FLUORESCENCE MICROSCOPY AS A ROUTINE METHOD FOR THE DETECTION OF M. TUBERCULOSIS AND M. LEPROAE

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Since the introduction of fluorescence microscopy (F.M.) into bacteriology as a diagnostic procedure for the demonstration of M. tuberculosis and M. leprae by Hagemann 4 various investigators have reported their experience with this method. Numerous modifications and improvements of Hagemann's original technique have been published, the most useful perhaps being the suggestion of Norman and Jelks 10 to mark suspicious areas on a slide containing fluorescent bacilli by means of a diamond marker and to re-stain the same film by the Ziehl-Neelsen method (Z.N.), in order to confirm the presence of acid-fast bacilli, and Hughes' improved stain, 6 which made it possible to use binocular microscopes for the examination.

For examination for tubercle bacilli most reports were favourable (Tanner, 12, 13, Lind and Shaughnessy, 9 Lempert, 6 Norman and Jelks, 10 Clegg and Foster-Carter, 2 Wilson 14 and others). The main advantages mentioned were: increased percentage of positives, speed of examination and, in some instances, lessening of eye-strain. Ritterhof and Bowman 11 regarded the method as oversensitive and not sufficiently specific, thus confirming Küster's statements. 7 Andrew et al. 1 noted that the method was of little advantage when series of examinations containing a large percentage of positives were dealt with.

Few publications are available assessing the value of F.M. for the examination for M. leprae. The largest series is that of Dubois and Swerts, 2 whose results were not sufficiently favourable to induce them to introduce the method as a routine procedure. Gohar 4 also found that, in spite of the slightly higher percentage of positives, the method had little advantage over the Z.N. technique.

SCOPE OF INVESTIGATION

Our main interest lay in exploring the possibility of using F.M. as a routine method for the microscopic examination of specimens for tubercle bacilli, either in conjunction with, or entirely replacing, the usual Z.N. technique. The results described in the literature seemed encouraging, but none of the conditions mentioned were strictly comparable with those obtaining at this Institute. The results described in the literature seemed encouraging, but none of the conditions mentioned were strictly comparable with those obtaining at this Institute.

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TECHNIQUE AND EQUIPMENT

Smears were made from the sediment of sputa digested with papain and later with Eusol. This was still later replaced by a digesting fluid of the following composition: calcium hypochlorite 160 g. and sodium carbonate 320 g., made up to 4 litres with distilled water, left to sediment and decanted. For use, the supernatant fluid was diluted 1 : 10 with distilled water.

For examination of the smears, a Cooke, Troughton and Simms 250 W high-pressure mercury-vapour lamp was used, in conjunction with a lamp-condenser, water-trough and deep-blue filter, which eliminated all but the extreme blue, violet and ultraviolet rays. The binocular microscope was fitted with yellow filters, placed on the field diaphragms of the eyepieces. With this light-source the objectives employed were: a 16-mm. 10x objective, N.A. 0.28, and a 4-mm. 40x metallurgical dry objective, N.A. 0.85. The former objective was used for a quick survey of the film and the latter for the morphological identification of the bacilli observed. It allowed the scrutiny of a relatively large field, evenly illuminated. Later on a third, 8-mm. 20x N.A. 0.5 objective was added, which was found more convenient for the survey of the film and detection of the organisms. The 10x objective was then used only for focusing.

With all these objectives, oculars magnifying 8x were used, which, in conjunction with the binocular attachment of the microscope, gave a magnification of 12x.

Z.N.-stained smears were examined with a Cooke fluorite oil-immersion objective N.A. 0.95 3.75 mm. 45x, using 10x Zeiss eyepieces in the binocular.

