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Quantitative fluorescent antibody technique in human and experimental schistosomiasis

BY

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Summary — Positive FA tests were found in 52 out of 54 patients with proven *S. mansoni* or *S. haematobium* infections. CF tests were positive in 21 on a total of 34 tested patients. *Fasciola hepatica* caused false positive FA reactions of a low titer. High FA titers were found in four recent human infections, in accordance with experimental evidence. Treatment had but few influence on fluorescent antibodies, while CF titers tend to decrease after an initial rise. The authors saw no advantages of quantitatizing the FA test in routine work.

The use of sections of paraffine embedded hepatopancreas of infected snails is proposed as an alternative to cercariae, in order to make the test less expensive and elaborate and to facilitate storage and expedition of antigenic material.

The indirect fluorescent antibody technique was applied to the serodiagnosis of schistosomiasis by Sadun and associates in 1960. They selected freshly shed, formalin fixed cercariae of *S. mansoni* as the most adequate antigenic material. The use of cercariae preserved by addition of rhodamine albumin and of dried blood on filter paper were later and major improvements by the same group (Anderson *et al.*, 1961a and 1961b; Sadun *et al.*, 1961).

We have applied the original technique with a few minor modifications both to infected mice and to human patients seen at the Institute for Tropical Diseases at Antwerp. The majority of the latter suffered of confirmed parasitological schistosomiasis, treated with nitrothiamidazol and were followed also by complement fixation tests.

Methods

The FA tests were performed as follows. Living cercariae of *S. mansoni* are obtained from infected *Australorbis glabratus* snails by phototactism. They are gently centrifuged and resuspended in 2 ml of 10 p. cent formalin in phosphate buffered saline at pH 7.2. One drop of lissamine-rhodamine conjugated bovine

(*) With technical assistance from P. Delange and R. Van Peer.

serum (Difco) is added per ml of suspension. Cercariae treated in this way remain in good condition for about one week at room temperature (becoming too brittle afterwards) and several months at least when frozen in at — 20 °C without peculiar precautions. Sera to be tested are routinely diluted 1/4 with P. B. S. for screening purposes or serially 1/4 up to 1/256 or higher for titration if screening proved positive. The reactivity of stored sera remains unaltered for one year at least when frozen in undiluted and not repeatedly thawed and frozen. A positive serum of known titer was always treated simultaneously and served as reference. The cercariae are washed twice in P. B. S. before use, gently centrifuged and this suspension brought to a concentration of approximately 100 cercariae per 0.1 ml as estimated by direct count. To 0.1 ml cercarial suspension 0.1 ml diluted serum is added and incubated for 30' at room temperature without agitation. The cercariae are then washed twice and fluorescein conjugated antihuman globulin of horse origin (Progressive Laboratories) added at an « optimal » dilution, which was later routinely taken as 1/2 with this brand of reactive (0.1 ml for 0.2 ml of washed cercarial suspension). After 15' incubation at room temperature, the cercariae are washed again, three times at least, and the total sediment is transfered carefully to an hollow ground slide for examination in a drop of P. B. S. We used a Reichert Zetopan-Binolux optical equipment with HBO 200 lamp, filters UG 1/2,5 BG 12/3 mm and GG 9/ mm and anoptral phase-UV condensor and objectives, at a magnification of 100 ×. In experimental work on mice we used elutions of standardized drops of tail blood on filter paper as described by Anderson *et al.* (1961).

To evaluate the preparations, we examined all the cercariae present and tried to comply with the criteria as given in table 1. The end point is a subjective appreciation of the degree of fluorescence presented by the largest percentage of cercariae. The titer was taken as the highest dilution with at least a ± reaction, but not a −/± one.

