THE EXAMINATION AND THE DETERMINATION OF THE VIABILITY OF *MYCOBACTERIUM LEPRAE* BY ELECTRONMICROSCOPY

by JAMES A. MCFADZEAN and ROBIN C. VALENTINE National Institute for Medical Research, London, N.W.7, and The Research Unit, Sungei Buloh Settlement, Malaya

The appearance of M. lepraemurium as seen by electronmicroscopy has been described by McFadzean and Valentine (1958–9). Two forms of the bacilli were observed, one the normal form (Fig. 1) and the other a degenerate non-viable form (Fig. 2). It was shown that an estimate of the percentage of degenerate forms present gave an indication as to the viability of a suspension. This paper describes the results of the application of similar techniques to M. leprae. A preliminary communication has been given of the results (McFadzean and Valentine, 1958).

Materials and Methods

Biopsies of skin were taken from patients with leprosy. The skin was sterilised with methylated spirit and anaesthetised by the injection of a local anaesthetic round the site. The biopsy was immediately processed by the technique described previously with the following modifications:

- 1. After grinding in phosphate buffer of pH 7.4 the material was centrifuged twice at approximately 1,000 r.p.m. for 2 minutes to remove tissue debris.
- 2. The material was then fixed in 4% formalin and centrifuged at 3,500 r.p.m. for 15 minutes. The deposit and bottom ml. were taken as the material to be examined and sent to London from Malaya by air. It had previously been determined that fixed suspensions of organisms showed no changes for periods up to 4 weeks.

Results

1. The Morphology of M. leprae

The appearance of the bacilli of M. leprae in the electron microscope showed no characteristic features to differentiate them from the bacilli of M. lepraemurium which we have described previously (McFadzean and Valentine, 1958; 1959). Two distinct forms of the organism were again observed. In one, the bacillus was uniformly filled with a relatively homogeneous protoplasm surrounded by the cell wall (Fig. 3). The well known denser bodies found in other mycobacteria were seen in some of the bacilli either forming darker polar regions, or clusters of dark granules. In the



Figure 1: Normal form of M. lepraemurium



Figure 2: Degenerate form of M, lepraemurium



Figure 3: Normal form of M. leprae



Figure 4: Degenerate form of M. leprae

other form observed, the protoplasm had shrunk from the cell wall either forming a dense spine along the organism or more usually was fragmented into a few dense round masses (Fig. 4). Such disordered protoplasm seems inconsistent with viability and we have demonstrated that organisms of *M. lepraemurium* having this appearance are in fact incapable of causing infection (McFadzean and Valentine 1958; 1959). The percentage of bacilli termed degenerate were assessed in samples of *M. leprae* in the manner described previously. As with *M. lepraemurium*, we believe that the degenerate count gives a useful measure of the viability of the preparation immediately prior to fixation for examination.

2. The percentage of degenerate forms of M. leprae present in patients with untreated lepromatous leprosy.

The patients in this group had never received any scientific treatment for leprosy. Unfortunately it was not possible to determine accurately the duration of clinical signs.

The percentage of degenerate forms found in each biopsy are shown in Table I. The average percentage of degenerate forms found was 56% (S.D. ± 24).

TABLE I

Patient's No.	No. of degenerate bacilli	% degenerate
13009	87/96	91
13015	40/60	67
13013	32/69	46
13010	58/284	20
13034	9/12	75
13014	3/11	27
13011	26/79	33
13097	29/62	47
13105	32/92	35
13099	2/7	29
13182	32/62	52
13154	6/16	38
13259	8/11	73
13257	36/54	67
13255	15/59	25
13258	30/48	63
13275	13/16	81
13274	24/25	96
13265	21/22	96

The percentage of degenerate forms of M. leprae found in untreated patients with lepromatous leprosy

3. The effect of treatment with DDS on the percentage of degenerate forms found

Eleven untreated patients were followed up for approximately one year during which time they received routine sulphone treatment. Biopsies were taken before treatment, between 7 and 9 months after treatment had commenced and after 12–14 months. The percentage of degenerate forms found on each occasion is shown in Table II.

TABLE II

The	percentage	of	degenerate	forms	of	Μ.	leprae	hefore	and	after
			treatm	ent wit	h I	DDS				

			D	egenerate	forms found	
				A,	fter	
Patient's No.	Befa	ore	7-9 m	onths	12-14	months
13009	87/96	91%	29/35	83%	0/0	
13015	40/60	67%	37/42	88%	0/0	
13013	32/69	46%	2/3	67%	0/0	
13010	58/284	20%	6/9	67%	0/0	
13034	9/12	75%	80/120	67%	14/16	88%
13014	3/11	27%	24/38	63%	0/0	
13011	26/79	33%	16/38	42%	0/0	
13097	29/62	47%	11/31	35%	0/0	
13105	32/92	35%	8/11	73%	0/0	
13182	32/62	52%	13/15	87%	0/0	
13154	6/16	38%	0/0	_	9/10	90%

There is an average increase of 18% in the degenerate forms found after 6 months treatment with sulphone. This is probably significant (5% level). The morphology of the degenerate forms was similar to those found in suspensions from untreated patients. After approximately 1 year bacilli could be found in only two samples. (Smears taken by the slit skin method still contained numerous bacilli at this time.) Examination by Ziehl-Neelsen of the suspensions prepared for the electron microscope showed a very few bacilli and acid-fast granular debris.

