

## Attempts to Obtain Mitsuda Reactions in the Skin of Leprosy Patients, using Fresh Suspensions of Nodules Produced in Black Mice by Inoculations of *M. leprae*: Greatly Increased Virulence of *M. leprae* by Passage through Black Mice

(with an Addendum on work using Boiled Inoculum)

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FUMIO HAYASHI<sup>1</sup> continued the work of KENSUKE MITSUDA on skin tests with human lepromin in leprosy patients, and reported in 1933 that the reactions produced by boiled and unboiled lepromins, which he called vaccines, showed no difference. In a great number of leprosy patients I used living or dead suspensions of bacilli from human and rat lepromas, and confirmed the finding of HAYASHI.

This year I succeeded in obtaining the transmission of infection by *M. leprae* to black mice of American race, and produced subcutaneous nodules with lepromatous structure, or abscessed lesions. Such nodules I triturated and diluted in saline, and used the unboiled suspension as the antigen for skin tests, similarly to my previous use of boiled and unboiled suspensions of murine and human lepromas, and cultures of *M. tuberculosis*.

In the present experiment I used 7 volunteers, of whom 3 were lepromatous cases and 4 neural. On 1st September 1959 I inoculated each patient with 0.15 to 0.2 ml. intradermally of the said suspension, the site being the antero-lateral aspect of the left thigh. The patients were followed up over 70 days, biopsies were taken of their lesions on one or two occasions for study of bacteriology and histology, and the bacteria were also studied in the skin sera and nasal mucosa for comparison.

### Material and Methods and Case Notes

One out of 8 black mice inoculated 3 July 1959 with suspension of human leproma (derived from Edgard, case 7) and showing a large subcutaneous inguinal nodule, was killed on 26th August at 53rd day after inoculation. The saline dilution of the triturated nodule was examined microscopically and kept for the inoculations and for electron micrographs. The inoculations were carried out as for the lepromin reaction.

**Case 1.** L.C. white male, aet. 70 years, leprosy patient since 1938, his disease having completed the "parabolic curve" of Muir, from A<sub>1</sub> to B<sub>3</sub> and A<sub>2</sub> within 40 years. He is now a "burnt-out" case, with bilateral high steppage gait (Scheube sign) and claw hands. There were innumerable bacilli and globi in smears from both sides of his nasal septum. This was on 29th August 1959, after he had taken Promin intravenously for 2 years without any improvement in his neural symptoms. On 1st September 1959 he was inoculated with 0.15 ml. of the inoculum from the black mice. On 5th September he had fever, and pain in the site, and an erythema 15 × 15 cm. in size. On 9th September the erythema reduced and nodulation began to form in its centre. On 24th September a biopsy of the lesion was taken at the third week. On 4th October four smears were taken

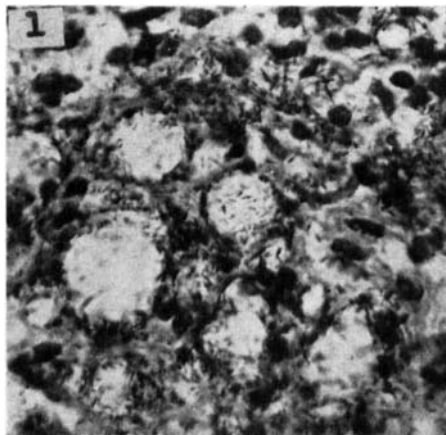


FIG. 1

*Photomicrograph of a section of the leproma of Edgard B. used to infect the black mice on 3rd July, 1959. Stained by Z.N. 700 x.*

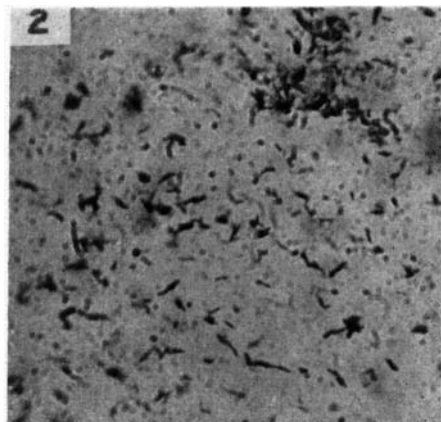


FIG. 2

*Photomicrograph of the smear of the suspension of the nodule of the black mouse killed on 26th August and used as inoculum for the skin tests. Suspension unboiled. Stained by Z.N. 700 x.*

