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The Degradation of *Mycobacterium leprae* by a Comparison of Its Staining Properties[1]

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Acid-fast stains whereby the disintegration of *Mycobacterium leprae* may be assessed have for many years provided a valuable means of indexing chemotherapeutic activity, and of estimating the cessation of active infection and risk of infectivity. Although the rate of degradation estimated by acid-fast stains formed the main basis of classification at the lepromatous end of the spectrum ([11]) little is known of the process of degradation of the organism or of the elimination of its degraded products. Such products are thought to play a role in the formation of extravascular immune complexes in the pathogenesis of erythema nodosum leprosum (ENL) reactions ([2]). Moreover, *in vitro* studies of lymphocyte transformation using whole or sonicated *M. leprae* antigen have demonstrated that hypersensitivity reactions may be dependent on bacterial disintegration. Reactions that involve skin have high lymphocyte responses to whole *M. leprae*. Reactions that involve mainly nerves respond to sonicated *M. leprae*, while mixed reactions involving both skin and nerves respond to whole and to sonicated *M. leprae* antigen ([1]).

This study was undertaken to follow the process of degradation of *M. leprae* by comparing the results obtained with a) acid-fast stains, b) methenamine silver to reveal cell walls ([5]), and c) the immunoperoxidase technique using an antimycobacterial antibody to identify non-particulate components. An immunoperoxidase technique was recently used to locate '*M. leprae* antigen' in tissues ([10]).

MATERIALS AND METHODS

Patients. Sixty-nine patients comprising different groups in the leprosy spectrum were available for study. They included 16 lepromatous (LL) patients, 8 active untreated patients (LLA) and 8 treated patients with advanced quiescent disease (LLR); 20 borderline lepromatous (BL) patients, 15 untreated and 5 treated; 8 untreated mid-borderline (BB); 8 untreated borderline-tuberculoid (BT), and 8 untreated tuberculoid (TT) patients. Five 'histoid' lepromas were also studied and four positive Mitsuda skin test biopsies, taken 30 days after intradermal injection of 0.1 ml of heat-killed *M. leprae* suspended in saline, were obtained from four BT patients. Twenty patients undergoing ENL reactions, 15 in the acute stage and five resolving, were also available for study.

The biopsies were received from the MRC Units at Sungei Buloh, Malaysia, and Addis Ababa, Ethiopia; from the Hospital for Tropical Diseases, London, and from Papua New Guinea.

Smears. Slit-skin smears from five patients treated for lepromatous leprosy in whom no acid-fast bacilli were detected were post stained with anti-BCG antiserum and then with methenamine silver.

Five smears of *M. leprae* from the armadillo were fixed, two in formol saline and two in formol-mercuric-chloride-acetic acid (FMA). These were stained with anti-BCG antiserum. The fifth smear (the control) was heat fixed and stained by Ziehl-Neelsen.

Tissues. Biopsies were fixed in FMA ([12]) for routine examination by hematoxylin-eosin and a modified Fite-Faraco acid-fast stain ([13]). Gomori-Grocott ([7]) methenamine silver impregnation was also used. The formol-mercuric-chloride-acetic acid mixture is used routinely in leprosy because of its excellent preservation of cytoplasmic detail. Recently it has been shown to be the fixative of choice for the immunoperoxidase technique ([3, 6, 9]), preserving both intracytoplasmic and some cell surface components.

Immunoperoxidase technique. Considerable experience is necessary to carry out this

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technique. The method described here has been successfully used by several technical assistants with precise reproducibility. The final preparation should have no background staining. Many heme-containing molecules, including hemoglobulin and catalase, oxidize diaminobenzidine-hydrogen peroxide (DAB) to form the insoluble polymer. This reactivity varies with fixation, incubation time, and pH. Therefore appropriate controls are essential for the specificity of the method.

Perfectly flat serial sections cut at 4 μm and air dried were used in this study. Excessive heat was avoided at all stages in the preparation of the tissue. The peroxidase-antiperoxidase (PAP) procedure of Sternberger, *et al.* ([19]) and Burns ([4]) was carried out with minor modifications. Trypsin did not intensify either the strength or the specificity of staining after FMA fixation and it was omitted.

Antiserum. Anti-*M. leprae* antiserum raised in the laboratory gave too weak a reaction to be of use, and pooled serum from five active LL patients was used instead as a source of antibody to *M. leprae*. These sections were examined directly after incubation with the antibody using peroxidase labelled anti-human Ig.

