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Stain Techniques and the Morphology of *Mycobacterium leprae**

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A comparative study of acid-fast stain techniques has shown that some methods in common use give divergent values for the MI, and smaller but significant discrepancies in the BI. The methods used by Rees and Valentine and by Shepard to equate solid staining with viability are in fairly good agreement.

The heating of carbol-fuchsin stain is the factor which causes the most variation in the MI, but fixation, time of staining, and method of differentiation all have some bearing on the results. It is thought that all the effective staining procedures produce some alteration of bacterial morphology.

The need for standardization and the suitability of certain methods for routine application are discussed.

Introduction

Although the morphological index (MI) or solid ratio is now widely employed as an index of viability of leprosy bacilli, it is not always recognized that the appearances are affected to a considerable extent by stain techniques. This was demonstrated in the case of *Mycobacterium lepraemurium* by Rees and Valentine (1962*b*) who found that with this organism a solid-stain morphology could be correlated with electron microscopic assessment of viability only when the time and temperature of the Ziehl-Neelsen stain were modified, though with *Myco. leprae* the correlation had been established while using Ziehl-Neelsen stain for 2 min (Rees and Valentine, 1962*a*). Shepard and McRae's (1965) correlation of morphology with viability as indicated by mouse footpad inoculation experiments was made by the use of a Ziehl-Neelsen procedure that was specifically devised for use with washed suspensions of bacilli (Shepard, 1962). It is also a cold method, and when used for routine smears it might be expected to give a lower MI than the hot techniques. The methods by which acid-fast staining has been correlated with viability appear to be relatively "weak", whereas for the routine screening of patients and the assessment of the bacterial index (BI) "strong" methods are needed.

The present study was undertaken to determine how the MI and BI are influenced by the various stain techniques in common use, and to discover whether it is possible to recommend a general-purpose method for routine use.

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Material and Methods

Skin biopsies were available from 11 patients suffering from lepromatous leprosy, and whose skin contained numerous bacilli; 5 of the patients had active infections and 6 were under treatment. In all, 50 impression smears made from each biopsy were used for the estimation of the MI by the method of Shepard and McRae (1965), and of the BI by the method of Ridley (1964). In addition, 2 "slit and scrape" smears were available from each of 6 to 8 sites from a further 10 patients with rather scanty leprosy bacilli. These were not suitable for determination of the MI because of the small numbers of bacilli, but they were a necessary supplement to the BI study, since they provided cases at the lower end of the BI scale. The results with this latter group, however, were unsuitable for mathematical analysis.

Five basic staining methods were compared, and with most of them a number of modifications were tested. Wherever possible, 3 identical smears were stained concurrently by each modification, one to be examined immediately, one to be examined after 24 h, and the 3rd after rinsing in absolute alcohol.

Allowances were made in times of differentiation for the thickness of slit and scrape smears; there was no optimal time. Impression smears required less differentiation because of the lack of contamination, and for the same reason the times could be standardized as stated below. To prepare carbol-fuchsin, 50 ml of 6% alcoholic basic fuchsin was mixed with 450 ml of 5% phenol in distilled water, filtered, and kept for one day in a dark bottle before use.

The following methods and modifications were evaluated.

1.(a) The Ziehl-Neelsen method. Heat-fixed smears were stained in carbol-fuchsin, differentiated in 1% acid-alcohol (1% HCl in 70% ethanol) and counterstained with 1% methylene blue. Unless otherwise stated staining was performed at 60°C for 15 min, and differentiation was carried out for 5 sec. The following time and temperature modifications of the Ziehl-Neelsen technique were employed: staining temperatures of 50°, 42°, 37° and 22°C (room temperature), and staining times of 5 min and 18 h (overnight).

(b) Modifications in differentiating agents and times in the Ziehl-Neelsen method were tested as follows: 1% acid alcohol for 3 min; 10% sulphuric acid for 3, 10 and 20 min; 25% sulphuric acid for ½ min, and 5 and 15 min.

The method of Rees and Valentine (1962*b*) consists in the Ziehl-Neelsen method (1a) as described but with staining at 37°C overnight.

2. Difficulty was experienced in adapting for use with smears the technique of Shepard (1962), which was devised for use on washed suspensions. The phenol gel caused a messy deposit of stain which obscured the bacilli. The gel was therefore omitted, but for the rest, Shepard's technique using formalin vapour fixation and staining at 22°C was followed exactly. As a further modification the smear technique was carried out at a staining temperature of 42°C. This method is referred to here simply as "formalin fixation".

