CHANGES IN THE MORPHOLOGY OF *MYCOBACTERIUM LEPRAE* IN PATIENTS UNDER TREATMENT

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There is still no direct way of determining the viability of *Mycobacterium leprae* in culture or by infectivity in animals. Nevertheless, characteristic variations in its morphology when stained by the Ziehl-Neelsen method have long been recognized, and their significance discussed. In fact, Hansen ([10]) was the first to suggest that leprosy bacilli with clear unstained areas were undergoing degeneration, and that granular bacilli were dead.

A reliable indirect method for determining the viability of *M. leprae* would be of the greatest value in assessing the progress of infection in a patient or its response to treatment. Although since Hansen's original suggestion very different interpretations have been given to irregularly-stained bacilli, more recently the weight of clinical and experimental evidence has supported his view. Before the introduction of sulfones, it was noticed repeatedly that a high proportion of the bacilli in smears from fresh, acute cases of lepromatous leprosy showed uniform staining. On the other hand, the bacilli in smears from patients with long-standing leprosy in which the disease had become relatively inactive, were nearly all irregularly stained.

With the advent of successful chemotherapy, Davey ([6]) observed a significant and rapid increase in the proportion of irregularly-stained bacilli in the smears from patients receiving active drugs; this increase occurred at the same time as the beginning of clinical resolution, and often preceded any fall in the bacterial index. In order to provide a quantitative assessment of the morphological changes in the bacilli during treatment, Ridley ([22]) has proposed using a "granularity index" based on 3 forms of organisms: "solid" (uniformly staining), "fragmented" (irregularly stained but intact bacilli), and "granular" (granules, either in line or in clumps, but not obviously belonging to intact bacilli).

More recently, in a series of experimental studies, McFadzean and Valentine ([13, 14, 15]) showed that degenerative changes in the morphology of the bacillus of rat leprosy, *Mycobacterium leprae murium*, seen in the electron microscope serve to identify dead forms, and could be

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used as a guide to the viability of the organisms. These observations were extended by Rees, Valentine and Wong ([17, 18]) using *M. leprae murium* and *Escherichia coli.* In both organisms, loss of viability was associated with similar morphologic changes identified in the electron microscope. Therefore, since electron microscopy could be used to identify loss of viability in two different species of bacteria, it was reasonable to conclude that human leprosy bacilli manifesting similar morphologic changes were also dead ([13–15, 17, 18]), particularly since rat and human leprosy bacilli are assumed to be closely related.

It is of course not possible to use electron microscopy in routine leprosy work. Rees and Valentine ([19]) however, have been able to examine, with both the light and the electron microscopes, the same individual leprosy bacilli stained by the Ziehl-Neelsen method. The results show that the irregularly-stained bacilli (including the "fragmented" and "granular" forms of Ridley) correspond exactly with the degenerate (dead) forms identified with the electron microscope, and only the uniformly stained bacilli ("solid" forms) correspond with normal and probably viable organisms.

It is concluded that at present the most reliable method for assessing the viability of organisms in patients with leprosy is that based on determinations of the proportion of uniformly-stained bacilli. The method is simple and could be carried out at most centers in the field. In order to establish the value of the method it should be used in different parts of the world on patients with different stages and manifestations of leprosy, and under different treatment schedules. Therefore, in the first of a series of drug trials (Macrocyclon Trial), undertaken by the British Medical Research Council in collaboration with the Malayan Ministry of Health at the Leprosy Research Unit, Sungei Buloh, the method was included in the protocol. It is considered that the results so obtained are of sufficient interest to warrant separate presentation, although full clinical details of the main drug trial will be published later. In this paper are described the results of serial smears from 39 new patients with lepromatous leprosy.

