DEMONSTRATION OF CAPSULES ON *M. LEPRAE* DURING CARBOL-FUCHSIN STAINING MECHANISM OF THE ZIEHL-NEELSEN STAIN

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In a preceding paper (2) I have set forth evidence that pathogenic mycobacteria are covered with exceptionally impermeable capsules and matrices, and that these must be modified or removed before the cells can be penetrated and stained by safranin 0, basic fuchsins, or crystal violet. Three factors found to modify and/or remove capsules from pathogenic mycobacteria were: heat, phenol and 5-10 per cent chloroform in aqueous suspension. These observations suggested that disruption of capsular structures by heat-fixation and by the dilute phenol in hot carbol-fuchsin might explain the rapid staining by the Ziehl-Neelsen method. It seemed also that removal of capsular components by this classical method might explain the failure of the usual light microscopy to suggest the presence of the extracellular structures which attract interest in electron micrographs. The results of this study support these premises, and define conditions which delineate capsules on *Mycobacterium leprae* after the application of carbol-fuchsin.

METHODS

Stocks of CO_2-frozen lepromatous skin were used, for which I am indebted to Dr. Chapman H. Binford. The simplest method of liberating the bacilli was to lay a fragment of skin on its epidermal surface in a small pool of water on a porcelain spot plate, and gently to scrape the subcutaneous surface of the tissue with a scalpel until readily-emulsified components had been freed from the insoluble collagen and other elements of the dermis. The resultant suspensions were transferred to small tubes. Rinsings from the tissue and spot plate were added to equal 10 times the original volume of tissue. Films were prepared as previously described (2) and dried for only three seconds before being immersed in formalin-alcohol for 5 minutes. As soon as these agents had evaporated, the coverslips were immersed in a dye, or subjected to modifying procedures or reagents which will be indicated in the tables and their footnotes. The special fixation was necessary to retain the films and bacilli during immersion in solutions lacking the fixative effects of cationic dyes (1).

Reagents: Formalin-alcohol: 1 part formalin solution (37-40% formaldehyde) in 9 parts of 95 per cent alcohol. Carbol-fuchsin: 0.3 per cent basic fuchsin (magenta III) in 4.5 per cent phenol/wt. and 8.6 per cent alcohol/vol. (1). The foregoing concentrations of phenol and alcohol, separately and combined, in water. $Sulf_4MB$ differentiator: 0.2 per cent methylene blue in 4 per cent/vol. of concentrated sulfuric acid (1). Safranin 0: 0.4 per cent in 0.02M PO_4 buffer, pH 6.5. Surface coater: 1 per cent nigrosin in water containing 0.01 per cent Tween 80.

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EXPERIMENTAL RESULTS

Before considering staining times and temperatures, it must be noted that preliminary fixation of films in formalin-alcohol causes the capsules of the leprosy bacilli to become tougher and less permeable than those on fresh bacilli ([3]).

As shown in Table 1, after exposure of unheated bacilli to carbol-fuchsin for 5 minutes at 37°C, approximately two-thirds of the rods are lightly stained. The majority of these show distinct capsular halos, usually with definite outer margins. Approximately one-third of the wider rods remain unstained. Heat fixation (98°C for one minute; actually less than that due to warming time) results in a higher proportion of stained rods, but these show less distinctive capsules and fewer sharply-defined capsular margins. Both heat fixation and hot carbol-fuchsin are required to stain 100 per cent of the cells. Only one-third of the cells are surrounded by narrow halos, and these shade into the nigrosin coat without definite outer margins. In the absence of a nigrosin coat the presence of these residual halos would not be suspected.

TABLE 1.—*The effect of heat-fixation and hot carbol-fuchsin solutions on modification or removal of capsules and on the staining of* M. leprae.

Heat [a] fixation 98°C	Carbol-fuchsin stain	$Sulf_4MB$ differentiation	Nigrosin coat	Per cent of bacilli			Total bacilli /strip	No. of globi with majority of bacilli [c]	
				Red w/o capsules[b]	Red w/ capsules[b]	White		Red	White
(None)	5 min. 37°C	4 min.	+	6	63	31	333	10	13
1 min.	5 min. 37°C	4 min.	+	7	79	14	312	16	4
1 min.	1 min. 98°C	4 min.	+	65	35	0	288	16	1
1 min.	1 min. 98°C	4 min.	—	100	0	0	(?)	19	0

[a] Coverslips were laid on a glass plate over boiling water, and then transferred directly to carbol-fuchsin solution at 37°C, or flooded with this solution and allowed to remain on the hot plate an additional minute.

[b] Capsules: w/o = rods narrow, red, nigrosin coats lying against the cell walls; w/ = stained rods with a white halo of capsular component, or white rods which may appear to be 2-3 times wider than decapsulated organisms.

[c] Most globi contain some red and some white bacilli.

Although classification of results in globi are necessarily less exact, similar effects are seen.

