The Carbol Fuchsin Staining of Mycobacteria in Leprosy Institutes of Southeast Asia and Japan, Especially the Effect of "Basic Fuchsin" used in the Carbol Fuchsin Formula

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Harada et al. (1976) has shown that batches of fuchsin having particular light absorbance characteristics give the most satisfactory acid-fast staining of mycobacteria.

One of the authors (Harada) went on a research trip to Phillipines, Singapore, Malaysia and Thailand as a dispatcher researcher of U. S.-Japan Cooperative Medical Science Program, from January 9 to February 8, 1978.

Harada inspected the staining procedure of carbol fuchsin stain of each institute and sanatorium and carried back samples of "basic fuchsin" used in the institutes to analyse the staining effects and absorption maximum of the dye.

We have also shown the staining procedure, staining effect and absorption maximum of the dye in Japanese institutes.

Materials and Methods

Mycobacteria used were tubercle bacilli in sputum, murine leprosy bacilli taken from murine leprosy nodules and leprosy bacilli taken from human lepromatous nodules. Staining procedure was done by Ridley's direction. Spectrophotometry was done under the direction of Lillie (1969) and Harada et al. (1976).

Results

Results are shown in table 1 and table 2.

1. Most of sanatorium used "hot method", while few sanatorium used "cold method".
2. 0.3% HCl-70% ethanol, 1% HCl-70% ethanol, 2% HCl-70% ethanol, 3% HCl-ethanol, 5% sulfuric acid, 10% sulfuric acid or 25% sulfuric acid were used as decolorizer. Decolorizing time varied from 5 seconds to 5 minutes.
3. Basic fuchsin used in the carbol fuchsin formula had an absorption maximum 545 to 556 μm .

In addition, batches of pararosanilin HCl had an absorption maximum varied from 545 to 550 μm .

4. The morphology and staining intensity of the bacilli were greatly affected by the composition of basic fuchsin used: When basic fuchsin having absorption maximum $<550\text{ }\mu\text{m}$ was used, almost all bacilli showed beading or weak staining and the number of stained bacilli was greatly reduced; with dye having $550\text{ }\mu\text{m}$, many bacilli show beading; with dye $>552\text{ }\mu\text{m}$, beaded bacilli were rare and the staining intensity of bacilli was increased.

Moreover, beaded staining could be demonstrated markedly in *M. tuberculosis* and *M. lepraemurium* than in *M. leprae*.

Discussion

Carbol fuchsin stain is used for demonstrating mycobacteria. However, it was initially developed for acid-fast bacilli such as tubercle bacilli. The leprosy bacillus is much less acid- and alcohol fast than the tubercle bacillus and frequently decolorized by the standard Ziehl-Neelsen technique. Therefore, in carbol fuchsin procedure the skin smears should be decolorized for few seconds in 1% HCl-70% ethanol (Ridley, 1964, Jopling, 1978). However, as shown in table 1, many workers did not use the Ridley's staining procedure.

The dyes marketed under the name of basic fuchsin may be either the chloride or acetate of pure pararosanilin, or mixture with its higher homologs, varying in color depth according to the proportion in which they are present.

Absorption maximum: pararosanilin $545\text{ }\mu\text{m}$; rosanilin $550\text{ }\mu\text{m}$; magenta II $554\text{ }\mu\text{m}$; new fuchsin $556\text{ }\mu\text{m}$ (Lillie, 1979).

The absorption maximum of basic fuchsin tested varies from 546 to $556\text{ }\mu\text{m}$. This indicates that basic fuchsin has no constant structure and composition. Some samples proved to be pararosanilin as a main component, while others showed deeper color than rosanilin and presumably contained appreciable quantities of magenta II or new fuchsin.

By the carbol fuchsin stain in which the carbol fuchsin formula having different absorption maximum was used, the bacterial and morphological index of leprosy bacilli in one laboratory could not be compared with against another.