MATERIALS AND METHODS

Selection of patients.—The 39 patients, selected from new admissions to the Sungei Buloh Leprosarium, were all adult males in the age range 17 to 60 years (only 9 were over 40 years old), and they included 28 Chinese, 4 Malays and 7 Indians. All of these patients had active lepromatous-type leprosy, and in most cases it was of relatively short duration. As far as could be determined, the majority had received no specific leprosy treatment. A few had, however, received from 1 to 3 injections of Dapsone prior to admission, and one patient, who gave a 7-year history of leprosy, had received courses of Dapsone injections 5 and 4 years before admission. A biopsy specimen was taken from each patient prior to commencing treatment and sent to Dr. D. S. Ridley ([23]) who classified them into pure lepromatous, 32 patients; or near-lepromatous, 7 patients.

Smears.—Before commencing treatment, smears were taken from both earlobes and from 4 to 6 other sites with active lesions. From 32 of the patients, smears were taken

every 3 months for one year; from 14 of these, smears were also taken after 18 months of treatment. The other 7 of the group had serial smears taken at 3 months and 6 months only. All smears were taken by the same person (W), using the scraped-incision technique ([25]). As far as possible they were made of uniform thickness, blood being excluded. Considerable care was also taken to ensure that serial smears were all from the same sites.

The smears were stained by a slightly modified Ziehl-Neelsen method, in which 1 per cent hydrochloric acid in absolute ethyl alcohol was used for decolorization. The smears were assessed in two ways:

(1) Bacterial index, estimated according to Ridley's logarithmic scale ([21]);

(2) The percentage of uniformly stained (i.e., "solid") bacilli, estimated as follows:

A number of different fields of the smear were examined rapidly, and the approximate "granularity" thereby assessed. Then 100 individually-distinguished bacilli were classified as showing uniform or irregular staining. Normally no difficulty was experienced in deciding whether a bacillus showed uniform or irregular staining, provided that bacilli in clumps and globi were not included. Very short uniform bacilli were classified as degenerate. There were only two sources of difficulty:

(*a*) Where a longer-than-average bacillus had a short clear gap approximately in the middle of the rod. It was then not certain whether the bacillus was dividing or whether it was just commencing to degenerate. But, provided that the two "halves" were each of the same length as were the majority of bacilli present in the smear, the former alternative was chosen.

(*b*) In smears taken from patients who had received 12 to 18 months' treatment, there were seen a few uniformly-stained bacilli of approximately normal length but in which the cell walls were not parallel. Although usually classified as "uniformly stained," some doubt was felt that such bacilli were viable.

The estimate of the percentage of uniformly-stained forms was not introduced until shortly after the commencement of the macrocyclon drug trial. As a result, the pretreatment smears from the first 7 patients were examined only for the bacterial index. Thereafter the morphology of the bacteria in all smears was recorded. The morphology of the pretreatment smears of 8 patients was assessed by Ridley. The morphology of all other smears, and the bacterial index of all smears, were estimated by one of us (W.) to ensure uniformity of interpretation. Although the reader of the smears was also the clinician in charge, it is not considered likely that clinical knowledge of the patients led to much bias in the assessment of the smears. Previous smear results were not reviewed before the assessment of each new set, and it was impossible to remember results over three-month periods.

Treatment: (*a*) *Dapsone* (*DDS*).—All 39 patients received twice-weekly injections of Dapsone in refined coconut oil. For the first 6 weeks of treatment the dose was 200 mgm. It was then increased to 300 mgm. twice weekly for the rest of the first year, thereafter the 14 patients who were followed until 18 months received 400 mgm. twice weekly.

(b) Macrocyclon.—Macrocyclon is the name given to the least toxic of a series of polyoxyethylene ethers known to have high chemotherapeutic activity against a standard tuberculosis infection in the mouse ([2]). Twenty of the 39 patients in the series received macrocyclon in addition to Dapsone. It was administered intravenously once a week for one year, in a dose of 50 mgm. per kgm. body weight.

