Mi 16

PIGMENT PRODUCTION BY M.LEPRAE IN VIVO AND IN VITRO CULTURES

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Taxonomically, Mycobacterium leprae is a member of Mycobacterium-Nocardia group of the actinomycetes. Other related groups are Aurantiaca, Gordona and Rhodecoccus. Almost all the members of these groups produce a variety of pigments : orange, pink, white, buff, lavender, salmon, red brown, brick-red, cream and other colours of pigments. These pigments, orange, yellow, buff-pink, tan, buff, chalky, malt, velvety, purple, grey, white, brown, pink, red can be found with or without O2 on exposure to light. The pigment metabolism of these mycobacteria or nocardia could be best studied on solid media but also to a lesser extent in liquid media. On protein rich media, melanoid, a tyrosine-based pigment is known to form. There has been very little information on pigment production by M.leprae. Preliminary reports show that M.leprae harvested from lepromatous armadillo livers showed a deep brown pigment.

Vaccine preparation with such M.leprae harvest was considered unacceptable and unethical. However, it was later realised that the iron-protein produced by M.leprae was responsible for this chromogenic effect. Our studies on the in vitro cultures of M.leprae on gelatin minimal agar and silicon minimal agar media showed that in the former medium, pigmentation started with creamish colouration, changed to light brown on prolonged aging. On silicon minimal medium, which had a glossy background, the pigment finally turned to pink-brown. M.lepraemurium in Fe-supplemented media, developed pigments of light brown colour after 3 weeks which changed to deep brown within 6 weeks and by 3 months to blackish brown or snuff colour.

The pigment production seems to be both of diagnostic and toxicological importance.

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Mi 27

ANTIBIOTIC-LIKE SUBSTANCES PRESENT IN THE SERA OF LEPROSY PATIENTS

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Many actinomycetes, particularly those belonging to streptomycetes are versatile producers of different antibiotics and related class of molecules, the bacteriocins or nocardiocins (from nocardiae)· However, there are hardly any report of antibiotic production by leprosy bacilli; this is possibly because of difficulties of growing the cultures in vitro and demonstrating its antibiotic activity by usual methods. This is, however, a widely but unexplained observation that cases of lepromatous leprosy with mutilation and open/exposed wounds, seldom get tetanus or other infections; this suggests that the patients lepra bacillary mass possibly offers a substantial protection due to antibiotic or such substances produced by it endogenously. Using a screening test based on dilutions of highly baccilliferous LL patients sera, it was found that only some sera but not all, caused inhibition of several mambers of a battery of sensitive test strains, e.g. B.pumilus, B.sphaericus, B.megaterium, B.cereus, S.aureus, E.coli, Cl.tetani. Cl.perfringens proteus and Pseudomonas spp. On investigation, it was found that LL cases which had already received some chemotherapy failed to show any inhibitory effects on these strains, while most of those LL sera of patients without any chemotherapy showed remarkable inhibitory effects.

We hypothesised that production of antibiotics by the LB in the LL cases stopped as a result of the bacteriostatic/bactericidal effects of these drugs on the leprosy bacilli circulating in the blood and tissues. We wanted to duplicate the inhibitory power of the serum of LL cases. For this purpose, several LL isolates available as CAN bacteria were cultivated in the GM medium fortified with guanine, hypoxanthene with optimum microaerophillc condition, incubating at 28 ^{0}C for various lengths of time and collecting the culture fluid at suitable intervals and looking for inhibitory effects by disc diffusion tests. The results showed a remarkable correspondence between the serum and culture fluid tests.

