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Two Methods of Demonstrating Leprosy Bacilli in Smears [1]

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The standard method of carbol fuchsin staining for mycobacteria was initially developed for use with strongly acid-fast bacilli such as tubercle bacilli. The decolorizer which is therewith used contains strong acid such as hydrochloric or sulfuric acid and these lead to over-decolorization of weakly acid-fast organisms such as the leprosy bacilli. Accordingly, it has been recommended that smears of leprosy bacilli should be decolorized for only a few seconds in 1% HCl in 70% ethanol or for five seconds in 0.5% HCl in 70% ethanol ([15, 16]). This staining procedure does not give consistently reliable results with leprosy bacilli.

Other reports have indicated that prolonged oxidation with periodic acid results in enhanced carbol fuchsin staining of leprosy bacilli ([3, 4, 11, 12]).

The effect of periodic acid oxidation on carbol fuchsin staining of leprosy bacilli, especially as related to use in the determination of Bacterial (BI) and Morphologic Indices (MI). The two methods here presented have been found to be suitably practical.

MATERIALS AND METHODS

Carbol-fuchsin/malachite green with acetic acid for acid-fast form of mycobacteria. *The stain.* One gram of basic fuchsin having an absorption of 552-556 mμ (we used Diamond fuchsin, C.I. 42510 maximum absorbance 555 mμ, chroma) is dissolved in 10 ml absolute ethanol. Five grams of phenol is dissolved in 100 ml distilled water. The two solutions are mixed and filtered just prior to use.

Procedure.

1. Stain with mixed solution flooding bacillary smear on glass slide with heating to steaming, 5 minutes.
2. Rinse in tap water.
3. Differentiate in 10% acetic acid for 0.5 minute, rinse in tap water. Dip slide for 1-2 minutes in aqueous 1% malachite green or differentiate in 1% malachite green in 10% acetic acid for 1-2 minutes.
4. Rinse in tap water, air dry.

The mycobacteria stain red against a counterstain of varying blue tones.

Periodic acid carbol pararosaniline stain (PACP) for acid-fast and chromophobic forms of mycobacteria. *The stain.* One gram of pararosaniline having an absorbance of 546-548 mμ (we used pararosaniline HCl, C.I. 42500, maximum absorbance 547 mμ, chroma) is dissolved in 10 ml absolute ethanol. This is mixed with 100 ml distilled water containing 5 gm phenol and filtered just before use.

Procedure.

1. Oxidize in aqueous 10% periodic acid, 18-20 hours.
2. Rinse in water and air dry.
3. Carbol pararosaniline at 37°C for 1 hour in Coplin jar or on flooded slide. Then warm to steaming for 5 minutes. Rinse in tap water.
4. Differentiate in 10% aqueous acetic acid, 0.5 minute or until bacillary smear is pink. Rinse in tap water and dip in malachite green, 1% aqueous, or 1% malachite green in 10% acetic acid for 2 minutes.
5. Rinse in tap water and air dry.

The mycobacteria stain a brilliant red against a varied blue tonal background. The stained bacilli resist fading for a long time and retain their staining even after strong decolorization.

Procedures. An unselected series of 200 leprosy patients from this hospital, and the National Airakuen Sanatorium and Naha Skin Clinic in the Ryuku Islands each provided slit skin smears from two to three sites of lesions on their torsos and extremities. They had been treated for leprosy for periods ranging from 1 to 35 years.

Skin smears were first fixed by heat and stained with the usual carbol fuchsin method

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([15]) utilizing the same fuchsin dye specified above.

After examination and evaluation of these stained smears they were restained by the following procedure.

1. The cedar wood oil, used for oil immersion examination, and the carbol fuchsin were removed by one minute in xylene, 10 seconds in absolute ethanol and about 10 seconds in 1% HCl/70% ethanol until the dye was removed. Slides then were rinsed in water and dried.
2. Slides were immersed in phenol gel (phenol 1 gm, gelatin 0.5 gm, distilled water 100 ml), 5 minutes, 37°C. Air dried, 37°C, 10 minutes.
3. Fixed in 10% formalin, 3 hours. Rinsed in water.
4. Retained with periodic acid carbol pararosaniline as above.

