SIGNIFICANCE OF CAPSULAR COMPONENTS OF *MYCOBACTERIUM LEPRAE* AND OTHER MYCOBACTERIA

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Early investigators ([1, 17]) of the microscopic properties of *Mycobacterium leprae* were puzzled by the nature of the material which binds this microorganism into such exceptional clumps and globi, and by the question whether these materials originated from the bacillus or the host. Since such components are removed by the usual heating of mycobacteria with carbol-fuchsin ([13]), study of these structures was interrupted by universal adoption of the Ziehl-Neelsen stain.

Interest in the significance of capsular components was aroused in the course of experiments in which dyes were employed to study factors which might decrease the impermeability of pathogenic mycobacteria to tetrazoles.[3]

Hence two conditions prevailed in the majority of the experiments: (*a*) the use of films containing metabolically active microorganisms, and (*b*) routine surface coating with nigrosin or Congo red to delineate 100 per cent of the bacilli, whether colored or white. The extraordinary widths of unstainable bacilli contrasted with the widths of those which stained, while clear halos were often seen around rods in which staining was being initiated.

Further experiments revealed that rates of staining were determined by the character, amount and distribution of capsules and capsular matrices, and that there are interesting associations between these structures and the capacity to persist in hosts. One of the interesting examples of an increase in permeability due to loss of capsules was afforded by the senescent populations of bacilli in sulfone-treated leprosy patients.

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[1] This investigation was supported in part by a Research Grant (E-827) from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

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[3] Cytologic evaluation of metabolic activity in individual organisms requires the penetration of tetrazoles (hydrogen acceptors) into internal sites of metabolic activity, and then their reduction to insoluble pigments (formazans). The problem of impermeability in pathogenic species was emphasized by the observation that optimal physiologic state and infectiousness lowered the proportions of cells in which such activity could be demonstrated.

MATERIALS AND METHODS

M. leprae from scraped skin incisions of patients and from CO_2-frozen fragments of excised lepromatous skin [4] were diluted approximately 1:50 in distilled water. For comparison, observations were made with 48-72 hour cultures of the HMS strain of *M. phlei*, 7-10 day cultures of the Phipps strain of BCG grown on the Petragnani medium, and with the Hawaiian and Wells strains of *M. leprae murium* recovered directly from animal lesions or from stocks which had been refrigerated in albumin and yeast supplement ([10]). Appropriate numbers of these organisms were provided by 1:200 or 1:400 dilutions of 10 per cent tissue suspensions.

In order to study natural properties, these mycobacteria were used immediately after dilution in distilled water. Spreads for microscopic study were not exposed to the usual drying and flame fixation. Aliquots of 0.000,3 cc. were transferred with a 28 gauge, 0.7 mm. loop, and arranged in rows on a series of 6×50 mm. coverslips or on clean glass slides, and dried for only 3 seconds at room temperature before rehydration by dyes or other solutions adjusted to pH 7.2. The slides or coverslips were then immersed in, or inverted on, the chosen solutions and incubated at 37°C for intervals of 3 minutes to 20 days before the films were differentiated. Safranin 0 at pH 6.5 required merely two rinses in 10 per cent formalin solution and two in water. Films exposed to crystal violet were differentiated by the Gram method: they were dipped 2 times in water, covered with Lugol's iodine for 60 seconds, rinsed in 95 per cent alcohol for 60 seconds, shaken and allowed to drain dry. Many studies on differentiation of crystal violet stains failed to produce a better method. Differentiation in the presence of acetone is not reliable.

Visualization of 100 per cent of the organisms and of macrocapsules [5] and capsular matrices was assured by surface coating with opaque dye substances which do not crystallize upon drying. Preparations which had been exposed to eosin, safranin or the fuchsins were flooded with 1 per cent nigrosin in water containing 0.01 per cent Tween 80, while those exposed to blue dyes were coated with 1 per cent Congo red in water. The lateral edge of the coverslip or slide was immediately touched to a blotter or absorbent paper to promote rapid and uniform drainage of the coating solution.