Also, surprisingly, many leading laboratories for leprosy research used pararosanilin for Ziehl-Neelsen preparations (Harada et al. 1976).

In carbol fuchsin stain of mycobacteria, the pararosanilin with only amino groups may react with acidic groups of the bacilli merely by condensation reaction, while the rosanilin, magenta II and new fuchsin containing methyl and amino groups react with acidic groups through salt linkage and condensation reaction (Harada et al., 1976).

In order to differentiate pararosanilin from basic fuchsin with higher homologs, we should test the sample of basic fuchsin by spectrophotometry.

In addition, it should be noted that even the basic fuchsin with same catalog No. sometimes has different absorption maximum.

In our laboratory, we usually use pararosanilin for preparation of Schiff's reagent, Gomori's aldehyde fuchsin and carbol pararosanilin (Harada, 1977), while fuchsin with higher absorption maximum $>552\text{ }\mu\text{m}$ are used for preparing carbol fuchsin and resorcin fuchsin.

Table 1 Staining procedure of carbol fuchsin in Southeast Asia and Japan

Institute	Staining, hot or cold, time	Differentiating agent and time	Counterstain and time
Leprosy Research & Training Center, Thailand	CF, hot, 5 min	1%HC1-70%EtOH, 1 min	
Phra-Pradang Hospital	CF, hot, 5 min	1%HC1-70%EtOH, 5 sec	
Skin Clinic, Bangkok	CF, hot, 5 min	1%HC1-70%EtOH, 1 min	
McKEAN Hospital	CF, hot, 10 min	0.3%HC1-70%EtOH, several sec	MB 2 min
Sungei Buloh Hospital	CF, hot, 10 min	25% H ₂ SO ₄ aq., 8 sec	0.1% MB 5 min
Trafalgar Hospital	CF, cold, 40 min	1%HC1-70% EtOH, 1/2 min	MB 5 min
Leprosy Research & Training Center, Manila	CF, cold, 15 min	2%HC1-70%EtOH, until slide to pink	Loeffler MB
Central Luzon Sanatorium	CF,	3%HC1-70% EtOH. 3 sec	Loeffler MB
L. Wood Memorial Research Laboratory, Cebu	CF, cold, 2-30 min	1% HC1-70%EtOH, 1/4-1/2 min	Loeffler MB 1 min
Chingelput, India	CF, warm-cold, 20 min or hot 5-10 min	3%HC1-70%EtOH 5 min	Loeffler MB 1/2-min
Jalma, India	CF, cold	2%HC1-70 EtOH	
Gov. Skin Clinic Sing.	CF, cold 5-10 min	1%HC1-EtOH or 5% H ₂ SO ₄ , unknown	MB
Fukusei Hospital	CF, hot, 5 min	3%HC1-EtOH	Loeffler MB 4x 3-4 min
Matsugaoka	CF, hot, 5 min	3%HC1-EtOH	Loeffler MB 10x 1-2 min
Miyako	CF, hot, 5 min	1%HC1-70%EtOH few sec	Loeffler MB 10x 1-2 min
Amami	CF, hot, 5 min	1%HC-EtOH	Loeffler MB 10x
Tohoku	CF, hot	3%HC1-70%EtOH 1/2-1min	Loeffler MB
Kikuchi	CNF, hot, 10 min	1%HC1-70% EtOH	Loeffler MB 4x
Kagoshima	CF, hot, 3 min	1%HC1-70%EtOH 5 sec	Loeffler MB 4x 1/2 min
Kusazu	CF, hot, 5 min	1%HC1-70%EtOH 15 sec	Loeffler MB 10x
Okinawa	CF, hot, 3 min	1%HC1-70%EtOH 5 sec	Loeffler MB 15 min
Nagashima	CNF, hot, 3-4 min	10%H ₂ SO ₄ aq., 1/2-1 min	Loeffler MB 4x
Oku	CF, cold, 10-30 min	5%H ₂ SO ₄ aq., several sec	Loeffler MB 10x
Oshima	CF, hot-cold, 30-40 min	1%HC1-EtOH	Loeffler MB 6-10x
Tama	CF, hot, 5 min	1%HC1-70%EtOH few sec	Loeffler MB
Kyoto University	CNF, cold, 20-30 min	1%HC1-70%EtOH few sec	Loeffler MB 4-5x