TABLE 1

Definition of the degree of fluorescence on counterstained cercariae

Quotation	Definition
−	Body uniformly red, without contrasting rim
−/±	Faint green fluorescence on tail bifurcation only
±	Faint rim all around the cercaria, visible at higher (200 ×) magnification only
±/+	Complete fluorescent rim all around the body, just visible at the usual magnification of 100 × (objective 10 ×)
+	Obvious, but still narrow fluorescent rim
+/++ ++ ++/+++	Intermediate degrees in which the green fluorescence progressively dominates the red counterstaining
+++	Fluorescence as in the control (serum with titer of 1/128 or higher)

Note : fluorescent precipitates on the oral succer are not taken into account.

Undiluted human sera were never used. A prozone effect was evident in sera with high titers (1/256 or more) in which fluorescence was brighter at 1/16 than at 1/4 or 1/8 dilutions. It is of no hindrance for the screening at 1/4 though giving sometimes an erroneous impression of rather low positiveness.

We found neither advantage nor difference in preliminary inactivation of sera as practised by Sadun *et al.* (1960) and advocated by Gane *et al.* (1964). Though the participation of complement is demonstrated (Cookson, 1964), its role in the enhancement of fluorescence has not been worked out. Experiments by Toussaint and Anderson (1965) appear rather unfavourable to inactivation.

The advantages claimed by Gane *et al.* (1964) of vital staining of the cercariae with rhodamine-albumine before fixation were not confirmed in our experiments, except the possibility of an immediate use of the cercariae, instead of a waiting time of 24 hours in order to guarantee a sufficient diffusion of the counterstain in the fixed material. The procedure eventually adopted of counterstaining freshly fixed cercariae gave equally good results and is less expensive in fluorescent counterstain.

The antigen for the CF tests was a buffered saline extract of adult *S. mansoni* worms from mice (Chaffee *et al.*, 1954). It was used at a 1/10 dilution. Complement was titrated in presence of antigen. Two exact units of complement and one exact unit of haemolysine were used. Serial two fold dilutions of serum in saline ranging from 1/2 were prepared and incubated during 15 hours at 4 °C together with the antigen and 0.4 ml of complement. Sensitized erythrocytes (0.4 ml of a 1,5 p. cent suspension) were then added and incubated for 1 hour at 37 °C. Only tubes showing no trace of hemolysis were recorded as positive.

Experimental results

Several lots of white mice, approximately three weeks old, underwent a single transcutaneous infection with 75, 150 or 300 cercariae.

Blood drops of standard dimensions were collected at the tail, eluted and titrated. Mice exposed to 75 cercariae showed but few infections, while those exposed to 300 cercariae died massively. The titrations on dried blood drops yielded maximal titers of 1/32, corresponding to approximately tenfold titers on serum as checked on sacrificed animals. This was rather deceiving as compared to the experiments of Bruijning who obtained titers up to 1/8000 (1965). Our results were not entirely satisfactory and are therefore quoted only briefly.

Mice became usually positive, whatever the number of infective cercariae, between the 37th and the 44th day; maximal titers were obtained around the 80th day. Titers dropped then quite sharply and remained afterwards unchanged or decreased slightly during a

maximal observation period of 392 days. The curve in function of time corresponds well to the experiments of Bruijning (1965) and Magalhaes Filho *et al.* (1965), and to the data of Sadun (1962). Bruijning found a very slight initial positive reaction (on undiluted serum only) three weeks after infection, followed by a sharp rise between the 30th and 40th day and a peak around the 60th to 80th day, after which there was a sharp drop and a levelling off. He considered this evolution as essentially similar to former results with the COP reaction (Bruijning, 1961), taking its lower sensitivity into account. Magalhaes Filho using the cercarienhullen-reaction, the cercarial FA test and the inhibition and indirect FA tests on perioval granulomas found a distinctive inhibition of fluorescence from the 20th day on, whilst the other techniques evidenced antibodies only from the 25-47th day on. The fate of one of our mice infected with 300 cercariae was however strikingly different. Here the reaction became clearly positive on the 21st day after infection, after being doubtful at 6 and 13 days, and the titer increased sharply till death occured on the 30th day.

