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Electron Microscopic Study of the Morphologic Index[1]

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The Morphologic Index (MI: the ratio of solid bacilli to the total counted bacilli) is widely used to follow the early therapeutic effects of antileprosy drugs. The reliability of light microscopic MI (LM-MI) has, however, not yet been established. In fact, the values of LM-MI are variable under different conditions of staining or fixation ([6]). The limited resolving power of the light microscope also presents difficulties. In order to check the reliability of the LM-MI, two samples for light and electron microscopy were prepared from each of a number of patients from the same leprosy lesions, and their light and electron microscopic MI's were calculated and compared.

MATERIALS AND METHODS

There were twenty-eight pure lepromatous (LL) and two borderline (BL) cases ([5]). The Bacterial Index of each patient was more than 3+ by Ridley's BI category. Thirty specimens for light and electron microscopy were taken from the same lesions and LM-MI and electron microscopic MI (EM-MI) were counted and 400 bacilli were counted in each light microscopic and each electron microscopic determination.

For light microscopy, smears of bacilli are fixed with formalin vapor for 20 minutes, stained with carbolfuchsin at room temperature for 30 minutes and differentiated with 1% acid-alcohol for 10 seconds. Counterstaining was with Löffler's methylene blue for 30 seconds.

The specimens for EM-MI were prepared by the previously reported ([3]) microsuspension method. A small amount of tissue from a leproma is teased into tiny pieces in physiological saline. Leprosy bacilli float in the saline. This suspension of bacilli is kept in the refrigerator for 24 hours after which the supernatant fluid is replaced with a small amount of fresh saline and mixed well to make a uniform suspension. A drop of this suspension is put on a 400-mesh copper grid, covered with collodion film, by means of a tuberculin syringe and allowed to stand for ten minutes. After ten minutes, the excess saline on the specimen grid is absorbed with a filter paper and the specimen grid is dried. Later, dried specimen grids are washed twice with distilled water. Leprosy bacilli on the grids are examined after shadowcasting. Bacilli seen in the holes of 400-mesh copper grid are counted on the fluorescent screen at a magnification of 10,000 to 15,000. The accelerating voltage

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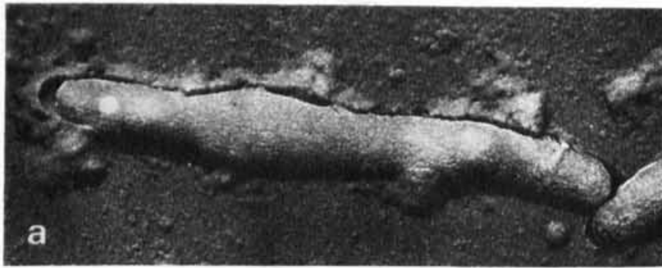
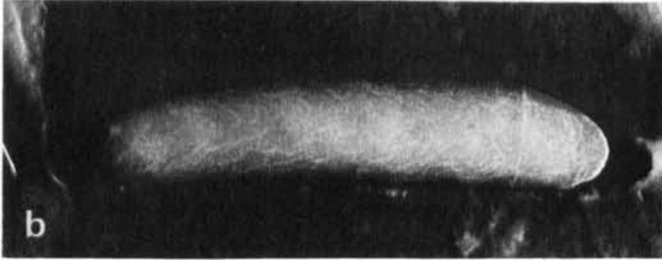


FIG. 1. Various electron microscopic forms of leprosy bacilli.
a. Bacillus filled with cytoplasm and swollen in the middle part of cell body (solid).



b. Plump bacillus (solid).

c. Slightly degenerated bacillus with detached cytoplasm (nonsolid).

d. Moderately degenerated bacillus with fragmented cytoplasm (nonsolid).

e. Moderately degenerated bacillus with fragmented cytoplasm (nonsolid).

f. Ghost bacillus without cytoplasm (nonsolid).

of the electron microscope utilized was 80 kv.

RESULTS

Various forms of leprosy bacilli have been reported from electron microscopic study ([4, 7]). In this study bacilli were classified by morphology as follows. Some bacilli are filled with cytoplasm and appear plump (Fig. 1-a, b). Among them there are long and short forms. A small number of solid bacilli are swollen in their midsections (Fig. 1-a). Other bacilli do not have the plump appearance, and their bacillary cytoplasm does not fill the bacillary bodies completely. When leprosy bacilli degenerate, the cytoplasm detaches from the cell wall and it is seen along the central axis of bacillary cell body (Fig. 1-c). When degeneration is more advanced, the cytoplasm of the bacillus becomes fragmented (Fig. 1-d, e). In the final stage of bacillary degeneration, only the cell wall remains (Fig. 1-f).

The criterion used for counting bacilli as solid under electron microscopy was that they be plump, filled with cytoplasm, and have cell walls not detached from the cytoplasm regardless of whether they were short or long. All other forms were regarded as nonsolid.