The different staining and different decolorizer with different time were used in different laboratories (table 1). Hence it is necessary to standardize the carbol fuchsin formula and staining procedure for demonstrating acid-fast leprosy bacilli.

As mentioned above, leprosy bacilli are easily decolorized by strong acid such as HCl-ethanol or sulfuric acid. Therefore, we recommended hot carbol fuchsin stain with acetic acid as decolorizer (Harada and Kasai, 1978).

It has been argued among leprologists that solid acid-fast bacilli are alive and non-solid are dead (Jopling, 1978).

Table 2 Samples of "basic fuchsin" used in the carbol fuchsin formula in the leprosy sanatorium of Southeastern Asia and Japan, and the effects of "basicfuchsin" on beading of mycobacteria

Sample	C.I. No.	Cat. No.	Lot. No.	Maker	Absorption maximum μm	Institute
Fuchsine Basic*	42500		0968540	B.D.H.	547	Leprosy Research and
Fuchsin (NB) Pulver	42520		622490	Merck	553	Training Institute, Thai-
Fuchsine Powder				Gov. Thai	553	land
Fuchsin (NB) Pulver			9011915	Merck	553	Phra-Pradang Hospital, Thailand
Basic Fuchsin				G.T. Gurr	554	Institute of Medical Research, Malaysia
Basic Fuchsin	42500			H. & WWK.	553	
Basic Fuchsin*			1681870	B.D.H.	547	
Bosic Fuchsin*			15046	Gurr	549	Sungei Buloh Hospital, Malaysia
Fuchsin basique			746685	Belgium	551	
Diamantfuchsin		490347	Reag. DAB7 (R405)		553	
Basic Fuchsin*			21223	G.T. Gurr	554	
Basic Fucnsin				Merck	546	Trafalger Hospital, Sing.
Fuchsin Basicum*		42517	24644	Carlo Erba	549	Leprosy Research and Training Center, Manila
Basic Fuchsine	42510		81853 B 65113	H. & Wm.	552	
Basic Fuchsine	42500		CF No. 48	C. & B.	550	
Basic Fuchsine	42500		CF No. 77A	C. & B.	551	Wood Memorial, Cebu
Fuchsin Basic*	42510		70201	Harleco	545	
Fuchsin Basic	42500	39402	L/927/63 36/2	B. D. H.	553	Chinglput, India
Fuchsin Basic	42500	39402	L 598/06 3706	B. D. H.	553	Jalma, India
Basic Fuchsin	42520	4040	70194490	Merck	553	Gov. Skin Clinic Sing.
Basic fuchsin*			406 $\times$ 5024	Wako	548	Fukusei Hospital
Fuchsin Basic*	42510		LDF 4530	Wako	549	Matsugaoka
Fuchsin Basic*			WH 1239	Wako	549	Miyako
Diamantfuchsin	42510	1A390	4.76	Chroma	550	Amami
Diamond Fuchsin	42510	1358	2489416	Merck	550	Tohoku
Newfuchsin Pulver	42520	782	5229266	Merck	554	Kikuchi
Fuchsine (basic)*			V5R1435	Hani	548	Kagoshima
Basic Fuchsin	42510			Merck	553	Kusatsu
Basic Fuchsin*			340279D 24	Ishizu	549	Okinawa
Basic Fuchsin	677 (1ed)		C.F. No. 43	Nat. An.	550	Oku
Fuchsin Basic*	677 (1ed)			C. & B.	549-50	
New Fuchsin		B-721	300361	C. & B.	554	
Fuchsin (NB) Pulver		4041	6411	Merck	554	Nagashima
Fuchsin Basisch	42510	1A308	6.75	Chroma	550	Oshima
Diamantfuchsin	42510	1A390	1.76	Chroma	550	
New Fuchsin	42520	1A390	3640831	Merck	554	
Fuchsin Basic*		4041	TP2873	Wako	549	Suruga