The percentages of bacilli stained were recorded while traversing the diameter of each film with the oil immersion objective, meanwhile recording the presence or absence of capsular halos around the bacilli and noting the number of clumps or masses (containing more than 10 bacilli) in which the majority of the bacilli were stained or white.

Other coverslips or slides were exposed to safranin or crystal violet at elevated temperatures (50°-98°C), to ensure staining of 100 per cent of the bacilli, while still others at times were stained by carbol-fuchsin and differentiated with 0.2 per cent methylene blue in 4 per cent concentrated sulfuric acid/vol. ([11]).

EXPERIMENTAL RESULTS

Exclusion of dyes.—Nine dyes, dissolved in water and adjusted to pH 6.8-7.4 were compared for their capacity to permeate and stain internal structures in *M. phlei* and *M. leprae murium* during incubation at 37°C for at least 24 hours. Each dye was used at concentrations which yielded clear stable solutions in water. Eosin (0.3%) caused

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[4] I am indebted to Dr. Rolla R. Wolcott and the laboratory staff at the Carville hospital for fresh samples, and to Dr. Chapman H. Binford for frozen specimens of skin from the Philippines.

[5] Wilkinson ([19]) distinguishes between macrocapsules which can be demonstrated by light microscopy and microcapsules which are less than 0.5μ wide and hence not visualized in light microscopy.

poor retention of films, stained only low percentages of *M. phlei* after 6-8 hours, and failed to stain *M. leprae murium*. Thionin, methylene blue and toluidine blue (all at 0.2%) imparted bluish tints to some rods of *M. leprae murium*, but produced no staining of internal structures. Malachite green (oxalate) always flocculated during efforts to neutralize its solutions to point where intact internal granules could be stained.

Safranin O (0.4%) afforded remarkable distinctions between *M. phlei* and *M. leprae murium*. It stained the granules in 100 per cent of *M. phlei* cells in one hour; but only 1-5 per cent of *M. leprae murium* in 24 hours and in but 25 per cent during 10 days incubation. Crystal violet and basic fuchsins (pararosanilin and Magenta III) caused very rapid staining of granules in *M. phlei* and slow but progressive staining of these structures in *M. leprae murium*.[6]

Comparison of impermeabilities.—The data presented in Fig. 1 permit two generalizations. Firstly, the rate of staining the saprophytic *M. phlei* accelerates with time, whereas staining of normal pathogens and BCG proceeds progressively more slowly. Secondly, within any species, the impermeabilities of individual cells differ so greatly that logarithmic time scales are required to plot the results.

If results with safranin were illustrated, the differences between *M. phlei* and the pathogenic species would be greatly magnified. The exclusion of crystal violet cannot be attributed to the dilute solutions employed. Only 60 per cent of the cells of *M. leprae murium* were stained during four days continuous incubation in Hucker's crystal violet. If, on the contrary, the capsules on this species were modified by heat-fixation on a hot plate (98°C) for 1 minute, this solution stained 90 per cent of the cells during 5 minutes at room temperature.

The curves in Fig. 1 may not reflect the true state of normal cells of clumped species (BCG and *M. leprae*) as they grow *in situ*. By the time suspensions have been prepared by suspending cultures or scraping skin, the natural relationships are disrupted. Many single cells stain readily, while those projecting from clumps often acquire dye from one end as though partially disrobed of capsular materials. Furthermore, it is difficult to enumerate or estimate the proportions of the rods which remain unstained in clumps and globi.

The time required to stain 100 per cent of *M. leprae* from the four untreated patients and one treatment failure could not be determined satisfactorily. After 24 hours in crystal violet, these unwashed preparations exhibited numerous stained extra-bacillary granules. The sharp flexure of curve (*f*) after one hour, however, indicates that the bacilli

[6] Safranin O is more soluble in water than in alcohol. Crystal violet and the fuchsins are triphenylmethanes, and more soluble in alcohol than water. Since the fuchsins would not yield stable solutions in distilled water without alcohol, crystal violet became the dye of choice for comparisons between pathogenic species.

remaining unstained at that time were very impermeable. *M. leprae* from treated patients did not present the difficulties cited for BCG and the more normal *M. leprae,* since 100 per cent of the cells stained in less than one hour, irrespective of clumping.