The usually observed increase in titer around the 40th day is admitted to be simultaneous with the beginning oviposition, approximately 4 weeks after infection, though oviposition is not a necessity for the appearance of fluorescent antibodies (Sadun, 1963). One of our mice reacted almost immediately with a sharp rise, which, considering the relatively low sensitivity of our FAT on mice, is probably due to an antigenic stimulus of the schistosomule itself. Cercarial penetration in humans has apparently the same effect, in some as yet undefined conditions at least (Sadun and Biocca, 1962) and anticercarial antibodies have furthermore been systematically demonstrated by immunodiffusion and immunoelectrophoresis methods (Capron *et al.*, 1966).

Results on Human Patients

One hundred thirty five quantitative FA tests were performed on sixty eight patients with parasitologically confirmed bilharziasis, thirty eight of whom were also assayed by a total of seventy seven CF tests. Each technique was performed and read by the same observer, independently of the other : the results being confronted at the end of the series.

The results are summarized in table 2. The first figure is the titer of the initial test and the number in brackets gives, as in table 3, the higher titers obtained in some patients, in later tests. Fluorescence of the type ± or ±/+ at 1/4 dilution was initially con-

sidered negative, but appeared to be at least indicative of schistosomiasis or fasciolasis (see table 2). Two out of fifty four patients with proven schistosomiasis reacted negatively. Three out of ten parasitologically negative patients reacted positively, but two of them had possible antecedents of urinary bilharziasis while the third one was not thoroughly investigated for parasites which makes these results inconclusive. *Fasciola hepatica* is cross-reacting as reported by Bruijning (1965) and Cookson (1963 — *F. gigantica*) and this may prove true of other trematodes as well. Titers of *S. haematobium* and *S. mansoni* infections could not be compared. Our material was too small and *S. haematobium* infections were likely to have been more chronic, as they were seen mostly in North African natives grown up in endemic areas, whereas *S. mansoni* infestations were observed usually in expatriates, returning from Central Africa after a limited time of residence.

TABLE 2

Quantitative FA tests for schistosomiasis as compared to parasitological data

FA Titers	Patients infected with :			Patients parasitologically negative	Total
	S. mansoni	*S. haematobium*	*F. hepatica*		
Neg.	3 (2)	1 (—)	2 (2)	7 (7)	13 (11)
1/4	5 (3)	—	2 (1)	—	7 (4)
1/8-1/16	10 (7)	2 (2)	— (1)	3[1] (3)	15 (13)
1/32-1/64	13[3] (18)	6[4] (6)	—	—	19 (24)
1/128 and higher	13[5] (14)	1[2] (2)	—	—	14 (16)
Total	44	10	4	10	68

1. Including two cases of supposed infection with *S. haematobium,* not confirmed in Antwerp.

2. Including one case of infection with *S. haematobium,* not confirmed but quite certain.

3. Including two cases of mixed infection with *S. mansoni* and *S. intercalatum.*

4. Including one case of old and cured infection, presenting now with bladder carcinoma.

5. Including one case anteriorly treated and negativated.

TABLE 3

Results of CFT as compared to parasitological and FAT data

CFT	Parasitologically negative for *Schistosoma*	Parasitologically positive for *Schistosoma*		FAT (positive cases)				
		mansoni	*haematobium*	Neg.	1/4	1/8-1/16	1/32-1/64	1/128 or more
Neg.	4[1]	15 (12)	2 (1)	1 (1)	4 (3)	5 (3)	6 (6)	1
1/2-1/4	—	4 (5)	— (1)	—	—	2	1 (3)	1 (3)
1/8-1/16	—	5[2] (4)	—	—	—	—	3 (3)	2 (2)
1/32-1/64	—	6[3] (8)	1[3] (1)	—	—	1 (2)	5 (4)	1 (2)
1/128	—	1 (2)	—	—	—	—	1 (2)	—
Total	4	31	3	1 (1)	4 (3)	8 (5)	16 (18)	5 (7)

1. Including two cases of infection with *F. hepatica,* one being positive by FAT, the other one negative.
2. Including one mixed infection with *S. mansoni* and *S. intercalatum.*
3. Including three atypical results with partial hemolysis in all tubes up to titer.