3.(a) Shepard's method. Shepard (personal communication) informed us that his 1962 technique could be applied to smears without any modification. All that was required was that, after application of the freshly prepared phenol gel (gelatin 0.5 g, phenol 0.5 g, distilled water 100 ml) to the smear, the surplus was drained off before re-exposure to formalin vapour; but neither at this stage nor at any

other time must the gel be allowed to dry. This method was followed exactly and compared with some of the other methods referred to; but a different batch of smears was used for this subsidiary trial because by the time Shepard's communication was received the original batch of means had been exhausted. When the results were analysed and the smears calculated, it was necessary to make small adjustments to bring them into line with the mean values obtained in the main trial.

(b) A modification of Shepard's method was tried in which 0.5% phenol was substituted for phenol gel. In all other respects the method was the same. As only a few cases were tested in this way the results are not tabulated.

4.(a) Aubert's (1950) technique. Heat-fixed smears were stained with a carbol-fuchsin-Tween-80 mixture at 22°C for 3 min. The stain was prepared as follows: basic fuchsin, 3.5 g in 12.5 g of pure phenol, was heated to 80°C and 25 ml of alcohol was added. It was allowed to cool, mixed, and made up to 300 ml with water, stirring well, after which 30 drops of Tween-80 were added slowly and mixed well. The stain was filtered before use. Differentiation was in 5% nitric acid followed by a wash in 70% alcohol.

(b) As a modification of Aubert's method, smears were stained for 10 min and differentiated in 1% acid alcohol.

5. Fluorescent methods. (a) According to Kuper and May (1960). The stain, an auramine-rhodamine mixture, was used at 60°C, with differentiation in 0.5% HCl in water.

(b) According to Mansfield (1969). Fixation was by heating in an oven at 65°C for 1 h, followed by formalin vapour for 15 min. The stain was phenol-auramine: 4 ml of phenol + 6 ml of glycerol poured over 0.3 g of auramine-C and mixed, to which 90 ml of distilled water was then added and mixed daily for 3 days. It was allowed to stand in the dark for 4 days before use. Staining was at 30°C for 15 min. Differentiation was in 0.5% acid alcohol and counterstaining in 0.5% potassium permanganate. For fluorescence, BG 12 excitation filters were used in conjunction with a 500 barrier filter.

Results

For the study of the MI the cases fell conveniently into 2 groups: 5 biopsies with MI's in the range 25-75 by the Ziehl-Neelsen (1a) method (Group I); and 6 biopsies with MI's in the range 1-20 (Group II). The means of the results when stained by various methods and modifications are given in Table 1. With Group I, the individual results with the different methods fell into a consistent pattern in line with the means. But with Group II, individual results were erratic and inconsistent with some (though not all) methods. The means, therefore, give only an approximate indication of staining performance in Group II.

The BI results, as already explained, were unsuitable for tabulation, but comments are made on the effect of each particular stain. In general the differences in the BI were smaller than those in the MI.

Temperature of staining. The one single factor which seemed to affect the MI considerably was heating in carbol-fuchsin: the higher the temperature, the higher the MI. The means of the 2 groups stained at various temperatures by the Ziehl-Neelsen method are shown in the table. In each group the MI was significantly higher at 60° than at 22°, but the difference was more pronounced

TABLE 1

Effect of temperature and special stain techniques on the mean MI

Smear group	Stain time	Technique and staining temperature (°C)											
		Ziehl-Neelsen (1a)					Formalin fixed (2)		Shepard (3)	Aubert (4a)	Aubert (4b)	Fluorescent (5a)	Fluorescent (5b)
		22°	37°	42°	50°	60°	22°	42°	22°	22°	22°	60°	30°
Group I	5 min	33		52		56				27			
Group II	5 min	0.2		3.5		6				0.3			
Group I	15 min	41	47	54	59	62	39	51	51		50	65	58
Group II	15 min	1.2	1.6	4.3	7	11	0.3	2.4	3.8		3.8	15	8
Group I	18 h	48	54	59		61							
Group II	18 h	3.5	4	7		11							

Group I = smears with high MI (5 cases).
Group II = smears with low MI (6 cases).
Rees and Valentine's method = ZN (1a) at 37°C for 18 h.

in the group with the low MI. A point which is not brought out by the table is that some fragmented organisms are apparently "reconstituted" by staining plus heat, whereas granular organisms remain granular. This was particularly true with mild heat (37°C) for short periods, which gave unpredictable results in the MI range 15-30.