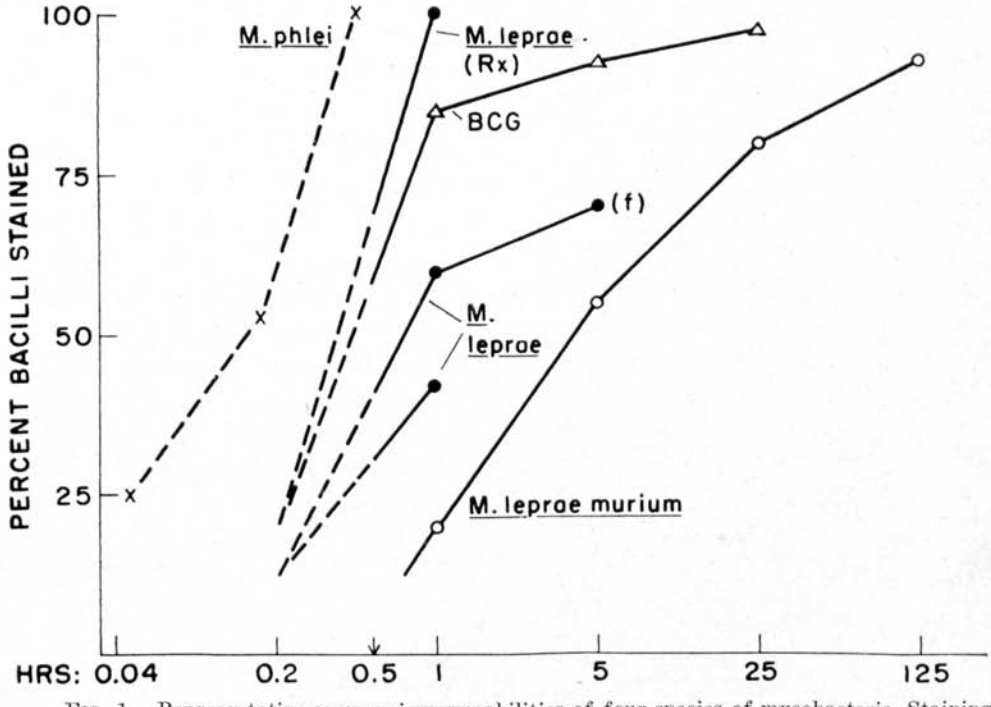

FIG. 1. Representative average impermeabilities of four species of mycobacteria. Staining rates in 0.04 per cent aqueous crystal violet, pH 7, 37°C, *M. leprae*: The curve marked (Rx) refers to bacilli obtained directly from 6 sulfone-treated patients; the unmarked curve refers to bacilli obtained directly from 1 untreated patient and 1 treatment failure; the bacilli of the (f) line come from 3 untreated patients but were frozen with solid CO_2 for 6 months.

Since 100 per cent of *M. phlei* cells stained in 27 mnutes, curves for pathogenic species are broken during the first 30 minutes in order to suggest the proportions of cells which may have been comparable to saprophytic cells. The proportions which stained more slowly (solid lines) may be considered to possess the attributes of pathogens.

The smoothest curves and slowest staining rates occurred with *M. leprae murium,* because of the uniform enclosure of individual bacilli in microcapsules ([6]). To stain 100 per cent of the cells required 10-14 days incubation at 37°C.

Capsules and impermeability.—The extracellular matrix of *M. phlei* promotes clumping but, even within clumps, it cannot be demonstrated by surface coating. The 85 per cent of BCG rods which stain within one hour are of normal width, while the remainder appear wider and many exhibit irregular outlines of matrix in Congo red coats. Between the first and 24th hours of staining some clumps are surrounded by a ball of matrix which prevents the delineation of individuals by surface coating.