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Mi 28

A PURE CULTURE LEPROSY VACCINE

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The basic problems of leprosy immunity in lepromatous and non-tuberculoid cases are defects in cell mediated immunity, a progressive hyperbacillimoea, a harmful persistent immune complex state, a macrophage granuloma reflecting a surrender of the host to the leprosy bacillus, and massive bacillary invasion of almost all tissue/organs. In theory and practice, experimental evi-

dences show that immunological unresponsiveness or anergy of LL cases to leprosy bacillary antigens can be partly/wholly changed to a positive response by exposure to various permutations and combinations with BCG, leprosy bacillus, other mycobacteria, and modulated by immunoboosters; some such responses may alhhhso be long lasting. As a result, the immunological failure reflected by lepromin anergy, immune complex formation, macrophage granuloma formation, may be corrected substantially; firstly, by reducing the bacterial load by appropriate chemotherapy(s); in the next stage, various combination immunotherapy will help achieving lepromin conversion of the cases by recruiting the appropriate subsets of the T-cells. An in vitro grown killed culture of the leprosy bacillus identified as CAN bacteria will be a most suitable ingredient of all combination vaccines. Our studies show that these in vitro cultures have same microbiological, molecular biological identity, and immunological specificity as that of the leprosy bacillus (Acta Lepr. 1999 (11) 105 - 112). Selective chemotherapy is a very suitable adjunct to vaccinotherapy based on in vitro sensitivity tests.

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Mi 33

MYCOBACTERIA AMD M.LEPRAE : SOME UNSOLVED CURIOUS PROBLEMS

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Two curious unexplained characteristics of growth of mycobacteria, including that of M.leprae are (i) Unusually varied growth rates : rapid, slow, very slow, non - growers. This is not known for any other group of bacteria. (ii) The traditional anti-biotics, streptomycin, kanamycin, chloramphenicol, tetracyclines, penicillins, etc. are ineffective compared with SuDDS, EMB, PZA, PAS, INH, ETH, CFZ, THA, MINOCYCLINE, QUINOLONES, RIFAMPICIN, etc. which are now known as non-antibiotics .

This preferential drug-susceptibility appears to be the result of this group belonging to the actinomycetes and serving as biochemical factories of numerous antibiotics having natural immunity against these antibiotics. It follows, therefore, that searches for antimycobacterials among non-antibiotics may be rewarding. This part we have described in a companion paper.

As regards finding an answer to the problem of highly variable growth rate and speed of growth, we have found that the mycobacteria, including M.leprae belong to two nutritional poles, i.e., either these are chemoautotrophs (requiring simple C and N sources) or heterotrophs (requiring complex C and N sources), or admixtures of these 2 characteristics in different proportion, giving rise in effect rapid growth (heterotrophic), and non-growers (chemoautotrophic); slow and very slow growers mix these characteristics in varied proportions.

Our hypothesis, if true, could be proved in practice by devising a biphasic medium : comprising a heterotrophic bottom layer (LJM, without malachite green, the toxic dye) oveflaid with a 2-mi layer of 7H10 medium (basically a hemoautotrophic medium) with the tetrazolium dye at a final concentration of 200 mg/ml. If clinical materials were used for cultivation, a combination of chloramphenicol (50 mg/l, polymyxin 25 mg/ l and carbenicillin 15 mg/l eliminated all contaminants. Fungi were avoided by using actidione @ 2 mgl/ l. We had a large miscellaneous bag of mycobacteria including M.TB and

M.leprae. Growth evident from appearance of colonies and tetrazolium reduction within 48 hours gave clear cut results on the merits of the biphasic medium which has a sound theoretical basis. We had presented preliminary results at Antwerp and Madrid recently. We present details in this Congress, on the usefulness and possibilities of this medium as a rapid cultivation and susceptibility testing tool.

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Mi 37

EVALUATION OF LEPROSY LESIONS BY SKIN SMEAR CYTOLOGY IN COMPARISON TO HISTOPATHOLOGY

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Skin smears of 25 clinically suspected cases of leprosy were evaluated cytologically - fine needle aspiration cytology done in 13 patients with raised or nodular lesions and slit skin smear done in 12 patients with flat lesions. Cytological subclassification was done across Ridley-Jopling spectrum by reading May-Grunwald-Giemsa stained smears in conjuction with Ziehl-Neelsen stained smears. The results were later compared with histopathological interpretation of skin biopsy taken from same site.

