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Immunofluorescent staining of *Treponema pallidum* and *Treponema pertenue* in tissues fixed by formalin and embedded in paraffin wax

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SUMMARY The main problems in identifying *Treponema pallidum* in tissues are optical definition, contrast, and specificity. In general, fluorochrome staining provides optical definition and contrast superior to that obtained by ordinary tinctorial staining, and in theory improved resolution. Specificity is lacking however, as with other stains. In contrast, immunofluorescence should combine the optical advantages of fluorochrome staining with the immunological advantages of specificity. Since the validity of such staining depends in part upon the integrity of the antigenic components of the micro-organisms, it is customary to avoid such drastic procedures as are involved in routine fixation and paraffin embedding. The manipulation, however, of unfixed cryostat material, in contrast with that of paraffin sections suffers from two disadvantages—namely, friability and infectivity. Published and unpublished work has shown antigenic stability in *T. pallidum* to a variety of procedures, both physical and chemical. Consideration of these facts led in this work to successful immunofluorescent staining after routine formalin fixation and paraffin embedding of tissues infected with *T. pallidum* or *Treponema pertenue*. Optical definition and contrast, were superior to that obtained with silver methods, but it was not possible to differentiate between these two organisms. Nevertheless immunofluorescence applied as described to paraffin sections should supply a convenient, safe, and sensitive means of reappraising the histopathology of treponemal disease in patients, necropsy material, and experimental animals.

Introduction

The discovery of a convenient stain for a micro-organism has, invariably, facilitated not only the identification of that organism and the diagnosis of the disease for which that organism is responsible, but it has promoted further study and understanding of the lesion and epidemiology of the disease. It is fundamental that there is sufficient contrast between the micro-organism and the host tissue. Satisfactory optical definition of the micro-organism by humoral staining however, has not always been concomitant with specificity.

In recent years, the technique of immunological staining as exemplified by the technique of immunofluorescence, has resulted in both satisfactory optical definition and specific identification of micro-organisms in pathological exudates and tissues. Since the validity of the staining depends upon the integrity of the antigenic components of the micro-organisms, it is essential that these are not modified or destroyed by fixation. Thus the usual processes of fixation, dehydration, clearing, and embedding in hot paraffin wax have been avoided and much of the histopathological work involving the immunofluorescence of tissue components, antigen-antibody complexes, the localisation and persistence of intact micro-organisms, or their dissociated antigens has been performed on frozen tissue sections unfixed, or fixed with less drastic fixatives such as acetone or alcohol.

Since the discovery of *Treponema pallidum* as the causative agent of syphilis (Schaudinn and Hoffmann, 1905), identification of the organism, in the

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absence of characteristic differences in staining or of confirmatory tests in culture, has depended on experienced observers recognising fine differences by dark ground microscopy. Although in general, the organism was refractory to tinctorial staining, Schaudinn and Hoffmann (1905) successfully used a modified Giemsa technique by which the organism stains pink. Subsequent attempts by others at improving optical definition resulted in the introduction of silver deposition methods, the best known of which are those of Fontana (for smears) and Levaditi (for tissues). Although optical definition is undoubtedly improved, albeit crudely, the methods lack specificity, in that saprophytic treponemes also stain, rendering this approach unsatisfactory for primary diagnostic purposes. Moreover, optical contrast by silver deposition, as between treponemal and tissue elements—in particular, argyrophil reticulin—is frequently lacking, rendering detection of the organisms difficult especially when scanty. In this respect the comments of Warthin (1929) are worthy of note. The problem of identifying *T. pallidum* in tissues is therefore one of optical definition, optical contrast, and specificity.

In general, staining by fluorochrome provides superior optical definition to that of ordinary staining, and in theory improved resolution, since each point on the object examined is its own light source. As with other stains specificity is lacking. Immunofluorescence combines the optical advantages of fluorochrome staining with the immunological advantages of specificity. Thus in relation to pathological exudates a direct immunofluorescent technique was used by Jue *et al.* (1967) and an indirect technique by Silverstein and Kent (1957), Garner and Robson (1968a, b), and Wilkinson and Cowell (1971). In both groups the later workers absorbed their sera with Reiter treponemes or their products in order to obtain greater specificity.

As an example of the optical sensitivity of immunofluorescence in relation to *T. pallidum* the work of Wells and Smith (1967) is pertinent. These workers demonstrated the presence of *T. pallidum* in the aqueous humour of monkeys with experimental ocular syphilis using acetone-fixed smears and direct immunofluorescence. Comparing their results with those obtained by silver deposition, these authors concluded that immunofluorescence was more specific and far more sensitive as it could detect very small numbers of *T. pallidum* in the aqueous humour.