RESULTS

Detailed analysis of the clinical results of the macrocyclon trial has shown no difference between the patients receiving dapsone alone and those on dapsone plus macrocyclon. Therefore, the results for this study are compiled from all 39 patients irrespective of their treatment. The results of the serial smears showing the bacterial index and the

proportion of uniformly-stained bacilli for all the patients and for the patients grouped into the histologic types, pure-lepromatous or near-lepromatous are summarized in Table 1.[2]

TABLE 1.—*Analysis of the bacterial index and proportion of uniformly-stained bacilli in serial smears from 39 patients, pure-and near-lepromatous, treated with Dapsone or Dapsone plus macrocyclon for 18 months.*

Type of lepromatous leprosy	Length of treatment (months)	Number of patients	Stained smears			
			Bacterial index		Proportion of uniformly stained bacilli	
			Range	Average	Range (%)	Average (%)
All patients	Pretreatment	32	5.0 - 2.3	4.3	88 - 8	54
		(39	5.0 - 2.3	4.4[a])	-	–
	3 months	39	5.2 - 2.0	4.3	39 - 1	12
	6 months	39	5.0 - 1.0	4.2	14 - 1	4
	9 months	32	5.2 - 1.3	4.0	7 - 0	3
	12 months	32	5.0 - 1.2	3.9	9 - 1	4
	18 months	14	4.7 - 1.0	3.6	8 - 0	3
Pure-lepromatous	Pretreatment	28	5.0 - 3.2	4.5	88 - 15	55
		(32	5.0 - 3.2	4.5[b])	-	–
	3 months	32	5.2 - 3.8	4.5	39 - 2	13
	6 months	32	5.0 - 3.3	4.4	14 - 1	4
	9 months	26	5.2 - 2.5	4.2	7 - 0	3
	12 months	26	5.0 - 2.5	4.2	9 - 1	4
	18 months	10	4.7 - 3.2	4.1	5 - 1	3
Near-lepromatous	Pretreatment	(7	4.5 - 2.3	3.7[c])	-	–
		4	4.3 - 2.3	3.2	70 - 8	43
	3 months	7	4.3 - 2.0	3.4	15 - 1	7
		(4	4.3 - 2.0	3.2	15 - 2	10[d])
	6 months	7	5.4 - 1.0	3.1	10 - 1	3
		(4	4.2 - 1.0	2.5	10 - 1	4[d])
	9 months	6	4.4 - 1.3	3.0	3 - 0	2
	12 months	6	4.3 - 1.2	2.9	5 - 1	3

[a]All patients, including 7 whose pretreatment smears were not examined for the percentage of uniformly-stained bacilli.
[b]All patients, including 4 whose pretreatment smears were not examined for the percentage of uniformly-stained bacilli.
[c]All patients, including 3 whose pretreatment smears were not examined for the percentage of uniformly-stained bacilli.
[d]Three months and six months averages for the 4 patients whose pretreatment smears were examined for the percentage of uniformly-stained bacilli.

Pretreatment smears.—The range of percentages of uniformly-stained bacilli in the pretreatment smears was 88 to 8, with an average of 54. However, only 4 of the patients had less than 30 per cent of uniformly-stained bacilli at that time. Two of these patients developed

[2]The complete table, giving details of serial smears from individual patients, together with the statistical analysis, is available on application to the authors.

typical erythema nodosum leprosum a few days before starting treatment. One of the other 2 patients, age 55 years, had light diffuse lepromatous leprosy which appeared to be only moderately active. The remaining patient was the oldest one in the series, and clinically he showed marked atypical features. He was admitted with a lepra reaction of moderate severity, although it is uncertain whether this had any effect on the percentage of uniformly-staining bacilli. Certainly in pure-lepromatous patients lepra reactions, unlike ENL, did not appear to affect the morphology of the leprosy bacilli.

