Some Investigations with the Fluorescent Antibody Technique

III. THE THRESHOLD FLUORESCENT ANTIBODY TEST IN SCHISTOSOMIASIS

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In this section the original experiments with the crude microscope equipment have been combined with the subsequent proving research described above.

The object has been to try and find a truly workable and dependable method for the fluorescent antibody technique in schistosomiasis using ordinary routine laboratory microscope with a minimum of minor inexpensive modifications and additions.

An attempt has been made to exploit the principles of threshold fluorescence, mentioned in the first part of this paper, whereby positive tests exhibit an unmistakeable specific fluorescence, but where the auto-fluorescence of negative is weak and at the same time also quite characteristic.

In addition, a second approach has been made to combine the fruits of the above independent research with the original brilliant work of Sadun and his co-workers (Sadun personal communication, and Sadun *et al.*, 1962) to produce a technique which employs a fluorescent counterstaining of the antigen by a prior coating of the cercariae with an immunological inert conjugate of albumen. In these investigations the tests described have been viewed by the simple apparatus described below, as well as with the research microscope and high intensity mercury vapour lamp.

This cross-checking has been carried out on two separate but essentially similar set-ups of the simplified microscope equipment at the Public Health and Bilharzia Research Laboratories respectively. These checks have included a series of positive fluorescent antibody tests done by one of us (D. de V.C.) with the high intensity U.V., and then passed to the Public Health Laboratory for examination with both the simplified and advanced U.V. research microscope and vice versa.

Three separate well-known makes of relatively modern routine microscopes have been tested and two makes of similar high intensity low voltage tungsten filament lamps. To these have been added a few inexpensive substage blue U.V. filters and orange eyepiece filters, and the same group of filters has been used for each experimental microscope set-up after some initial tests to find the optimum filter combinations for each microscope and lamp. These are described below under materials and methods.

MATERIALS AND METHODS

Microscope—

Reichert Biozet with two lens condenser N.A.: 1.20 with X10 and X20 achromatic objectives and X8 and X10 plano eyepiece. (Alternatively a Watson "Bactil" microscope with 2/3 and 1/6 in. apochromatic objectives with like condenser and eyepieces, and a similar routine Leitz microscope were tried with good results.)

Lamp—

This was a "Wild" low voltage high intensity microscope lamp with a transformer supplying a 6 volt 30 watt bulb which was run at 8 volts for the short periods of actual examination. The Reichert "Lux F N 1" lamp employing a similar bulb and transformer was also effective.

The main consideration here is to have a sufficiently intense source of illumination to produce sufficient blue U.V. radiation to excite the requisite secondary fluorescence in the conjugated antibody. Because tungsten lamps are poor in such radiation, the source of light needs to be utilised to the maximum in the microscope optical train by very carefully adjusted Köhler illuminations. If possible, a plano aluminised microscope substage mirror should be used, because this causes less loss of U.V. or blue U.V. light than the usual glass mirrors.

Filters—

Reichert dark blue substage pass filters were used for the weak blue U.V. light, and orange, blue-exclusion eyepiece filters with the following respective Schott and Gen. specifications: B.G./12/6 mm. and GG9/1 mm. + OG1/1.5 mm.

Although it was possible to see the positive tests quite clearly with a binocular body, a much brighter image was obtained with an inclined monocular, and the brightest of all with the old-fashioned straight body tube which has no intervening prism components.

Sera and Antigens—

Only the fluorescent Coombs or anti-globulin test was employed, and Lissamine Rhodamine RB200 fluorescein isothiocyanate and D.A.N.S. conjugated anti-human globulin sera with the techniques already described were used.

Controls—

These were as described.

Results

A. *Threshold Fluorescence Technique* (Plate IX)

1. *Positive Tests*

(a) RB200 conjugated sera Coombs gave a yellow to orange fluorescence against a near black to brownish-black background.

(b) Fluorescein Coombs sera gave a much more brilliant fluorescent yellow-green image.

