The Effect of Antibacterial Drugs on the Ultrastructure of *Mycobacterium leprae* in Human Skin

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Biopsies of skin from patients with lepromatous leprosy were taken before treatment, after 1 year's treatment with DDS, and after 3 and 6 weeks' treatment with DDS, rifampicin or streptomycin, and sectioned and examined in the electron microscope. Longitudinally sectioned *Myco. leprae* were classified into grades of morphological intactness. Analysis of the results showed a significant shift towards less-intact bacteria after 1 year of DDS treatment and after 6 weeks of rifampicin treatment. The assessments agreed qualitatively with conventional morphological indices measured by light microscopy. Some details of the process of degeneration were noted.

Introduction

The first experiments to distinguish between morphologically intact (presumed viable) and degenerate (presumed dead) *Mycobacterium leprae* were made with whole bacteria observed in the electron microscope (Rees, Valentine and Wong, 1960). It was subsequently shown that there was a good agreement between the appearance of each bacterium in the electron microscope and the identical organism stained and observed under the light microscope (Rees and Valentine, 1962). The relation between morphological intactness (solidity) and infectivity has been confirmed (Shepard and McRae, 1965), and the disappearance of solid organisms from tissues has been used to follow the progress of effective antileprosy therapy (Water and Rees, 1962). We have attempted to study the details of the process of degeneration in ultra-thin sections of skin from leprosy patients, both before and after periods of chemotherapy.

Methods

Two series of skin biopsies, A and B, each series from 3 patients with previously untreated lepromatous leprosy, were kindly supplied by Dr J. M. H. Pearson of the (British) Medical Research Council Leprosy Research Unit, Sungei Buloh, Malaysia. Series A consisted of biopsies taken before and after 1 year of chemotherapy with 4,4′-diamino-diphenyl-sulphone (DDS, 600 mg per week); Series B came from patients before, and then 3 and 6 weeks after starting treatment with DDS (600 mg per week), rifampicin (600 mg daily), or streptomycin (1 g daily). The biopsies were divided into halves; one half was used to prepare a suspension of bacteria (Rees, 1964) and the other was fixed for electron microscopy. Biopsies in Series A were fixed in 4% neutral formaldehyde (Richardson, 1960), while material in Series B was fixed in 2.5% glutaraldehyde in 0.085 M-cacodylate buffer, pH 7.4 (Glauert and Thornley, 1966), washed in 2.5% (w/v) sucrose in cacodylate buffer, pH 7.4, containing 0.1 M-calcium chloride, and post-fixed in 1% OsO_4 in veronal buffer (Kellenberger, Ryter and Séchaud, 1958). After fixation both series of biopsies were embedded in Araldite, sectioned, and stained with saturated aqueous uranyl acetate and lead citrate. Sections were examined at 80 kV in a Siemens Elmiskop 101.

Prints of electronmicrographs were examined for longitudinally sectioned bacteria, which were classified visually into 9 grades of morphological intactness, from "apparently intact" to "cell wall and minimal cytoplasm remaining". Examples of organisms from various grades are shown in Fig. 1. Two assessments were made of each biopsy, separated by a period of 21 months, as a test of the objectivity of the classification. In all, 50 sections were classified in each assessment of each biopsy. Comparisons between the assessments before and after treatment were made in two ways. In the first, the grades were scored 1 for Grade 1, 2 for Grade 2, and so on, and mean values and standard deviations calculated. A "standardized normal deviate" was calculated, this being the difference between 2 means divided by the standard error of the difference. This statistic was used to test whether there was a true difference between the 2 means. A value of 1.96 or more, which has only a 5% probability of occurring if there is in fact no difference between the true means, was taken to indicate a real difference.

The second method of comparing findings before and after treatment involved dividing the graded sections into "Grade 1" and "the rest" (corresponding roughly to the "solid"–"degenerate" classification used in light microscopy) and using either χ^2 or Fisher's exact test as appropriate, to determine whether the difference between the numbers of solid bacteria in the 2 groups before and after treatment could be ascribed to chance. A number of transverse sections of bacteria were examined to ascertain if there was any relation between (minimum) diameter, treatment, and apparent morphological intactness.

All the biopsies in Series B were examined "blind", the nature of the treatment, if any, being not known until afterwards.