Anti-BCG antiserum was used to detect *M. leprae* antigen since *M. leprae* is known to share antigenic determinants with this *Mycobacterium* ([8]). Anti-BCG was obtained from DAKO (Mercia Brocades, Watford, England). It was raised without Freund's complete adjuvant and the specificity of antisera obtained from this source has been described by a number of workers [reviewed by Mason, *et al.* ([9])].

Serial sections of two active LL granulomas were used to standardize the technique. Optimal dilutions were obtained using a checkerboard system of testing serial dilutions of antiserum against other reagents. The other reagents were similarly tested for optimal efficiency. Anti-BCG was used at a dilution of 1/200 in Tris buffer.

Staining method. Sections are adhered to acid-cleaned, dry slides.

1. Take sections to water removing all trace of wax.
2. Remove mercury with iodine, 2 min.
3. Wash. Remove iodine with 5% sodium thiosulphate.
4. Block endogenous peroxidase with 30 volumes hydrogen peroxide in methanol, 1% H_2O_2 in 99 ml methanol for 30 min (room temperature).
5. Wash very well in running water followed by distilled water, 15–20 min.
6. Block excess reaction with normal swine serum diluted 1/5 in Tris, 45 min.

Tris

60.57 g Tris/500 ml H_2O
376 ml N/HCl
Adjust pH 7.6
Make up to 1 liter with H_2O
Use: dilute 1/10 with normal saline

7. Tip off swine serum, without washing.
8. Treat sections with optimally diluted rabbit antiserum, 30 min.
9. Wash several times in Tris buffer.
10. Treat sections with swine anti-rabbit Ig 1/20 in Tris, 30 min.
11. Wash very well at least 30 min, changing buffer 3 times.
12. Treat sections with PAP 1/50 (optimum dilution), 30 min.
13. Wash in Tris.
14. Make up DAB.

DAB (Sigma)

Dissolve 0.006 g DAB/10 ml Tris
Dilute 30 volumes H_2O_2 : 1 ml H_2O_2 + 10 ml Tris
Add 3 drops dilute H_2O_2 to DAB

15. Treat sections with DAB-peroxide for up to 5 min. Wash in tap water as soon as they turn brown.
16. Wash in running water, 10 min.
17. Counterstain nuclei in Mayer's hemalum, 15 sec.
18. Wash to blue.
19. Dehydrate, clear and mount.

All sections stained with anti-BCG were treated as described. However, Mitsuda skin biopsies required overnight staining with anti-BCG; the sections being kept in a moist chamber at 4°C.

Controls. One section was stained for endogenous peroxidase by a solution of DAB-peroxide. A second control was normal rab-

bit serum used in place of the antiserum. A third section was treated with serum after absorption of antibody by BCG (Glaxo, vaccine); 5 μg/ml BCG was incubated, mixed, centrifuged at 2500 × *g*, filtered, and used.

Finally, the method has been tested through the spectrum of leprosy and it was felt that this provided a control within the system ([15,16,17]).

Enumeration of bacilli. The Bacterial Index was determined as follows:

6+ 1000+ AFB in every field
5+ 100–1000 in every field
4+ 10–100 in every field
3+ 1–10 in every field
2+ 1 AFB in every 10 fields
1+ 1 AFB in every 100 fields

The solid, fragmented, and granular (SFG) index ([14]) was used to assess the ratios of solid-staining bacilli, to fragmented rods, to granular debris.

RESULTS

Control sections were all negative.

Sections stained with pooled LL serum as antibody were usable but gave weaker results than those obtained with anti-BCG serum.

Intact bacilli stained equally well by all three methods. BCG-positive bacilli had a beaded appearance. Bacillary products could only be demonstrated by special techniques—methenamine silver for cell walls, acid-fast stain for cytoplasm, and anti-BCG for degraded or soluble cytoplasmic components. The means of the Bacterial Indices are recorded in Figure 1.

Spectrum of leprosy. There was a gradual rise in numbers of bacilli from BT to BL, and in untreated patients equal numbers of bacilli were seen as rod-shaped organisms by all three staining methods. BCG-positive bacilli consistently appeared granular. Some macrophages contained diffuse BCG-positive debris, which was especially notable in the BL group. In this group after chemotherapy there was a diminished number of bacilli still recognizable by all these methods, but fragmented and granular acid-fast forms were increased, and the abundant intracellular BCG component was diffuse rather than granular. Some cells contained only this degenerative product.