Table 2 (continued)

Sample	C.I. Na.	Cat. No.	Lot. No.	Maker	Absorption maximum μm	Institute
Fuchsin (NB) Pulver	42520		7021224	Merck	554	Tama
Diamantfuchsin	42510	10504		Chroma	554	
Diamantfuchsin	42510	1358	3640026	Merck	550	
Diamantfuchsin	42510		4607428	Merck	550	
Diamantfuchsin	42510		52338	Merck	552	
Fuchsin Basisch*	42510	10755		Chroma	549	
Fuchsin Basisch*	42510	1A 308	1.75	Chroma	546	
Fuchsin bas. for* Schiff's reagent	42500	10757		Chroma	547-8	
Basic Fuchsin*	677 (1ed)		CF No. NF 51	Nat. An.	546	Tama
Fuchsin for bacteria stain	42510	10760	11.76	Chroma	553	
Newfuchsin	42520	1B 467	7.73	Chroma	556	
Diamond Fuchsin		1358	21163	Merck	550	
Diamantfuchsin	42510	10540	7002	Chroma	554-5	
Diamantfuchsin	42510	10540		Chroma	554-5	
Diamantfuchsin	42510	10540	6712	Chroma	554-5	
Diamantfuchsin	42510	1A 390	7.76	Chroma	550-1	
Diamantfuchsin	42510	1A 390	8.76	Chroma	550-1	
Diamantfuchsin	42510	1A 390	1.76	Chroma	550-1	
Diamantfuchsin	42510	1A 390	11.76	Chroma	551	
New Fuchsin	42520		3640831	Merck	554	Kyoto University
Fuchsin (NB) Pulver	42520			Merck	553	Osaka University
Diamond Fuchsin	42510	1A 390	1.76	Chroma	550	Tokyo Womens Medical College
Diamond Fuchsin	42510	1858	1418977	Merck	550-1	Institute for Medical Research, Japan
Fuchsine Basic*			012B 0233	Kanto	550	
Diamantfuchsin	42510		2574632	Merck	549-50	
p-Rosaniline HCl*	42500	1B 297	5.76	Chroma	546	Tama
p-Rosaniline HCl*	42500	1B 297		Chroma	546	
p-Rosaniline HCl*	42500	224	711-1	Eastman	545	
p-Rosaniline HCl	42500	1B 297	9.76	Chroma	550	
p-Rosaniline HCl	42500	1B 297	4.77	Chroma	550	

* intense beading

Addendum: Basic fuchsin used in leading leprosy research laboratory for carbol fuchsin formula cited from Microscopia Acta 78, 21, 1976. Barksdale, New York University, Basic fuchsin C. I. 42500, Allied Chem. NF 86. Chang, National Institute of Health, Newfuchsin C. I. 42520, Allied Chem. 1165-0971, 555 μm . Basic fuchsin C. I. 42510; Chroma ZF-2, 550 μm . Hanks, John Hopkins University, New fuchsin. Kirchheimer, U. S. Public Health Service Hospital, Carville, Basic fuchsin C. I. 42500, Allied Chem. CF 72. Murry, U. S. Public Health Service Hospital, San Francisco, Basic fuchsin C. I. 42500, Coleman & Bell. 546-7 μm . Nyka, V. A. Hospital, Baltimore, Basic fuchsin C. I. 42500, Allied Chem. NF 88, 546 μm . Rees, National Institute for Medical Research, London, Basic fuchsin, E. Gurr, Cat. No. MN 412 554 μm . Ridley, Hospital of Tropical Disease, London, Basic fuchsin G. T. Gurr 22902, 547 μm . Shepard, Communicable Disease Center, Atlanta, Basic fuchsin C. I. 42500, Coleman & Bell, CF 63, 552 μm .