T. pallidum has also been demonstrated in cryostat sections of infected tissue by direct and indirect immunofluorescence (Yobs *et al.*, 1964, 1965; Smith *et al.*, 1967). The organism however, has not to date been demonstrated in sections of tissues fixed in formalin and embedded in paraffin. The manipulation of unfixed cryostat material in contrast with that of paraffin sections suffers from two disadvantages—friability and infectivity. A study was made of the possibility of using immunofluorescence to identify *T. pallidum* in tissues fixed by a standard routine method of fixation, and embedded in paraffin wax by a standard routine method. That certain of the immunologically-reactive components of treponemes were sufficiently stable to withstand such treatment was indicated by D'Alessandro and Dardanoni (1953) who isolated a thermostable polysaccharide from the Reiter treponeme, and also by Hardy and Nell (1957) in work on virulent *T. pallidum* in which they too detected a thermostable antigen. Our unpublished work concerning the effect of heat and chemicals on the serological activity of *T. pallidum* antigens agreed with these findings.

It is emphasised that the results reported here were not derived from single experiments but are representative of batches of experiments which were remarkable in the uniformity of their reproducibility.

Materials and methods

TREPONEMAL SUSPENSIONS

T. pallidum suspensions

Suspensions of *T. pallidum* were obtained from rabbit testicular syphilomas 8–10 days after inoculation as follows.

The particular strain used in these studies was the Nichols strain of virulent *T. pallidum* (Nichols and Hough, 1913). This organism used in the *T. pallidum* Immobilisation (TPI) test was obtained from the Venereal Diseases Reference Laboratory, The London Hospital, Whitechapel, London E1, by the courtesy of Dr A. E. Wilkinson.

Animals

Adult male New Zealand white rabbits weighing approximately 3–4 kg were obtained from commercial suppliers. Before inoculation the animals were allowed to acclimatise to the animal house for at least three weeks. They were kept in individual cages with food and water always available. They were fed on a pellet diet (Oxoid laboratory animal diets). The temperature of the room was usually below 22°C.

Before inoculation approximately 5 ml of blood was drawn from the middle artery of the ear of each rabbit. The serum was separated, inactivated at 56°C for 30 minutes, and examined by the TPI test, or the fluorescent treponemal antibody absorbed (FTA-ABS) test.

Inoculation procedure
A suspension of *T. pallidum* (Nichols pathogenic strain) containing approximately 10^8 treponemes per ml was inoculated into the body of the rabbit testis, in a dose of 0·5 ml for each testis. Corticosteroid was given to the inoculated animals to suppress immunological response and increase the yield of treponemes (De Lamater *et al.*, 1952; Turner and Hollander, 1957). This was administered intramuscularly as cortisone acetate (Cortisul, Roussel Laboratories, London), 25 mg per ml. Daily doses were given of 0·2 ml/kg body weight, starting from the first day of inoculation until full development of syphilitic orchitis.

Harvesting of treponemes
When the testes became red, indurated, and nearly twice their normal size the rabbits were killed by intravenous injection of 5 ml pentobarbital sodium solution (Nembutal, Abbott Laboratories) 60 mg/ml. The testes were removed asceptically via scrotal incision. In order to increase the surface area and thereby facilitate elution of the treponemes, the testes were cut into small fragments which were placed in an extraction flask to which was added 5–10 ml of sterile phosphate buffer saline pH 7·2 per testis.

The air in the flask was extracted and replaced by 5% carbon dioxide and 95% nitrogen mixture, a procedure which was repeated three times. The flask was then shaken on a mechanical shaker (Griffin flask shaker) for one hour at room temperature. Red cells and gross testicular debris were removed by centrifugation at $1000 \times g$ for 10 minutes. Continuity of the strain was ensured by immediate inoculation of a fresh batch of rabbits.

Treponema pertenue *suspensions*
The strain of *T. pertenue* used in this work was received in hamsters, through the courtesy of Professor J. Pillot, Pasteur Institute, Paris. Treponemes were harvested from skin ulcers as follows. Animals were killed by chloroform, crusts were removed from the ulcers, after which ulcers were excised, finely minced with scissors, suspended in 2 ml of phosphate buffer saline, and shaken mechanically for 30–60 minutes. Tissue fragments, hair, and blood cells were removed by centrifugation at $1000 \times g$ for 10 minutes. Attempts were made to harvest treponemes from regional lymph nodes, but since fewer organisms were obtained than from skin ulcers, this source was abandoned.

Preliminary experiments in animal passage in which intradermal injections of phosphate buffer saline suspensions in 0·2 ml amounts into the unshaven thighs of hamsters were made, also failed to produce a satisfactory treponemal harvest. For maintenance of strains in the laboratory, therefore, these methods were abandoned in favour of direct animal to animal passage, as described below.

T. pertenue—*laboratory maintenance*
T. pertenue was maintained in the laboratory by passage in hamsters at intervals of between five and 10 weeks using the method of skin scarification (Hill and Gordon, 1954). Thus, animals were anaesthetised lightly by ether. The skin of the inner side of the unshaven thigh of the recipient was cleansed with normal saline, then five or six small scratches were made with a scalpel in a small circumscribed area approximately 1 cm in diameter in one or both thighs. The infected ulcer of the anaesthetised donor was rubbed on the scarified area of the recipient. Approximately 10 frictional passages were made, during which the ulcer began to ooze. During the procedure the donor and recipient areas were inspected, and it was considered that satisfactory passage had been accomplished when the recipient area had been well coated with blood and exudate from the donor. In this way up to six recipients were inoculated from one donor.