Skin smear interpretation was conclusive in 18 (72%) patients and these were placed in Ridley-Jopling classification. On comparison with skin biopsy results;

complete cytohistological correlation was seen in 16(64%) and this comprised of 7 cases of Tuberculoid group (2 TT, 5 BT), one of BL and 8 of LL group. Cytohistological concordance was 100% in Polar leprosy.

Skin smear cytology, being a simple, relatively non traumatic, cost and labour effective technique with a high degree of histopathological correlation is recommended for routine use in diagnosis and follow-up of leprosy cases.

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Mi 39

NERVE TRUNK DESTRUCTION IN LEPROSY - A MORPHOLOGICAL STUDY

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The impact of leprosy is on nerves. The peripheral nerve trunks are the ones that generally get affected and damaged. While their involvement is detected clinically by thickening and tenderness with resultant loss of function, it is difficult to assess the histo-pathological changes in them. This is because these nerve trunks cannot be subjected to biopsy. However, it was possible to get 25 biopsy specimens from specially selected cases in a study of nerve grafting. The histological findings in these specimens are reported.

The proximal and distal ends of the specimen were marked while taking out the specimens at biopsy. A comparative assessment of qualitative and quantitative changes of the two ends was thus possible.

With the exception of 3 specimens, all the rest shared an epithelioid cell granuloma indicating the cases to be TT or BT types of leprosy. As characteristic of these types, many of the funicles were totally or partially destroyed, while a few remained intact. In 8 specimens, there was almost total destruction of the nerve with hyalinization or fibrosis. The destruction was more marked at the distal end than at the proximal end.

Cellular infiltration varied considerably. In some there was total fibrosis with minimal cellular infiltration, while in others there was varying degree of inflammatory exudate. The presentation is purely on morphological findings.

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Mi 61

STUDIES ON QUANTITATIVE RELATIONSHIP AMONG NORMAL MOUSE PAD, PCR AND ATP BIOLUMINESCENCE METHODS

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Various techniques have been used for monitoring of treatment in leprosy. These include commonly used method mouse foot pad (MFP) as well as other techniques like PCR and ATP bioluminescence. In our earlier study we reported on the overall qualitative relationship between these methods. While there was general correlation in the trends, there were differences in the actual positives reported by these methods. It would be thus important to understand quantitative relationship among these methods. In the analysis being reported in the present study, an effort has been made to understand quantiative relationship among these methods in BL/LL cases being treated with different therapeutic regimens. Biopsies were collected at various intervals of treatment and were processed for viability assessment by MFP (Shepard et al), PCR (Williams et al and Hartskeerl et al) and ATP by (Katoch et al) by techniqes already established at this institute. Biopsies were initally divided into two parts- one part was processed for mouse foot pad whereas the other part was processed for assessment of ATP levels as well as PCR positivity by either of two methods. ATP levels were measured as pg/ million acid-fast bacilli. Comparison of the results of these three techniques suggest that there is overall relationship between these methods when the ATP levels are in higher range. However, the relationship between the ATP vs PCR or ATP vs MFP is not straight in the lower ranges. The clinical significance of these findings needs to be debated.

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Mi 103

FALL IN THE BACTERIOLOGICAL INDEX IN LEPROSY SKIN SMEAR

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Objectives : To observe trend in fall of B.I. in bacteriological positive cases, registered in the Leprosy Mission Hospital, Naini.

Design : Retrospective study, reference from individual case charts, 100 cases of the year 1997 were followed up till the date released from treatment.