The fields examined were, under these circumstances, as illustrated in Table I.
TABLE I

<table>
<thead>
<tr>
<th>Magnification</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4 (oil)</th>
</tr>
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<tbody>
<tr>
<td>Field diameter</td>
<td>10x</td>
<td>20x</td>
<td>40x</td>
<td>45x</td>
</tr>
<tr>
<td>in mm.</td>
<td>1·11</td>
<td>0·59</td>
<td>0·27</td>
<td>0·205</td>
</tr>
<tr>
<td>Area in sq. mm.</td>
<td>0·967</td>
<td>0·264</td>
<td>0·057</td>
<td>0·033</td>
</tr>
<tr>
<td>Relative area</td>
<td>29·3</td>
<td>8·0</td>
<td>1·7</td>
<td>1·0</td>
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INVESTIGATIONS AND TRAINING OF EXAMINERS

To begin with, a certain number of smears were examined daily by F.M., the results carefully recorded, and the slides then stained by Z.N. and handed to one of the staff normally doing the routine examination. This second examiner was not aware that the slides had already been examined. Comparison showed that, after some experience had been gained, the results obtained by F.M. were at least as accurate as those obtained by the Z.N. technique. In fact, slightly more positive results were obtained by the former method. In addition, the time consumed in examining the smears was considerably less. The method proved very tiring to begin with, apparently due to difficulties in accommodating the eyes to the unfamiliar focussing and colour scheme, but this disappeared very soon with practice. These preliminary examinations seemed promising enough to pursue the investigation further, and for this purpose more workers were trained.

With increasing experience, it was deemed safe to discard slides found negative by F.M. without re-staining them by Z.N., and this stage was generally reached after examination of approximately 300 slides. To save time, therefore, the first 300 slides of each new recruit were re-examined by one of us by F.M., the negatives discarded, and the positives and suspicious ones re-stained by Z.N. and re-examined by the recruit. That this procedure was justifiable was shown by the results of periodic re-examinations of discarded negative slides, as will be described later. In this manner an adequate team was trained to deal with a fairly large number of examinations daily.

RESULTS

*M. tuberculosis*

Results were recorded over a period of approximately one year.

Of a total of 60,747 sputa received for examination during this period, 37,917 were examined by F.M. Of these, 5,335 (14%) proved to be positive. Only those figures obtained after establishment of an adequate fluorescence team will be included here, covering exactly 10 months. During 5 months no diamond marker was available, and in the following tables it will be shown to what extent its lack handicapped the confirmation of scanty positives.

To illustrate the value of, and need for, experience in the use of F.M., comparative figures will be given of the results of one worker, who had already examined approximately 3,500 slides, and the rest of the team, which included beginners, who had examined only 300 specimens, although others had a great deal more experience.

The figures in brackets in both tables indicate how many of the F.M. positive and Z.N. negative smears contained only very scanty bacilli.

It will be noted that in the case of the single worker no more possibly false-positive results were recorded when a diamond marker was available. In all cases summarized in Tables II and III slides found negative were discarded without re-staining by Z.N.

As described above, before allowing new workers to discard their negative slides about 300 slides of each were checked by F.M. or by Z.N. In this manner, 1,489 slides were checked by F.M. Of these, 17 (1.5%) thought negative by the initial examiner were found positive. Of 1,144 slides checked by Z.N., 22 (1.9%) thought negative by the examiner were found positive. Practically all these positive result were missed during examination of the first 150 slides, and the majority were very scanty. A technical point which at first causes some difficulty is that of focussing on the correct plane of the slide, but this disappears with experience.

To ensure that a reasonably high standard of efficiency was maintained, batches of slides discarded by various examiners as negative, were periodically re-examined by Z.N. Of 1,162 slides checked in this way, 11 (0.9%) were found positive.

<table>
<thead>
<tr>
<th>TABLE II.—NO DIAMOND MARKER AVAILABLE</th>
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<tbody>
<tr>
<td>+FM</td>
</tr>
<tr>
<td>+ZN</td>
</tr>
<tr>
<td>Single worker</td>
</tr>
<tr>
<td>% of Total</td>
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<tr>
<td>Other workers</td>
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<td>% of Total</td>
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<td>+FM</td>
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<tr>
<td>Other workers</td>
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<td>% of Total</td>
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In order to compare the relative accuracy of the 2 methods, 2,730 specimens were examined in duplicate, one slide of each stained for fluorescence, and one for Z.N. As this manner of examination introduced some element of chance, only those smears on which, on re-examination, acid-fast bacilli could be found were counted in this comparison. In half the cases, routine Z.N. slides were checked by F.M., in the other half routine fluorescence slides by Z.N. In no case did the person being checked know this, whereas it could not be avoided that the person checking knew, which very likely increased their care in examining the slides. The results were as follows: 18 positives (0.66%) were missed by fluorescence workers whereas 49 positives (1.79%) were missed in examining Z.N.-stained films.