Our 96 p. cent positive FAT in proven carriers of schistosomes, differ from the data of Pellegrino (1963) and of Foster (1965), who respectively found but 86 and 63 p. cent positive FAT in proven cases.

Results of CF tests are compared in table 3 to parasitological and FAT data (not always simultaneously performed however). The sensitivity of the CF test is quite low in this series, with about 40 p. cent of proven cases reacting negatively (13 on 34). The FAT of these patients proved to be positive in nine (titer 1/8 or more), slightly positive in three and negative in but one patient. These results are discordant with other reports. Pellegrino (1963) found for example 96 p. cent positive CF tests in confirmed chronic *S. mansoni* infections. In the same conditions, a figure of 93 p. cent positive CF tests was quoted by Anderson and Naimark (1960), who therefore considered it as the most sensitive serological test for schistosomiasis together with slide flocculation. The same figure is found in the appreciation by Jackowsky and Anderson (1961). The causes of these discrepancies were not elucidated.

We were able to assess FA and CF titers in four recent human infections (*). These four adult european males bathed in an artificial pool in eastern Congo in the first days of November 1965. They soon presented cercarial dermatitis and a general infestation syndrome. Stools became positive about mid-December. The FA and CF titers are given in table 4. These early FA titers are definitely

TABLE 4

Serology of four recent human infections

Patient	8-10 weeks after infection	12-16 weeks after infection	20 weeks after infection	13 months after infection
B ... FAT	1/512 (30/12/65)	1/256 (11/2/66)	1/128 (21/3/66)	—
CFT	1/8	—	—	—
C ... FAT	1/2048 (10/1/66)	1/2048 (7/2/66)	—	—
CFT	1/16	1/8	—	—
F ... FAT	1/256 (5/1/66)	1/256 (14/2/66)	—	1/128 (14/12/66)
CFT	1/4	1/32	—	—
W ... FAT	1/512 (12/1/66)	1/256 (21/2/66)	—	—
CFT	1/64	1/64	—	—

(*) Observations of P. Limbos to be published later.

higher than those usually observed. It may be assumed that titers in human beings follow a curve identical with that of experimental infections in mice and are directly related to the recentness of the infection. The opinion of Gane *et al.* (1964) that it takes some two to three months for the FA test to become positive, is not substantiated by these findings.

The worm load in human infestations is difficult to appreciate. We tried to relate whenever possible the FA titers to the mean number of eggs of *S. mansoni* per g of stools, on three successive quantitative parasitological examinations before treatment. As suggested from table 5, such a correlation is possible but the data are so rough and subject to bias that it cannot be considered as proven.

TABLE 5

Relation between the maximal FA titer and the quantity of eggs of *S. mansoni* per g of faeces

Titer	Eggs/g	N° of patients
Neg.	69	2
1/4-1/16	55	11
1/32-1/64	162	17
1/128 or more	180	10

TABLE 6

Effect of treatment on the quantitative FAT and CFT

Effect of treatment	FAT		CFT	
	after 3-8 weeks	after 9-17 weeks	after 3-8 weeks	after 9-17 weeks
No differences	15	8	10	2
Increase	4	1	3	—
Decrease	3	1	1	5

1. Delays are given from the last day of treatment.
2. The groups 3-8 weeks and 9-17 weeks do not concern the same patients.
3. All patients were treated with nitrothiamidazole, with one exception treated with thioxanthone.
4. A difference of one degree in titer for the CFT and two for the FAT is reported either as an increase or as a decrease.

Treatment with nitrothiamidazole has apparently no rapid effects on FA titers (table 6). There was no significant decrease in titer up to 17 weeks after treatment, but possibly a slight increase in the first weeks. The CF titers appear more sensitive, as they increase during the first eight weeks and decrease more pronouncedly afterwards, but the number of observations is too small.

