THERAPEUTIC TRIALS IN LEPROSY USING SERIAL BIOPSIES Dr. D. S. Ridley

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Therapeutic trials in leprosy are beset with two difficulties: (1) clinical impressions of progress are subjective, and photographs are not amenable to statistical analysis; and (2) various bacterial indices are in use which, having no mathematical basis, are inconvertible one to another; none of them reflects accurately either the clinical or the total response to chemotherapy. Neither of these difficulties was an impediment to the finding of an active drug when there were no alternatives; now that there are many new drugs to be compared with one that is proved, better methods of testing are needed, especially since the number of untreated lepromas suitable for trial is becoming limited.

The fallacy of the bacterial index of skin smears is that it reflects the density of bacilli in the leprous lesion from which it was made, but not the size of the lesion (Ridley, 1955). The point is crucial since, as can be shown by serial biopsies, response to sulphones proceeds partly by solution of the leprous granuloma (which is apparent clinically), and partly by a reduction of the bacilli therein (which is noted in skin smears), and the two processes are independent; soemtimes one, sometimes the other is predominant according to the race of the patient and the stage of treatment. It would be desirable therefore to have an index not only for bacterial density but for size of lesion; but it is not possible to measure size clinically or from smears: the whole of the leprous infiltration would have to be scraped out, spread to a uniform thickness, measured, and its area multiplied by the bacterial index, in order to determine the number of bacilli.

Serial biopsies provide the basis of an index which takes account of both size and density of the bacterial mass (Ridley, 1957). It was found that the mean rate of fall of this index in a group of lepromatous patients on sulphone therapy was remarkably constant at all stages of treatment, irrespective of the initial severity of the infection; if confirmed this observation would provide a basis for an absolute comparison of different drugs. For European and Eurasian patients on sulphones the mean rate of fall of the index in each period of 6 months was about 25% of the index at the beginning of the period. The results have since been extended and reassessed by a slightly modified system, and the rate remains the same as before from beginning to end of treatment. The progress of individual cases is erratic; the mean rate of improvement of individuals in this series varied between 18% and 40% per 6 months period. By contrast with these figures 8 similar patients on izoniazid progressed at a mean rate of 9% per 6 months; while 3 patients in the sulphone group, who were excluded from the trial because their treatment was interrupted by prolonged spells of severe erythema nodosum, made no progress: their indices fell 0.5% per 6 months. The results are given in more detail in the earlier paper.

The procedure and the methods used, slightly modified, are here described in detail in the hope that the system will be given the wider trial which is necessary to establish confidence in its reliability.

Calculation of the Biopsy Index

In sections of uniform thickness stained for acid-fast bacilli, the density of bacilli in the parts of the section occupied by leprous granuloma is estimated in much the same way as in a smear; areas of healthy dermis are ignored. For this purpose Cochrane's (1952) index has been modified in such a way that each figure represents 10 times as many bacilli as the figure below it; the scale is defined as follows, according to the number of bacilli in a field of view under the 1/12th inch (2 mm.) objective:

2 + 1 or more bacilli, on average, in 10 fields

1 + 1 or more bacilli, in 100 fields.

Sometimes the density of bacilli in different areas of one section is not uniform, in which case the density in each part of the granuloma is estimated separately, and the mean taken.

To obtain the bacterial index of the section, "biopsy index " for short, the figure for the bacterial density is multiplied by the fraction of the section occupied by the leprous granuloma. This fraction is estimated by observation of a haematoxylin-eosin section under a low power magnification: a 11 inch (32 mm.) objective is ideal; by considering whether the granuloma occupies much more or less than one quarter, half or three-quarters of the dermis, it is possible to estimate the fraction to the nearest 1/10. with fairly good agreement between different observers. Excised lesions should be divided and each half examined for this purpose. If the entire dermis is occupied by leprous infiltrate the factor is I; the theoretical maximum for the biopsy index therefore is 6.0; if the bacterial density is 5 + in a granuloma which occupies 7/10of the dermis, the index is 3.5, which is an average figure for a fully developed untreated leproma. If the initial index is 4.0 and after 6 months' treatment it falls to 3.6, the improvement, 0.4 out of 4.0, is 10%; if after another 6 months it falls to 2.4 the improvement is 1.2 on 3.6 or 33%; mean $21\frac{10}{2}\%$.