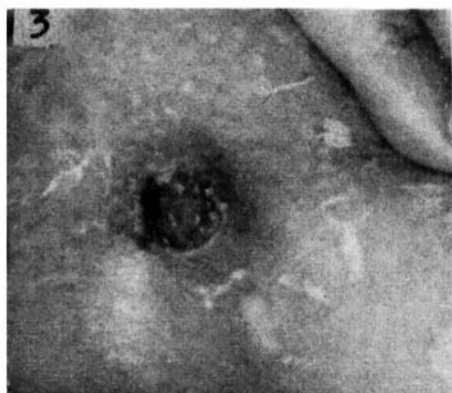


FIG. 3

*Photograph of the lesion of patient 2 after 66 days incubation: crater of  $1.5 \times 1.5$  cm. upon an infiltration of  $3 \times 4$  cm.*



FIG. 4

*Photograph of the lesion of patient 5 after 66 days incubation: deep crater of  $2 \times 1$  cm. upon an infiltration of  $4 \times 4$  cm.*

from the lesion and from the nasal mucosa. On 17th October there was a small scar with a crust on a nodule  $1 \times 1$  cm. On 24th October the nodule was small in size, at the 54th day after inoculation. On 5th November the lesion had subsided, On 14th November the nodule was  $1 \times 2$  cm., but no exacerbation of symptoms. and a smear was taken of the nasal mucosa.

**Case 2.** A.B.A., a white female, aet. 45 years, leprosy patient,  $L_1$  type, since 1953, when she had a few nodules on her legs and positive nasal mucosa. After a long period of standard treatment the skin lesions (lepromas and macules) disappeared and nerve signs started in both hands and feet. In 1950 she was  $N_2$  and began with Promin treatment, but there was no improvement in the nerve signs. On 30th April 1957 she presented double claw hand, perforating plantar ulcers on both feet, and there were a few bacilli in the nasal mucosa. On 1st September 1959 she was inoculated with 0.15 ml. of the suspension derived from the black mice. On 3rd September she had fever, pain in the site, and erythema of  $5 \times 5$  cm. locally. On 8th September nodulation in the site began. On 10th and 15th September the erythema was less and the nodule  $1 \times 1$  cm. On 17th September there was infiltration of  $3 \times 3$  cm. On 24th September a biopsy was taken of the lesion at the third week, and smears. On 10th October smears of the lesion were positive. On 24th October at the 54th day after inoculation the lesion was crateriform and bled easily. On 29th October the crater was  $1.5 \times 1.5$  cm. in size, and smears were taken. On 7th November the crater was still open, and was photographed. There was no exacerbation of skin symptoms and no lepra reaction at all.

**Case 3.** R.C.R., white male, aet. 35 years. In 1957 he was  $L_1-N_1$  with lepromas on both elbows, erythematous spots on both legs, impairment of sensation in arms, hands, and feet. Smears from skin and nose were positive for bacilli. He showed good improvement after 90 intravenous injections of Promin. On 1st September he was injected with 0.10 ml. of black mouse inoculum. On 3rd September he had fever and showed  $10 \times 10$  cm. local erythema. On 5th September the erythema was regressing and nodulation had begun. On 9th September there was a nodule of  $0.5 \times 0.5$  cm. and this increased by 15th September. On 22nd September, 3 weeks after inoculation, biopsy and smears were taken. On 29th September a crater had formed. On 1st October 4 smears were taken of the lesion and the nasal mucosa. On 27th October at 57 days after inoculation, there was a large crater with 2 cm. of detached skin around it, and the patient complained of pain. On 29th October new smears were taken, at a time when the lesion was regressing slowly. There was no exacerbation of skin lesions and no lepra reaction.

**Case 4.** G.W., white male, aet. 34 years, weight 126 kg. His father is a case of  $L_2$  leprosy, and his mother had early leprosy years ago. On 20th August 1959 he showed an erythematous infiltration on left frontal region, which was positive for bacilli, as also the nasal mucosa. He also had axillary and inguinal eczema marginatum. He was given Promin treatment, with a good response. On 1st September he was injected with 0.2 ml. of the black mouse inoculum. On 3rd September he had fever and pain, an erythema of  $10 \times 10$  cm., and swelling of left inguinal lymph glands which impeded his walk. On 5th September there was less pain and the erythema had increased to  $20 \times 20$  cm., without a nodule. On 10th September there was an infiltrated swelling turning to nodulation. On 15th to 22nd September there was a hard infiltration of  $10 \times 10$  cm. On 24th September, the 54th day, the infiltration had disappeared leaving a small scar with a slight secretion in its centre. There was no exacerbation of skin symptoms nor lepra reaction. A biopsy was taken of the experimental lesion.