Response to treatment.—The slow rate of decrease in the bacterial index of the pure-lepromatous patients is in keeping with the general experience at Sungei Buloh. The small number of near-lepromatous patients included in this series showed a wider individual variation, but improved on the average at approximately twice the rate of the lepromatous cases. This again is in accord with local experience.

The rate of decrease in the percentage of uniformly-stained bacilli showed a remarkable uniformity. There was a very rapid fall during the first 3 months. Some further fall occurred over the next 3 months,

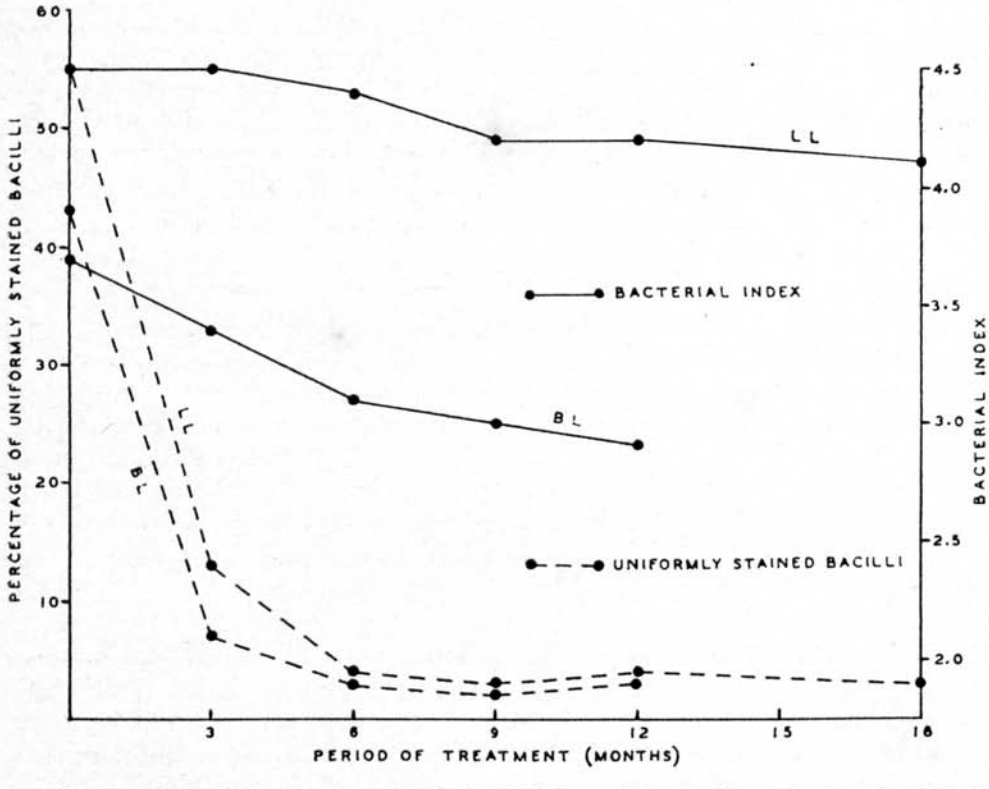

FIG. 1. Effect of treatment on the bacterial index and proportion of uniformly stained bacilli in lepromatous and near-lepromatous patients.

but thereafter in most cases there was little change, and a lower limit of between 2 and 5 per cent was reached. Although the numbers of Indians and Malays, 7 and 4 respectively, were too small to be significant there was no suggestion that race affected the response to treatment. Likewise, the rate of decrease in the percentage of uniformly-stained bacilli in smears from the small number of near-lepromatous patients did not appear to be significantly different from that of the pure-lepromatous patients. The divergence between the effect of treatment on the bacterial index and the proportion of irregularly-stained bacilli is shown in Fig. 1. No difference was detected between the patients who received dapsone alone and those who in addition were treated with macrocyclon.