Initial experiments using nose to nose passage in hamsters were considered unsatisfactory, for the treponemal harvest was poor, and the suffering caused to the animal disproportionate.

SEROLOGICAL REAGENTS

Human sera
Sera from patients with syphilis and yaws were obtained through the courtesy of Dr A. E. Wilkinson, The Venereal Diseases Research Laboratory, The London Hospital Research Laboratories, Ashfield Street, London E1.

Rabbit antisera
Antisera to the pathogenic *T. pallidum* (Nichols strain) were produced in rabbits experimentally infected with this organism. Sera were collected between four months and two years after the primary manifestation of the disease, by bleeding from the artery of the ear (25 ml–40 ml). Sera were pooled, preserved by the addition of sodium azide to a final concentration of 1 in 1000, and stored in 1 ml amounts in screw-capped bijoux bottles at −20°C.

Pooled human syphilitic serum from patients with strong TPI and FTA-ABS reactions was also used.

Hamster antisera
Hamsters were infected with *T. pertenue* by scarification of the skin of the nasal area and later that of the inguinal region, as described above. Blood was obtained by cardiac puncture four months later and

the sera were pooled.

All sera—that is, both human and animal—were distributed in aliquots of 0·25 ml and kept at a temperature of −20°C until used.

Titration of antisera

Samples of pooled human patients' sera and animal antisera were titrated against air-dried films of *T. pallidum* fixed in methanol (5 minutes) or acetone (10 minutes) using an indirect fluorescent treponemal antibody technique. Bright immunofluorescence was obtained at dilutions of 1 in 800 with human patients' sera and rabbit antisera, and 1 in 400 with hamster antisera. In these experiments smears of *T. pallidum* were used in preference to smears of *T. pertenue*, as they contained less debris and more numerous treponemes per high power field. In indirect fluorescent treponemal antibody experiments on fixed cryostat tissue sections from rabbits and hamsters infected with *T. pallidum* and *T. pertenue* respectively, bright immunofluorescence was ensured when such sera were used at a dilution of 1 in 5 in phosphate buffer saline pH 7·2, or in 'sorbent'.

IMMUNOFLUORESCENT CONJUGATES AND REAGENTS

Anti-globulin conjugates

Anti-globulins (human, rabbit, or hamster) conjugated with fluorescein isothiocyanate were stored at a temperature of −20°C until used.

Anti-human globulin immunofluorescent conjugate as Fluoroscan Diagnostic Reagent was obtained from Winthrop Biologicals Ltd, Winthrop House, Surbiton-upon-Thames, Surrey, England. It was used as supplied, undiluted.

Anti-rabbit globulin immunofluorescent conjugate as 'SwAR/FITC' was obtained from Nordic Pharmaceuticals and Diagnostics, Langestraat 57-6, Tilburg, Holland (agents: Fraburg Ltd, 54 Furze Platt Road, Maidenhead, Berkshire). It was used at a dilution of 1 in 80.

Anti-hamster globulin immunofluorescent conjugate as 'SwAH/FITC' was also obtained from Nordic Pharmaceuticals and Diagnostics. It was used at a dilution of 1 in 80.

Pooled human, rabbit, or hamster immune sera were used with the corresponding conjugated anti-globulin, in indirect immunofluorescent staining experiments, on air-dried fixed films of *T. pallidum* and *T. pertenue*, cryostat sections, and paraffin sections of tissues from rabbits and hamsters experimentally infected with *T. pallidum* and *T. pertenue* respectively.

Liver powder

Acetone dried rat liver powder (made in the Department of Rheumatology at the Royal Free Hospital) was used to absorb immunofluorescent conjugates so as to remove non-specific staining.

Sorbent

This is a concentrated supernatant of heated Reiter treponema culture, obtained from the Venereal Diseases Research Laboratory, The London Hospital Research Laboratories, Ashfield Street, Whitechapel, London E1.

Absorption of sera

Pooled human patients' sera or pooled rabbit or hamster immune sera were used unabsorbed or absorbed with 'sorbent', according to the principles of the standard FTA-ABS test for syphilis. Anti-globulin conjugates were used unabsorbed or absorbed with liver powder.

PREPARATION OF TISSUES FOR IMMUNOFLUORESCENT STAINING

Tissues examined

Tissues included testis, lymph nodes, and skin from rabbits infected experimentally with *T. pallidum* intratesticularly or intradermally. Skin and lymph nodes from hamsters infected with *T. pertenue* by skin scarification (Hill and Gordon, 1954) were also used. Paraffin blocks of syphilitic human tissue, which included liver with congenital syphilis and lymph nodes and skin from suspected syphilitics, were examined together with paraffin blocks of skin from cases suspected of having yaws in Ghana. (These paraffin blocks were received through the courtesy of the late Professor K. R. Hill, Department of Pathology, The Royal Free Hospital, Gray's Inn Road, London WC1).

Fixation of tissue

The following fixatives allowed the satisfactory immunofluorescent staining of tissues:

(*a*) 5% acetic acid in ethanol (Wolman and Behar, 1952).

