A STUDY OF MALACHITE GREEN STAINING OF LEPROSY BACILLI[1]

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Malachite green-fuchsin staining was reported by Murohashi ([2, 3, 4]), as a method for differentiating living and dead tubercle bacilli. He suggested that it might be applicable to *M. leprae* as well as *M. lepraemurium*. The assumption underlying this method is that malachite green can combine specifically with highly polymerized desoxyribonucleic acid (DNA) at a pH of about 4. On this assumption, green-stained bacilli are thought to be viable and pink-stained bacilli dead. If the assumption is true for leprosy bacilli, this method might give more reliable data than the method of Rees and Valentine ([6]), in which viability and nonviability of leprosy bacilli are surmised from the bacillary morphology in Ziehl-Neelsen-stained specimens.

In the course of examination of leprosy bacilli by malachite green-fuchsin staining, however, it was noted that more green-stained bacilli were found when malachite green staining was performed at room temperature than when the original staining was carried out at 66°C. This phenomenon suggests that malachite green may combine also with substances other than DNA in leprosy bacilli.

In this paper, the reason why many more green-stained bacilli were found on staining at room temperature is analyzed, and histochemical characteristics of the substances found in leprosy bacilli are described.

MATERIALS AND METHODS

Freshly smeared bacilli were obtained from various stages of lepromatous type or borderline group patients treated with Ciba 1906 or DDS. Samples were obtained from untreated cases also.

Some samples were obtained from materials taken at biopsy for other experiments, which had been stored for several months in suspension in 0.85 per cent NaCl or in Hanks' solution in a freezer at about −10°C. In this case, a drop of the suspension was put on a slide, dried at 60°C and fixed with flame.

As controls, tubercle bacilli ($H_{37}Rv$, BCG) and murine leprosy bacilli (Hawaiian strain) were used. Tubercle bacilli were grown on Petragnani medium for four weeks at 37°C, and murine leprosy bacilli were obtained from a mouse (C_3H) inoculated 12 weeks previously. Leprosy bacilli, tubercle bacilli and murine leprosy bacilli were put on the same slide in all experiments.

Calf thymus DNA and human peripheral blood cells were used in some experiments as controls. A drop of aqueous solution of thymus DNA (5 mgm./ml.) was spread over an egg albumin-coated slide and dried.

Two methods (I and II) were employed. The steps in the staining were as follows:

Method I.—

1. Smears were dried on slides and fixed by flame.

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2. The slides were immersed in 1 per cent malachite green, dissolved in 0.2 M acetate buffer at pH 4.2, and stained for 5 minutes at 60°C. The dye solution was warmed to 60°C beforehand in an incubator. (In the original method, an aqueous solution of 1 per cent malachite green, warmed to 50°-60°C, was used at a pH of about 4.)

3. The slides were taken out and a small amount of the malachite green solution used in the second step was poured on them to prevent drying. They were then cooled for 20 minutes at room temperature. After cooling the slides were washed.

4. Pfeiffer's solution, i.e., diluted carbol-fuchsin with ten times its volume of water, was poured on the slides and allowed to remain for 5 minutes at room temperature. Then the slides were washed.

5. The slides were next put in 8 per cent HNO_3 or 25 per cent sulfuric acid at room temperature for 1 minute.

6. Finally the slides were washed, dried, and examined.

Method II.—In Method II, the step of malachite green staining was performed at 23°C for 30 minutes. Other procedures were the same as in Method I.

For the analysis of the step of malachite green staining, slides were put in 25 per cent sulfuric acid for one minute after procedures 1-3 in Method I or II, and washed in water and examined.

In experiments on the effect of temperature on malachite green staining, slides were stained for 5 minutes at 55°, 50°, 45°, 40° and 35°C, respectively, and cooled at room temperature for 20 minutes as in the staining procedure at 60°C.

Feulgen, murexide, periodic acid-Schiff (PAS), and peracetic acid-Schiff reactions and staining with Sudan III, Sudan black B and toluidine blue, were performed according to the methods described in Lison's text book (1).