The criterion for solid bacilli under light microscopy was that there be uniform deep staining of the bacillary bodies without any clear defect in morphology. All other bacilli are classified as "nonsolid." These criteria were essentially similar to those of other workers ([1, 7, 9]).

The results are presented in Table 1. Only three cases among the thirty showed higher LM-MI values than EM-MI. However, the ratio of EM-MI to LM-MI (EM/LM ratio) varied from 1.04 to 12.33 in the other 27 cases. The statistical correlations between EM-MI and LM-MI are diagrammed in Figure 2. The sample points are distributed along the belt zone as shown in the diagram, and it is suggested that there is a positive linear correlation between LM-MI and EM-MI. In such linear correlations, Pearson's "simple correlation coefficient" is most suitable to express the correlation quantitatively, and this was calculated at 0.72.

Regression analysis may also be used as a means of determining statistical correlation. The regression equation was calculated on the hypothesis[3] of the linear correlation of LM-MI and EM-MI. The result of the calculation was:

$y = 1.84^{4} + 0.23x$, or $x = 4.38y - 8.08$.

This equation means that:

EM-MI(%) = (LM-MI) x 4.38 − 8.08.

DISCUSSION

In this study only formalin vapor fixation and staining at room temperature were used for LM-MI, because the heating of carbolfuchsin is the factor which causes the chief variation in determining the values of LM-MI([6, 8]). When formalin-vapor-fixed smears were compared with heat-fixed ones, bacilli were found to be stained more clearly with the former method than with the latter, and it is easier to determine whether the bacilli were solid or nonsolid by the former method.

Waters and Rees classified very short uniform bacilli as degenerated ([9]), but, by observation with the electron microscope, some bacilli of very short length were found to be completely filled with cytoplasm. Therefore in the EM-MI dterminations these short bacilli were counted as solid bacilli.

No cases showed zero value in EM-MI. This fact seems natural as these 30 cases examined in the present study were all active lepromatous or borderline-lepromatous cases with BI's of over 3+. Usually in clinical practice we regard lepromatous cases with zero LM-MI to be noninfectious. However, according to the results of this study, two cases with zero

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[3] The validity of this hypothesis was checked by the analysis of variance of regression coefficient. Calculated variance ratio was 30.59. This value is larger than the variance ratio ($F^{1}_{28}(0.01) = 7.636$). Thus, the calculation proves the validity of the hypothesis with the reliability of more than 99%.

[4] This term of the regression equation is called the "intercept." The significance of the value was checked under the null hypothesis H_0: the intercept = 0. The calculated t value was 1.615, and as the value was smaller than the acceptance region t_0 (28; 0.05) = 2.048, the null hypothesis was not rejected.

TABLE 1. *Comparative table of the values of light microscopic MI and electron microscopic MI.*

Case No.	MI(%) LM	MI(%) EM	Case No.	MI(%) LM	MI(%) EM
1	0.00	3.75	16	5.75	28.25
2	0.00	9.50	17	6.25	6.50
3	1.00	8.00	18	7.00	34.00
4	1.25	1.00	19	8.25	38.75
5	1.50	18.50	20	8.50	14.50
6	1.75	10.00	21	9.25	7.00
7[a]	2.25	5.75	22	10.00	22.50
8	3.25	2.25	23	10.25	26.00
9	3.25	14.50	24	12.00	28.00
10	3.75	33.00	25	12.00	33.00
11	4.00	9.25	26	14.00	45.50
12	4.25	8.25	27	14.25	50.75
13	4.25	46.00	28	14.25	63.25
14[a]	4.50	9.00	29	15.25	31.00
15	4.50	12.00	30	21.00	46.00

[a] BL cases.

LM-MI showed EM-MI values of 3.75 and 9.50 respectively. Therefore the evaluation of noninfectivity based only on zero LM-MI would appear unreliable.

Electron microscopy is more reliable than light microscopy in studying the leprosy bacilli because of higher resolving power and its more simple procedure of specimen preparation. The electron microscopic MI, therefore, seems to be more reliable than the light microscopic MI. However, the electron microscope is not available in all clinical laboratories and in such a situation, the regression equation may be useful for estimating the value of EM-MI based on LM-MI values. As the