(c) D.A.N.S. conjugated Coombs sera were disappointing, probably because the blue U.V. from the tungsten lamp did not possess sufficient spectral energy to elicit sufficient secondary fluorescence.

2. *Negatives*

These were a very pale ghost-like greyish green and quite unmistakeable in contrast to the positive tests with RB200 and fluorescein conjugated anti-human globulin sera.

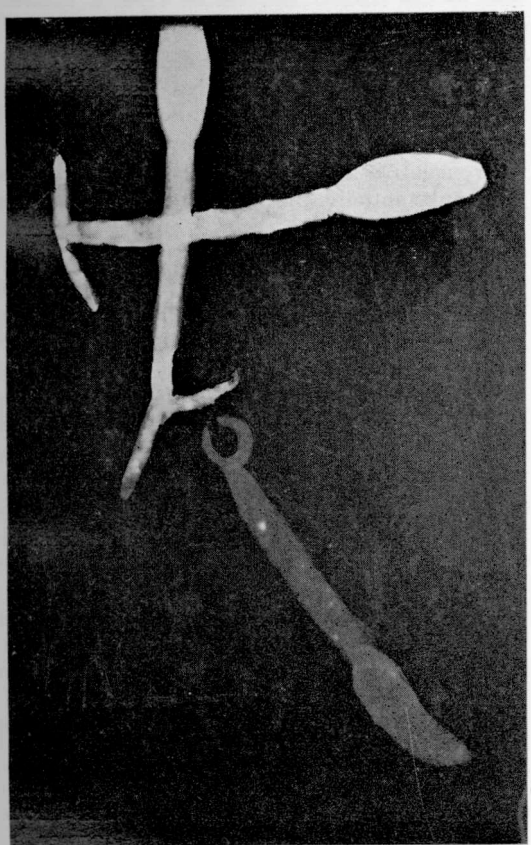

Plate IX.—Threshold fluorescent antibody test mixed slide, showing two positive and one negative cercariae. Fluorescent Coombs test with fluorescein isothiocyanate conjugated rabbit anti-human globulin serum.

Comment.—Of these two conjugates, using untreated fixed cercaria as a test antigen, the fluorescein anti-human globulin gave the best results because of the very much higher fluorescent index of this dye over Rhodamine and any other possible conjugated fluorochromes. Fluorescein isothiocyanate conjugated anti-globulin sera are therefore the best choice for this threshold fluorescent antibody test, and this applies also to a limited number of tests done with bovine sera and an anti-bovine globulin fluorescein Coombs serum.

B. *The Enhanced Fluorescent Anti-Globulin Test*

Rhodamine Albumen Coated Antigen

It has been shown by Smith *et al.* (1959) that the non-specific staining of tissue components with fluorescein conjugated specific antibodies can be largely anulled by counterstaining with an immunologically inert Lissamine Rhodamine RB200 conjugated serum of rabbit, bovine or ovine origin. This produces a reddish-orange background, while the specific antigen is unaffected and is immunologically stained the typical brilliant yellow-green of the fluorescein conjugated antibody.

Sadun (personal communication) and Sadun *et al.* (*ibid.*) have applied this principle to the fluorescent antibody test for schistosomiasis in which they have coated *S. mansoni* cercariae, fixed in 5 per cent. formalin in F.T.A. buffered saline, with RB200 conjugated bovine albumin.

The cercariae so coated are stored in this solution and, after washing in F.T.A. buffer, are employed as a substrate for the fluorescent Coombs or anti(human)-globulin test.

The coated cercariae *per se*, and in negative tests, are a brilliant orange-yellow.

In positive tests this colour is immunologically overlaid with the fluorescein conjugated anti-globulin serum. The result is to produce a very much enhanced fluorescent image. In addition, the distribution and intensity of the overlying fluorescein anti-globulin serum in positive tests has been used by Sadun (*ibid.*) as a quantitative method of scoring such tests.

The technique is well suited to the "poor man's" fluorescent antibody microscopic equipment described above.