This situation is confused by the fact that some workers use pararosanilin; while others use fuchsin with higher homologs (Harada et al. 1976). Moreover, some workers seem actually to prefer the beaded staining (Lillie, 1969).

Mycobacterium seems to be a two phase organism which can appear in either acid-fast form or chromophobic form, depending upon its environment. The chromophobic bacilli can only be demonstrated by periodic acid-carbol pararosanilin stain. Of course, the acid-fast bacilli can be stained more intensely by periodic acid-carbol pararosanilin than with classic carbol fuchsin (Nyka and O'Neill, 1970, Harada, 1977).

As a result, smears stained with periodic acid-carbol pararosanilin give significant greater bacterial and morphological index than those with classic carbol fuchsin (Harada and Kasai, 1978).

Similar findings were found in tissue sections (Harada, unpublished).

Because of the two phase property of mycobacteria, the classic carbol fuchsin stain cannot be sufficient for estimating the stop of treatment and for considering the leprosy patients as non-infectious.

Accordingly, the periodic acid-carbol pararosanilin stain is the most reliable and preferable technique for demonstrating leprosy bacilli.

Summary

We analysed the staining effect of carbol fuchsin, especially the composition of basic fuchsin present in the carbol fuchsin formula which is used in the leprosy institutes in Southeast Asia and Japan.

The classic carbol fuchsin stain is unstable for demonstrating leprosy bacilli.

The carbol fuchsin stain using the basic fuchsin with low absorption maximum (Pararosanilin) shows beaded staining bacilli in contrast to roded staining bacilli with high absorbance basic fuchsin.

Different institutes use different staining methods—hot or cold—with basic fuchsin having an absorption maximum varying 546 to 556 μm and different decolorizers with varying decolorizing times.

Therefore, it is difficult to give a constant result of the bacterial and morphological index in different institutes.

Accordingly, the classic carbol fuchsin staining technique is not sufficient for estimating the stop of treatment and for considering the patients as non-infectious.

It is essential to standardize the staining procedure by adapting basic fuchsin with high absorbance for the assesment of the bacterial and morphological index of the leprosy bacilli.

Moreover, the problem lies on the present of chromophobic bacilli that does not stain by the classic carbol fuchsin stain. In this instance the periodic acid-carbol pararosanilin stain is the most reliable and preferable method.

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東南アジア及び日本の癩施設での抗酸性菌の 石炭酸フクシン染色について

特に石炭酸フクシン溶液に使用される塩基性
フキシンの効果について

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我々は石炭酸フキシンの染色効果とくに東南アジア及び日本の癩施設に於て使用されている石炭酸フクシン溶液の中の塩基性フキシンの組成のそれについて研究した。

異った施設は異った染色法, hot 又は cold, 546~556 μm の吸収曲線をもった塩基性フクシンを使用, 異った脱色剤, 脱色時間を使用している。

低い吸収をもった石炭酸フクシンでは菌は Bead 状の染色を示し, これに反し, 高い吸収性をもつフクシンでは Rod 状を示す。だから異った施設で同一の結果を

得ることは困難であり又 BI 及び MI も同様である。

この問題は石炭酸フクシン法によって染色されない chromophobic 菌の存在により混乱する。従って古典的
石炭酸フクシン法は感染性を決定したり治療を止めるためには信頼出来ない。

癩菌の BI と MI を決定するためには高い吸収をもつ塩基性フクシンを使用する染色法を標準化することが必須である。抗酸性と chromophobic 菌の両者を染める過沃度酸・パラロザニン染色はだからもっとも信頼出来, そして適切な方法である。