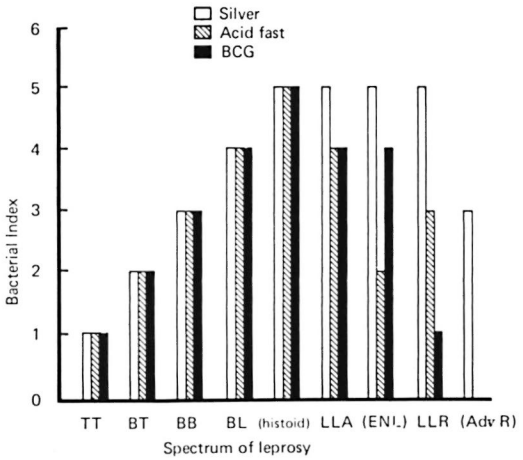

FIG. 1. The degradation of *M. leprae* in untreated patients through the spectrum of leprosy, histoid lepromas and in ENL.
LLA = active lepromatous leprosy
LLR = lepromatous leprosy in long-treated patients in regression
Adv. R. = advanced resolution in lepromatous leprosy

In all the active LL lesions there was an equally high density of bacilli which were solid by acid-fast stains, intact by silver, and granular by anti-BCG. In most cases no diffuse BCG material was evident, although in a few cases it was striking. Following prolonged chemotherapy, regressing lepromas had negligible numbers of solid bacilli and fewer fragmented or granular acid-fast bacilli. The level of diffuse BCG material in cells was much lower, although on occasion it was extracellular. There was no difference between active and regressing lesions in the large number of cell walls demonstrated by silver impregnation (Fig. 2). Advanced resolution was marked by clumps of aggregated and diffuse silver, cell-wall deposit.

Localization of antigen. The TT and BT lesions were marked by epithelioid cell granulomas, while the lepromatous group comprised bacteria-laden macrophages. Intermediate in the BB group, the lesion was composed of activated macrophages.

TT-BT-BB. Solitary acid-fast organisms with intact cell walls were found in the subepidermal zone or in small nerve trunks. In general, macrophages contained intact acid-fast rods which stained with a beaded appearance by anti-BCG. In some BT and BB

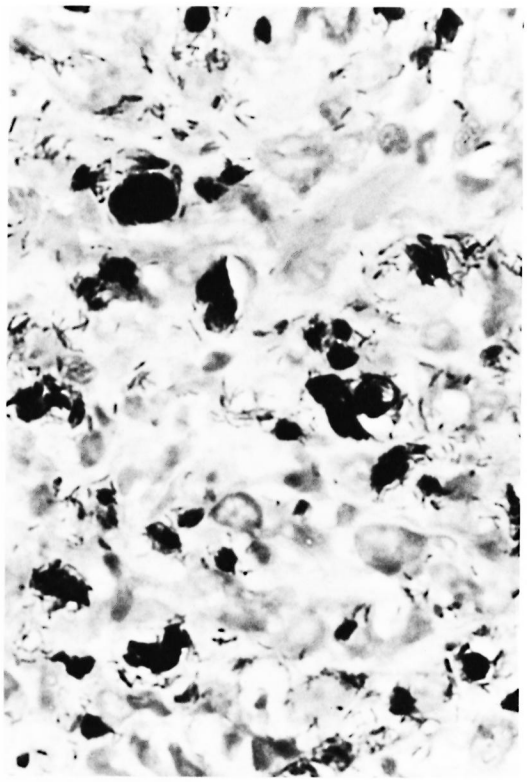

FIG. 2. Active lepromatous leprosy. Large numbers of intact cell walls are seen among aggregates of cell wall material. Methenamine silver (×750).

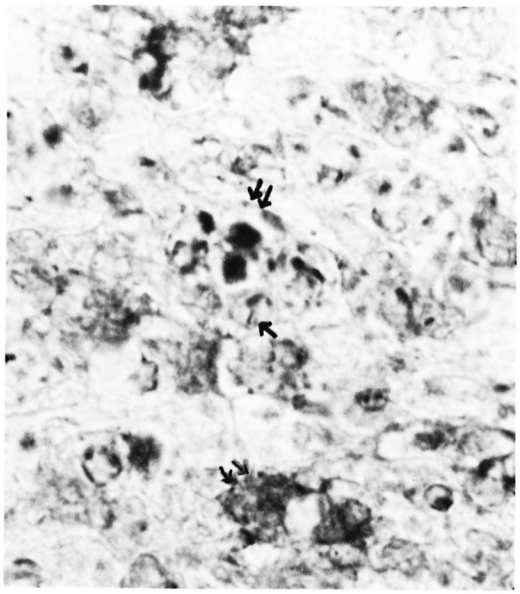

FIG. 3. BL leprosy. Diffuse BCG-positive debris (⬟⬟) appears in the phagocytic vacuole on disintegration of the bacillus (⬟). Immunoperoxidase anti-BCG (×750).