Although both figures are small, it will be seen that nearly 3 times as many positives were missed on the Z.N.-stained smears.

It may be of interest here that during this double check the single worker of Tables II and III missed one slide containing one acid-fast bacillus in 420 slides, whereas 8 positives were missed by the examiners doing the counter-check with the Z.N.

**Leprosy**

Leprosy smears numbering 2,320 were examined during this period, each one by both methods, by one single examiner. Of these, 345 (14.9%) were positive by F.M. and Z.N., 21 (6.1%) of which were very scanty. Forty-six (1.9%) were found positive or doubtful by F.M., but could not be confirmed by Z.N. Three slides, found negative by F.M., showed acid-fast bacilli when stained by Z.N. All these were missed during examination of the first 150 slides of this series.

**CONCLUSIONS**

**A. Tuberculosis**

It was felt that the series of examinations briefly described above sufficed to answer the question set out at the beginning, namely, whether F.M. could with advantage be used as a routine method for the detection of *M. tuberculosis* in direct smears from sputa.

Assuming a correctly-adjusted lamp and consistently good staining, the main points in favour of the method appeared to be:

1. **Reduction of examination time in the hands of experienced workers.** The time can be reduced to \(\frac{1}{4}\) of that needed for examination of Z.N.-stained smears. This is partly due to the fact that the field examined is larger and partly to the increased contrast in fluorescence-stained slides.
2. **Cleaner, easier and simpler staining technique.** Even should, as sometimes happens, the staining be unsatisfactory, the smears being either under-stained or under-decolorized, this is almost bound to be noticed during examination, and the preparations can then be re-stained for F.M. or Z.N., as desired. Badly-stained, scanty Z.N. smears might very easily be discarded as negative.
3. **Slightly higher percentage of positive results, particularly where acid-fast bacilli are scanty.**

4. **Lessening of eye-strain.** It was the consensus of all our fluorescence workers that, after the initial, rather tiring, stage of learning to accustom the eyes to the new method was overcome, F.M. is far more restful than the routine Z.N. method.
5. **Use of dry objectives only.** This also increases the speed of examination and facilitates re-staining of smears where required.

Against the adoption of fluorescence microscopy as a routine method were the following considerations:

1. It is difficult to train and maintain a team of fluorescence workers capable of dealing with so large a number of specimens, unless non-medical personnel are utilized for the purpose, which is not done at present.
2. The equipment is costly, although, once installed, the maintenance costs are not prohibitive, taking into account the number of specimens dealt with and the saving in medical officers' time.
3. The possibility of false-positive results must be borne in mind. Re-staining and re-examination of all positive and doubtful smears by the Z.N. technique, as practised during this investigation, largely offsets the initial gain in time—an important consideration, as approximately 14% of our smears proved to be positive and many more doubtful.

In spite of these disadvantages, it was decided that, although at present impracticable as a sole routine method, F.M. could be used for the examination of a proportion of our specimens, and since these investigations were concluded, at least 40,000 more smears have been examined by this method, about 50% of all sputa received for examination. Experienced examiners are allowed to discard definitely-positive slides as well as negatives, and only scanty-positive and doubtful slides are checked with Z.N. Periodical checks on discarded positive and negative slides are carried out. They have proved satisfactory, and it appears that the policy of discarding definite positives does not introduce any error.