In the case of the leprosy bacilli from the treated patients, no halos have been seen. The Congo red coats lie directly against the walls of the stained bacilli, even when in clumps. Of bacilli derived from untreated patients, the lower proportions which stain within the first hour are of similar appearance. The cells which resist dye permeation look larger. If partially or lightly stained, these bacilli are surrounded by halos which sometimes are as wide as the diameter of the enclosed rod. Although globi usually contain some stained organisms, they occasionally are outlined in surface coats as pure white masses.[7]

In the preparations discussed above the differing widths of stained and unstained rods of *M. leprae murium* are apparent in surface coats, but capsules cannot be seen, The first demonstration of capsular structures required ultra-thin sections and electron microscopy ([6]). In the micrographs it can be seen that the cells tend to be covered by individual microcapsules. Further studies have permitted demonstration of the microcapsules by light microscopy. Following exposure to 0.4 per cent crystal violet for 30 minutes at 50°C some of the bacilli are stained and some are not. The stained bacilli are decapsulated. The rods which are just acquiring a purple tinge in the granules exhibit extrabacillary structures in two forms, which are dependent upon two preliminary treatments. If they are from clean suspensions refrigerated in albumin and yeast supplement and then diluted in 0.5 per cent serum, clear halos are seen. If cruder suspensions have been digested in 0.5 per cent bile plus 1 per cent pancreatin for 10 minutes at 37°C, and the films then fixed in formalin-alcohol, the Congo red coats are thinned and ruffled for a distance which is several times greater than the diameter of the rods.

The stability of capsules on pathogenic species.—Many unsuccessful attempts to modify the permeability of capsules, or to remove them by methods which leave the cells metabolically active, emphasize the fact that the extracellular components are infinitely more inert and rugged than internal structures and functions. The relatively impermeable cells in highly infectious suspensions of *M. leprae murium* have not been modified by any reasonable exposures to drying; to acids and alkalies; to Tween 80 or saponin; to trypsin, pancreatin, ribonuclease or lysozyme; or by 24 hour immersions in petroleum ether, carbon tetrachloride or 1:1 mixtures of ethyl ether in absolute alcohol; or by 7-day exposures to petroleum ether and carbon tetrachloride.

None of the solvents mentioned facilitates staining of capsules or internal lipids by Sudan black. Failures to stain with Sudan black in

[7] Differences in the encapsulation of *M. leprae* in clumps and globi from untreated and sulfone-treated patients can be recognized after carbol-fuchsin staining and differentiation in sulfuric methylene blue. The point is to observe the spacings between the clumped rods. Those from untreated patients are usually not in contact with one another; those from treated patients lie side by side.

70 per cent alcohol at elevated temperatures, or during incubation at 37°C for 7 days, perhaps are due to the fixative effect of alcohol, which toughens capsules and impedes the entry of other compounds ([12]). Whether the exclusion of safranin and tetrazoles from encapsulated pathogenic cells and the failure to obtain staining with certain dyes is due to fixative effects has not been investigated.

At present, experimental modification or removal of capsules from pathogens is limited to four conditions. They are modified slowly during incubation in triphenylmethane dyes (many cells thereafter are permeable to safranin), more rapidly during incubation in 5 per cent phenol in water, and very promptly during heating at 98°C. They are removed by brief shaking with 5-10 per cent chloroform in aqueous suspension.

Control experiments.—Since the staining of nuclear materials in many bacteria is possible only after hydrolysis of DNA complexes, it may be argued that the foregoing observations on granule staining do not depend upon impermeability, but upon modification of nucleoproteins. Other criteria, therefore, are required to evaluate the proposition that impermeability is a significant determinant of the rates at which mycobacterial granules are stained by safranin, the fuchsins and crystal violet.

