Modifications in the fluorescence microscopy technique as applied to identification of acid-fast bacilli in tissue and bacteriological material

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SYNOPSIS The principles and techniques involved in fluorescence microscopy as related to the demonstration of acid-fast bacilli are discussed. The auramine-rhodamine staining method, for both tissue and bacteriological preparations, is described as well as the necessary equipment for both microscopy and photomicroscopy. Fluorescence staining is no more difficult nor time consuming than a Ziehl-Neelsen stain yet it is more effective in demonstrating acid-fast organisms.

Interest in fluorescence microscopy for demonstrating acid-fast bacilli has waxed and waned since first described by Hagemann (1937, 1938). As a result, it seems that a large number of pathologists and laboratory directors are either unaware of the fluorescence technique or reluctant to introduce it into their laboratories. Yet, this method has been shown to be simple, particularly with present-day equipment, and most effective for use with both tissue and bacteriological material.

The reluctance to introduce this procedure may partly stem from a lack of a concise, clear-cut description of the necessary equipment and staining technique as well as failure to point out various difficulties which may be encountered. The sole purpose of this paper is to attempt to remedy this as well as to introduce some technical modifications both in the staining procedure and in the equipment previously described (Kuper and May, 1960; McClure, 1953).

Fluorescence microscopy, in our own experience, with the modifications described below, has proven decidedly more effective than the Ziehl-Neelsen method and it is more easily performed. There is convincing evidence in the literature of the increased effectiveness of fluorescence microscopy in demonstrating acid-fast organisms when this procedure is compared with the more generally accepted and the more generally used Ziehl-Neelsen method (Braustein and Adriano, 1961; DeGroat and White, 1964; Freiman and Mokotoff, 1943; Koch and Cote, 1965; McClure, 1953; Tanner, 1941; Truant, Brett, and Thomas, 1962; Yamaguchi and Braunstein, 1965). We have found only one study (Needham, 1957) which reported equivalent results with the two procedures with no distinct advantage to the use of the fluorescence microscopy technique. The method is now used routinely in our laboratories for identification of tubercle bacilli in both tissue and bacteriological specimens.

BASIC PRINCIPLE

A combination of auramine and rhodamine dye is used as a stain, and potassium permanganate is used as a counterstain after the material is decolorized with acid alcohol. The acid-fast organisms do not decolorize after staining. A light source producing a suitable wavelength is used to excite fluorescence of the stain retained by acid-fast organisms and this fluorescence can then be detected by use of either dark field or bright field microscopy. A filter system which absorbs background light but transmits the light caused by fluorescence results in easily visible organisms.

METHODS AND MATERIALS

TISSUE STAINING The staining method for tissue is basically the one described by Matthaei (1950) as modified by Kuper and May (1960). Further modifications are detailed in the following description of the procedure.

Routinely processed, unstained, paraffin sections mounted on glass slides are used. They are deparaffinized
in three changes of xylene leaving the slide in each change for one and a half minutes and they are then hydrated by dipping four or five times in (1) absolute alcohol, (2) 95% alcohol, (3) 10% alcohol, (4) water, in succession. This is then followed, immediately, by staining with an auramine-rhodamine fluorescent stain which consists of:

1. Auramine O (Allied Chem., Nat. Analine Div.) CI4100D . . . . 1.5 g.
2. Rhodamine B, O (Matheson, Coleman, and Bell) DI4517D . . . . 0.75 g.
3. Glycerol . . . . 75.0 ml.
4. Phenol crystals liquefied at 50°C . . . . 10.0 ml.
5. Distilled water . . . . 50.0 ml.

The stain should be thoroughly mixed before use and preheated to 20 minutes to a temperature of 60°C. The slides are placed in the heated stain and kept in a 60°C incubator for 10 minutes. Preheating provides a convenient and time-saving means of applying the stain at the proper temperature. Upon removal of the slides from the stain they are:

1. Washed for two minutes.
2. Decolorized in 0.5% HCl in ethanol for two to three minutes.
3. Rinsed in a slow running tap water bath for two minutes.
4. Counterstained with 0.5% potassium permanganate for one minute (this gives the sections a pale brown appearance grossly).
5. Washed for two minutes.
7. Dehydrated in absolute alcohol for 15 seconds.
8. Cleared in xylol.

Lung tissue from a proven case of pulmonary tuberculosis was used as a control.

Although others (Lind, 1949; Richards and Miller, 1941) have stated that with or without refrigeration the stain is stable indefinitely, we have found it best to prepare fresh stain after approximately two months of use. For longer periods results in a marked decrease in the rhodamine staining property as much of the reddish tinge rhodamine imparts is lost after extended periods of storage.