**Case 5.** M.R., a white female, aet. 66 years, who had leprosy for 30 years as  $L_2$  case. She became clinically cured but relapsed and is intolerant to sulphone. Now she is  $L_2-N_2$  with widespread large elevated spots and perforating plantar ulcers in both feet, and skin and nose bacterially positive. On 1st September she was injected with 0.2 ml. of the black mouse inoculum. On 5th September she had fever and local pain and showed  $5 \times 5$  cm. of erythematous infiltration. On 19th September there was an abscessed lesion of  $5 \times 5$  cm. on the thigh. On 22nd September the third-week biopsy was taken, also smears. On 29th September pus was collected for bacteriology. On 6th October four smears were taken. On 17th October the lesion was better and the infiltration almost gone. On 3rd November smears were taken of skin and nasal mucosa. On 7th November, at the 67th day, the lesion was photographed. The ulceration was  $2 \times 2$  cm. She complained of pains and the skin lesions were exacerbated.

**Case 6.** N.A., a female mulatto, aet. 34 years, L<sub>3</sub> case for 9 years. On 1st September she was injected with 0.2 ml. of the black mouse inoculum. On 13th October she complained of fever and had increased general pains for the last 2 weeks, with exacerbation of skin lesions. As she suffers constantly from lepra reaction it is difficult to accept that the situation is due to the inoculum. Biopsy was taken. Smears of biopsy at the margin of the lesion were strongly positive for acid-fast bacilli which is not important because she is a case of diffuse leprosy. On 14th November a 2 × 2 cm. black scar remained in the lesion. The scar was opened with the galvanic needle and compressed to obtain secretion for culture and bacilloscopy.

**Case 7.** Edgard B., white male, aet. 37 years, who in 12 years had taken about 15 litres of sulphones, mostly intravenous Promin. After a period of good improvement, for 2 years he has suffered periodic lepra reaction. Now he is a L<sub>3</sub>-N<sub>1</sub> case, covered with plaques and large and small lepromas, and the nasal mucosa strongly positive for acid-fast bacilli and globi. About 6 months ago he started on 6 to 8 tablets of Ciba 1906, without change in the positivity of the nasal mucosa but with considerable change in the morphology of *M. leprae* from the skin. On 1st September he was injected with 0.2 ml. of the black mouse inoculum (the black mouse was infected on 3rd July 1959 with the patient's own leproma suspension). On 10th September there was nodulation of 2 × 2 cm. increasing to 3 × 3 cm. on 15th September. On 29th September in the 4th week after inoculation biopsy of the lesion was taken, and the secretion sown in culture media, and smears were taken. On 6th October the lesion was much better, with a crater of 1 × 1 cm. Four smears were taken. On 5th November the crater was reduced in size; smears were taken of the secretion of the lesion and nasal mucosa. On 7th November new biopsies were taken, and skin emulsion and nasal mucosal secretion inoculated in 15 black mice. Though this patient was an L<sub>3</sub> case he had stronger general and skin reactions with the inoculations.

### Bacilloscopy

Smears taken from the lesion at 3 weeks were surprisingly poor in acid-fast bacilli, and mostly showed free nodules. The nasal mucosa was positive in only 2 out of 7 patients. The scars, or eliminating foci, were poor in purulent secretion and in phagocytised elements.

### Histopathology

Biopsies of the lesions were made from all 7 patients, 4 being in the 3rd week after inoculation, 1 in the 4th, 1 in the 6th, and 1 in the 8th. The reports of Prof. C. B. MAGARINES TORRES (Chief of the Div. of Anatomopathology of the Oswaldo Cruz Institute). Reports Nos. 21,616 to 21,621 say "Moderate and discontinued infiltration by large mononuclear cells and lymphocytes around the superficial plexus of the subpapillary layer, and absence in all 6 specimens of nodules with tuberculoid structure". The material from one case (No. 6) was unsuitable for histological examination.

### Comments

The dose of the inoculum was about 0.2 ml., which is the dose needed to infect mice of 20 g. weight. This dose was given to patients of an average weight of 60 kg. and it could be 3,000 times greater. The mice support 0.2 ml. of fresh emulsion of human leproma without ulceration of the skin (except in passage). The leprosy patients, whether their disease was active or quiescent, suffered a heavy local reaction, with crater formation, lasting for more than

2 months, and the final state is as yet unknown. Two hypotheses are under consideration, one of the great increase of virulence of *M. leprae* cultivated in the bodies of these murines, and the other of the hypersensitivity of the leprosy subjects. It is not easy to obtain volunteers for such experiments, but I shall try to repeat the test with boiled inoculum.