Discussion

The FAT has proved to be a reliable test for the diagnosis of infestations, active or latent, with Schistosoma species.

A variety of antibodies are probably thus detected, accounting for the sensitivity of the method, its relative lack of specificity and its failure to give much information on the activity or the intensity of the infection. The fluorescent antibodies, whatever their nature, appear persistent and stable, as demonstrated by experimental data, by the relatively steady titers in positive individuals, exclusive of recent infections and the lack of immediate reaction to treatment. Buck (1964) demonstrated in the course of a field survey in Ethiopia that the age distribution of positive stools *(S. mansoni)*, positive skin tests and four serological tests are in close agreement, with a peak in the younger groups, while the positive FAT progressively increase with age. In the opinion of the author this is a proof of the non-specificity of the FAT. Nevertheless the cumulative percentage could be explained also by the persistence of fluorescent antibodies once they have appeared.

If this were true, the FAT could be an easy method to exclude schistosomiasis as a possible pathogenic factor in a given patient, more reliable as a screening than a parasitological examination in an all purpose laboratory. In mass surveys the FAT performed on whole blood drops, dried on filterpaper, is a real advance, but it will give more information on the prevalence of infection than on the incidence of active disease. Doubts about the specificity of FAT have also been expressed by Kagan (1965). We agree with Sadun (1963) and Ganc (1964) about the use of a 1/4 dilution in order to avoid many non-specific positive reactions. Even at this dilution some common antigens with other trematodes (e.g. Fasciola) do interfere, but by raising the titer one would eliminate a certain number of schistosomiasis cases (table 2). The immunisation by cercarial penetration, as demonstrated by Sadun and Biocca (1962), poses a puzzling problem, which could necessitate a reassessement of the significance of FA tests in areas

where non-human schistosomes are highly prevalent (which is in fact the rule).

While quantitating the test makes the appreciation of the degree of positiveness easier and permits a clear differentiation between doubtful, chronic and recent infections, the time and expenses involved seem exaggerated for routine work. At a 1/4 dilution differentiation between the doubtful (± to ± / +) and decidedly positive (++ or +++) reactions is not too difficult. In our experience even the slightly positive reactions are significant of a trematode infection, but further investigations are necessary before concluding. We may mention here that a few negative sera came from patients with filariasis, which were said to interfere.

The actual problems are probably the relative elaborateness of the techniques using cercariae and the difficulties of standardization of results between different laboratories. Several modifications have been proposed. Cookson aimed at the enhancement of sensitiveness and the use of a less expensive optical equipment. His suggestions include the direct conjugation of cercariae with lissamine-rhodamine, the ingenious use of fluorescent antiglobulins absorbated on bentonite particles and the derived bentonite fluorescent complement fixation test (Cookson *et al.* 1963 and 1964).

Rivera de Sala (1962) elaborated a fluorescent variant of the COP reaction without immediate practical implications. Sato *et al.* (1964), aside from demonstrating the applicability of the test to *S. japonicum* infections, pointed to the different mechanism of reaction between the direct and indirect tests on living cercariae, the latter differing mainly in the presence of precipitates around the oral succer, a feature we observed also with formalin fixed cercariae and which we considered non-specific, though of unknown significance.

Toussaint and Anderson (1965 and 1966) have proposed a fluorometric test with the use of a soluble antigenic fraction, which provides a quantitated and reproductible reading in addition to eliminating non-specific fluorescence. This obviates the subjective element. The different nature of the antigen, necessarily more purified than whole cercariae, does not make up to now a direct comparison with the usual test possible.