cases these macrophages also contained a small amount of diffuse BCG-positive debris. Very noticeable was the appearance of abundant diffuse BCG material in endoneurial and perineurial macrophages of nerves in the three groups, more than was apparent elsewhere in the granuloma or in the nerve itself.

BL-LL. Intact, acid-fast, BCG-positive bacilli were found in macrophages. Diffuse BCG material when present was seen in phagocytic vacuoles which sometimes appeared at the macrophage cell margin (Fig. 3). Granular or diffuse BCG components corresponding to acid-fast bacillary products were demonstrated in nerve trunks and in cells infiltrating the endoneurium or perineurium. Silver impregnation revealed intact and aggregated cell walls. Some endothelial cells of medium-to-large blood vessels contained only BCG-positive debris, and some of this material was seen close to the nucleus in muscle cells of the larger blood vessels.

In the granuloma of the epidermal-dermal junction, especially in LL lesions, solid-staining, acid-fast bacilli were located in the center of the lesion with more granular organisms at the periphery. Peripheral macrophages contained more BCG-positive debris and dense deposits of silver-impregnated, cell-wall material. This arrangement of cellular activity was reversed on passing lower into the dermal-subcutis region, where the most heavily infected macrophages were found in the advancing edge of the lesion. Extracellular diffuse BCG debris was sometimes present in active lesions but silver-impregnated, cell-wall debris was not seen outside the macrophage. Isolated macrophages in intercollagenous spaces contained few granular acid-fast bacilli and more abundant diffuse BCG-positive material (Fig. 4).

Histoid leproma. There was a uniformly high density of intact and solid-staining, acid-fast bacilli which appeared granular with anti-BCG. Extracellular diffuse BCG debris was demonstrated especially in the subepidermal border and in discrete foci

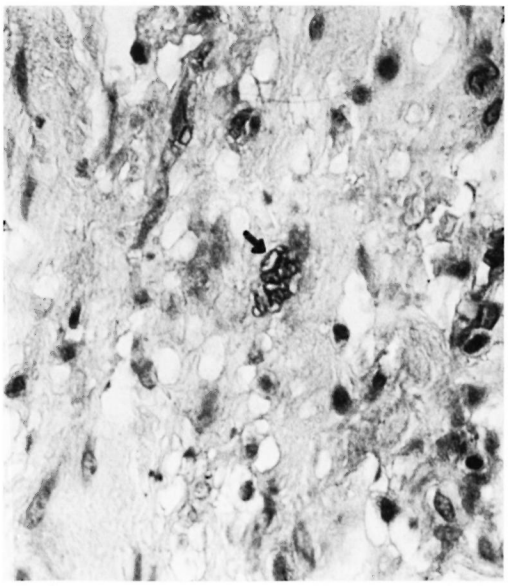

FIG. 4. Lepromatous leprosy. Isolated macrophages in intercollagenous spaces contain diffuse BCG-positive debris (⬟). Immunoperoxidase anti-BCG (×250).

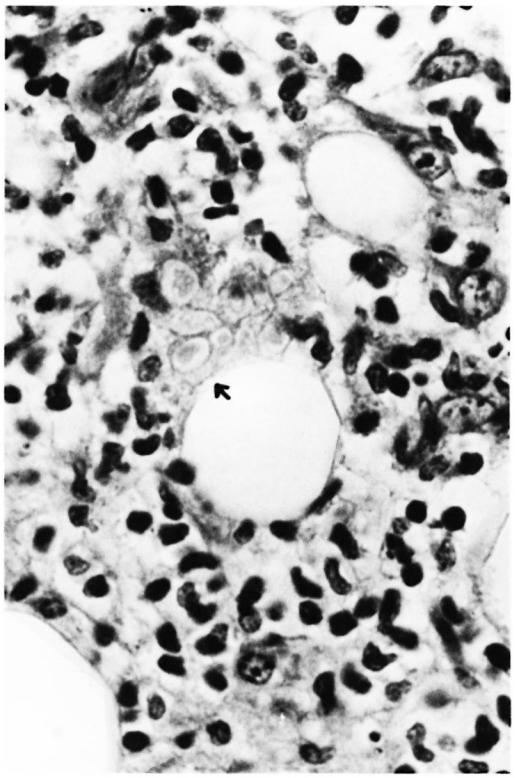

FIG. 5. ENL. Diffuse BCG-positive material is seen around fat lobules in the subcutis (⬟). Immunoperoxidase (×400).

within the granuloma. The material was seen in isolated neutrophils in these lesions.