Fig. 1. Examples of longitudinal sections of *Myco. leprae* in skin biopsies, showing some of the grades of degeneration used in quantitative investigation. (a) Intact bacteria (Grade 1) (EM x 70,000). (b) Early stage of degeneration; cytoplasmic membrane damaged, granular cytoplasm (Grade 2) (EM x 80,000). (c) Considerable degeneration of longer organism (Grade 5) (EM x 80,000). (d) Cell wall and minimal contents only remaining (Grade 9). Note electron-transparent part of wall revealed by contiguous organisms. (EM x 80,000)

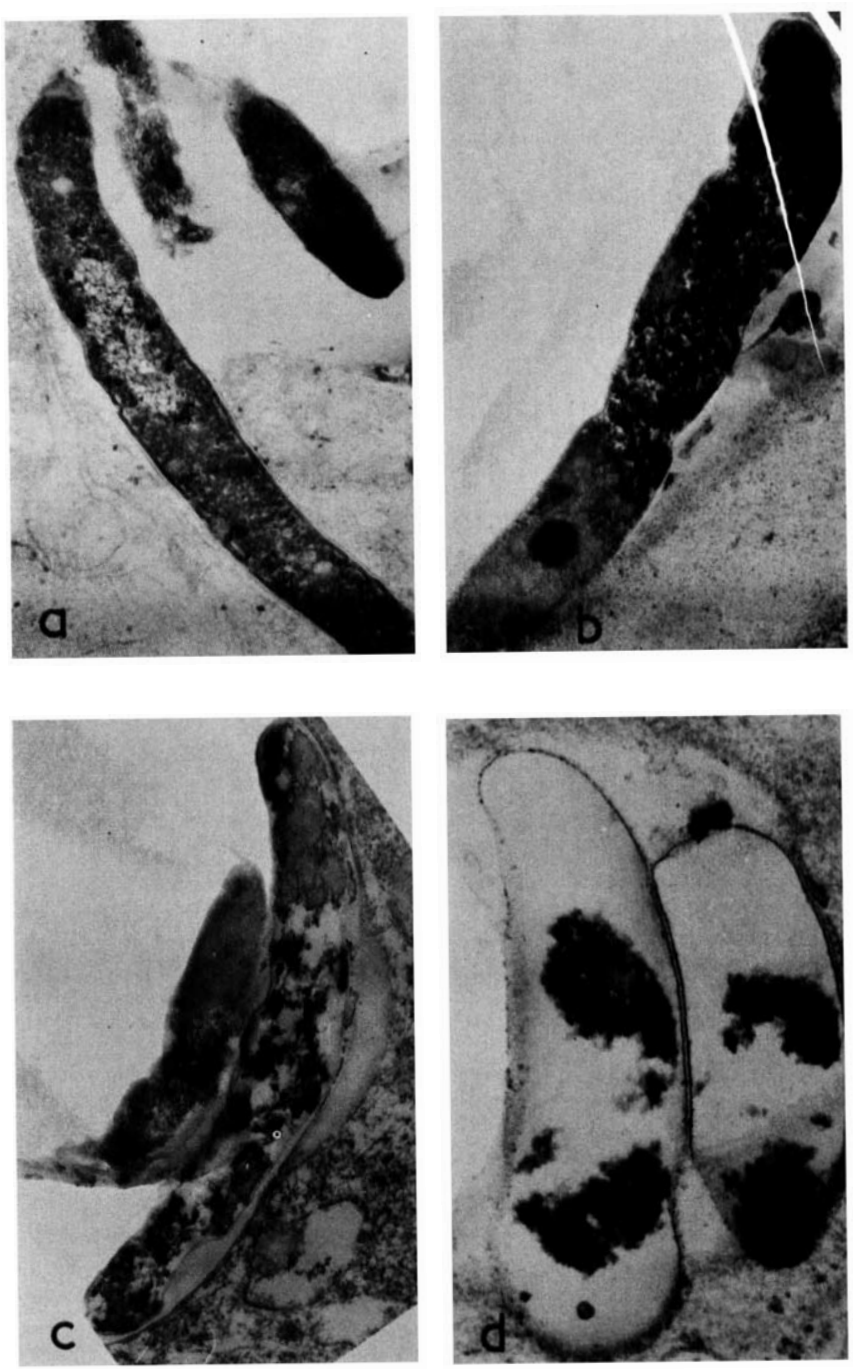

Fig. 1

Morphological indices of the bacterial suspensions (Waters, Rees and Sutherland, 1967) were kindly provided by Dr R. J. W. Rees.