Examination after staining at room temperature was always difficult because of pallor, which caused a low MI. At this temperature, the BI was also low especially in cases where bacilli were few, and some were missed altogether. Examination after 24 h showed a further fall in both the MI and BI due to fading. This did not occur after staining and heating.

Time of exposure to carbol-fuchsin. The influence of the staining time on the MI was dependent on the temperature of the stain (see Table 1). At 42°C there was a minimal difference in the result whether the staining time was 5 or 15 min. But at room temperature there was a marked lowering of the MI in Group II smears when these were stained for 5 instead of 15 min. Prolonged exposure to the stain resulted in higher MI values, especially in Group II.

The time of staining was more important in assessing the BI. At 5 min some bacilli failed to stain at all, and others, being poorly stained, were difficult to detect; staining at higher temperatures corrected this to some extent. Prolonged exposure to warm stain caused much dropping out of bacilli and skin-scrape smears were difficult to handle. This was the disadvantage of Rees and Valentine's method.

Differentiation. Neither the time of differentiation nor the choice of the agent (whether 1% acid alcohol or 10% or 25% sulphuric acid) caused much variation in the BI or MI as long as smears were stained in steaming carbol-fuchsin for a minimum of 15 min, and provided large numbers of bacilli were present. The time of differentiation was critical only when either of these requirements was not met. The BI fell after prolonged differentiation in either acid alcohol or 25% sulphuric acid following staining at 22°C and/or rinsing in absolute alcohol.

With sulphuric acid differentiation, there was a fall of 5-10% in the MI when examination was made after 24 h. The stain resulted in a purplish background, against which scanty bacilli were often difficult to detect. Over-differentiation was relatively easy when bacilli were scanty. We preferred 1% acid alcohol.

Padma's (1963) 3% acid alcohol was, in our experience, too strong a differentiator for the "weak" stain produced after staining at room temperature (22°C) as recommended. A quick rinse in absolute alcohol before drying gave a cleaner result with more precise morphology.

The above results all relate to the Ziehl-Neelsen method, though they probably are of more general application. The staining quality of other methods, and the effect on the MI and BI of the special features associated with these methods, are dealt with separately.

Formalin fixation. Fixation in formalin vapour (Method 2) caused some slight fall in the MI compared to heat fixation (see Table 1). At low levels the MI was very inconsistent, often falling to zero. Bacilli were pale, there was much dropping out of bacilli, "ghost" forms were seen, and identification was difficult. The BI was considerably lower than that seen in heat-fixed preparations; when bacilli were few, none of them might be stained. Examination after 24 h showed a further fall in the MI as compared with heat-fixed smears.

Shepard's method (3a), although it differed from Method 2 only in the use of phenol gel, Shepard's method gave a much better staining quality and the MI was

higher and more consistent. When bacilli were scanty and granular, however, they were pale and not easy to detect.

The substitution of phenol for phenol gel (3b) produced brightly stained bacilli and granular organisms were better stained. In a small series of smears the MI was not significantly different from those obtained by the use of Shepard's method (3a).

Aubert's method (4a). Using Tween-80 in the stain, had one serious drawback, namely the deposition of the stain in oily blobs, which made it difficult to distinguish globi with certainty. Overwashing in water after staining removed much of the colour from the bacilli, although the slide was somewhat cleaner. This was a critical step and had to be controlled carefully. The bacilli were pale, the MI was low, and there was much "dropping out". At low values, the BI also was lower than in the Ziehl-Neelsen method. There was much loss through fading due to the nitric acid differentiation, and examination had to be made at once.

A modification (4b), using Aubert's stain for 10 min and differentiating in acid alcohol, was easier to control and showed much improvement on Aubert's method (4a). Bacilli were bright and the morphology easy to study. The MI was higher than with method 4a and almost identical to that in Shepard's method. There was still some dropping out of bacilli from the slide, but this did not affect the BI to the same extent as in Aubert's method (4a). The result was still decidedly messy with slit and scrape smears.

Fluorescent methods. The method of Kuper and May (5a) resulted in brightly fluorescent bacilli with clear precise morphology easily distinguishable on both impression smears and skin-scrape smears. The MI was exceptionally high relative to all other methods, particularly at low values. The BI was in the same range as that by the other methods, and examination of routine smears with scanty bacilli was very easy. However, it could not be stated with certainty that the fluorescing organisms were *Myco. leprae* and not skin contaminants. One case showed some fluorescence of non-alcohol-fast organisms.