Since the staining of pathogenic mycobacteria proceeds in carbol-fuchsin much more rapidly than in aqueous basic fuchsins, the effect of dye solvents was assayed by exposing films to the diluents at 37°C for 30 minutes, and then immersing them in safranin 0 overnight. As shown in Table 2, the combination of dilute phenol and alcohol caused the greatest increase in permeability and the lowest total counts. Dispersion of internal granules was indicated by diffuse rather than granular staining.

TABLE 2.—*Modification of the permeability of* M. leprae *by the solvents employed in carbol-fuchsin solutions, assayed by means of safranin O.*

Solvents [a] (37°C, 30 min.)	Safranin[b] 0.4% 37°C	Per cent of bacilli			Total bacilli /strip	No. of globi with majority of bacilli	
		Red w/o capsules	Red w/ capsules	White		Red	White
4.5% phenol, 8.6% alcohol	18 hrs.	1	70	29	278	11	10
4.5% phenol, —	18 hrs.	0	65	35	316	11	12
— 8.6% alcohol	18 hrs.	0	10	90	336	6	12
Water	18 hrs.	0	13	87	394	8	16

[a] After incubation in the solutions listed, the coverslips were rinsed in water and inverted on safranin.

[b] Following exposure to safranin, the slips were rinsed 2 × in water (2. min.) and nigrosin coats applied.

Dilute phenol alone was almost as effective as the combination in modifying permeability, and definitely less disruptive to internal granules. Exposure to dilute alcohol alone did not modify permeability or granules significantly. After each treatment the corresponding effects were seen in globi. As described in the section above, factors which prepared the bacilli for dye permeation decreased the size and distinctness of capsular halos.

After applying the respective treatments, similar results and much more brilliant red stains were obtained by exposing *M. leprae* to carbol-fuchsin for 5 minutes at 37°C. However, the difference between the first two preparations (presence of dilute phenol) and the second two (exposed to alcohol and water) was smaller. This was due in part to the phenol and alcohol in the fuchsin solution, and in part to the more progressive staining action of the triphenyl methane dyes ([2]).

DISCUSSION

The fact that capsular components on pathogenic mycobacteria must be modified or removed in order to stain 100 per cent of the cells is not an insurmountable impediment to the study of capsules by light microscopy. Two methods apply the principle of staining less than 100 per cent of the cells and then delineating capsules and the least permeable cells by surface coating. These are: immersion of briefly dried, unheated films in aqueous crystal violet for one hour at 37°C ([2]) and exposure to carbol-fuchsin for 5 minutes after fixation of unheated films in formalin-alcohol for 5 minutes. Because of the solvent anl lytic action of the dilute phenol and alcohol in carbol-fuchsin, differences in impermeability can be shown more dramatically by the use of aqueous crystal violet or fuchsin. Whether aqueous reagents will permit com-

parable expansion of capsular halos following the fixation used in this study has not been determined.

SUMMARY AND CONCLUSIONS

When films containing *M. leprae* were fixed in formalin-alcohol rather than by heat, exposed to carbol-fuchsin for 5 minutes at 37°C, and then coated with nigrosin, the majority of the rods lightly stained by carbol-fuchsin were surrounded by capsular halos with definite margins.

Analysis of factors which modify capsules and prepare the bacilli for dye permeation has shown that heat and dilute phenol are the primary capsular solvents in the Ziehl-Neelsen procedure. The combination of dilute phenol and alcohol as solvents for fuchsin converts the mycobacteria from granular to diffuse staining, because of the dissolving of internal granules.

RESUMEN Y CONCLUSIONES

Cuando se fijaron en formalina-alcohol más bien que al calor las películas que contenían *M. leprae* y se expusieron al carbol-fuchsina por 5 minutos a 37°C. y se recubrieron luego con nigrosina, la mayoría de los bastoncillos teñidos ligeramente por el carbol-fuchsina fué circundada por aureolas capsulares de bordes bien definidos.

El análisis de los factores que modifican las cápsulas y preparan los bacilos para la penetración en seco ha demonstrado que el calor y el fenol diluído son los solventes capsulares primarios em el procedimiento de Ziehl-Neelsen. La combinación del fenol diluído y del alcohol como solventes para la fuchsina, convierte a las micobacterias de la coloración granular a la difusa, debido a la disolución de los gránulos internos.

RESUMÉ ET CONCLUSIONS

Lorsque des frottis de *M. leprae* sont fixés par un mélange de formol et d'alcool plutôt que par la chaleur, exposés a la fuchsine phéniquée pendant 5 minutes à 37°C, puis recouverts de nigrosine, la plupart des bacilles colorés légèrement por la fuchsine sont a present entourés d'un halo capsulaire à bords nets.

L'étude des facteurs qui modifient les capsules et préparent les bacilles pour la pénétration des colorants a démontré que la chaleur et le phénol dilué sont les solvants majeurs des capsules lors du procédé de Ziehl-Neelsen. La combinaison de phénol dilué et d'alcool comme solvants pour la fuchsine dissout les granules internes des mycobactéries et, pour cette raison, transforme la coloration granulaire en une coloration diffuse.

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