(*b*) 95% ethanol.

(*c*) 10% freshly prepared formol saline.

(*d*) Neutral buffered formalin prepared as follows:

Formalin	100 ml
Sodium dihydrogen phosphate (monohydrate)	4 g
Disodium hydrogen phosphate (anhydrous)	6·5 g
Distilled water	900 ml

The neutral buffered formalin was preferred as it allowed brighter immunofluorescent staining,

especially when tissue blocks were left in fixative for a long time. Most experiments, however, were carried out using formol saline since it is a widely used routine tissue fixative.

In experiments tissues were exposed to fixative for periods measured in minutes, in hours, days or weeks.

Temperature of fixation

The effect on immunofluorescent staining of fixation at 4°C and ambient temperatures for various periods was studied, as was also quick fixation at 80°C (Robinson and Fayen, 1965). In the latter method, portions of tissue 2–3 mm in thickness are placed in 10% formol saline at a temperature of 80°C for three minutes before being processed for sectioning.

Cryostat sections, unfixed tissue

Small portions of tissue approximately 5 mm × 3 mm were snap frozen in dry ice/ethanol mixture. Sections 2 μm thick were then cut in the cryostat, after which they were dried under a fan at an ambient temperature. This approach was adopted in initial experiments, after the successful demonstration of immunofluorescent staining of *T. pallidum* in tissues by Yobs *et al.*, 1964, using similar methods.

Cryostat sections fixed tissue

Subsequent studies of the effect on immunofluorescent staining of *T. pallidum* in tissues, after preliminary fixation of tissue blocks in different fixatives, were also carried out in cryostat sections as described above. Before being sectioned, tissue blocks which had been immersed in fixative were washed in phosphate buffered saline pH 7·2.

Paraffin embedding of fixed tissues

After the successful demonstration of *T. pallidum* in cryostat sections cut from blocks of fixed tissue, experiments were continued in which a rapid paraffin embedding technique was compared with a standard routine paraffin embedding technique, for their effects on immunofluorescent staining. When formalin fixatives were used, tissue blocks were first washed and then soaked in phosphate buffered saline for between two and 24 hours before the embedding process. This step was not required for the 95% ethanol or the 5% acetic acid in ethanol fixatives.

Rapid method

The method used was a modification of the technique of Robinson and Fayen (1965). In this procedure dehydration, clearing, and wax impregnation were hastened by shaking the tissue and reagents in a mechanical shaker. The entire procedure took two hours. The procedure is as follows:

1. Cut fixed tissues in slices 3 mm in thickness or less.
2. Immerse in 95% ethanol in a 30 ml screw cap glass universal container and shake. Two changes of 10 minutes are required.
3. Immerse in absolute ethanol and shake. Two changes of 10 minutes each are required.
4. Immerse in a mixture of equal volumes of absolute ethanol and acetone, and shake for 10 minutes.
5. Immerse in acetone and shake for 10 minutes.
6. Immerse in a mixture of equal volumes of acetone and xylene and shake for 10 minutes.
7. Immerse in xylene and shake. Two changes of 10 minutes each are required.
8. Immerse in filtered paraffin at a temperature of 65°C and agitate gently and intermittently by hand for five minutes.
9. Immerse in filtered paraffin at 58°C and agitate intermittently by hand. Two changes of 10 minutes each are required.
10. Finally, embed in paraffin at 56°C.

Routine method

A standard automatic tissue processing technique occupying approximately 20 hours was used. During the process, tissues were exposed to the dehydrating and clearing agents and the molten wax for periods ranging from between five and eight hours in each. Chloroform was substituted for xylene as the clearing agent. The method is as follows:

1. 70% ethanol, one hour.
2. 95% ethanol, one hour.
3. Absolute alcohol, four changes of 1½ hours each.
4. Chloroform, three changes of 1½ hours each.
5. Wax bath at a temperature of 56°C, three changes as follows:
   (*a*) Six hours.
   (*b*) Two hours.
   (*c*) Transfer to fresh wax at a temperature of 56°C for embedding.

Paraffin blocks were stored at a temperature of 4°C until sectioned.

Paraffin sections

These were cut at 2 μm and floated on to glass slides that had been previously coated with 0·5% gelatin (Nairn, 1969). They were then thoroughly dried in an oven at a temperature of 56°C for 20 minutes. Microscopical examination of *T. pallidum* was optimum in sections of 2 μm, but not in sections of 5 μm.

IMMUNOFLUORESCENT STAINING, INDIRECT METHOD

Preliminary processing of sections

Immune sera or their dilutions were inactivated at a temperature of 56°C for 30 minutes. Before staining, paraffin was removed from sections by soaking in three consecutive baths of xylene for 15 seconds each. Xylene was removed by soaking in three consecutive baths of 95% ethanol. Sections were brought to water by soaking in phosphate buffered saline pH 7·2 for two consecutive changes of 10 minutes each. Finally they were dipped in distilled water for a few seconds, drained, and the excess moisture removed from around the sections with absorbent tissue, or they were allowed to dry in air.

