasis and from 61 not parasitized individuals. Venous blood was also collected at the same time and both materials kept, as described, for at least one week before immunofluorescence tests were performed. As already observed for the hemagglutination test [7], qualitative results were the same for the corresponding serum specimens and blood eluates in the 78 patients. In positive cases, titers obtained with both materials did not differ significantly, the same titers occurring in three cases, differences of one dilution in 12 and of two dilutions in two. When different titers were obtained, higher values were seen for serum specimens in 12 cases and for blood eluates in two cases.

DISCUSSION

In the search for practical serologic tests for the diagnosis of Manson's schistosomiasis, which would be sensitive and specific, a com-

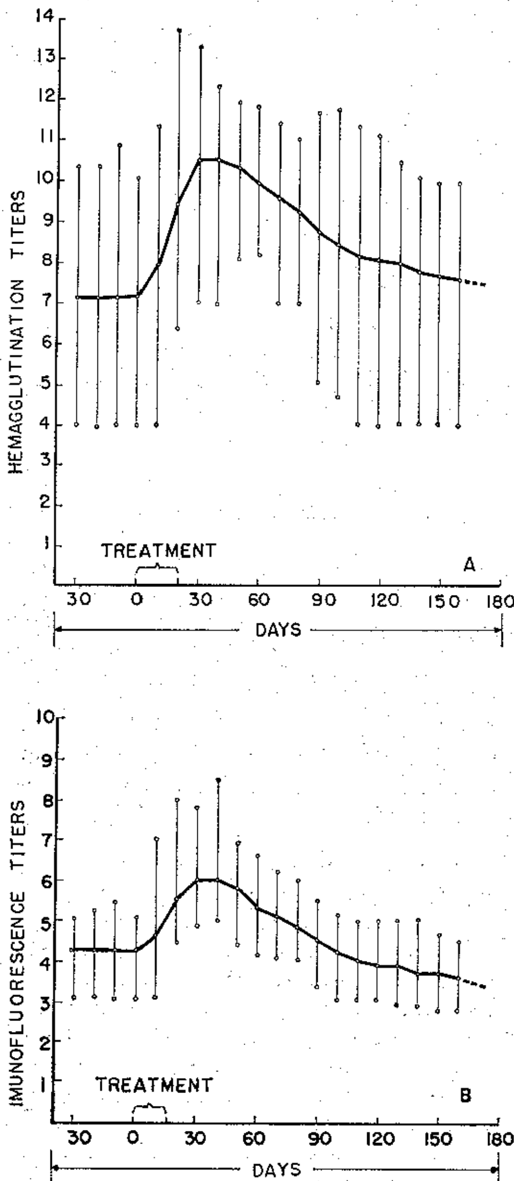

Fig. 2 — Medium titers and range of titers observed in 8 patients before and after treatment. A — hemagglutination titers. B — Immunofluorescence titers. Titers expressed as $2^n \times 5$ (see text)

parison was made between two tests previously developed in our laboratory. Both techniques, the immunofluorescence test with worm particles[4] and the hemagglutination test with formalin-treated human erythrocytes coated with worm antigens[7], are practical enough for populational studies. Relatively small amounts of antigens are necessary for a large number of tests, as many as 50 worms being sufficient for about 1,000 tests in any of the two techniques. Antigens are stable at normal storage conditions and can be kept ready for use. Both tests are easily performed and eluates from blood samples dried on filter paper can be employed. In this way, venous punctures are not necessary and samples can be sent by post to central laboratories.

To compare the sensitivity of the tests, they were performed in serum specimens from 100 patients with parasitologically proven Manson's schistosomiasis, presenting chronic forms of the disease. In every case a positive fluorescence test was seen, and in 96, positive hemagglutination tests were also observed. In four patients with acute forms of the disease or recent parasitisms, negative tests were obtained, only a positive fluorescence test of low titer (1:20) occurring in one of these cases. Higher titers were always observed for the hemagglutination test, but in triplicate testing of 60 positive serums, a correlation could be established between corresponding fluorescence titers (Y) and hemagglutination titers (X), expressed as $Y = 5.02 + 0.4031 (X - 8.51)$.