The dyes used except for basic fuchsin, were products of E. Merck AG, Darmstadt. Basic fuchsin used for the Feulgen reaction was the product of Matheson Co., Inc., East Rutherford, New Jersey.

RESULTS

In comparing the results of Method I and Method II, applied to many samples obtained in various clinical stages of leprosy patients, it was found that almost all showed many more green-stained bacilli by Method II than by Method I. Many green-stained bacilli, about 20-40 per cent of the total bacilli, were seen even in the cases in which all bacilli were stained pink by Method I.

In order to analyze this phenomenon, the single step of malachite green staining was applied to many samples. Several samples showed many more green-stained bacilli after staining at 23°C than at 60°C. One sample was found which had bacilli stainable at 23°C but not at 60°C. This sample was obtained from a lepromatous patient treated with Ciba 1906 for one month. It had been homogenized in Hanks' solution and stored in a freezer for about 6 months. The sample had been melted several times for other experiments. In order to obtain clear results, this sample was used mainly in the following experiments.

If malachite green could stain only polymerized DNA of leprosy bacilli, two possible factors might explain the failure to stain at 60°C: (1) insufficiency of time for staining due to inadequate permeability of the dye, and (2) facilitation of decoloration by sulfuric acid due to modification of cell walls of the bacilli during the treatment at 60°C.

With reference to the first of these possibilities it was noted that the number of green-stained bacilli did not increase even by increasing

the time of staining. No difference was found in 15, 30, and 60-minute-staining at 60°C. Actually, on staining at 23°C, faintly stained bacilli were found as early as 5 minutes and clear green-stained bacilli after 10 minutes of staining. From these results it was concluded that failure to stain was not due to inadequate permeability of the dye into leprosy bacilli.

With reference to elimination of dye supposedly combined with DNA by sulfuric acid, it was noted that 30 minute treatment with tertiary butyl alcohol at 32°C after malachite green staining at 60°C also did not yield any green-stained bacilli. In this connection it was noteworthy that thymus DNA stained with malachite green was not decolorized by treatment with tertiary butyl alcohol. Moreover, the slides that were treated with 0.2 M acetate buffer at pH 4.2 at 60°C for 5 minutes and later cooled to room temperature for 20 minutes, have shown many green-stained bacilli on malachite green staining at 23°C. From these results, the possibility of elimination by sulfuric acid of the dye supposedly combined with DNA, as a result of modification of cell walls of the bacilli during the treatment at 60°C, was excluded.

The assumption of Murohashi and Yoshida ([4]) that the dye can combine specifically with highly polymerized DNA cannot be supported from the above results.

Studies on the effect of temperature of staining revealed that green-stained bacilli appeared on staining at 50-55°C and were present after staining at and below 40°C as frequently as on staining at 23°C.

In Table 1, the results of experiments performed for analysis of the failure to stain at 60°C are summarized. To facilitate understanding of the results, a schematic representation of the phenomenon noted in Table 1 is shown in Figure 1, which is based on the following hypothesis. The basophilic substances combine with some elements of the bacillary body by weak strength and the dye molecules can combine with a part of the basophilic substances at 23°C (A). But, at 60°C the dye molecules moving actively can penetrate into loosened spaces between the basophilic substances and bacillary body. Then the basophilic substances, surrounded by dye molecules, are liberated from the bacilli (B). When green-stained bacilli stained at 23°C are put into acetate buffer at 60°C, dye molecules move away from bacilli (A → C). But the basophilic substances remain in bacillary bodies because a small quantity of the dye can not surround the basophilic substances. In this case decolorized bacilli can be stained again at 23°C (C → A).

CHARACTERISTICS OF THE BASOPHILIC SUBSTANCES

Reference is made by Lison ([1]) to the following basophilic substances: uric acid and its derivatives, acid mucopolysaccharides, nucleic acids and chromolipoids. In the following experiments, various tests for these substances were performed.