Staining of sections

One or two drops of diluted human patients' serum or animal antiserum were placed on the section so as to cover it completely. Thus treated, the section was placed in a moist chamber at an ambient temperature for 30 minutes, after which, having been rinsed briefly and gently in running tap water, it was washed twice in phosphate buffered saline pH 7·2 for 10 minutes each, before being dipped in distilled water and drained of excess moisture.

The appropriate anti-globulin immunofluorescent conjugate optimally diluted was applied to the tissue section, which was treated exactly as described above for human patients' serum or animal antiserum. For fluorescence microscopy stained sections were mounted in buffered glycerol (2 volumes of phosphate buffered saline pH 8·0 and 8 volumes of glycerol).

Control of immunofluorescent staining

Each immunofluorescent staining experiment was accompanied by two negative controls. In one, immune serum was replaced by normal human, rabbit, or hamster serum (without any treponemal antibodies), whichever was appropriate. In the other, normal uninfected animal tissue was processed as for the infected tissues, treated with serum containing treponemal antibodies, and stained with the appropriate anti-globulin fluorescent conjugate. In none of these controls were treponemes or objects resembling treponemes ever seen.

The effect of variables on immunofluorescent staining

The effect of variables on the immunofluorescent staining of treponemes in tissues was studied in order to define the limitations and usefulness of the method. Towards this end, therefore, tissues as blocks in the above-mentioned fixatives, or in paraffin, or as sections on slides, were kept for varying periods at ambient temperatures, or at 4°C, before being processed and subjected to immunofluorescent staining.

Recording of intensity of immunofluorescence

A simple method of scoring the intensity of immunofluorescence was adopted, since it was not the intention to record minor changes in intensity as in the measurement of immunological activity, but merely to record gross changes as the result of fixation, dehydration, clearing, and embedding in paraffin wax.

The degree of fluorescence was scored as follows:

1. Intense fluorescence ++++
2. Moderate fluorescence +++
3. Slight fluorescence ++
4. Faint fluorescence, but organism clearly visible +

In general, the intensity of immunofluorescent staining observed in sections was slightly less than in the case of films.

COMPARISON OF STAINING BY IMMUNOFLUORESCENCE AND SILVER IMPREGNATION

Staining by silver impregnation

In order to study the sensitivity of the immunofluorescent technique as applied to treponemes in tissues, and also the facility with which the organism could be identified, a comparison was made between the technique and two of the silver impregnation methods of treponenal staining applicable to tissues.

(*a*) *Levaditi's method*

Dobell's modification of the method, as described in Disbrey and Rack (1970) was used. In the application of this technique to the current study, portions of tissue were taken from tissue blocks about to be studied by immunofluorescence, and prepared for silver impregnation. This method, however, did not allow the comparison by microscopical examination of closely adjacent areas. The following technique was therefore applied.

(*b*) *The method of Warthin and Starry*

This method, described in Drury and Wallington (1967), is applicable to sections and allows the study of closely adjacent areas of tissue. To allow a more valid comparative study, alternating serial sections were submitted to immunofluorescent staining or silver impregnation.

OPTICAL EQUIPMENT, FLUORESCENCE MICROSCOPY

Two binocular fluorescence microscopes were used:

(*a*) Watson 'Hilux 70'.
(*b*) Reichert.
The latter was preferred.

(*a*) *Watson 'Hilux 70', fluorescence microscope*
This microscope was equipped with a halogen tungsten lamp (100 W) and a dark field condenser. The excitation filters consisted of a DB 545 with a Schott filter 1·5 mm BG 18. A Wratten no. 12 filter was used as a barrier filter. A fluorite oil immersion objective (× 90) was used in conjunction with compensated oculars (× 7).

(*b*) *Reichert fluorescence microscope*
In this microscope a halogen tungsten lamp (100 W) and a dark field condenser were used in conjunction with a Balzer FITC interference filter. The barrier filter consisted of a Wratten no. 12. Two immersion objectives were used: a glycerol immersion objective (× 60), and a fluorite oil immersion objective (× 100). Compensated oculars (× 6) were used with both objectives.

Results

IMMUNOFLUORESCENT STAINING OF *T. pallidum* IN TISSUES OF EXPERIMENTALLY INFECTED RABBITS

Unfixed tissue
In cryostat sections of unfixed tissue treponemes always fluoresced brightly, and although the tissues showed some non-specific fluorescent staining, this was never of such a degree as to interfere with the optical definition of the treponemes.

Fixed tissue embedded in paraffin
Immunofluorescent techniques failed to demonstrate *T. pallidum* in paraffin sections of infected testis, skin, or lymph nodes fixed in formol sublimate, mercuric chloride in Schaudinn's fluid, glutaraldehyde, or osmium tetroxide. By contrast, when fixed in 10% formol saline, neutral buffered formalin, 5% acetic acid in ethanol, alcohol 95%, or when heated to 80°C for three minutes, the treponemes retained their ability to react with specific antibody and fluoresced brightly even after many days of fixation, as described below.