Setting : The Leprosy Mission Hospital, Naini, Allahabad

Participants : M.B. cases registered in 1997 and were released from treatment before March 2000

Main Outcome Measures : Percentage of cases, which will show a standardised fall of Bacteriological Index

Results : 15% had a B. I. fall of 0.31. 20% had a B. I. fall of 0.61, 42% had a B. I. fall of 1.01, 12% had a B. I. fall of 2.3 1, and 3% had a B. I. fall of 1.64, at the time of release from treatment.

Conclusion : 42% cases which had a B. I. fall of 1.01 per year, proves by this study that the average fall in B. I. is 1.01 per year.

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Mi 172

COMPARATIVE STUDY OF BI OF SKIN SMEARS AND BACTERIAL INDEX OF GRANULOMA (BIG) IN LEPROSY

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For the purposes of therapy, leprosy patients are grouped into Paucibacillary & Multibacillary leprosy based on clinical & bacteriological criteria. As all skin smear positive patients irrespective of their classification are grouped as MB leprosy, skin smears were considered as the final arbiter. More recently, in view of the general poor quality of skin smears there has been a de-emphasis on skin smears. Although histopathology remains as a standard diagnostic tool in the diagnosis of leprosy, it has no assigned role in treatment of leprosy.

The aim of the present study was to compare the bacterial load in the skin biopsy (expressed as BI of granuloma - BIG) to the BI of slit skin smears and to look for any predictable patterns and their probable value as a parameter in therapy of leprosy. This study was conducted in the Department of Dermatology of Osmania Medical College, Hyderabad, India between January & December, 1998.

108 patients (males 80; females 28) were included in the study. The clinical classes were TT-1; BT-61; BL-24; LL-12 and Ind. Leprosy-4. Skin smears were positive in 23 out of 108 patients (21.3%), whereas 42 biopsies were positive for bacilli (38.8%). Out of the 61 patients clinically classified as BT, only one patient was positive in skin smear, whereas 10 were positive for BIG in skin biopsies with value ranging from 1+ to 4+. In comparison, in 12 patients of LL group, 11 were positive for both skin smears and for BIG.

The difference between BI of skin smear and BIG in the present study was observed to be 1+ in 18 patients, 2+ to 3+ difference in 8 each, 4+ difference in 6 and 5+ difference in 1 patient.

This study identified 8 patients with less than 5 lesions, who revealed AFB with BIG ranging from 1+ to 4+ on skin histology. 4 of them revealed BL on histology with a BI ranging from 3+ to 4+. This study raises the issue whether BI of granuloma is a better indicator of the bacterial load and a true indicator of multibacillary or paucibacillary status.

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Mi 296

EXPERIENCES WITH QUALITY CONTROL OF SLIT SKIN SMEARS IN WEST NEPAL

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Green Pastures Hospital in Pokhara, Nepal has been functioning as a tertiary leprosy referral centre for over 20 years. After the integration of leprosy control programmes into the general health system of Nepal in 1987, the laboratory of Green Pastures Hospital was appointed with the task to set up a quality control network for slit skin smears within the Western region of Nepal for basic health service laboratories.

A survey was done by sending out all required materials such as slides and reagents to the peripheral laboratories through the quality control network in the Western region of Nepal. The basic health service laboratories were requested to send back 10% of their positive and negative slides for quality control. However, 52 slides were received during this survey. All slides were rechecked, comments on the quality were made and send back to the peripheral laboratories. Practical training and quality checkups were organized accordingly.

The following results were seen during the first survey. Regarding the quality of smearing,

30.7% of the slit skin smear slides were found to be spoiled with blood and did not have sufficient tissue material. Staining was not done adequately in 26.9% of the slides showing no sufficient decolounzation and also precipitation of carbol fuchsin. The bacteriological index (BI) of the positive slides correlated with the grading of the surveyors in only 22% cases. Positive slides reported as negative were found in 15.4%. After

follow up activities, insufficient smearing and staining was seen in only 8% respectively 2% of the slides and correlation of BI values occured in 93%.