1. The murexide reaction did not occur. (Test for uric acid.)

TABLE 1.—*Summary of experiments on failure of bacilli to stain at 60°C.*

Pretreatment	Staining	Bacilli		
		Tubercle	Murine leprosy	Human leprosy
None	23°C, 30 min. in dye solution	+++[a]	+++	+++
None	60°C, 5 min.[b] in dye solution	+++	+++	−
60°C, 5 min.[b] in 0.2 M acetate buffer, pH 4.2	23°C, 30 min. in dye solution	+++	+++	+++
23°C, 30 min. in dye solution	60°C, 5 min.[b] in dye solution	+++	+++	−
	After removing the cedar oil by xylene, the above slide was stained again at 23°C for 30 min.	+++	+++	−
23°C, 30 min. in dye solution	60°C, 5 min.[b] in 0.2 M acetate buffer, pH 4.2	−	−	−
	After removing the cedar oil by xylene, the above slide was stained again at 23°C for 30 min.	+++	+++	+++

[a] Symbol +++ means more than 100 green-stained bacilli per field.
[b] After treatment at 60°C for 5 minutes, slides were taken out and a small amount of the solution used was poured on them. They were then cooled at room temperature for 20 minutes, as described in the text.

2. Faintly stained bacilli were seen after staining at 23°C for 30 minutes with 1 per cent malachite green adjusted to pH 2.1 with HC1. (Acid mucopolysaccharide containing sulfuric acid should be stained strongly with this solution.)

3. Distinct metachromasia was not observed on staining with 0.05 per cent toluidine blue, dissolved in 0.02 M acetate buffer at pH 4.2, at 23°C for 10 minutes and 45 minutes. (Acid mucopolysaccharides containing sulfuric acid or hyaluronic acid are stained metachromatically.)

4. PAS reaction. Faintly stained bacilli were observed, but their number was less than that after malachite green-staining. The treatment with periodic acid in this test did not eliminate the basophilic substances. (Polysaccharides are PAS-positive, but chromolipoids may be positive.)

5. After treatment with N-HC1 for 10 minutes at 60°C, with 5 per cent trichloroacetic acid (TCA) for 15 minutes at 90°C, or with 10 per cent perchloric acid (PCA) for 20 minutes at 70°C, the malachite green staining carried out at 23°C for 30 minutes did not show green-stained