Medium titers and standard errors, expressed as 8.5 ± 1.22 for the hemagglutination test and 5.02 ± 1.04 for the immunofluorescence test, when titers (x) were normalized (n) through the equation $x = 2^n \times 5$, indicate a good reproducibility of results for both tests. A unit corresponding to one doubling dilution, titer differences of two dilutions can be considered as significant, since confidence intervals of two standard errors, which would include 95% of the means likely to be obtained, would barely exceed such a difference in the hemagglutination and immunofluorescence tests.

In a group of 152 non-schistosomiasis individuals, 2.6% positive results were obtained for both tests. However, such positive results occurred in different cases for the two tests, positive fluorescence tests being found in one case of Kala-azar and in three cases of malaria and a positive hemagglutination test in one case of Chagas' disease and in three cases of *Pemphigus foliaceus.*

Such tests were always of low titers, 1:40 or less for the fluorescence and 1:80 or less for the hemagglutination.

Presence of intestinal parasites did not seem to interfere with results.

In a group of 23 patients who did not show clinical, parasitological and epidemiological evidences of schistosomiasis but presented positive or doubtful intradermal tests with *S. mansoni* antigens [15], only three cases showed fluorescence positive tests of very low titers (1:10). Six other showed doubtful fluorescence tests. In every case the hemagglutination test was negative. Other tests, as the complement fixation test and the cholesterol-lecithin flocculation test, had been positive in a few patients in this group.

Certainly, results obtained in the present groups of schistosomiasis and non schistosomiasis patients are only initial, a much larger experience with the tests being necessary before a final evaluation of their diagnostic value can be advanced.

Especially in the case of the immunofluorescence test, false positive results are sometimes referred in high percentage, from 30% to 60% [8], mainly when cercariae are used as antigens. With adult worm antigens, different reports reveal such percentages as far situated as 0% [17] and 19% [19]. Since positive serological tests have been found in individuals immunologically sensitized to cercariae of *Schistosoma bovis* and of fowl schistosomes [9, 18], and since serum from patients with Manson's schistosomiasis reacts with free-living cercariae and with *S. bovis* cercariae [6, 14], it is possible to suppose that, at least in certain population groups, high percentages of false positive results could be related to sensitizations of this kind.

In one non schistosomiasis patient having hidatidosis, we have observed fluorescence and hemagglutination titers of 1:40 and 1:80, with *S. mansoni* antigens. However, by absorbing the serum with human AB cells, both tests became negative. Identical absorptions of serum from schistosomiasis patients did not modify titers found in the tests. OLIVER-GONZALEZ et al. [10, 11, 12, 13] have referred to the presence in *S. mansoni* and other parasites of polysaccharide substances antigenically related to the A_2 human agglutinogen. In this sense, it seems that we cannot discard the possibility that some false positive tests could be related to the presence of anti-H antibodies in serums, natural or resulting from immunological responses. Also, heterophile antibodies, found in serum from patients with schistosomiasis or with other diseases, of parasitic origin or not [1, 16] could perhaps be responsible for certain false positive results.

We think, however, that the practical aspects of the present techniques, especially the hemagglutination test, as well as the initial results here obtained, invite their extensive trial in the immunodiagnosis of schistosomiasis.

RESUMO

Estudo comparativo das reações de hemaglutinação passiva com hemácias formolizadas e de imunofluorescência com partículas de vermes, para a esquistossomose

Compararam-se as reações de hemaglutinação com hemácias formolizadas e de imunofluorescência com partículas de vermes, para a pesquisa de anticorpos anti-*S. mansoni*, nos soros de 105 pacientes parasitados e de 175 não parasitados. A facilidade de execução de ambas as técnicas, utilizando soros ou mesmo amostras de sangue colhidas em papel de filtro, permite sua utilização em inquéritos populacionais. Houve estreita concordância de resultados, com positividade de 100% para a imunofluorescência e de 96% para a hemaglutinação nas formas crônicas da esquistossomose e com apenas 2,6% de reações positivas no grupo não parasitado. Encontraram-se títulos mais elevados para a hemaglutinação. Estudos de reprodutibilidade indicaram como significativas, nas duas provas, variações de títulos de uma diluição de razão 4. Em pacientes submetidos a tratamento, curvas sorológicas semelhantes foram obtidas pelas duas reações.

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