Plan for a Therapeutic Trial

Cases are selected for the trial according to the usual criteria. The only experience of the biopsy index so far has been with lepromatous cases, free from any borderline features. Although one would prefer to use untreated cases, I cannot see that any serious error would arise from the use of previously treated patients provided the trial was arranged to commence 3 months after the change of drugs. If treatment is seriously impeded for any length of time by reactions the case is excluded from the trial; otherwise reactions are ignored.

The selection of sites for biopsies is important and should be undertaken by the same doctor throughout the period of trial. The lesions chosen should be the most active that are also representative; solitary ulcers and exceptional nodules should be disregarded as comparable lesions will not be available later.

The most useful period for observations is 6 months. The published results were based on biopsies taken every 3 months; and to minimize random variation between one biopsy and another they were subsequently paired and the means taken to provide a 6-monthly analysis; even then there were many ups and downs in indices. In order to find out whether it is always necessary to base each idex on two biopsies, I have made separate analyses of the results on the basis of (I) double biopsies at 6-monthly intervals, (2) single biopsies at 6-monthly intervals, and (3) single biopsies at 3-monthly intervals. Surprisingly, whereas there was good agreement between the results based on single and on double biopsies at 6-monthly intervals (they are summarised in the table), the means of results at 3-monthly intervals, based on the same sets of indices, were discrepant; the reasons for this are made apparent later. The conclusion is that single biopsies at 3-monthly intervals are unsatisfactory, but that single biopsies at 6-monthly intervals should give a good index of progress for a drug provided (1) each case is observed for at least 2 periods, and (2) a total of at least 50 periods of observation is obtained from all cases, e.g. 20 cases observed from 12-18 months. If it is desired to obtain a result in 6 months, or from a very small number of cases, it is necessary to make a pair of biopsies in each period of 6 months. It is necessary that each biopsy should be made within I month of the date it is due.

The termination of the trial may be either by set period of time, or by cure of the cases; even in the first method some cases may become bacteriologically negative before the termination is due. As far as can be seen in sulphones the index falls at a constant rate, in a group, until bacilli are no longer to be found; but very low indices cannot be accurately estimated, and it is necessary to decide

TABLE

Comparison of Results based on Single and on Double Biopsies at 6-monthly intervals

Case	I	2	3	4	5	6	7	8	9	10	II	12	13	14 al	Mean 1 cases
Initial Index	4.5	4.4	3.9	3.5	3.5	3.3	3.0	2.5	2.2	2.0	1.5	1.0	0.9	0.8	
Previous treatment	_		_		+	+	÷				+		÷	÷	
Duration of trial in months	48	30 42	18	36	18 30	36	30	12	1.8	30	12 24	12 24	12 18	12 18	
% Mean improvement (Double biopsies)	19	26	20	$22\frac{1}{2}$	39	23	18	38	19	28	37	22	$2I\frac{1}{2}$	$20\frac{1}{2}$	25.3
% Mean improvement (Single biopsies)	18	23	23	23	45	18	16	33	20	30	36	27	22	22 <u>1</u>	25.5
Trial completed (C) or incompleted (I)	С	С	Ι	С	С	С	C	Ι	Ι	С	С	С	С	С	