EFFECT OF STORAGE IN DIFFERENT FIXATIVES ON THE IMMUNOFLUORESCENT STAINING OF *T. pallidum*

Comparison of the effects of fixation in 10% formol saline and 10% neutral buffered formalin at an ambient temperature and at 4°C
In paraffin sections of tissues which had been fixed in formalin fixatives for periods of up to 14 days, and examined within 24–48 hours of embedding, *T. pallidum* was readily stained by indirect immunofluorescence, the intensity of the staining at both temperatures varying from ++++ at 24 hours to +++ at 72 hours. The ability to respond to immunofluorescent staining with at least +++ intensity persisted for 14 days at both temperatures. At 30 days the ability to fluoresce with this intensity was retained only by those tissues fixed in neutral buffered formalin at a temperature of 4°C, while intensities of ++ were shown by tissues fixed in formol saline at a temperature of 4°C, or neutral buffered formalin at an ambient temperature (Table 1). Thereafter the ability to respond to immunofluorescent staining fell until it became absent at 67 days. At the lower levels of intensity, occurring after prolonged fixation, fluorescence was slightly better with neutral formalin at 4°C.

Comparison of the effects of fixation in 5% acetic acid in ethanol and 95% ethanol
With these two fixatives temperature had little if any effect on the intensities of immunofluorescence. Intensities were of the order of ++++ at 14 days, +++ at 30 days, ++ at 40 days, and + at 67 days. Tissue shrinkage after fixation with 95% ethanol was marked, but this had no effect on the intensity of staining in *T. pallidum*, or the ease with which the organisms were identified (Table 1).

Comparison of the effects of fixation with formalin fixatives with alcoholic fixatives
Comparison of the above results shows that better intensities of fluorescence were obtained with the alcoholic fixatives than with the formalin fixatives. Thus at 14 days intensities were ++++ and +++ respectively. At 30 days they were +++ and ++. Finally, at 67 days in the alcoholic fixatives, fluorescence still persisted with an intensity of +, but in the formalin fixatives it was extinct. Although non-specific staining of tissue was marked, this was removed by absorption of conjugates with liver powder.

In conclusion, although the antigenic constituents of *T. pallidum* appear to be least altered by fixation in alcoholic fixatives regardless of temperature, as shown by their response to immunofluorescent staining, from the practical point of view, satisfactory staining invariably results after fixation at room temperature for between 12 and 24 hours with formalin fixatives.

Effect of storage in paraffin wax blocks of fixed tissues, on the immunofluorescent staining of T. pallidum *in these tissues*
As with fixation, the immunofluorescent staining of

Table 1 *Formalin and alcoholic fixatives: effect on intensity of immunofluorescence in* T. pallidum *in rabbit testis after different lengths of exposure*

	Results			*Results*	
Fixative	*4°C*	*Ambient*	*Fixative*	*4°C*	*Ambient*
24 hours			24 hours		
Neutral buffered formalin	++++	++++	Acetic acid ethanol	++++	++++
Formol saline 10%	++++	++++	95% ethanol	++++	++++
72 hours			72 hours		
Neutral buffered formalin	+++	+++	Acetic acid ethanol	++++	++++
Formol saline 10%	+++	+++	95% ethanol	++++	++++
14 days			14 days		
Neutral buffered formalin	+++	+++	Acetic acid ethanol	++++	++++
Formol saline 10%	+++	+++	95% ethanol	++++	++++
30 days			30 days		
Neutral buffered formalin	+++	++	Acetic acid ethanol	+++	+++
Formal saline 10%	++	+	95% ethanol	+++	+++

T. pallidum in fixed tissues embedded in paraffin wax was affected by the passage of time, and in the case of formalin fixatives the ability to respond to immunofluorescent staining was retained for longer periods at 4°C than at an ambient temperature. It is uncertain, however, whether this may also be true for the alcoholic fixatives (Table 2). Comparison of the intensities of immunofluorescent staining after treatment with formalin fixatives showed no difference between those obtained at an ambient temperature and those at 4°C (Table 2). A greater intensity of staining at 4°C compared with that at an ambient temperature was, however, observed with alcoholic fixatives (Table 2). Prolonged fixation in formalin fixatives and prolonged storage in paraffin did not favour the production of bright immunofluorescence which was extinct at 34 days (Table 3). By contrast, when alcholic fixatives were used, ++ fluorescence was obtainable after 34 days' storage in paraffin (Table 3). In general, brighter intensities of immunofluorescence were obtained after between 12 and 24 hours' fixation followed by embedding, section, staining, and examination in 48 hours.

Effect of fixation and embedding in paraffin wax on the immunofluorescent staining of T. pertenue *in tissues*

Tissue blocks of skin and lymph nodes from hamsters infected experimentally with *T. pertenue*, which had been fixed in 10% formol saline and 95% alcohol at a temperature of 4°C for seven days were embedded in paraffin, and subjected within 24 hours to indirect immunofluorescent staining using *T. pertenue* antiserum from hamsters, and swine anti-hamster globulin immunofluorescent conjugate. Results (see Table 4) show immunofluorescent intensities of ++ with fixation in formol saline and +++ with fixation in 95% alcohol.