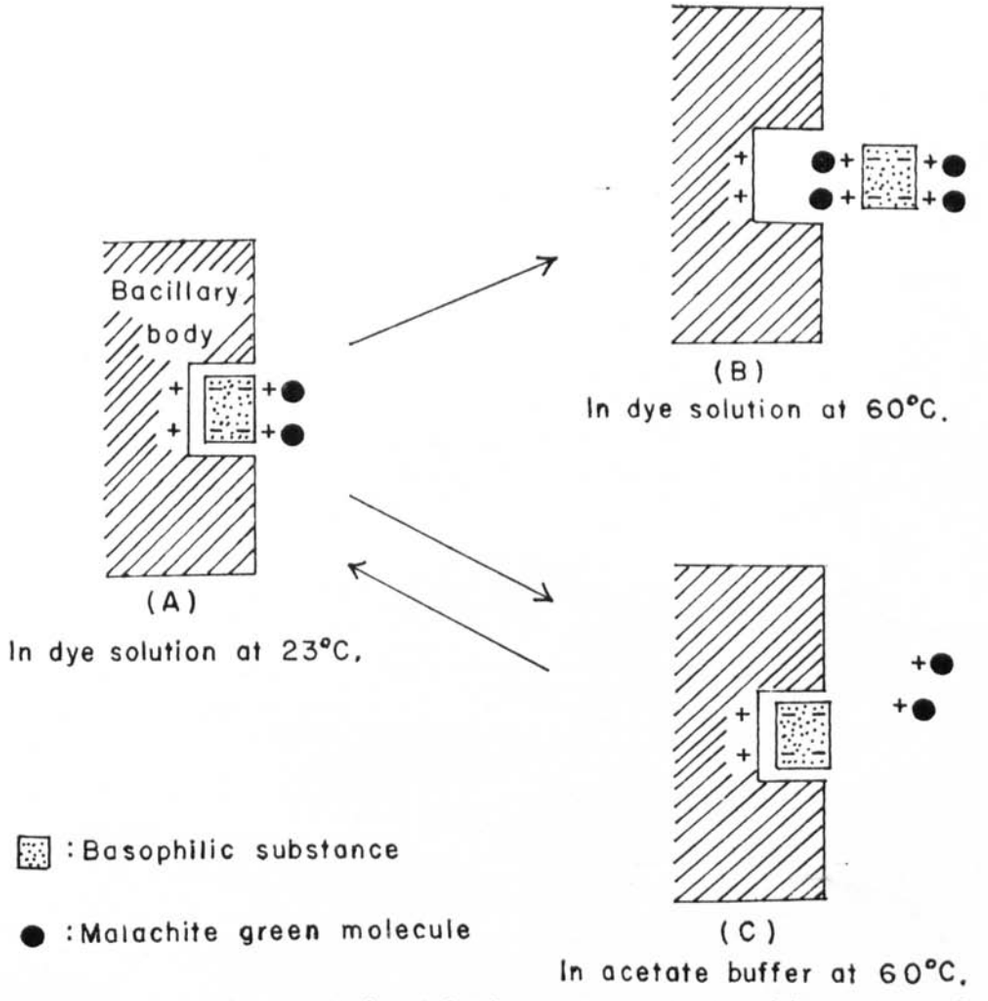

FIG. 1. Schematic representation of the phenomenon occurring in stainings at 23°C and 60°C. Explanation is given in the text.

bacilli. But treatment with 10 per cent PCA for 18 hours at 4-5°C did not affect the staining. (Test for nucleic acids.)

6. The Feulgen reaction was performed after treatment with N-HCl at 60°C for 5, 10, 15, and 20 minutes, and no distinctly positive results were observed, although tubercle bacilli, murine leprosy bacilli, thymus DNA and human leucocytes showed positive results. (Test for DNA.)

7. Staining with Sudan III and Sudan black B, saturated in 60 per cent ethanol, at 23°C for 30, 60, and 90 minutes did not give distinct results. (Chromolipoids can usually be stained with these solutions.)

8. Effects of organic solvents. After treatment with ether, acetone, ethanol, benzene, chloroform or chloroform-ethanol (1:1) at 23°C or higher temperatures for 15-180 minutes, malachite green-staining

TABLE 2.—*Results of various treatments on malachite green staining.*

Treatment			Bacterial index [a]
—			5+
Test No. 5			
N-HCL	60°C	10 min.	0
5% TCA	90°C	15 min.	0
10% PCA	70°C	20 min.	0
10% PCA	4°C	18 hrs.	5+ - 4+
Test No. 8			
Organic solvent			
Ether	23°C	60 min.	5+ - 4+
		180 min.	4+
	35°C	60 min.	4+
		180 min.	4+
Ethanol	23°C	30 min.	5+ - 4+
		60 min.	5+ - 4+
		120 min.	4+
		180 min.	4+
	60°C	15 min.	5+
		30 min.	4+
		60 min.	3+
		180 min.	2+
Chloroform	23°C	30 min.	3+ - 4+
		60 min.	3+
		120 min.	3+ - 2+
		180 min.	3+ - 2+
	60°C	15 min.	3+ - 2+
Chlorofrom (cont'd)			
	60°C	30 min.	3+ - 2+
		60 min.	2+
		180 min.	1+
Benzene	23°C	60 min.	4+
		180 min.	4+
	60°C	60 min.	4+
		180 min.	4+
Acetone	23°C	60 min.	5+ - 4+
		180 min.	4+
	56°C	60 min.	4+
		180 min.	4+
Test No. 10			
Acidfastness			
25% sulfuric acid			
	23°C	30 min.	5+
		60 min.	5+
		180 min.	5+
		24 hrs.	3+ - 4+
		48 hrs.	3+
1% HCl-alcohol	23°C	1 min.	5+
		30 min.	5+
		60 min.	3+
		180 min.	2+
		24 hrs.	0

[a]Bacterial index (7) of green-stained bacilli:
5+ Many bacilli in 1 average $1/12$ inch objective field (estimated over 100 bacilli).
4+ Ten or more bacilli in 1 average $1/12$ inch objective field.
3+ One or more bacilli in 1 average $1/12$ inch objective field.
2+ One or more bacilli in 10-$1/12$ inch objective fields.
1+ One or more bacilli in 100-$1/12$ inch objective fields.

carried out at 23°C for 30 minutes showed a reduced number of green-stained bacilli. The effects of chloroform (23°C, 60°C) and ethanol (60°C) were especially prominent. The effect of chloroform-ethanol was the same as that of chloroform. (Chromolipoids are scarcely soluble in organic solvents.)