Camargo *et al.* (1965) objected to the elaborate procedures and the high cost of the cercarial FA test and proposed a slide test on fragments of minced adult worms. Anyone who has seen the amorfous fluorescent particles which do sometimes occur, even in carefully performed tests will have somewhat prejudicial feelings against such a method. Furthermore we found the fluorescent antigen in bouin fixed, paraffin embedded, counterstained sections of adult

schistosomes in liver tissue of infected mice, strictly limited to the coecal lining, in but a minimal part of the whole worm's mass. This is incidentally conflicting with von Lichtenberg findings (1964); who described also, on cryostat, not counterstained, sections, bright fluorescence of the cuticle of adult worms. It is possible that antibodies to adult worms appear late and at variable moments in the parasitosis — when worms begin to die ? — as immunoelectrophoretic data suggest (Capron *et al.*, 1966). This might explain discrepancies when adult worms are used as antigen. Intact schistosomal eggs are antigenically unreactive (Sadun, 1963, in discussion).

The FA test is feasible on paraffin embedded experimental liver granulomas, preferably after counterstaining. The miracidia and circumoval diffusing antigens become brightly fluorescent. Such an antigen was used by Magalhaes Filho (1965) but we found too much variability in the reactivity of granulomas, depending on their age, on the degree of degeneration of the miracidium, on the amount of autofluorescent egg shell material, etc. to use it satisfactorily in practice.

We have recently been experimenting with hepatopancreas of snails in the later stages of schistosomal infection as antigenic material. It represents in fact a mass of agglomerated cercariae which can be obtained and processed easily, as routine histological material. In preliminary trials, sections of hepatopancreas were found to be a well-suited material, which might take the place of isolated cercariae routine tests. It spares reagents and time (centrifugation or sedimentation being avoided) and is of easy preservation and expedition. The practical implications of the antigenic communities between the worm and its intermediate host (Capron *et al.*, 1966) have not yet been investigated in regard to the test on snail material.

Résumé — Des réactions d'immunofluorescence positives furent obtenues chez 52 patients sur un total de 54 reconnus atteints de bilharziose à *S. mansoni* ou à *S. haematobium*, alors que la fixation du complément n'était positive que chez 21 patients sur un total de 34 cas reconnus atteints. *Fasciola hepatica* provoque des réactions faiblement positives dans le test d'immunofluorescence pour bilharziose. Des titres élevés furent trouvés chez quatre patients récemment infectés par *S. mansoni*, en concordance avec les données expérimentales. Le traitement n'a que peu d'influence sur les titres en immunofluorescence, mais fait baisser les titres de la réaction de fixation du complément, après une élévation initiale. Les auteurs ne voient guère d'avantages à pratiquer des réactions d'immunofluorescence quantitatives en routine de laboratoire.

L'emploi de coupes d'hépatopancréas de planorbes infectées comme antigène est proposé au lieu de cercaires. Le test devient ainsi plus rapide et plus économique tandis que la conservation et l'expédition de l'antigène sont facilitées.

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DISCUSSIE — DISCUSSION

M. Capron — Je voudrais demander à M. Gigase quel antigène et quelle technique de fixation du complément il utilise ?

M. Gigase — Je vais passer la parole à Monsieur Van Meirvenne qui s'est occupé plus spécialement de la fixation du complément.

M. Van Meirvenne — L'antigène a été préparé selon la méthode de Chaffée. Le complément a été titré en présence de l'antigène. Pour le test même des dilutions de sérum à partir de moitié ont été incubées avec l'antigène et deux unités de complément pendant dix-huit heures à 4° C. Le système hémolytique a été préparé avec une seule unité d'hémolyse. Uniquement les tubes ne montrant aucune trace d'hémolyse ont été considérés comme positifs.

M. Capron — Il est intéressant de noter que vous ayez ainsi une réaction toujours positive avec la réaction de fixation du complément; je sais bien que nous n'en avons pas une large expérience à Lille mais les résultats sont discordants dans la mesure où l'on considère la nature de la bilharziose. Dans la bilharziose à *S. haematobium* on retrouve un peu les mêmes phénomènes qu'avec l'immuno-électrophorèse, c'est-à-dire qu'il y a 60 p. cent seulement des sujets qui sont positifs, alors que dans la bilharziose à *S. mansoni* pratiquement on obtient des taux élevés chez 90 ou 95 p. cent des malades.