Negative tests show orange-yellow cercariae, while positives are overlaid in degree by the

brilliant yellow-green of the fluorescent conjugated anti(human)-globulin serum.

The method has on the whole worked well here when commercially available Rhodamine counterstains have been used.* But attempts to prepare these in the Public Health Laboratory have hitherto been disappointing, not the least disadvantage being the difficulty of obtaining bovine or ovine serum locally which is immunologically inert to schistosomiasis. The cercariae are fixed by adding equal volumes of live cercariae in water to 10 per cent. formalin in buffered F.T.A. saline. Generally speaking, the commercially prepared bovine serum counterstain is then added at the rate of 1 ml. per 100 ml. of the resulting 5 per cent. formalin F.T.A. buffer cercarial suspension.

It is difficult to be dogmatic about these proportions because no details of the conjugated protein content of the counterstain are given by the makers and each batch has to be assessed by trial and error.

Too high a concentration of counterstain produces bright orange-red cercariae which are antigenically inert, and the counterstain ultimately flocculates out of the formalin buffer solution.

If the counterstain is too weak there is minimal or no coating of the cercariae, which tend to give anomalous results apart from vitiating the whole principle of this technique, which is to enhance the fluorescence of the antigenic particles so that the weak excitation of the tungsten lamp may be significantly increased.

C. *The Enhancement of Fluorescence by Addition of Fluorochromes*

An attempt was made to increase the fluorescence of formal buffer fixed cercariae by adding a variety of fluorescent dyes in F.T.A. buffer to the initial and final stages of the test, i.e., to the test serum or the fluorescent anti-globulin serum. Among the dyes tried at final dilutions of 1/50,000 to 1/100,000 were acridine orange, thioflavine, acriflavine, primuline, berberine, phosphine and Rhodamine. Of these, only the first two showed minor promise and all tended to extinguish the fluorescence of the fluorescein or RB200 conjugated anti-sera. This is in keeping with observations that when unlike fluorochromes are mixed in solution they tend to lower rather than enhance their mutual fluorescence.

* F.A. Rhodamine counterstain: Difco Laboratories, Detroit, 1., Michigan, U.S.A.

D. *Enhancement of Fluorescence by Conjugation of Antigen*

At the Public Health Laboratory cercariae freshly fixed in 5 per cent. formalin F.T.A. buffered saline were washed free of buffer and suspended in cold isotonic saline.

To this was added an equal volume of cold carbonate-bicarbonate buffer and the suspension refrigerated to just above 0° C. This suspension was then conjugated for 18 hours in the refrigerator with Lissamine Rhodamine RB200 after the method of Chadwick *et al.* (*ibid.*). 0.1 ml. of acetone extract of the RB200 sulphonyl chloride was used for each 1 ml. of cercarial suspension and the mixture was gently agitated by using a Matburn mixer. After conjugation the cercariae were washed with F.T.A. buffered saline. Most of the undamaged cercariae showed successful conjugation to the RB200 and varied from a brilliant orange to orange-red fluorescence.

The conjugated cercariae did not lose their antigenicity and gave positive tests with the fluorescent Coombs test employing fluorescein conjugated anti-human globulin serum.

In negative tests and controls the cercariae gave substantially the same appearance as the Rhodamine bovine serum coated cercariae described above.

In positive tests a similar appearance was evident, except that the overlaying effect of the fluorescein anti-globulin serum was not so pronounced.

This was probably due to the underlying cercariae being more saturated with RB200 molecules through actual conjugation than the mere coating effect of the Rhodamine bovine serum counterstain.

These experiments suggest that a possible alternative exists to the RB200 bovine albumen counterstain for producing a fluorescent antigenic particle which will both enhance the final fluorescence of positive tests and differentiate the negative results. The method is also more directly controllable because the various components and factors of the conjugation procedure are capable of wilful experimental variation.