Evaluation. The BI was evaluated by the logarithmic scale ([15]). The MI was recorded as the number of solid forms per 100 bacilli examined, or if there were fewer than 100 bacilli per smear the result was recorded as the number of solid forms in the total number of bacilli found.

RESULTS

The PACP restained preparations not only showed the bacilli intensely colored but also some of the previously nonsolid appearing bacilli now appeared solid and previously chromophobic forms appeared as solid or nonsolid rods. Of the 200 patients studied, 104 demonstrated bacilli by the carbol fuchsin method while the PACP method demonstrated bacilli in 173 patient specimens. By the latter method bacilli were found in 69 of 96 skin smears from leprosy patients in which no bacilli could be demonstrated on the same smears by the CFMG method. The results are summarized in Table 2. The comparative staining effects of the prior periodic acid oxidation, fuchsin type and decolorizer used are summarized in Table 1.

DISCUSSION

Stains marketed as "basic fuchsin" may be either the chloride or the acetate of pararosaniline or its admixture with higher homologues. In carbol fuchsin staining of mycobacteria it seems that beaded staining is

TABLE 1. *Effect of prior periodic acid oxidation, fuchsin type and decolorizer on the carbol fuchsin stain of leprosy bacilli.*

Prior oxidation	Fuschin type	Decolorizer and counterstain		Staining intensity[a]
No	pararosaniline HCl[c]	1% HCl-70% EtOH	1% malachite green aq.	1[b]
	diamond fuchsin[d]			2
	pararosaniline HCl	10% acetic acid	1% malachite green aq.	2[b]
	diamond fuchsin	or 1% malachite green in 10% acetic acid		3
10% $HIO_4 \cdot 2H_2$ aq 18 hours	pararosaniline HCl	1% HCl-70% EtOH	1% malachite green aq.	5
	diamond fuchsin			4
	pararosaniline HCl	10% acetic acid	1% malachite green aq.	6
	diamond fuchsin	or 1% malachite green in 10% acetic acid		4

[a] Numerals from 1 to 6 show staining intensity of acid-fast leprosy bacilli.
[b] Beading shown.
[c] Pararosaniline HCl, C.I. 42500, absorption maximum 547-8 mμ, Chroma.
[d] Diamond fuchsin, C.I. 42510, absorption maximum 555 mμ, Chroma.

most apt to occur with pararosaniline samples whereas in its admixture with higher homologues more uniform staining of the individual bacilli is usually obtained ([6,8]). Moreover, with pararosaniline preparations the staining of bacilli may be so poor that some lose their acid-fastness. Accordingly, reported results of acid-fast staining of *M. leprae* are confused by the fact that some leprologists use pure pararosaniline while others use the mixed preparations and carbol fuchsin prepared with these two types of basic fuchsin present quite different staining properties. Cook ([1]) recommended the use of the relatively coarse granule preparation rather than the more purified type generally specified for Schiff's reagent. We find that the best results are obtained with basic fuchsin having an absorbance of 552-556 mμ when BI and MI determinations are to be made in leprosy studies.

Pottz ([13]) described a staining technic, utilizing acetic acid as decolorizer, by which excessive decolorization is minimized. We have tested a variety of weak acids, including acetic, formic, citric and lactic acids, as decolorizers and find that acetic acid gives the best results.

When periodate oxidation is employed intense staining of bacilli, including chromophobic forms, results with the use of carbol fuchsin prepared from either pararosaniline or commercially available basic fuchsin dyes containing pararosaniline as the chief component, providing the absorbance is 546-548 mμ. Inferior results occur with basic fuchsin having an absorbance range of 552-556 mμ ([4]).