DISCUSSION

Both Davey ([4, 6]) and Doull ([9]) have drawn attention to the importance of following the morphology of the bacilli in chemotherapeutic drug trials in lepromatous leprosy. Davey has suggested that only patients showing a high percentage of uniformly-stained bacilli in their smears are suitable for inclusion in such trials. It is considered that this criterion for the selection of patients is of considerable importance, in view of the finding that irregularly-stained bacilli are almost certainly dead. The present series suggests that untreated patients with moderately or very active lepromatous leprosy do not in general, have a proportion of uniformly-stained bacilli lower than about 30 per cent, although this requires further investigation. The few patients in the series who on clinical grounds were considered at least suitable for the drug trial (older patients with only mildly active disease) tended to have the lowest figures, but it was often not possible to anticipate the results for individual patients.

After commencing chemotherapy the change in the morphology of the bacilli was rapid. The 3-months smears of every patient showed a reduction in the percentage of uniformly-stained organisms, and after 6 months 36 of the 39 patients had smears with more than 90 per cent of the bacilli "fragmented." Davey's account of the rapid change in the morphology of the bacilli is fully confirmed. Accepting the view that irregularly-stained bacilli are dead, then treatment with Dapsone results in 6 months in the killing on average of all but 4 per cent of the leprosy bacilli in the skin lesions. At first sight, this result may seem difficult to accept in view of the persisting, and almost undiminishing, positivity of the bacterial population in direct smears from the lesions, not only in the first 6 months but even after 18 months of treatment. In fact, however, it is quite consistent with what is already known of the fate of dead mycobacteria in animal tissues. For example, the studies of Rees and Hart ([20]) on the population of tubercle bacilli in closed lesions in the lungs of mice showed that while active treatment

resulted in a very rapid fall in the number of viable organisms, the total number of stained organisms remained almost stationary. Mycobacteria, unlike most other microorganisms, do not readily undergo lysis or lose their staining properties when they die *in situ, in vivo.* Dead and still stainable mycobacteria will persist in the tissues for long periods unless they are in sites (in "open" lung lesions or in superficial ulcers) in which they may be easily shed from the body. Successful chemotherapy in human pulmonary tuberculosis may sometimes result in the production, for a time, of culture-negative sputa which repeatedly contain stainable bacilli. Even more commonly, operation specimens resected from lungs of treated patients with culture-negative sputa may show closed lesions with persisting but nonviable tubercle bacilli ([26]). It is therefore not surprising that in leprosy, after successful treatment has been given, the closed skin lesions show persisting positivity for stained organisms even though a very high proportion of them may be dead.

Although it is our intention to report clinical details of the patients in this series in the account of the macrocyclon drug trial, it is relevant to note that all showed improvement of a greater or lesser degree, and that none appeared to relapse during the period of observation. At the same time, in no patient did the percentage of uniformly-stained bacilli increase once the lower limit had been reached. On the other hand, Davey has reported the reappearance of uniformly-stained bacilli in patients treated with thiambutosine (DPT or Ciba 1906) ([6]), and with ditophal (Etisul) ([5]), who subsequently developed clinical evidence of relapse.

Similar observations have been made by Hart, Rees and Valentine ([11]) in experimental studies using rat leprosy as a model. These workers have shown that by following the morphology of stained organisms in animals treated with isoniazid the reappearance of increasing proportions of well-stained organisms is a very sensitive measure of the development of drug resistance. It is concluded, therefore, that determinations of the proportion of uniformly-stained organisms (equating these with viable organisms) offers at present the most sensitive way of determining and comparing the activity of new drugs for the treatment of leprosy, and at the same time of detecting the development of drug resistance.

The finding that successful treatment with Dapsone (DDS) results in a very rapid fall in the proportion of viable organisms gives support to the view, now increasingly held, that treatment renders "open" cases noncontagious long before cure is obtained ([12]). It also helps to explain the very similar speeds of response of lepromatous leprosy to the proven effective chemotherapeutic drugs. Thus an analysis of the results of a number of trials undertaken by the Leonard Wood Memorial shows that Dapsone, Diasone, and dihydrostreptomycin were of

approximately equal clinical value, and the giving of two drugs in combination was no more effective than one alone ([8]). Experience with DPT suggests that this drug, too, has a speed of action of the same order as that of Dapsone, and Davey and Currie ([3]) have noted that DPT also causes rapid changes in the morphology of the bacilli.