Results

Data about longitudinal sections of bacteria in the three pairs of biopsies in Series A are shown in Tables 1 and 2. Agreement between the two assessments was very good, and both methods of statistical analysis showed that there was a significant shift towards more-degenerate categories after 1 year's treatment with DDS.

TABLE 1

Use of standardized normal deviate (d) to compare morphology of sectioned bacteria in biopsies taken before and after 1 year's treatment with DDS

		First assessment					Second assessment				
Patient no.	Biopsy	No. of sections	Mean[a] grade	± S.D.	d	*P*[b]	No. of sections	Mean grade	± S.D.	d	*p*
16101	Before	50	3.16	2.64	12.29	<0.001	50	3.18	2.62	10.6	<0.001
	After	50	8.16	1.15			50	8.14	1.14		
16052	Before	50	2.96	2.34	9.9	<0.001	50	2.38	1.96	13.1	<0.001
	After	49	6.82	1.49			51	6.88	1.45		
16136	Before	50	5.36	2.41	6.17	<0.001	50	5.34	2.33	6.4	<0.001
	After	50	7.76	1.33			50	7.76	1.32		

[a] Grades were scored from 1 for "intact sections" to 9 for "cell wall and minimal cytoplasm remaining".
[b] *P* is the probability that the observed difference in mean grade would occur by chance if there were no true difference.

TABLE 2

Use of χ^2 or Fisher's exact test to compare proportions of "intact" and "degenerate" sectioned bacteria in biopsies taken before and after 1 year's treatment with DDS

		First assessment				Second assessment			
Patient No.	Biopsy	Grade 1	Grade 2-9	χ^2	*P*[a]	Grade 1	Grade 2–9	χ^2	*P*
16101	Before	22	28	25.7	<0.001	17	33	18.1	<0.001
	After	0	50			0	50		
16052	Before	24	26	28.5	<0.001	28	22	36.2	<0.001
	After	0	49			0	50		
16136	Before	9	41	–[b]	0.001	8	42		0.006
	After	0	50			0	50		

[a] *P* is the probability that the observed difference between proportions of Grade 1 would occur by chance if there were no true difference.
[b] Where no χ^2 is given, Fisher's exact test was used to calculate *P*.

The results of 3 and 6 weeks' treatment with DDS, rifampicin, or streptomycin are shown in Tables 3 and 4. Agreement between the two assessments was good except for the first biopsy from patient 16316 (treated with streptomycin). Also, for the biopsies from this patient the two statistical methods used for comparing

TABLE 3

Use of "standardized normal deviate" (d) to test effect of short periods of treatment on morphology of sectioned bacteria

Patient No. Drug	Week after treatment started	First assessment: No. of sections	Mean grade ± S.D.	d	P[a]	Second assessment: No. of sections	Mean grade ± S.D.	d	P
	0	50	4.72 ± 2.73			50	4.8 ± 2.64		
16308	3	50	4.66 ± 2.25			50	5.00 ± 2.09		
	6	50	6.24 ± 2.37			50	6.2 ± 2.34		
Rifampicin	0 *v* 3			0.12	high			0.42	high
	0 *v* 6			2.98	<0.01			2.82	<0.01
	0	50	6.06 ± 2.2			50	6.26 ± 2.07		
16305	3	50	6.64 ± 1.41			50	6.82 ± 1.23		
	6	50	6.26 ± 1.7			50	6.32 ± 1.69		
DDS	0 *v* 3			1.57	high			1.86	high
	0 *v* 6			0.51	high			0.08	high
	0	50	3.42 ± 1.54			50	2.86 ± 1.68		
16316	3	52	4.37 ± 2.81			50	4.44 ± 2.73		
	6	50	5.06 ± 1.88			50	5.1 ± 1.8		
Streptomycin	0 *v* 3			2.13	<0.05			3.51	<0.001
	0 *v* 6			4.78	<0.001			6.4	<0.001

[a] *P* is the probability of the observed difference between mean grades occurring by chance in the absence of a true difference.