4. The effect of centrifugation on the percentage of degenerate bacilli found

The standard sampling process adopted was designed to compare as directly as possible with the observations on *M. lepraemurium* and also to obtain the maximum number of bacilli and cleanest preparations. It was thought, however, that centrifuging might influence the percentage of degenerate bacilli found, e.g. different densities of normal and degenerate bacilli. Samples were taken from 6 biopsies at different stages of processing. The average percentages of degenerate organisms found were:

- 1. Immediately after fixation-58%
- 2. In the supernatant after centrifuging at 3,500 r.p.m.-67%
- 3. In the standard sample—68%

These figures are not significantly different.

5. The influence of the site of biopsy on the percentage of degenerate bacilli found

Two biopsies were taken from a number of patients and the bacilli extracted separately. The results are shown in Table III.

TABLE	Ш	
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Effect of sampling differences on the percentage of
degenerate organisms found

	ish indian	Ľ	Degenerate or	ganisms	
Patient's No.	Details of sample	Num	bers	%	0
13009	Different ears	47/47	40/49	100	82
13015	,, ,,	17/28	23/32	61	72
13013	,, ,,	16/24	16/45	67	36
13010	,, ,,	48/243	10/41	20	24
13097	Different arms	8/11	21/51	73	41
13105	Different sides of back	16/60	16/32	27	50

There is no significant overall difference between the samples. The samples from both ears in patient 13013 are significantly different (1% level) and in patient 13009 probably significantly different (5% level), but are not different in patients 13015 and 13010. Samples from different sites in patients 13097 and 13015 are probably significantly different (5% level).

6. Comparison of the results by electronmicroscopy and the light microscope

It seemed possible that the percentage of degenerate forms observed by electronmicroscopy might correspond with bacilli showing irregularities of staining by conventional techniques. To investigate this, a number of biopsies were halved, and one half prepared for electronmicroscopy and the other fixed and sectioned as described by Ridley (1954). Dr. D. S. Ridley examined the bacilli in the sections and classified them into (a) solid and (b) fragmented and granular. The bacilli in the suspensions were also examined by the standard Ziehl-Neelsen technique and classified into uniformly staining organisms and those showing irregularities of staining. Table IV gives the results. There is no significant difference between the results of the smears stained by Ziehl-Neelsen and the electronmicroscope findings but the percentages of fragmented and granular forms found in the sections are probably significantly lower than the percentage of degenerate forms found by electronmicroscopy.

TABLE	I	٧
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Comparison of observations with the electron microscope	and
observations with a light microscope	

Percentage of degenerate bacilli					
	Ziehl-Neelsen				
Electronmicroscopy	Smear	Section			
25	38	20			
81	96	90			
67	58	40			
96	91	30			
63	45	50			
	Percentage Electronmicroscopy 25 81 67 96 63	Electronmicroscopy Smear 25 38 81 96 67 58 96 91 63 45			

Discussion

As stated previously (McFadzean and Valentine, 1958) more than half of the organisms from untreated patients with lepromatous leprosy appear degenerate. This is in contrast to some 5% in suspensions of *M. lepraemurium* prepared by a similar technique. It is known that killed *M. leprae* persist for considerable periods after intradermal inoculation in man and it is likely that the high percentage of degenerate forms results from the slow removal of organisms which have died during the protracted course of the disease.

The increase found in the percentage of degenerate forms after some 6 months therapy was probably significant (5% level). It is puzzling as to why difficulty was found in obtaining bacilli for examination after some 12 months therapy. (The concentration of bacilli necessary for examination by the electronmicroscope is approximately 10^7 /ml.). According to Ridley (personal communication) the bacillary index falls by more than 50% after twelve months therapy in Malayan patients, and at that time all the bacilli are fragmented or granular. This would suggest that the organisms are friable and are possibly broken up by the extraction processes. This is substantiated by finding acid-fast material in the suspension.

The close correlation between the findings with the electronmicroscope and the light microscope would suggest that the latter technique can be applied to following the state of the bacilli during treatment. The observations with the electronmicroscope on M. VIABILITY OF MYCOBACTERIUM LEPRAE BY ELECTRONMICROSCOPY 11

lepraemurium and *M. leprae* lend weight to the significance of the morphological appearances which have been reported for many years with the light microscope and *M. leprae* and described recently by Davey (1959).

Summary

It has been shown that M. leprae is morphologically similar to M. lepraemurium when examined by the electronmicroscope. As with M. lepraemurium two forms of the organism were observed one of which we termed the normal form and the other a degenerate form which could not possibly be viable. It had previously been shown that the degenerate form of M. lepraemurium was incapable of producing infection in animals.

Samples taken from untreated cases of lepromatous leprosy showed that an average of 56% of the bacilli were degenerate. This is in contrast to *M. lepraemurium* where only some 5% were degenerate.

The percentage of degenerate forms increased after 6 months treatment with sulphone but difficulty was experienced in obtaining sufficient bacilli for examination after 12 months treatment with sulphone.

The percentage of granular and fragmented forms found in the suspensions and on sections with the light microscope in general agreed with the percentage of degenerate forms found by electron-microscopy.

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