9. Peracetic acid-Schiff reaction. Faintly stained bacilli were observed, but their number was less than that after malachite green staining, as in the case of the PAS reaction. The treatment with peracetic acid in this test did not eliminate the basophilic substances. (Test for fatty substances containing unsaturated groups.)

10. Acidfastness. Staining with malachite green was performed at 23°C for 60 minutes. Malachite green was not eliminated by 25 per cent sulfuric acid in 3 hours, but many bacilli were decolorized within 24 hours, although a few bacilli could hold the dye for 48 hours. The bacilli were decolorized by 1 per cent HCl-alcohol within 60 minutes, although there were many green-stained bacilli after 30 minutes. (Ceroid, a kind of chromolipoid, is acid-fast.)

11. No pigments were observed distinctly in unstained samples. (Chromolipoids are usually seen as yellow, brown or black granules.)

The detailed results of tests (Nos. 5, 8, and 10) are shown in Table 2.

DISCUSSION

Basophilic substances include uric acid and its derivatives, acid mucopolysaccharides, nucleic acids, acid proteins, polyphosphates, chromolipoids, etc.

Several histochemical tests for purines are not specific, except for the murexide reaction, and a negative result in the murexide test does not give any decisive conclusion. But the effects of organic solvents may exclude uric acid as one of the basophilic substances.

Acid mucopolysaccharides containing sulfuric acid may be excluded from the basophilic substances possibly present because of the faint staining at pH 2.1, acidfastness in the presence of 25 per cent sulfuric acid, the results of the experiment on metachromasia, and the effects of organic solvents. The presence of other acid mucopolysaccharides containing hyaluronic acid also may be excluded as a result of the experiment on metachromasia and the effects of organic solvents.

The results of treatment with MCl, TCA, and PCA suggest the presence of DNA, but a negative Feulgen reaction and the effects of organic solvents are contradictory to it. Moreover, in the analysis of the step of malachite green-staining, combination of the dye with DNA was not supported by the experiments. According to Murohashi and Yoshida (4) ribonucleic acid cannot be stained by malachite green.

The presence of proteins and polyphosphates may be excluded by the effects of organic solvents.

In view of the exclusion of all the above possibilities, chromolipoids are believed, on the basis of the effects of organic solvents, to

be the basophilic substances in leprosy bacilli, although the staining with Sudan III and Sudan black B does not give distinctly positive results. In bacteria, in contrast to sections of animal tissues, one is not likely to observe distinct color because the amount of lipoids present may be very small and decoloration may occur during washing in 60 per cent ethanol for 30 seconds. The result of the staining therefore does not mean the absence of lipoids. Some lipo-pigments give such negative results ([5]). Some chromolipoids also may show negative results in the PAS reaction and peracetic acid-Schiff reaction ([1,5]). Various degrees of solubility in organic solvents and of acidfastness suggest that the oxidation of lipoids containing unsaturated fatty acids progresses gradually in leprosy bacilli, and a complex of various stages of chromolipoids is formed. As the basophilic substances were seen also in cases of patients treated over a long term, these substances may be formed in the course of degeneration of leprosy bacilli, under circumstances similar to those occurring in animals in the formation of ceroid.

Malachite green molecules that combine with the basophilic substances at 23°C are not easily replaced by fuchsin in the step of fuchsin staining of malachite green-fuchsin staining. After treatment with Pfeiffer's solution for 5 minutes, many green-stained bacilli can be seen, although only a few green-stained bacilli are seen after 10-20 minutes of treatment. On the contrary almost all green-stained bacilli, observed on malachite green staining at 60°C in many samples, become pink after fuchsin staining of 5 minutes. These facts may explain the presence of many more green-stained bacilli in the case of staining at room temperature.