**B. Leprosy**

As regards leprosy, the position is slightly different. The results obtained by an experienced worker using F.M. appear equally good, even slightly better than those obtained by the Z.N. technique. The disadvantage here lies in the fact that most of the routine smears submitted for examination are poor and often contain debris, which retains the stain, so that there is always a risk of missing scanty acid-fast bacilli. We have hesitated to issue even a negative report without checking the result by the Z.N. technique. Consequently we have abandoned F.M. for this type of investigation, although we have no evidence that it is not equally useful in skilled hands.

**SUMMARY**

The possibility of using fluorescence-microscopy as a routine method for the examination of large numbers of specimens for *M. tuberculosis* and *M. leprae* was investigated. Fluorescence-microscopy yields a slightly higher proportion of positive results than the routine Ziehl-Neelsen technique, the examination-time is considerably reduced, the method appeared ultimately to cause less eye-strain and it is cleaner and easier than the Ziehl-
Neelsen method. These advantages outweigh the disadvantages of cost of equipment and training and maintenance of a suitable team of workers. The danger of obtaining false-positive results can be overcome by experience and checking of scanty positive and doubtful smears by the Z.N. technique. The method is at present used for about 50% of sputa received for examination.

Examination of leprosy smears by F.M., although it appears to give equally good results as the Z.N. technique, has been abandoned, mainly because routine smears submitted for this examination are often unsuitable for this method and have to be restained and re-examined by Z.N. to reach a diagnosis of reasonable certainty.

Our gratitude is due to the many members of our staff, medical and technical, who so patiently and willingly assisted with the preparation of the smears and their examination. Without their help this work would have been impossible.

THE SIGNIFICANCE OF LOW SERUM CALCIUM VALUES IN THE SOUTH AFRICAN BANTU

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According to accepted standards, the diet of the South African Bantu, in common with that of many indigenous peoples dwelling in tropical and semitropical countries, is markedly deficient in calcium9, 10. Moreover, as we have mentioned elsewhere,2 the dietary intake of vitamin D is negligible, that of phytate phosphorus is high, the calcium-phosphorus ratio is adverse (occasionally as wide as 1 : 10), and the intakes of protein and fat are less than are usual among White communities. These dietary factors are listed by many authorities, for example Bicknell and Prescott,3 as being unfavourable for the absorption of calcium. There is, of course, plenty of sunlight (an average of 9 hours per diem in the Transvaal) although some authorities like Hess4 consider that a dark skin militates against the production of vitamin D from radiation. According to current views, stigmata of calcium deficiency should be apparent, and low levels of the element in the blood serum have been listed by some investigators.10, 11 That low levels are common among Natives of Southern Africa, who are habituated to a low intake of calcium, is borne out by local evidence.12, 13 Nevertheless, the view that low serum calcium values are evidence of calcium deficiency, and of reduced body stores, etc., is of questionable validity and open to a number of criticisms which will be discussed in this paper.

For a number of years at this centre we have been interested in the subject of calcium metabolism under conditions of low intake, and consequently we have determined serum calcium values on children and adults as opportunity offered. Particular attention has been given to pregnant and multiparous long-lactating mothers, among whom the effects of the drain of the element on serum values are of obvious interest.

We wish to stress that our studies concern Bantu children and adults in outward good health, and that subjects with altered serum calcium concentrations arising from various metabolic disorders, including rickets, have been excluded. The metabolism of calcium in the latter disease will be discussed in another paper.

SUBJECTS AND METHODS

(1) Boys. (a) 23 subjects, aged 7-12 years, were out-patients at Coronation Non-European Hospital, Johannesburg, suffering from cuts, minor burns, and other ailments not likely to influence serum calcium levels. (b) A further group of 54 boys, aged 14-15 years, were delinquents at Diepkloof Reformatory, Johannesburg. They had been detained there for some months, and were employed on farm lands producing food (including milk) for their own consumption.

(2) Adult Males. 48 men, aged 18-40 years, were newcomers from different Southern African territories examined in Johannesburg and passed as medically fit for service on the gold mines.

(3) Adult Females. The 33 adult women were either out-patients or newly-admitted in-patients at Bara-