The solution of auramine and rhodamine, as prepared, is almost saturated (Kuper and May, 1960), and there is no advantage in increasing the concentration. When the phenol is added, a scum appears which can be removed by filtration through a large-pore filter paper or glass wool. The scum does not apparently interfere with staining but if filtration is preferred it must be repeated each time, before using the stain.

STAINING OF BACTERIOLOGICAL PREPARATIONS The staining method utilized for bacteriological material is the one described by Truant et al. (1962) and Koch and Cote (1965) with some modifications.

Both undigested and digested materials can be stained (sputum, bronchial secretions, early morning voided urine, exudates, caseous material from guinea-pig inoculation site etc.). Material digested by 3% NaOH (U.S. V.A. Handbook of Tuberculosis Laboratory Methods, 1962) or by the acetyl-N-cysteine method (Kubica, Dye, Cohn, and Middlebrook, 1963; Kubica, Kaufmann, and Dye, 1964) was found to be equally satisfactory for staining for fluorescence microscopy. The combination of Auramine O and Rhodamine B (as described under tissue staining) is used. The stock solution is kept at room temperature and filtered immediately before use through W. and R. Balston Whatman filter paper no. 2. The stain is not heated. Slides are air dried at 37°C. and subsequently heat-fixed with a Bunsen burner. Staining is performed at 37°C. for 30 minutes on a staining rack.

The stained slides are decolorized for 10 minutes with the following solution:

- HCl . . . . 0.5 ml.
- Ethyl alcohol, 70% . . . . 99.5 ml.
- NaCl . . . . 0.5 g.

The slides are then flooded with a counterstain for two minutes. The counterstain consists of:

- Potassium permanganate . . . . 0.5 g.
- Distilled water . . . . 100.0 ml.

Counterstaining for longer than two minutes decreases fluorescence of the organisms. As a control, a guinea-pig virulent strain of M. tuberculosis, isolated from a human being, was employed.

MICROSCOPIC EQUIPMENT AND ACCESSORIES With the more widespread use of fluorescent antibody techniques and fluorescent staining for amyloid, the equipment necessary for fluorescence microscopy is being found more generally in laboratories. The optical and illumination systems for the procedures are interchangeable, particularly when the copper sulphate solution which had been frequently recommended previously (Kuper and May, 1960; McClure, 1953) is eliminated from use as the excitation and heat-absorbing filter. For our purposes we use an E. Leitz, Ortholux research microscope with binocular or monocular observation and straight photographic tube. The optical system consists of an apochromat dry objective 12.5:0.30, an achromat dry objective 25 : 0.65, an achromat dry objective 45 : 0.65, a fluorite oil objective with iris diaphragm 95 : 1:32, periplanar 10 : eyepieces and an immersion dark field no. D 1:20. A condenser, and a bright-field Berch-A 0:95 condenser with field and aperture diaphragms.

The illuminating system consists of a universal lamp no. 250 with an HN 200 high pressure mercury burner type L II. There is also an alternate illumination system with a 6 v., 5 amp. tungsten lamp attached.

The filter system consists of two heat-absorbing filters, a 2 mm. KG-1 and a 4 mm. BG-38, a BG-12 (4,150 A) excitation filter and an OG-1 (K530) barrier filter which is attached to the oculars.

PHOTOGRAPHIC EQUIPMENT Although photomicrographs can be used to provide a visual record they are not a necessity. For our purposes basic photographic equipment is used. Photomicrographs in both colour and black and white are taken with a Leitz 35 mm. photo unit. This consists of a micro camera attachment (MIKAS-M) with lateral observation and focusing telescope, a time and
instantaneous shutter, two cable releases, a periplanatic 10 × eyepiece, and a 1/3 × conical tube with bayonet mount. The camera body is an M2X with focal plain shutter and a bayonet flange for attachment to the MIKAS-M. There is also a double release for operating the deflecting prism and shutter.

For colour slides Kodak EHB 135-20 film is used and for black and white prints Kodak FX 135-20 is used. Exposure times with the above photographic system (as well as with the Leitz Orthomat which was also tried) are not critical and can be varied to obtain the desired effect. Exposure times of from one to nine minutes were tried, and the most generally satisfactory exposure time was found to be five minutes.

A single field should be photographed only once as fluorescence fades with prolonged exposure. Photomicrography as described above was successful with either the dark field or the bright field condenser.