Pertinent data are contained in Table 1. The rates at which metabolic activity of dense suspensions of mycobacteria can lead to extracellular reduction of artificial hydrogen acceptors [e.g., methylene blue ([4]) and monotetrazoles ([9, 10])] depend in part upon the rates at which reduced compounds are exuded from the microorganisms. The proportion of cells exhibiting internal granules of formazan after anaerobic incubation with tetrazolium blue does not test for nuclear struc-

TABLE 1.—*Impermeabilities of five mycobacteria compared by additional criteria.*

Species	Rates of hydrogen transfer by dense suspensions	Proportion of bacilli exhibiting stained granules			Time required for damage by:	
		Time (37°C)	TzB [a] 0.1%	Safranin 0.4%	KOH	Drying
M. phlei	rapid	1 hr.	97%	100%	0.03N, 15 min.	3 sec.
BCG [b]	slow	48 hr.	60%	66%		3 min.
H-37Rv [b]	slower	48 hr.	33%	38%		
M. leprae [c]		48 hr.	9-47%			
M. leprae murium	slowest	48 hr.	4%	5%	1.0 N, >15 min.	>3 min.

[a] Tetrazolium blue 0.1 per cent, glycerol 0.3M,M/10 PO_4 pH 7.5; strict anaerobiosis.

[b] Results for BCG and H-37Rv show the highest permeabilities which have been observed in these two species.

[c] All samples from patients after some degree of treatment. Note the variable permeabilities, and that in the most permeable (i.e., largely decapsulated) sample at least 47 per cent of the bacilli were metabolically active.

tures but for penetration of tetrazoles into the major sites of reduction, which are mitochondrial equivalents. Failures of this compound to reach mitochondrial sites agree closely with those obtained in the presumed nuclear staining by safranin 0. The results with KOH afford a more conventional test of impermeability. Resistance to drying in thin films tests primarily for ability to retain water and osmotic equilibria in the midst of the supersaturated salt solutions near the centers of such films.

DISCUSSION

For some time it has been known that the differing responses of mycobacteria to neutral red ([8]) and other tests for dye adsorption ([7]) are due to surface characteristics which are associated with pathogenicity. The results of this study indicate that these properties are attributable to true capsules and capsular matrices.

In order to understand the influence of capsules on pathogenicity or behavior *in vitro* one must consider properties of the species or strain, and also those determined by the physiologic state of cells.

As an example of special properties within a species one may emphasize the unique arrangement and ruggedness of the microcapsules on *M. leprae murium* from active lesions. These have been shown to explain the remarkable impermeability to tetrazoles and safranin 0 and the slow staining rates with crystal violet. The infectiousness of this species has exhibited unexpected tolerances to acids and alkalies ([2]), to antiformin ([14]), to carbol-fuchsin solutions for one hour ([14]), to incubation in methylene blue for 24 hours ([3]), and to heating at 60°C for 30 minutes ([15]). Under the conditions of the present study the usual acids and alkalies did not increase permeability, and carbol-fuchsin did not stain 100 per cent of unheated cells within 24 hours. After heating suspensions or films at 60°C for 30 minutes, safranin stained only 92-96 per cent of cells within 24 hours and crystal violet did not stain 100 per cent within one hour.

Incubated suspensions of *M. leprae murium* remain infectious for susceptible rats for only 12-14 days ([16]). This is the period required to stain 100 per cent of cells during continuous incubation in crystal violet.

In respect to physiologic state, many studies have shown that bacterial capsules tend to be abundant and effective during active synthesis ([19]) and to be lost from senescent cultures. The pathogenic mycobacteria seem not to be an exception. Bloch's investigations of surface properties related to the infectiousness of H-37Rv showed that three-day cultures were less permeable ([4]) and more virulent ([5]) than 21-day cultures containing equivalent numbers of viable organisms. Werner ([18]) observed that young cultures of virulent strains of tubercle bacilli tolerated hexane, while cells from older cultures were damaged by it.