When the malachite green-fuchsin staining was applied to tubercle bacilli and murine leprosy bacilli as control samples, no observations were contradictory to the assumption as to the method presented by Murohashi.

There were many samples that showed numerous green-stained bacilli after malachite green-staining at 60°C. But after treatment of these samples with chloroform-ethanol for 60 minutes at 60°C, almost all bacilli could not be stained with malachite green. As to the demonstration of the polymerized DNA by histochemical methods other than the Feulgen reaction in leprosy bacilli, the effects of various pretreatments with HCl, TCA, PCA, etc., cannot give any conclusive results. Only the effect of purified desoxyribonuclease may be conclusive.

SUMMARY

1. Malachite green fuchsin staining, devised by Murohashi to differentiate living and dead acid-fast bacilli, was applied to leprosy bacilli, and the effects of temperature at the step of malachite green-staining were examined.

2. Staining of leprosy bacilli with 1 per cent malachite green, dis-

solved in 0.2 M. acetate buffer at pH 4.2, revealed the presence of basophilic substances that were liberated from leprosy bacilli at 60°C in the presence of the dye.

3. From the results of various histochemical examinations, it is concluded that such basophilic substances were not DNA but lipoids, presumably derived from oxidation of unsaturated fatty acids.

4. The basic assumption in malachite green-fuchsin staining that malachite green can combine specifically with highly polymerized DNA at a pH of about 4 is not valid for leprosy bacilli, and for this reason malachite green-fuchsin staining cannot be applied to leprosy bacilli for the differentiation of living and dead bacilli.

RESUMEN

1. La coloración de verde de malaquita-fucsina ideado por Murohashi para diferenciar bacilos acido-alcohol-resistentes vivos y muertos, fué aplicado al bacilo leproso y fueron examinados los efectos de la temperatura en el paso de la coloración con verde de malaquita.

2. Coloreanda los bacilos leprosos con 1 por ciento de verde de malaquita disuelta en 0.2 M acetato bufer a un pH 4.2 se reveló la presencia de substancias basofílicas que fueron liberadas desde el bacilo leproso a 60°C en la presencia del colorante.

3. Por los resultados de varios examenes histoquímicos, se ha concluido que tales substancias basofílicas no eran DNA sino lipidos, presumiblemente derivados por la oxidación de acidos grasos insaturados.

4. La presunción basica con la coloración de verde de malaquita-fucsina, de que el verde de malaquita combina especificamente con el DNA altamente polimerizado a un pH de alrededor 4, no es valido para el bacilo leproso, y por esta razon la coloración de verde de malaquita-fucsina no puede ser aplicada al bacilo leproso para la diferenciación del bacilo vivo del bacilo muerto.

RESUMÉ

1. La coloration par la combinaison vert de malachite-fuchsine, telle qu'elle a été conçue par Murohashi pour différencier les bacilles acido-résistants vivants de ceux qui sont morts, a été appliquée aux bacilles de la lépre. Les effets de la température sur cette phase de la coloration par le vert de malachite ont été examinés.

2. La coloration des bacilles de la lèpre par le vert de malachite à 1% dissous dans 0.2 M de tampon acétate au pH 4.2 a révélé la présence de substances basophiles libérées des bacilles de la lèpre à 60°C en présence du colorant.

3. Les résultats de divers examens histochimiques permettent de conclure que ces substances basophiles ne sont pas de l'ADN mais des substances lipoïdiques, probablement dérivées par oxidation des acides gras non saturés.

4. L'hypothése de base sur laquelle repose la coloration par la vert de malachite-fuchsine, à savoir que le vert de malachite se combine spécifiquement avec des polyméres élevés d'ADN à un pH d'environ 4, n'est pas valable en ce qui concerne les bacilles de la lèpre. Pour cette raison, la coloration par le vert de malachite-fuchsine ne peut être appliqué aux bacilles de la lèpre pour la différenciation des bacilles vivants et morts.

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