Erythema nodosum leprosum (ENL). The non-reacting area of the lesion was marked by large numbers of intact cell walls, a lesser number of which were granular with the acid-fast stain. The demonstrable BCG component was variable, mostly intracellular, granular and diffuse. This contrasted with the appearance of bacilli in the reacting areas where much diffuse BCG-positive material could be detected extracellularly and in macrophages, especially around fat lobules (Fig. 5). Very little acid-fast granular debris was seen in the degenerating macrophages, although there were some intact rods and large aggregates or a diffuse material by silver impregnation. Neutrophils in the reacting area contained only BCG or silver-stained diffuse components.

In resolving ENL lesions there were a few residual foam cells containing degraded bacillary products, either cell walls or acid-fast or BCG-positive debris. Isolated residual macrophages in intercollagenous spaces contained BCG-positive debris.

Mitsuda skin tests. Small amounts of diffuse BCG-positive material were found in a few macrophages scattered among accumulations of epithelioid cells. No cell walls or acid-fast organisms were detected.

Smears. The five smears negative by acid-fast stains did not stain with BCG. However variable numbers of cell walls were seen after treatment with silver.

Smears from the armadillo contained solid or fragmented acid-fast bacilli. FMA fixation gave an enhanced granular staining of *M. leprae,* compared to formalin, after anti-BCG.

DISCUSSION

The immunoperoxidase technique has been used previously to compare BCG-positive *M. leprae* with those that stain by Fite-Faraco's acid-fast stain. No quantitation was attempted but the acid-fast technique was thought to be slightly superior ([10]). The difference in the results with anti-BCG in that study compared to those in this study may be due to technique.

In the present investigation it was difficult

to stain *M. leprae* with *M. leprae*-specific antibody. With BCG antiserum granular staining was obtained. This suggests that there is difficulty of access to the mycobacterial determinant in the cytoplasm of an intact bacillus.

Degradation of *M. leprae* by the macrophage is seen here in three stages: acid-fast (solid, fragmented, and granular); non-acid-fast cell walls revealed by silver impregnation, and non-acid-fast, BCG-positive debris. "Solid" bacilli stain by all three methods but their appearance with anti-BCG is always granular. When the acid-fast organism becomes fragmented, diffuse BCG debris can be seen. In LL the cell wall is still intact and remains so often until the terminal stage of disintegration, by which time acid-fast and BCG staining is negative.

The rates of degradation vary at different points on the leprosy spectrum, reflecting cell-mediated immunity. Thus they are more rapid in the borderline than in the lepromatous group. In BT, BB, and BL the degradation of cell walls, loss of acid-fastness, and total elimination of bacilli are an almost simultaneous process. In the borderline part of the spectrum the last component of the bacillus to disappear was the BCG-positive debris; whereas in the lepromatous part of the spectrum, it was the cell wall. In regressing lepromatous patients, it is evident that the macrophages are effete and nonfunctional, as demonstrated by the lack of immunological factors shown by immunoperoxidase ([15]). They would therefore lack the enzymes required to digest the cell walls, and the leakage of BCG-positive material from damaged cell walls would be a slow process. In BB and BT the viable and active granuloma cells possess the enzymes required to digest the bacterial cell walls. Total absorption follows rapidly.

In BB and BT disappearance of BCG-positive material is, however, slower in nerves than in the epithelioid cell granuloma of the dermis, no doubt due to the barrier to absorption presented by the nerve and its perineurium. This would explain the observation that in reversal reactions associated mainly with nerves the response to sonicated *M. leprae* is greater than that to intact organisms, and vice versa in reversal reactions associated mainly with skin ([1]). Sonicated and non-sonicated organisms would both exhibit cell walls, but only in the former would the BCG-positive matter be exposed.

Although histologically a 30-day Mitsuda reaction is similar to the granuloma of a tuberculoid lesion, BCG-positive material was still present. This indicates that the breakdown of the bacilli had only recently been accomplished. The Mitsuda reaction is presumably a response to the event.