DISCUSSION

Fluorescence microscopy depends on the ability of certain dyes to reradiate visible light when illuminated by light of a shorter wavelength. When auramine and rhodamine are used, ultra-violet light is not required since the excitation maxima of these dyes do not lie in the ultra-violet range. They can be excited by light of up to 4,960 Å. A mercury vapour lamp, although not necessary, can be used to produce light of suitable wavelength. Use of the lamp increases versatility of the apparatus. The use of other illuminating sources such as a 500 watt projection bulb has been described (Graham, 1943; McClure, 1953) but suitable modifications must be made to equipment if the bulb is to be employed. Auramine fluoresces at about 4,320 Å. and rhodamine at about 5,560 Å. With insertion of filters which absorb direct illuminating radiation but which transmit the differently coloured light caused by fluorescence, organisms retaining the stain become visible. A blue light source is needed for illumination. A K530 barrier filter (OGI, orange), placed in the microscope ocular, filters out the blue light of the background allowing fluorescing organisms to stand out on a darker field. The transmission requirements of this filter are not critical but the filter should not transmit too much of the green light which may be transmitted by the excitation filter. As much light as possible on the long wave side of 5,200 Å. should be transmitted.

While staining at 60°C for 10 minutes has been recommended (Truant et al., 1962) and results in adequate brilliance, this is not critical. Staining time can vary from 10 to 60 minutes and it can be performed at either 37°C or 60°C (Truant et al., 1962).

Decolorization with 0·5% hydrochloric acid in ethanol is recommended for Mycobacterium tuberculosis. Other mycobacteria, and in particular M. leprae, which are acid-fast but less alcohol-fast than M. tuberculosis, should be decolorized with either 25% sulphuric acid for 20 to 30 minutes (Gray, 1953) or 0·5% hydrochloric acid without alcohol (Kuper and May, 1960). Alcohol dehydration of M. leprae should also be avoided because of the danger of over-differentiation of this organism by alcohol.

Counterstaining with potassium permanganate serves to suppress the autofluorescence of tissue, background material, and debris but excessive exposure causes loss of brilliance or even complete decolorization of bacilli in spite of exposure to acid alcohol (Kuper and May, 1960). Acid alcohol is unnecessary if the material is treated with permanganate for more than a half minute but less than four minutes (Kuper and May, 1960). The mode of action of the permanganate is not known. Counterstaining for one minute was preferred in our preparations. Some background fluorescence persisted but this served a useful purpose in so far as some of the histological features of the tissue could be distinguished. Yet, there was still excellent contrast between organisms and background. Longer periods of counterstaining, although adequate, were not found to be quite as satisfactory and, in fact, tended to decrease some of the staining qualities imparted by the rhodamine.

Before applying the mounting media, the slides are dehydrated in absolute alcohol and cleared in xylol to allow for better penetration of the mountant. This eliminates minute air bubbles. These two steps should be omitted when staining M. leprae for reasons already discussed.

The use of a satisfactory mounting medium for coverslipping tissue preparations (bacteriological material is generally not coverslipped), although briefly mentioned in one report (McClure, 1953) but generally not discussed in others, is an important consideration in order to obtain the maximum degree of fluorescence and contrast. Fluormount™, in our experience, was found to be completely unsatisfactory. It does not readily solidify and thus the coverslip is easily movable. This causes obvious difficulties. It also imparts excessive fluorescence to the entire slide resulting in loss of contrast. Relatively rapid fading of the stain and an increase in autofluorescence also occurs. Permmount was found to be adequate but the most satisfactory preparations were those in which the coverslip was mounted with D.P.X. The preparations mounted with D.P.X. have excellent contrast and fluorescence with a minimum of generalized fluorescence.

The slides of tissue are best examined within one to four days of staining but fluorescence is maintained for at least several months although there is some slight loss of brilliance as well as some increase
bacilli. Also, be often the mixed tinge reddish fast bacilli which the tissue to tend is black.

Although working in a darkened room is not necessary while examining slides for fluorescing bacilli, it does help to decrease fatigue as well as to heighten contrast. Light shields or even dim room lighting can be substituted quite adequately for a darkened room.

The material is examined for acid-fast bacilli using a 10x ocular and 25x objective lens (Fig. 1). This provides for accurate and rapid examination. Typical morphological characteristics are confirmed with the 45x objective lens (Figs. 2 and 3) or 95x oil immersion although the latter usually is not necessary in examining tissue preparations after some experience is obtained with the procedure. In our laboratory oil immersion is always used for final confirmation in bacteriological specimens. Binocular observation is quite adequate with present day lens systems and either dark-field or bright-field (Fig. 4) examination can be used. Fluorescence and contrast between organism and background is more prominent with dark-field examination and its use is less fatiguing than the bright-field examination. We have also found dark-field examinations to be more convenient. It should be mentioned that the inconvenience of using oil on the substage condenser is avoided and general field illumination is greater with use of the bright field.