We conclude that the experience in the Western region of Nepal demonstrates the importance of tracing problems and providing practical training in the area of slit skin smears in order to improve the performance in the peripheral laboratories.

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Mi 381

ENDOTHELIAL CELL INFECTION IN THE PATHOGENESIS OF LEPROMATOUS NEURITIS

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M. leprae is the only bacterial pathogen that infects peripheral nerves, often leading to defomity and thus to the stigma associated with leprosy. The mechanisms responsible for localization of M.leprae to peripheral nerves and therefore responsible for initiating specific nerve injury, have eluded detailed investigation due to the serious limitations in the biopsy of human peripheral nerves and the lack of good animal models.

Recent studies have revealed that experimentally infected armadillos develop a neuropathy which closely resembles human lapromatous neuritis. Evidence from this model suggests that an early event in the infection of peripheral nerves by M. leprae is the infection of endothelial cells of the epineurial blood vessels and lymphatics.

In order to study the mechanisms of infection of endothelial cells, we have examined the interaction between human umbilical vein endothelial calls (HUVEC) and M. leprae in vitro. HUVEC bound and ingested freshly obtained M. leprae and control BCG in a time and concentration-dependant manner. Uptake increased slowly, peaking at 18-24 hr. for M. leprae, and at 12-18 hr for BCG. Optimal uptake of M. leprae requires a ratio of bacilli: HUVEC of approximately 100:1. Assays using radiolabelled bacilli have revealed that uptake is slightly accelerated with heat killed or aldehyde fixed

M. leprae. Ultrastructural and confocal microscopic studies have indicated that some bacilli are internalized soon after binding to HUVEC. This suggests that the delay in uptake is probably due to low levels of binding initially by mechanisms which may be upregulated after prolonged exposure to mycobacteria.

We propose that M.leprae first colonize the surface of nerves, infecting lymphatic and vascular endothelium, then extend inward along blood vessels into the endoneurium. This is consistent with many previous reports of endothelial cell infection in leprosy, but contrasts sharply with the classical view that the bacilli initially bind and enter Schwann cells, ascend within the nerve, and explode outward . The possibility that nerve involvement by M.leprae begins with endothelial cells offers new approaches to understanding the pathogenesis of nerve injury in leprosy, viewing this as a dynamic sequence of adhesion, immunologic and inflammatory processes involving endothelium. The identification of specific mechanisms in this pathway of infection may offer new opportunities for intervention to treat or prevent nerve injury in this disease, and thus to prevent permanent nerve loss and subsequent deformity.

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Mo 26

CAN DNA HOMOLOGY BE THE ABSOLUTE YARDSTICK TO IDENTIFY M.LEPRAE?

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Before the nucleic acid characteristics of the leprosy bacilli (LB) could be adequately understood, there was a general belief that all LB possibly belong to a single genetic type, and identification of isolates of LB would depend on their conforming to this type. Studies on DNA relatedness of LB strains, however, revealed a wide diversity among different isolates. These genetic diversities could be due to the leprosy bacillus being prevalent for thousands of years over widely separated geographical regions like India, China, Egypt, Africa, Europe, as well as, the New World with chances of segregation and multi-centric evolution. These genotype differences are similar to those existing among the salmonellae, the plague bacilli, cholera vibrios and the tubercle bacilli; these differences in leprosy bacilli correlate well with distinct clinical diseases these types or subtypes produce. These often have a geographical segregation. In recent times, application of numerous taxonomic parameters showed that the so called M.leprae forms a dense cluster of human pathogenic strains, yet are divisible into numerous genetic subtypes, as evident from the work of different workers. These had thrown new light on several distinctly different biological types, e.g. those with long/short generation time (slow/fast growers) those with low or high yields in vitro; slow or fast growers in the mouse footpads; multibacillary, clinical LL or TT types; lucio type, histoid type, alopecia type, hyperbacillary single nodule type, pure nuritic