Cross-reactions in T. pallidum *and* T. pertenue *as demonstrated in paraffin sections by immunofluorescence*

Paraffin sections of skin and testis of rabbits experimentally infected with *T. pallidum* were treated within 24 hours of embedding with *T. pertenue* antiserum prepared in the hamster, followed by an anti-hamster gamma globulin immunofluorescent conjugate. In the same way paraffin sections of skin and

Table 2 *Formalin and alcoholic fixation with paraffin storage: effect on intensity of immunofluorescence in* T. pallidum *in rabbit testis*

	Results			*Results*	
Fixative and storage	*4°C*	*Ambient*	*Fixative and storage*	*4°C*	*Ambient*
7 days' storage			7 days' storage		
Neutral buffered formalin	+++	+++	Acetic acid ethanol	++++	+++
Formol saline 10%	+++	+++	95% ethanol	++++	+++
14 days' storage			14 days' storage		
Neutral buffered formalin	ND	ND	Acetic acid ethanol	+++	++
Formol saline 10%	+++	+++	95% ethanol	+++	ND
21 days' storage			21 days' storage		
Neutral buffered formalin	++	ND	Acetic acid ethanol	++	++
Formol saline 10%	++	++	95% ethanol	+++	++
60 days' storage					
Formol saline 10%	ND	—			
96 days' storage					
Formol saline 10%	+	ND			

Fixation: 7 days at 4°C or an ambient temperature.
ND = Experiment was not done
— Negative results

Table 3 *Effect on intensity of immunofluorescence in* T. pallidum *in rabbit testis after prolonged fixation in formalin or alcoholic fixatives with prolonged storage in paraffin at 4°C*

Fixative	*Fixation (days)*	*Storage (days)*	*Results*
Neutral buffered formalin	34	34	–
Formal saline 10%	34	34	–
Acetic acid ethanol	34	34	++

lymph nodes from hamsters infected with *T. pertenue* were treated with *T. pallidum* antiserum prepared in the rabbit followed by an anti-rabbit gamma globulin, immunofluorescent conjugate. In both sets of experiments the treponemes stained equally well, in the heterologous systems. No difference in the

Table 4 *Formalin and alcoholic fixatives with paraffin storage (4°C) effect on intensity of immunofluorescence in* T. pertenue *hamster tissue (homologous system)*

Tissue	*Fixative 7 days 4°C*	*Results*	*Fixative 7 days 4°C*	*Results*
Skin	Formol saline 10%	++	95% ethanol	+++
Lymph node	Formol saline 10%	++	95% ethanol	+++

quality of the intensity of immunofluorescent staining was noted when homologous and heterologous systems were compared. Thus it was not possible to differentiate between *T. pallidum* and *T. pertenue* in paraffin sections using these antisera (Table 5).

Table 5 *Immunofluorescence in paraffin stored tissues (24 hours) cross-reactions of* T. pallidum *and* T. pertenue *(heterologous systems)*

T. pallidum *in rabbit tissue*			
Tissue	*Fixative (7 days 4°C)*	*Antiserum (hamster)*	*Results*
Skin	Formol saline 10%	*T. pertenue*	+++
Skin	Acetic acid ethanol	*T. pertenue*	+++
Testis	Formol saline 10%	*T. pertenue*	+++
Testis	Acetic acid ethanol	*T. pertenue*	+++
T. pertenue *in hamster tissue*			
Tissue	*Fixative (7 days 4°C)*	*Antiserum (rabbit)*	*Results*
Skin	Formol saline10%	*T. pallidum*	+++
Skin	Acetic acid ethanol	*T. pallidum*	+++

Immunofluorescent re-staining of sections routinely stained by haematoxylin and eosin

Attempts were made to extend the use of the immunofluorescent methods of staining *T. pallidum* in tissues by decolourising routinely stained and mounted haematoxylin (Cole's) and eosin sections and re-staining by indirect immunofluorescence. The object of the procedure was to enable a retrospective diagnosis of treponematosis to be made in circumstances where further material might not be available for examination. Coverslips and mounting medium were removed by immersing slides in xylol, sections were taken to phosphate buffer saline as previously described, and indirect immunofluorescent staining applied. None of the sections examined showed any evidence of specific immunofluorescence although non-specific fluorescence was heavy in areas. Autofluorescence was not prominent.

Comparison of the optical sensitivity of immunofluorescence and silver deposition in the microscopy of T. pallidum *in tissues*

The treponemal content of the testes of rabbits experimentally infected with *T. pallidum* was examined by indirect immunofluorescence, and silver deposition. Levaditi's method was applied to tissue blocks and that of Warthin and Starry to tissue sections. Sections stained by indirect immunofluorescence and examined with the immersion objective showed many areas where treponemes were present in large or uncountable numbers and the optical definition allowed a single treponeme in a field to be easily identified. By contrast, in sections treated with silver deposition, it was difficult to identify unequivocally an occasional treponeme amid the mass of argyrophil matter. The gross insensitivity of silver deposition as compared with immunofluorescence in the identification of *T. pallidum* in tissue sections was thereby clearly demonstrated, and the observations of Warthin and Starry (1920) were confirmed.