Mycobacteria are two-phased microorganims which, depending on the environment, can appear either in metabolically active, acid-fast forms or as inactive chromophobic forms ([12]). The acid-fastness has been thought to signify the presence of the wax, mycolic acid. It appears that carboxyl and chiefly hydroxyl groups of these lipids are essential for the acid-fast response ([5]). Additionally, chromophobic mycobacteria in tuberculous or leprous lesions and acid-fast bacilli made nonacid-fast by extraction or blocking can be visualized as being acid-fast with the periodic acid pararosaniline method. The mechanism of this staining procedure depends on the oxidation of hydroxyamino groups in the cell walls to produce free aldehydes. The reaction is related to that forming Schiff's bases by arylamines condensing with aldehydes: thus, $R\text{-}CHO + 2\ ArNH_2 \rightarrow R\text{-}CH(NHAr)_2$ ([4]).

In practice, carbol fuchsin frequently fails to demonstrate any bacilli even in lesions known to be tuberculous or leprous. The problem actually lies with the character of the mycobacteria which may be very weakly acidfast or chromophobic. Tuberculous or leprous lesions containing only chromophobic forms will remain "negative" when stained with the usual carbol fuchsin, while with the periodic acid carbol fuchsin method bacilli may be demonstrated in large numbers ([10-12]). This was well demonstrated in the present study where the same slides, restained by the periodic acid method, then visualized many more bacilli.

It is significant that in the restained sections there was increase in both nonsolid and

TABLE 2. *Bacterial Index with carbol fuchsin and periodic acid-carbol pararosaniline in the same skin smears.*

Bacterial Index	Tuberculoid		Borderline		Lepromatous		Total	
	CF	PACP	CF	PACP	CF	PACP	CF	PACP
6+					4	10	4	10
5+					12	10	12	10
4+				1	16	20	16	21
3+			1	0	22	24	23	23
2+			2	4	8	15	10	19
1+	1	8	2	13	36	68	39	84
0	13	6	17	4	66	17	96	27
Total	14	14	22	22	164	164	200	200

CF = carbol fuchsin stain (Ridley, 1964).
PACP = periodic acid-carbol pararosaniline stain.

solid visualized forms. These results contrast with the findings of Levy *et al* (7) who reported that there is no evidence that the standard acid-fast staining technic results in a gross underestimation of numbers of *M. leprae* as compared with carbol fuchsin staining preceded by periodic acid oxidation. Harada (4) also noted that there is no quantitative difference between bacilli visualized by the two methods.

It is likely that the apparent discrepancy in findings results from studies employing a high initial proportion of acid-fast bacilli which are not affected by periodate oxidation. If, however, the preparations contain a high proportion of chromophobic organisms, then the oxidation technic will reveal greater numbers than will the usual carbol fuchsin method. Under treatment with antileprosy drugs, the leprosy bacilli may lose their acid-fastness and become chromophobic. In this case periodic acid oxidation will restore their staining.

Nyka and O'Neill (12) reported that chromophobic tubercle bacilli are alive, that they may recover their acid-fastness and may play an important role in the pathogenesis of relapse in tuberculosis. They noted that the antituberculosis drugs in current use are effective only against metabolically active bacilli and that this would indicate that chromophobic bacilli have low metabolic activity and are beyond the reach of effective chemotherapy.

Delville (2) demonstrated that the Ziehl-Neelsen technic does not detect acid-fast bacilli in all forms of leprosy. In most leprous lesions, other staining technics (Gram, Ziehl-periodic acid, Fite-Faraco) reveal also non-acid-fast bacilli as well as typically acid-fast organisms, the former appearing first.

It has been argued that in leprosy solid acid-fast bacilli are living while nonsolid forms are dead. Thus, the ratio of nonsolid forms is used as an indication of the effect of antileprosy drugs, the greater the proportion of nonsolid forms the greater the effect of the drug is presumed to be (9, 14).