Application of this knowledge could improve the precision and significance of tests for infectiousness and dye reactions. The differing impermeabilities of individual cells from each of the species studied emphasize the lack of homogeneity in populations from colonies or tissues, and suggest that, even with cultivable species, observations with suspensions will be less instructive than microscopic assays of interactions between dyes and mycobacteria.

Since, within any species, capsules must be regarded as accessories of the physiologic state, rapid staining and lack of capsules are taken to denote senescent populations of *M. leprae* rather than specific effects of sulfone therapy. Other drugs, and also natural factors in untreated patients, may be expected to produce comparable changes in these qualities of the bacilli.

SUMMARY AND CONCLUSIONS

The impermeabilities of five species of mycobacteria (*M. phlei,* BCG, H-37Rv, *M. leprae* and *M. leprae murium*) were examined by drying microspots of suspension for only 3 seconds, followed by prompt rehydration in dye solutions adjusted to neutrality and by incubation at 37°C. Capsules and 100 per cent of cells were revealed by surface coats of Congo red or nigrosin.

The staining of mycobacterial granules by safranin 0 permitted essentially qualitative distinctions between the saprophytic *M. phlei* and capsule-bearing cells of the pathogens. Crystal violet (and basic fuchsins) caused slow but progressive staining of granules in encapsulated pathogenic cells. End-points for staining 100 per cent of cells (not determined for H 37Rv or *M. leprae*) were: *M. phlei,* 1 hour; BCG, 24 hours; *M. leprae murium,* 10-14 days.

In each species the cells which stained readily were devoid of capsules. Those which resisted dye permeation possessed demonstrable capsules or matrices. It was only as capsular components were modified or lost during incubation that crystal violet staining could proceed.

The rugged microcapsules of *M. leprae murium* occur on individual cells, confer exceptional resistance to chemical and physical agents, and are associated with infectiousness.

M. leprae was recovered in two states: readily stainable and devoid of capsules, and highly impermeable with large capsules. The nonencapsulated states in sulfone-treated patients were taken to indicate senescent populations rather than lack of viability or specific effects of these drugs.

RESUMEN Y CONCLUSIONES

Se estudiaron las impermeabilidades de cinco especies de micobacterias (*M. phlei,* BCG, H-37Rv, *M. leprae* y *M. leprae murium*) por la desecación de microplacas de suspensión solamente por 3 segundos, seguida de rápida rehidratación en soluciones

colorantes ajustadas a la neutralidad y de la incubación a 37°. Con capas superficiales de rojo del Congo o nigrosina se revelaron cápsulas y 100 por ciento de las células.

La coloración de los gránulos micobacterianos con safranina 0 permitió trazar distinciones esencialmente cualitativas entre el saprofito *M. phlei* y las células capsulóferas de los gérmenes patógenos. El violeta de cristal (y las fuchsinas básicas) produjeron una coloración lenta, pero progresiva, de los gránulos en las células patógenas encapsuladas. Los puntos terminales para la tinción de 100 por ciento de las células (no se determinaron para el *M. leprae*) fueron: *M. phlei*, 1 hora; BCG, 24 horas; *M. leprae murium*, 10-14 días.

En cada especie, las células que se colorearon fácilmente estaban desprovistas de cápsulas. Las que resistieron la penetración por el colorante poseían cápsulas o matrices observables. Fué solamente cuando se habían modificado o perdido los componentes capsulares durante la incubación que podía actuar la coloración con violeta de cristal.

Las bastas microcápsulas del *M. leprae murium* se presentan aquí y allí en células dadas, otorgan extraordinaria resistencia a los agentes físicos y químicos y se relacionan con la infecciosidad.

Se recuperó el *M. leprae* en dos estados: fácilmente colorable y desprovisto de cápsulas, y sumamente impermeable con cápsulas grandes. Los estados sin encapsulación en los enfermos tratados con sulfonas se interpretaron como indicativos de poplaciones envejecientes, más bien que de falta de viabilidad o de efectos específicos de dichas drogas.

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