The effect of fixation by heat at 80°C for 3 minutes on the immunofluorescent staining of T. pallidum

For these experiments, portions of testes from rabbits experimentally infected with *T. pallidum* were cut into blocks approximately 5 mm square by 2–3 mm in thickness. Each portion of tissue was placed in a glass test-tube containing 10 ml of 10% formol saline previously heated to 80°C, and further heated at 80°C for a period of three minutes with agitation, or 30 minutes without agitation. After embedding in paraffin by the rapid two-hour method, blocks were stored at a temperature of 4°C for 24 hours or seven days, and examined by indirect immunofluorescence.

In sections of tissue fixed for three minutes and examined at 24 hours, *T. pallidum* fluoresced with ++++ intensity. On storage for seven days at 4°C, however, this fell to ++ (Table 6).

Rapid heat fixation (three minutes at 80°C), followed by rapid embedding in paraffin, provides, therefore, a quick diagnostic method for examining selected material for the presence of *T. pallidum*.

Table 6 *Fixation by heat at 80°C with paraffin storage effect on intensity of immunofluorescent staining in* T. pallidum *in rabbit testis*

Heat fixation (minutes)	*Paraffin storage 4°C*	*Results*
3	24 hours	++++
3	7 days	++
30	24 hours	++

Immunofluorescent staining of old paraffin wax blocks
Although previous experiments had shown that the immunofluorescence of *T. pallidum* became weaker with time and disappeared after three months, in order to complete the study of the effect of ageing on the response to immunofluorescent staining of *T. pallidum* in tissues embedded in paraffin wax, a paraffin block of indefinite age, but at least several years old, from a case of congenital syphilis, containing numerous *T. pallidum* as demonstrated by Levaditi's silver deposition technique, was sectioned, processed as previously described, and stained by indirect immunofluorescence. Treponemes were not seen in any of the sections.

Furthermore, a series of paraffin blocks, containing tissue from cases of clinical yaws, 15 years old, were similarly examined. Treponemes were not seen, either by silver deposition (Warthin and Starry) or by indirect immunofluorescence. Moreover, in none of these experiments was there any specific staining of amorphous antigenic material of treponemal origin.

Finally, there was no non-specific staining of any degree in any of the sections examined, either from the syphilitic liver or from the cases of yaws. Tissue autofluorescence, however, was marked, being of a brilliant white colour, but this has never given rise to any difficulties of observation or interpretation in earlier experiments where treponemes were identified and where autofluorescence became more marked as time progressed.

Although the experiments described above were entirely negative as expected, they extend and confirm what had been noted in earlier experiments—namely, that never at any time in the course of these experiments were any artefacts observed which even remotely resembled treponemes. It is considered, therefore, that the indirect method of immunofluorescence as applied to *T. pallidum* in tissues fixed and embedded in paraffin wax in a routine manner, produces results which are morphologically specific and, probably antigenically specific, generically so at least, although experiments were not conducted with treponemal sera against bacteria belonging to other genera.

Discussion

These results show that *T. pallidum* can be stained by the indirect immunofluorescent technique in tissues fixed by a routine fixative and embedded in paraffin wax by a routine automated method. Unfortunately immunofluorescent staining of *T. pallidum* in old paraffin wax blocks of tissue was not successful. Nevertheless, optimum immunofluorescence may be obtained for up to two to three weeks, when fixation and storage have been at ambient temperatures. This time may be extended to five weeks at 4°C. These times are adequate if it is desired to examine sections for *T. pallidum* by immunofluorescence after routine histopathological examination. If the primary intention is to examine the tissue for treponemes, then fixation at 4°C in buffered formalin, 5% acetic acid in ethanol or 95% alcohol, will provide even brighter immunofluorescent staining of *T. pallidum* and, in the case of 5% acetic acid in ethanol and 95% alcohol, for periods up to 10 weeks at least. The speed and sensitivity of the method compared with silver deposition methods make this immunofluorescent technique eminently suitable for routine histopathological examination.

In the past, much of the histopathological research concerning the entry and passage of *T. pallidum* through the body has been carried out on sections using silver deposition, or by dark field examination of samples of tissue homogenates. Where microscopical examination has been negative, animal inoculation of tissue homogenates has on occasion revealed the presence and location of the treponeme. This type of approach is laborious, and indirect immunofluorescence applied to the staining of *T. pallidum* in paraffin sections has an optical sensitivity so that a single treponeme in a high power field of tissue can be detected with ease, which is a considerable advance on previous methods, where visualisation (as opposed to specific identification) of the organism in deep tissues is required. Lack of species specificity, however, lessens the value of the method as applied to paraffin sections of superficial lesions in areas where saprophytic treponemes are part of the natural flora, unless the antisera are previously absorbed with the Reiter treponeme. In this case there will be a slight loss in the intensity of immunofluorescence.

Nevertheless, this technique as applied to paraffin sections supplies a convenient, safe, and sensitive means of reappraising the histopathology of syphilis in the patient, in the post-mortem room, and in the experimental animal.

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