on some point beyond which the trial of any patient will be discontinued. If an index figure, say 0.2, is taken as the signal it will be found that the last period of observation, on average, will produce a greater fall in the index than it should, since each case will be terminated by a significant fall; to avoid this error a figure is chosen beyond which one more period of observation will be made, and the trial of that case then terminated. The procedure I have used is to wait till the index falls to 0.4 or lower and then to terminate the trial after the next biopsy, which may be higher or lower than 0.4; but if in any biopsy there are no bacilli, or the area of the lesion is estimated to occupy less than one tenth of the skin, the biopsy is discounted and the trial of that case is stopped immediately. The termination of the cases in the Table was different in many cases according to whether the analysis was by single or double biopsy; in the latter case account was taken of all biopsies (which happened to be at 3-monthly intervals), but in the analysis of single biopsies only every other one was noted and the first signal of 0.4 was sometimes missed. This explains the double set of figures in column 4 of the Table. Even so the discrepancies are not great. The trials of 3 cases were not completed: one patient discharged herself and two others were progressing when the analysis was made.

Analysis of Results

The 14 cases in the Table include all lepromas which have been observed by serial biopsy for at least 1 year. The mean improvement in all 6-monthly periods of observation was 24.3%per period. The mean of the mean rates of progress of the cases, assessed individually, was 25.3% per period (25.5% with single biopsies). The difference of 1% is accounted for by the bias in the former instance due to the fact that more observations were recorded from slow cases than from those who made a quick response; the bias, however, appears to be unimportant and the mean of this series can be taken as 25%. It would be interesting to see whether other workers with patients of different race obtain a comparable figure for sulphones. This mean is the only figure that need be calculated to determine the result of the trial; but there are some other points of interest.

Individual biopsies vary considerably, as would be expected. A simple comparison of two biopsies is never of any value. At 6-monthly intervals much of the irregularity in the movement of the biopsy index is due to the ups and downs of the patient's progress and the effect of reactions, although when the index reaches a low figure inaccuracies of estimation make their mark; in general, however, the progress of the patient outweighs other factors. At 3-monthly intervals random irregularities become preponderant and lead to curious results; an example is the progress of case 13 during the last 15 months of the trial:—

Index 0.6 0.9 1.05 0.25 0.4 Total fall, 0.6 - 0.4 = +33%% Fall -33 - 14 76 -38 Mean % fall, 76 - 85= -2.25%

It will be noticed that when the index goes up instead of down, the rise is expressed negatively as a percentage of the index at the end of the period instead of at the beginning; this usually leads to a sensible answer: 0.6, 0.9, 0.6 gives percentage movements of -33 + 33 which cancel each other, just as do the movements of the index. But in the exceptional instance given above the three negative percentages outweighed the single positive fall which was actually the greater: several small steps, expressed as percentages, outweigh one large one. This did not happen with biopsies at 6-monthly intervals.

Although the mean improvement of 25% applies to all stages of treatment, this figure does, of course, represent a greater absolute fall in the index at the beginning of treatment than at the end when the value of the index is smaller. The index is multiplied every 6 months by 0.75; at the end of I year it would be expected to be 0.75^2 times the initial figure, and at the end of 4k years (9) periods) it will be multiplied by 0.75^9 (Log 0.75 = -0.125; ×9 = -1.125; antilog = 0.075); this answer (0.075 = 7.5%) represents an improvement of $92\frac{1}{2}$ %. The formula to express the progression is $x^n = b/a$, where a = the initial index, b = the final index, x = the multiplication factor for each period, and n = the number of periods; it can be applied to individual cases or to means, and will give the expected improvement, theoretically, for shorter as well as for longer periods, e.g. if the ratio of b to a is 0.75 to 1 for 6 months, it will be the same for 2 periods of 3 months, and the factor x for 3 months will be found from $x^2 = 0.75/1$; then .75 = 0.866, which represents an improvement of 13.4% per x =3 months.

Technical Methods

No special virtue is claimed for the methods described except that of simplicity. A full description of the removal of skin tissue for biopsy is given by Khanolkar (1951), and by Freudenthal and Haber (1951) who describe also some refined histological processes.