It is therefore suggested that all of these drugs are equally able to kill off the vast majority of the leprosy bacilli present in the body within a few months of starting treatment. The giving of two drugs at once is unlikely to increase the rate of killing of leprosy bacilli sufficiently to affect the rate of clinical progress, although such combinations may prevent the emergence of drug resistance in the case of DPT or Etisul. It has, however, been claimed that Etisul is bacteriocidal, unlike Dapsone and DPT which are considered to be bacteriostatic. It is therefore of some interest to know whether, on Etisul therapy, the rate of decrease in the percentage of uniformly-stained bacilli is greater than with Dapsone. This question is at present being investigated.

Nevertheless, although successful treatment with Dapsone, and presumably with the other proven effective chemotherapeutic drugs, results in the rapid death of the majority of the viable leprosy bacilli, yet the lesions and manifestations of leprosy remain very apparent. The general clinical response is extremely slow. Such observations raise the question of the relative importance of viable organisms to the clinical manifestations of leprosy. It would seem that gross lesions can persist as a result of a high proportion of dead organisms, and that complete resolution of these lesions will not take place until the final removal of the dead organisms or their debris from the local site. This again is not surprising, since it is known experimentally that dead mycobacteria or even extracts of such mycobacteria will produce in animals typical granulomatous reactions at the site of inoculation ([24]).

It is not as yet possible to assess the full significance of the small numbers of uniformly-staining bacilli (on average 3-4%) found in the smears taken after 9, 12 and 18 months of treatment. Although the results obtained by studying experimentally the morphology of *M. leprae murium* and *E. coli* gave a slight over-estimate of the order of 5 per cent of the viable count ([18]), it cannot be assumed that the same is true for *M. leprae*. Cessation of treatment after only 6 to 12 months' chemotherapy almost invariably leads in lepromatous patients to reactivation of the disease. Indeed, relapse may still occur after a very much longer course of chemotherapy ([16]), so that it has been suggested ([1]) that treatment of lepromatous leprosy with sulfones should be continued, certainly for 2 years after all activity has ceased, and perhaps for life.

Viable bacilli must therefore remain in the body for a number of years after the commencing of effective treatment. Moreover, when

relapse does occur after treatment with Dapsone has been prematurely stopped, then the disease responds once more, at the normal rate, to a fresh course of Dapsone. To date there has been little published evidence of the emergence of Dapsone-resistant bacilli. This would suggest that the residual viable bacilli must be present in the body at sites or in tissues either where the concentration of sulfone is too low to be effective, or else where the drug is inactivated in some way, and from which the bacilli can spread should treatment be stopped.

Since lepromatous leprosy produces vascular granulomatous lesions, as opposed to the avascular granulation tissue of tuberculosis, it would at first seem difficult to envisage any particular site or type of cell in which these residual bacilli could survive. Nevertheless, it is of interest to note that, in treated patients said to have become smear-negative, skin biopsies often reveal the presence of scanty bacilli. These bacilli are frequently found to be in the small nerves of the dermis, and Dharmendra ([7]) says that they may occur in either uniformly- or irregularly-stained form. Thus there is some evidence that live bacilli may still be present in these nerves when both living and dead bacilli have been removed from the other tissues of the dermis. It is therefore tentatively suggested that, whereas a high proportion of the leprosy bacilli in lepromatous granulation tissue are killed within a few months of starting treatment, live bacilli remain in the superficial nerves. Here they may be in a resting phase, or alternatively they may still be able to multiply although perhaps at a reduced rate, and it is possible that live bacilli are continuously being "shed" in small numbers into the surrounding dermis, where they are rapidly killed. Gradually, over many years these bacilli protected in the nerves decrease in numbers and eventually die. This may be either as a result of the long-continued presence of suboptimal concentrations of sulfone, or else in the course of the natural history of the disease. This is analogous to the "burnt-out cases" of leprosy seen in the presulfone era.