TABLE 4

Use of Fisher's exact test to compare proportions of bacteria in biopsies

Patient No. Drug	Weeks after treatment started	First assessment Grade 1	First assessment Grade 2–9	First assessment P[a]	Second assessment Grade 1	Second assessment Grade 2–9	Second assessment P
	0	7	43		9	41	
16308	3	3	47		2	48	
	6	0	50		0	50	
Rifampicin	0 v 3			0.205			0.029
	0 v 6			0.012			0.001
	0	5	45		4	46	
16305	3	0	50		0	50	
	6	0	50		0	50	
DDS	0 v 3			0.056			0.117
	0 v 6			0.056			0.117
	0	1	49		7	43	
16316	3	8	44		7	43	
	6	1	49		0	50	
Streptomycin	0 v 3			0.031[b]			—[c]
	0 v 6			—[c]			0.012

[a] *P* is the probability that the observed difference between proportions of Grade 1 would occur by chance if there were no true difference.

[b] Increased intactness.

[c] No change in intactness.

pre- and post-treatment findings gave discrepant results. There was no significant shift in the bacterial population after 3 or 6 weeks' treatment with DDS (patient 16305); nor after 3 weeks of rifampicin treatment (patient 16308), but a significant change, by both methods of statistical analysis, after 6 weeks' treatment with this drug.

The morphological indices of the bacteria from biopsies of Series A and B, measured by light microscopy, are shown in Table 5. The results agree qualitatively with those from electron microscopy. No clear-cut pattern was seen in the measurements of transverse sections.

The process of degeneration appeared to follow a definite pattern in the sections. First the cytoplasmic membrane of the bacteria broke down; at the same time the mesosome appeared to disintegrate and small pieces could be seen mingling with the cytoplasmic and nuclear material as it too disintegrated. Finally, the bacterial contents disappeared except for small fragments of cytoplasmic material. The cell wall remained intact throughout; both the inner electron-dense and the outer electron-transparent parts could be observed. If bacteria in the final stage of degeneration were contiguous, the electron-transparent part of the walls could be seen separating them.

Discussion

The use of the morphological index as an estimate of bacterial viability is important clinically as indicating, more rapidly and sensitively than do total

TABLE 5

Morphological indices, by light-microscopy, of Myco. leprae *in biopsies from patients in series B (Data kindly supplied by Dr R. J. W. Rees)*

Patient No. Drug	Weeks after treatment started	Degenerate bacteria (%)
16308	0	87
	3	98
Rifampicin	6	98
16305	0	86
	3	87
DDS	6	87
16316	0	86
	3	95
Streptomycin	6	91

counts, the progress of therapy and the infectiousness of the patient. It has been criticized (Chang and Andersen, 1969) because, at least in some other species of mycobacteria, the appearance of stained organisms can vary with different growth conditions and staining techniques. It is therefore encouraging to find that an ultrastructural degeneration of *Myco. leprae* occurs and corresponds to the changes indicated by light microscopy (and originally observed in unsectioned bacteria in the electron microscope), during antileprosy treatment. The results from the patient treated with streptomycin are equivocal, though they appear to indicate removal of bacteria; streptomycin is, however, regarded as a poorly effective drug against leprosy.

Two sources of error prevent quantitative comparison of light- and electron-microscopical results. Ultra-thin longitudinal sections include only about one-fifth of the whole organism, so that the first stages of the degenerative process may be missed. Further, the sections of necessity include only a very tiny part of the whole biopsy specimen, which in turn represents only a small sample of the patient's parasites. On the other hand, the bacteria are examined *in situ* in their tissue habitat, so that possible artefacts produced by homogenizing do not occur in the electronmicrographs.

Our study did not provide the hoped-for details of the early stages of the degenerative process. It did however draw attention to the persistence of bacterial walls more-or-less empty of cytoplasm; these would not be stained by carbol fuchsin. Although these walls can play no part in the spread of infection, their presence may cause a continuing pathological process in the patient freed from living bacteria by chemotherapy. Such a process cannot be controlled by existing antileprosy drugs.

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