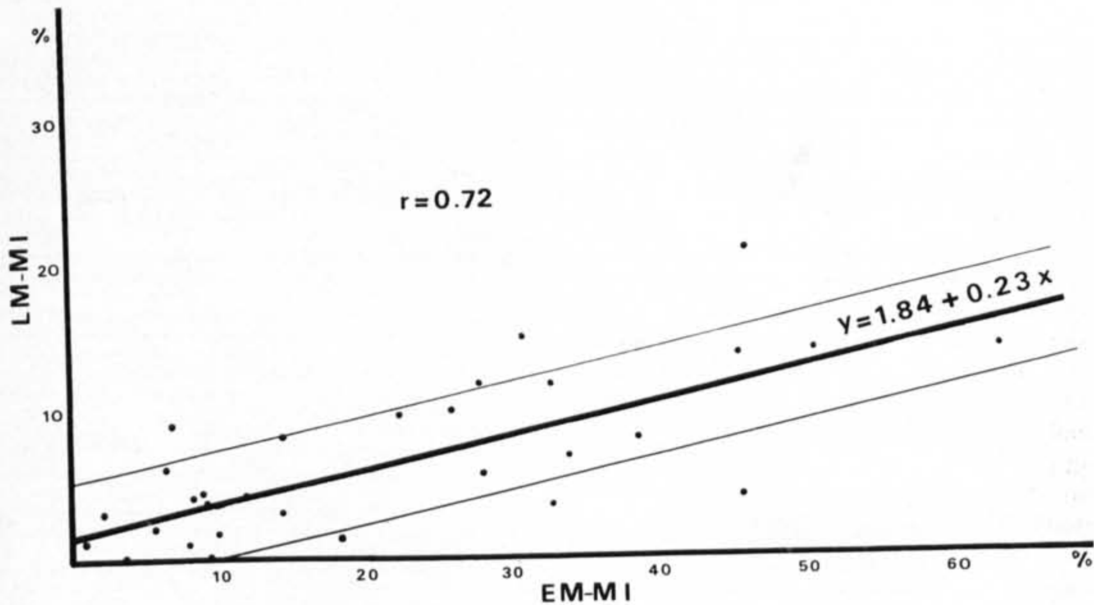

FIG. 2. Correlation diagram between light microscopic MI (LM-MI) and electron microscopic MI. (EM-MI); r: simple correlation coefficient.

accuracy of the regression is still not satisfactory, it is important to use LM-MI values as accurately as possible in using this equation for estimating the EM-MI value based on the value of LM-MI. At least 400 bacilli should be counted for the LM-MI as has been previously suggested ([2]).

SUMMARY

A new method of examining the Morphologic Index of leprosy bacilli by electron microscopy, using the microsuspension method, is reported. The Morphologic Index determinations of 28 lepromatous and two borderline-lepromatous cases were determined by comparative light and electron microscopy. In almost all cases the electron microscope gave higher values than the light microscope. A correlation between the two kinds of MI can be expressed by the formula: EM-MI (%) = (LM-MI) × 4.38-8.08. By using this equation, it is possible to estimate more reliable MI value from the data obtained by the light microscope and then it is possible to more precisely evaluate the early therapeutic effect of antileprosy drugs than by use of the light microscopy values alone. It was also found that MI negative values by light microscopy may be positive by electron microscopy.

RESUMEN

Se presenta un nuevo método para estudiar el Indice Morfológico de los bacilos de lepra por medio del microscopio electrónico, utilizando el método de microsuspensión. Se determinó el Indice Morfológico de 28 casos lepromatosos y dos casos lepromatosos-borderline, comparándose los resultados obtenidos por medio del microscopio de luz y el microscopio electrónico. En casi todos los casos el microscopio electrónico dió valores mayores que el microscopio de luz. La siguiente fórmula sirve para expresar la relación entre los dos tipos de IM : ME − IM (%) = (ML − IM) × 4,38 − 8,08. Por medio de esta ecuación se puede estimar en forma más exacta el valor del IM a partir de los datos obtenidos por medio del microscopio de luz, siendo así posible evaluar el efecto terapeútico de las drogas antileprosas en forma más precisa que por medio de los valores obtenidos con el microscopio de luz únicamente. Se encontró también que valores negativos de IM, pueden ser positivos con el microscopio de luz.

RÉSUMÉ

On rapporte ici une nouvelle méthode pour l'examen de l'Index Morphologique des bacilles de la lèpre au microscope électronique, utilisant une méthode de microsuspension. L'Index Morphologique a été déterminée sur 28 lépromateux et dans deux cas de lèpre lépromateuse-dimorphe, en comparant les résultats obtenus par la microscopie optique et par la microscopie électronique. Dans presque tous les cas, le microscope électronique a fourni des valeurs plus élevées que le microscope optique. Une corrélation entre les deux résultats des Index Morphologiques peut être exprimée par la formule : EM-MI (pour cent) = (LM-MI) × 4,38 − 8,08. En utilisant cette équation, il est possible d'estimer de façon plus fiable la valeur de l'Index Morphologique, à partir des données obtenues par le microscope optique. Il devient dès lors possible d'évaluer de façon plus précise l'action thérapeutique précoce des médicaments anti-lépreux, que l'on ne peut le faire en ayant recours seulement aux valeurs fournies par la microscopie optique. On a également observé que des valeurs négatives de l'Index Morphologique pouvaient être positives par le microscope optique.

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