The present study suggests the necessity of reconsidering the contention that nonsolid bacilli are dead and also suggests that there may be nonacid-fast bacilla in healing and apparently healed lesions. It is very likely that variations in the stain affinity of leprosy bacilli are influenced considerably by their environment and that leprosy bacilli can survive in apparently healed and near-healed lesions, even after prolonged chemotherapy, remaining latent for long periods and maintaining a threat of relapse of the disease.

SUMMARY

Two methods, the carbol fuchsin with acetic acid differentiation and the periodic acid-carbol pararosaniline, were used for demonstrating leprosy bacilli in skin smears. Bacillary smears from 200 long-treated patients with tuberculoid, borderline and lepromatous leprosy were stained with periodic acid-carbol pararosaniline. There were significantly greater BI and MI determinations than with classic carbol fuchsin staining. With the former stain bacilli were found in 69 of 96 skin smears in which no bacilli could be seen by the latter stain. It is suggested that under the action of antileprosy drugs some leprosy bacilli may lose their acid-fastness and become chromophobic; chromophobic bacilli can be restored to their staining with periodic acid pretreatment.

Leprosy bacilli in their chromophobic form can survive in healing and apparently healed lesions even after prolonged chemotherapy and can be a possible source of relapse.

RESUMEN

Se utilizaron 2 métodos, el de carbol-fucshina con ácido acético como diferenciante y el del ácido peryódico-carbol pararosanilina, para demostrar los bacilos de la lepra en extensiones de piel. Los frótis preparados de 200 pacientes con lepra tuberculoide, intermedia o lepromatosa se tiñeron con ácido peryódico-carbol pararosanilina. Los índices bacteriológico y morfológico fueron significativamente más grandes que los obtenidos por el método clásico de tinción con carbol-fucshina. Con la primera coloración, se encontraron bacilos en 69 de 96 frótis de piel en los que no se pudieron observar bacilos con el último método de tinción. Se sugiere que bajo la acción de las drogas antileprosas, algunos bacilos de la lepra pueden perder su ácido-resistencia y llegar a ser cromofóbicos; la ácido- resistencia de los bacilos cromofóbicos puede ser restaurada por pretratamiento con ácido peryódico.

Los bacilos de la lepra en su forma cromofóbica pueden sobrevivir en las lesiones en regeneración y en aquellas aparentemente "curadas" aún después de quimioterapia prolongada y pueden ser una posible fuente de reinfección.

RÉSUMÉ

Deux méthodes ont été utilisées en vue de démontrer la présence de bacilles de la lèpre dans des frottis cutanés, à savoir la carbol fuchsine avec une différentiation par l'acide acétique, et l'acide périodique-pararosaniline. On a coloré des frottis bacillaires obtenus chez 200 malades traités pendant longtemps, et atteints de lèpre tuberculoide, borderline ou lépromateuse, par l'acide périodique-carbol pararosaniline. L'Index Bactériologique (BI) et l'Index Morphologique (MI) déterminés dans des frottis colorés de cette manière étaient significativement plus élevés que ceux obtenus par des colorations classiques avec la carbol-fuchsine. Avec la coloration par l'acide périodique-carbol pararosaniline, des bacilles ont pu être observés dans 69 frottis cutanés sur 96, alors que la coloration par la carbol-fuchsine ne permettait de mettre les bacilles en évidence dans aucun de ces cas. On suggère qu'à la suite de l'action des médicaments anti-lépreux, certains bacilles de la lèpre peuvent perdre leur acido-résistance et devenir chromophobes. Les bacilles chromophobes pourraient recouvrer leur acido-résistance habituelle avec un pré-traitement par l'acide périodique.

Des bacilles de la lèpre, sous forme chromophobe, peuvent surgir dans des lésions en voie de guérison ou apparemment guéries, et ceci même après une chimiothérapie prolongée. Ces bacilles pourraient constituer une source éventuelle de récidive.

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