Mansfield's modification of Kuper and May (5b) overcame the problem of non-specific staining by lowering the staining temperature. Staining was bright and examination easy. The MI was slightly lower than with (5a), but the BI was about the same.

Reproducibility of smears. It was noted that impression smears gave reproducible results with the MI, but that there was some variation in the BI because different amounts of material were used in making the smears. With slit and scrape smears, a pair of smears made from a knife blade after a single scrape were not always uniform as regards either the MI or BI, presumably because of drying on the knife before making the second smear. When 2 separate scrapes were made from the same slit in order to make a pair of smears, the MI and BI were more or less identical if there was heavy infiltration and plenty of dermal fluid in the lesion, but the results were apt to be discordant when the lesions were small and dry. Separate slits made close together within the same lesion gave reproducible results for MI and BI in one patient, but were found too irksome for the patient to be generally acceptable. They were not employed in this study.

Discussion

The object of determining the BI is to indicate the density of *Myco. leprae* in a smear; the higher the value, the better the stain technique. As regards the MI, our

results confirm the finding of Rees and Valentine (1962*b*) that heating in carbol-fuchsin causes a redistribution of acid-fast material in the body of the bacillus and so raises the MI; our results also confirm the observation of Nakamura *et al.* (1968) that heat fixation gives a higher MI than formalin gas. There are 2 possible objective assessments of any MI, whatever its apparent level: Rees and Valentine's (1962*a*) comparison with electron-microscopic morphology (using *Myco. lepraemurium*) and Shepard and McRae's (1965) comparison with footpad viability. In our experience in this study, the acid-fast stain techniques used by Shepard and by Rees give fairly good agreement, though the MI is somewhat lower by Shepard's method. Some of the other methods in regular use gave widely divergent results, especially when the MI was low.

Any stained specimen is an artefact, and this applies no less to acid-fast stains than to others. As regards Shepard's method, we had at first assumed that the object of the phenol-gel was to make the washed bacilli adhere to the slide. Our results prove, however, that phenol-gel is an essential component of the technique, without which bacilli are pale and the MI is low. The technique requires that, after application of the phenol-gel, while the gel is still moist, the slide is warmed on the lid of a boiling water-bath for several minutes. This reproduces precisely the conditions which, according to Rees and Valentine, caused a re-distribution of acid-fast material in the substance of the bacillus, with the artificial production of "solids", namely, heat in the presence of phenol solvent. All the methods that we have found to produce MI levels of the sort that have been equated with viability employ heat in the presence of phenol, with 2 exceptions: Aubert's (4b) method uses Tween-80, which might well facilitate the liquefaction or mobility of acid-fast substance. The other satisfactory relatively cold (30° C) method is the fluorescent method of Mansfield (5b), which incorporates glycerol. The mechanism here is not quite clear. It seems likely therefore that a small re-distribution of acid-fast substance during staining is necessary in order for the MI to be equated with viability, using the present criteria for estimating the MI. The difficulty is to achieve just the right degree of adjustment. Weak staining methods which could not possibly alter acid-fast morphology would be unsatisfactory for general use, since the staining is pale and some granular organisms are not stained at all.

Rees and Valentine's method is not convenient for routine use, and was indeed never intended for this purpose. A very similar result can be obtained using the Ziehl-Neelsen (1a) procedure, with staining at 42° C for 15 min. The only disadvantage of this is that in order to produce the temperature accurately a special water-bath or incubator is required. With experience, however, the correct amount of direct heat can be judged fairly well: the slide is heated until the stain just begins to steam. We found Shepard's method at first impossible to apply to skin smears, but the difficulty was overcome. The only practical disadvantage of this method is that fresh phenol gel has to be prepared each day. This problem could be overcome by substituting 0.5% phenol for phenol gel; and from a small series of tests it seems that this modification would make a good alternative to Ziehl-Neelsen at 42° C and would be suitable for routine use. Any of these 3 methods give reasonably good staining quality and a fairly reliable BI. But for the certain staining of all leprosy bacilli there is no substitute for the Ziehl-Neelsen method using hot stain (60° C). The modification of Aubert's method (4b) can be recommended as simple and reliable, and it gives results almost identical to those

by Shepard's method; it also has advantages under field conditions. But with slit and scrape (as opposed to impression) smears, the result is messy.

For the routine management of patients it is not necessary for the MI to be absolutely equated with viability, but in view of the importance attached to the MI in drug trials, standardization of technique is important.

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