In ENL there is increased degradation of bacilli represented by a rapid fall in acid-fast organisms with a concurrent elevation of BCG-positive debris, relative to the situation in non-reacting lesions at a corresponding stage of regression. The release of BCG-positive debris into the extracellular space by altering the antigen-antibody ratio precipitates the ENL reaction ([18]). The reason why there should be a rapid build up of degradation products at this point is less clear. It seems likely that the first stages of decay following death affect the morphology of *M. leprae* but not the ratio of acid-fast to BCG-positive products. At some point, often perhaps related to time after commencement of chemotherapy, the organisms lose their acid-fastness with a proportional increase of BCG-positive debris. By this time the macrophages are also degenerate, and if they then release their degradation products the ENL cycle is likely to supervene, subject to there being a suitable antigen-antibody ratio.

As a final technical point, the non-specific staining of plasma cells ([15]) (and reported by other workers) can be blocked by adequate exposure to normal swine serum. This point needs critical examination when setting up the technique.

SUMMARY

The disintegration of *Mycobacterium leprae* is revealed by a study of its acid-fast component, its non-acid-fast cell walls using methenamine silver, and its BCG-positive cytoplasmic component. Solid bacilli stain by the three stain techniques used to identify these products, but the BCG component is demonstrated only with difficulty and appears granular.

Degradation of *M. leprae* is fairly rapid in BT, BB, and BL, and clearance of bacillary products occurs almost simultaneously because of the destruction of the cell walls.

However, clearance is slower in nerves and BCG-positive material persists. The breakdown of cell walls is slow in LL and their clearance is delayed, but BCG-positive material is cleared as fast as it leaks out. ENL appears to coincide with a more rapid breakdown and release of disintegration products from degenerate macrophages. The Mitsuda reaction appears as an epithelioid cell granuloma after complete degradation of *M. leprae* with residual BCG positive material at 30 days.

RESUMEN

Se visualizó la degradación del *Mycobacterium leprae* en base al estudio de su componente ácido-resistente, de sus paredes celulares no-ácido-resistentes (usando metenamina argéntica), y de su componente citoplásmico BCG-positivo. Los bacilos sólidos se tiñen por las tres técnicas usadas para identificar estos productos pero el componente BCG sólo se demuestra con dificultad y aparece granular.

La degradación del *M. leprae* es bastante rápida en los casos BT, BB, y BL, y la eliminación de los productos bacilares ocurre casi simultáneamente debido a la destrucción de las paredes celulares. Sin embargo, la depuración es más lenta en nervios, persistiendo el material BCG positivo. El rompimiento de las paredes celulares es lento en los casos LL y su eliminación es retardada, pero el material BCG positivo es eliminado tan rápido como se libera. Durante el estado reaccional tipo ENL el rompimiento y la liberación de productos de degradación a partir de macrófagos en degeneración parecen ocurrir más rapidamente. La reacción Mitsuda aparece como un granuloma de célula epitelioide después de la degradación total del *M. leprae* con material positivo residual BCG a los 30 dias.

RÉSUMÉ

On peut mettre en évidence la désintégration de *Mycobacterium leprae* en étudiant le constituant acido-résistant du bacille, les parois cellulaires non-acido-résistantes colorées par le methenamine d'argent, et le constituant cytoplasmique positif pour le BCG. Les bacilles solides se colorent par les trois techniques de coloration utilisées pour identifier ces produits, mais le constituant BCG ne peut être mis en évidence qu'avec difficulté et révèle un aspect granulaire.

La dégradation de *M. leprae* est plutôt rapide chez les malades BT, BB, et BL. L'élimination des produits bacillaires survient presque simultanément par suite de la destruction des parois cellulaires. Néanmoins, l'élimination est plus lente dans les nerfs, et du matériel positif au BCG persiste. La dégradation des parois cellulaires est lente chez les malades LL, et leur élimination est retardée, tandis que le matériel positif au BCG est éliminé au fur et à mesure, aussitôt qu'il est rejeté à l'extérieur des bacilles. Il semble que l'Erythème Noueux Lépreux coïncide avec une accélaration de la dégradation et de la libération des produits de désintégration provenant des macrophages dégénérés. La réaction Mitsuda apparaît comme un granulome de cellule épithélioide après la dégradation complète du *M. leprae* avec du matériel positif de résidu de BCG après 30 jours.

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