If this concept of the response of lepromatous leprosy to chemotherapy is correct—and histologic evidence for or against it should not be difficult to obtain—then the ability of the infection to become active again when treatment is stopped after many years' chemotherapy is readily understood. Although the outlook for leprosy patients has completely altered since the introduction of sulfones, yet there remains a great need to reduce the length of time required to effect a cure. On the evidence here produced it would seem that the present standard drugs are able to kill rapidly the majority of leprosy bacilli. It is therefore suggested that in the search for new chemotherapeutic agents in leprosy special attention should be given to drugs that are able to penetrate nervous tissue, and to drugs or other agents which enable the human body to break down and eliminate dead mycobacteria. Success in either of these two ways would, it is anticipated, lead to a

further major advance in the medical treatment of lepromatous leprosy.

SUMMARY

Serial smears were made from 39 pure-lepromatous and near-lepromatous patients, first before commencing chemotherapy and thereafter at three-month intervals for 12 to 18 months. The percentage of uniformly-stained bacilli in each smear was determined, as an assessment of the proportion of viable bacilli present. From a pretreatment average of 54 per cent uniformly-stained organisms, a rapid fall occurred in every case, and after only 6 to 9 months treatment an average of 96 per cent of the bacilli showed irregular staining. Thereafter little change occurred, although the bacterial index declined slowly. The significance of these findings is discussed in relation to the known behavior of dead mycobacteria in tissues, the slowness of clinical response to treatment with the proven drugs, and the tendency of leprosy to relapse even after several years of chemotherapy.

RESUMEN

Se hicieron frotes seriados de 39 enfermos lepromatosos y cuasi-lepromatosos, primero, antes de iniciar la quimioterapia y después a plazos de tres meses durante 12 a 18 meses. Se determinó el porcentaje de bacilos teñidos uniformemente en cada frote, como valoración de la proporción de bacilos viables presentes. Partiendo de un promedio pretratamiento de 54 por ciento de microbios teñidos uniformemente, hubo una rápida baja en cada caso y al cabo de no más de 6 a 9 meses de tratamiento un promedio de 96 por ciento de los bacilos mostraba coloración irregular. A partir de ahí, hubo poca alteración, aunque el índice bacteriano descendió lentamente. Se discute el significado de estos hallazgos en relación con el conocido comportamiento de las micobacterias muertas en los tejidos, la lentitud de la respuesta clínica al tratamiento con las drogas comprobadas y la tendencia de la lepra a recidivar aun tras varios años de quimioterapia.

RESUMÉ

Des frottis en série ont été faits chez 39 malades lépromateux, d'abord avant le début de la chimiothérapie, ensuite à trois mois d'intervalle durant 12 à 18 mois. Le pourcentage de bacilles colorés de façon uniforme, pris comme indice de la proportion de bacilles viables présents, a été déterminé dans chaque frottis. A partir d'une valeur moyenne de 54% pour les bacilles colorés de façon uniforme dans les examens pratiqués avant le traitement, une chute rapide a été enregistrée dans chaque cas, et après 6 à 9 mois seulement de traitement 96% des bacilles en moyenne ont montré une coloration irrégulière. Par après, peu de changements ont été notés, encore que l'index bactériologique ait décliné lentement. La signification de ces observations est discutée, d'après ce que l'on sait du sort des mycobactéries mortes dans les tissus, et en rapport avec la lenteur de la réponse clinique au traitement par des médicaments bien établis, et avec la tendance de la lèpre à récidivier après plusieurs années de chimiothérapie.

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