It has also been possible to conjugate cercariae in this manner to D.A.N.S. and Rhodamine isothiocyanate. Although the former has been found to work with the powerful U.V. microscope, it is not suited, as has been noted, because of the lack of spectral energy of the lamp in the simple modified microscope equipment described here.

Discussion and Conclusion

In the above experiments the principal consideration has been simplification and an attempt to bring the fluorescent antibody methods in schistosomiasis within the scope of almost any laboratory.

It should be remembered, however, that the conversion has been from a very specialised and powerful type of ultra-violet microscopic technique which is capable of a relatively great latitude in defining fluorescent objects.

The simple equipment described above is relatively insensitive in that it can only define gross fluorescence in large objects adequately coated with fluorescent antibody molecules as would occur in a positive test. It is this very insensitivity which is its chief virtue, because negative or doubtful marginal results only give the very weak and typical greyish microscopic image of autofluorescence.

For this reason the simplified blue U.V. equipment described above will be less subject to error the less complicated the technique employed.

We conclude, therefore, that the threshold fluorescence method using 5 per cent. formalin buffer fixed cercariae and a fluorescein species anti-globulin serum is the test of choice. Apart from being quite straightforward, it is less expensive because it entails the use of only one fluorescent anti-globulin serum for any animal, including human, species to be tested.

The coating of cercariae with Lissamine Rhodamine RB200 conjugated bovine or other serum in the enhanced fluorescence test introduces more elements of preparative experimental error and the commercial counterstain is fairly expensive for any large-scale use.

The conjugation of the cercarial antigen to RB200 sulphonyl chloride, while attractive and cheap as a possible alternative, must be regarded as experimental and warrants further investigation and proof before it can be universally adopted for either the simplified or the advanced U.V. microscopic equipment. It does, however, suggest a new line of approach and gives scope for producing fluorescent antigens with other dyes and of other colours.

In this connection it does seem that the summative fluorescent effect of the yellow-green fluorescein overlying the orange-red of RB200 albumen coated cercariae is somewhat lessened by the extinguishing effect which the green component of the former exerts on that part of the red component of the latter. It should be possible to use a fluorochrome for the antigen which gives a higher degree of summation to either fluorescein or Rhodamine when these are subsequently immunologically applied. The coating or conjugation of antigen to D.A.N.S. which fluoresces yellow does in part fulfil these conditions, but unfortunately, for the purpose of the simplified test, it does not fluoresce sufficiently brightly with the low-energy blue U.V. tungsten lamp employed, although it works quite well with the mercury vapour lamp.

We feel that this work is but a pointer for the possibilities of the simplified blue U.V. fluorescent antibody technique, and in particular there appears to be much scope in finding and utilising relatively cheap but more powerful sources of illumination and of devising the optimum filter combinations to fit them. There is a limitation to the size of the antigen particles which can be seen with this simplified apparatus and this technique. But experience has shown that small objects such as trypanosomes are quite distinguishable using fluorescein conjugated antisera. Smaller objects, such as bacteria, cannot be seen except in large aggregates.

In conclusion, we should mention that the simplified blue U.V. apparatus described can be used for the identification of certain micro-organisms which have been stained with fluorochrome dyes, and in particular for the Auramine staining of *M. tuberculosis* devised by Keller over 20 years ago with similar apparatus.

The acridine orange stain for D.N.A. and R.N.A. in cytological smears for tumour and other cells is also a practical possibility with this equipment.

Summary

The principles of the fluorescent antibody technique are described, with the early investigations of this method in human schistosomiasis.

This subsequent proving research of the validity of the technique in schistosomiasis with advanced fluorescent microscopic equipment is described, with some observations on the cross-antigenicity of related and unrelated parasites.

The use of the method as an ideal diagnostic technique for schistosomiasis is discussed, including some of the difficulties which may be expected to be encountered.

In the final section the conversion of the results of the proving research inexpensive and

readily available microscopic equipment is described and discussed in the context of making the technique available for most routine laboratories in the diagnosis of mammalian, including human, schistosomiasis.

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