Excision of Skin. The operator wears gloves which can if necessary be kept on the hands and soaked in dettol in between operations. The skin is disinfected with a colourless antiseptic. The part to be excised is surrounded by sterile towels—the number is reduced to one by using a towel with a circular hole in the centre. To avoid a wait for the local anaesthetic to take effect,

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Dr. W. H. Jopling, at the Jordan Hospital, Redhill, mixes hyaluronidase (1500 unit ampoule) with only 1 ml. of 2% procaine or similar preparation and injects it under the skin to be excised; the effect is immediate. A suture is inserted but not tied and the ends are carried up through the shaft of a punch; he finds that for firm or infiltrated skin a punch of 5 mm. diameter is satisfactory and its incision can be closed usually by a single suture; for soft skin overlying fat a punch of 6-7 mm. is needed, and it will require 2 to 3 stitches. Some prefer a scalpel. Whatever instrument is used it is important that the incision should extend down to the subcutaneous fat, and should be square with the skin surface, not sloping. After the incision is made the tissue is held up by the loose suture for excision.

Fixation and Embedding. The specimen is dropped into 3-5 ml. of the following mixture (Lowy, 1956): 40% formaldehyde 10 ml., Mercuric chloride 2 g., glacial Acetic acid 7 ml., water 100 ml. After about 3 hours (not more than 4 hours) the specimen is transferred without washing to about 10 ml. 70% alcohol, in which it can be kept for several weeks, and if desired despatched to a central laboratory. Alternatively, Zenker's fixative gives good results but is not quite so simple or flexible. The suture is pulled out, and the specimen divided in half; later, the two halves are embedded in the same block. Dehydrate in alcohol as follows: 70%, overnight; 90%, 2 hours; 95%, 2 hours; absolute, 2 changes of 2 hours each. Transfer to cedarwood oil overnight, wash in benzene 30 minutes and impregnate with paraffin wax at 56° C., 3 changes in 4 hours, and finally embed.

Section cutting. A sharp knife is more important than the type of microtome, and sharpening the knife is perhaps the most skilled operation of all. The best hone is a Belgian yellow stone; the best strops are impregnated with jeweller's rouge on one side while the reverse is plain or lightly oiled for finishing. A small knife needs frequent stropping. Sections should be of uniform thickness; 7μ is satisfactory.

Staining of sections for bacilli is done by the method of Lowy (1956), or Wade (1952) which is probably simpler:

- 1. One part paraffin oil in 2 parts pure turpentine, 2 changes in 15 minutes. Blot.
- 2. Lugol's iodine, 5 minutes. Wash.
- 3. Sodium thiosulphate (5%), I minute. Wash thoroughly.
- 4. Concentrated carbol-fuchsin (do not heat), 20 minutes. Wash.
- 5. Differentiate with 20% sulphuric acid (about 30 seconds), until the connective tissues are a pale pink. Do not overdifferentiate. Wash.

- 6. Counterstain (about 30 seconds) with 0.1% toluidine blue to give a pale purple-blue background. This shows up nuclei and structure better than methylene blue.
- Blot, and dry in air. (Do not use alcohol.) Clear in xylol. Mount in synthetic medium or balsam.

Two sections of each block are stained for bacilli and the better is used for calculating the bacterial density. Another section is stained with haematoxylin and eosin. Alternatively the section stained for bacilli can be counterstained lightly with Ehrlich's haematoxylin (about I minute, and no differentiation) and the same section is then used for calculating both parts of the index.

Conclusion

The technique of assessing the progress of leprosy patients by the use of serial skin biopsies, which has been fully described, appears to offer the best method available at present of estimating the activity of an anti-leprous drug.

The expected improvement factor obtained by this system for sulphones is constant at all stages of treatment because acquired resistance to these drugs by *M. leprae* is a rare event. This may not be so with other drugs.

The performance of serial biopsies need not be arduous, but requires organization; in compensation the need to use large numbers of patients is obviated. As a matter of opinion I can see no necessity to undertake serial skin biopsies and smears in parallel; after an initial examination by each method, biopsies are the most useful method of assessing progress until the patient is nearly better; thereafter multiple smears are needed as a test of cure.

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