Identification of *M. tuberculosis* is made by both the colour and the morphology of the stained organism (Koch and Cote, 1965; Kuper and May, 1960; Wellmann and Teng, 1962). Although the bacilli may appear slightly larger than would be expected due to the fluorescent glow, they retain the slender, often slightly curved, rod-like structure and beading can be readily seen. They vary from 2 to 5 μ in length. The organisms fluoresce a reddish, golden yellow while the tissue appears to be a dark, pale green. The background is black. Artefacts, on the other hand, tend to be a hazy yellow or gray green and lack the reddish tinge which auramine imparts to the organisms. They are poorly delineated, in contrast to acid-fast bacilli which are seen clearly. If the stain is not mixed before use, staining variations occur and the bacilli may appear gray to silvery or bright yellow and often the reddish tinge is not present. It may then be difficult to distinguish artefacts from acid-fast bacilli. Also, the tissue as well as the background may appear black which results in difficulty with focusing in addition to difficulty in distinguishing background from tissue.

Either auramine or rhodamine can be used individually for staining. Auramine alone will result in a golden yellow fluorescence and rhodamine alone results in a less satisfactory preparation in which the organisms appear reddish and morphologically indistinct (Truant et al., 1962). The combination of dyes results in the greatest contrast between the acid-fast organisms and the background as well as in the greatest intensity of fluorescence.

Slides of tissue which have been originally stained with carbol fuchsin can be decolorized in acid alcohol and restained with auramine-rhodamine. The restained organisms fluoresce clearly and contrast is excellent. There is apparently no decrease in the number of bacilli which take the stain. Restaining of tissue with carbol fuchsin following staining with auramine-rhodamine on the other hand is not as satisfactory. In this instance, bacilli appear pale red and hazy and many do not retain the stain at all. Attempts at removal of the auramine-rhodamine and/or the potassium permanganate did not improve the quality or degree of staining. Bacteriological slides stained for fluorescence microscopy, in contrast, can be restained with the Ziehl-Neelsen stain without prior decolorization or other intervening procedures. Unlike the tissue preparations described above and unlike the experience of Truant et al. (1962), we were able to obtain brilliant restaining of bacteriological fluorescence microscopy slides with carbol fuchsin. Bacteriological material stained initially with carbol fuchsin can also be restained with auramine-rhodamine without loss of the fluorescent properties of the organisms.

For the determination of the specificity of auramine-rhodamine staining for bacteriological preparations, the following organisms were stained: *Aerobacter, E. coli*, *Proteus*, *Pseudomonas*, *Clostridium sp.*, *S. aureus*, *S. pyogenes*, and a partially acid-fast strain of *Nocardia asteroides*. None of these organisms displayed any degree of fluorescence either separately or in mixed cultures. Group III (Battey) and group II (scotochromogen) anonymous mycobacteria fluoresced brilliantly when stained with auramine-rhodamine.

After trials on a number of specimens we cannot help but agree with investigators who have found fluorescence microscopy, when applied to the detection of acid-fast bacilli, to be more accurate as well as easier to use than the more commonly used Ziehl-Neelsen technique. Organisms are more readily seen and more appear to take the auramine-rhodamine stain than take the carbol fuchsin stain (Gray, 1953; Richards and Miller, 1941; Tanner, 1941). In
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FIG. 1. Dark field of lung tissue containing acid-fast bacilli. Auramine-rhodamine × 250.

FIG. 3. Dark field of sputum containing acid-fast bacilli. Auramine-rhodamine × 450.

FIG. 2. Dark field of lung tissue containing acid-fast bacilli. Auramine-rhodamine × 450.

FIG. 4. Bright field of liver tissue containing acid-fast bacilli. Auramine-rhodamine × 250.
material where organisms are scarce, they are more easily, as well as more frequently, found following fluorescent staining. Thus the number of positive identifications is increased. The use of lower magnifications decreases the time necessary for examining the material and accuracy is increased.

A word of caution is necessary. A considerable number of specimens should be examined by the microscopist before putting the procedure into operation. This will enable him to gain confidence in the procedure as well as to gain experience in identifying acid-fast organisms. Experience is also helpful in deciding what is and what is not artefact.

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