### Conclusions

1. My impression is that *M. leprae* multiplied exuberantly in the black mice, increasing greatly in virulence, and this should be tested.

2. The living inoculum used caused strong local reaction in all 7 patients, which reaction differed clinically and histopathologically from that of the Mitsuda skin test.

3. These experiments should be repeated with boiled identical inoculum to allow of a definite conclusion.

4. The inoculated patients, save one, did not show the classical lepra reaction, but they will be observed further.

5. The lesions obtained in these 7 patients were comparable with those obtained with living cultures of acid-fast bacilli isolated from leprosy patients.

### Addendum on later work with boiled inoculum

Following up the 3rd conclusion above, I prepared *boiled antigen* from a skin lesion of another black mouse of the same batch, and gave a dose of 0.2 ml. by intradermal injection in 11 adult persons, being 10 leprosy patients and 1 contact, with the following results:—

**Case 1.** L.C., male aet. 70 years (the same as No. 1 of the previous experiment). On 24th December he was inoculated in both thighs. On 26th December there was an early reaction and on 31st December regression. After 2nd and 3rd weeks both sides reactive and positive for bacilli.

**Case 2.** A.B.C., female aet. 46 years (the same as No. 2 of the previous experiment). On 3rd December she was inoculated with 0.15 ml. below the previous crater, which was still open. There was a strong reaction. By 14th January the crater reduced to 2 sq. cm. The lesion was positive for bacilli and the nasal mucosa negative.

**Case 3.** E.B., male aet. 37 years (the same as No. 7 of the previous experiment). On 5th December he was inoculated in his left thigh, 15 cm. above the previous lesion, which was still open, excreting abacillary pus. On 7th December at 48 hours there was a strong reaction, with erythema and infiltration of 5 sq. cm. On 12th December the infiltration had reduced to 3 sq. cm. On 7th January in the 4th week infiltration had reduced to 2 sq. cm. On 12th January there was abscess formation and positive smears for bacilli, also in nasal mucosa, +. (In November 7 was ++.)

**Case 4.** A.S., male aet. 67 years. He was L<sub>3</sub>, now with only residual dark spots. The nasal mucosa is negative. Long ago he had repeatedly negative Mitsuda Test. On 3rd December he was inoculated with 0.2 ml. in the left thigh. On 5th December at 48 hours he showed infiltration of 3 sq. cm. and it so continued during 3 weeks. There was no pain. On 7th January the nodule ulcerated spontaneously. The Mitsuda Test was strongly positive clinically.

**Case 5.** R.R.C., black male, aet. 47 years. Was  $L_3$  for many years and is now  $N_3$ . On 7th December he was inoculated in his left forearm with 0.2 ml. On 9th December at 48 hours it was negative, and after 2nd week positive ++. After 3rd week +++. On 7th January the nodule ulcerated, smears positive +, and nasal mucosa negative.

**Case 6.** M.N.C., female aet. 25 years. She was  $L_3$  case, now bacterially negative. On 5th December was inoculated with 0.15 ml. in left thigh. On 7th December at 48 hours there was an erythema upon a hard infiltration of 10 sq. cm. On 31st December there was a large nodule and incision and aspiration were carried out. Bacilloscopy ++. On 7th January there was a scar of 0.5 sq. cm. On 14th January there was slight ulceration of 0.5 sq. cm.

**Case 7.** J.A., female of 45 years.  $L_1$ - $N_1$  case. The previous Mitsuda Test was slight +. On 3rd December she was inoculated in the left thigh with 0.2 ml. On 5th December at 48 hours was negative. After the 4th week there was erythema and infiltration. On 5th January the nodule opened. Bacilloscopy ++. On 12th January there was suppuration. On 14th January appearance of a good positive Mitsuda.

**Case 8.** E.R., white female of 65 years.  $L_2$  case. On 7th December was inoculated with 0.2 ml. in left thigh. After 1 week there was erythema of 3 sq. cm. On 29th December erythema was reduced. On 5th January the infiltration was reduced. On 12th January no nodule formed.

**Case 9.** H.L., white male, 58 years.  $L_3$  case. On 11th November 1959 the Mitsuda test was negative (Prof. H. Portugal). On 3rd December he was inoculated with 0.2 ml. in each thigh. On 5th December at 48 hours, negative and remained so up to 14th January.

**Case 10.** A.G., white female,  $L_2$  case 2 years ago, since 1 year becoming a neural case with great enlargement of the left cubital nerve and neuralgia in the right foot. Bacilloscopy of the cubital nerve ++. Nasal mucosa + on both sides. On 24th December she was inoculated with 0.2 ml. in her left thigh. On 26th December at 48 hours, negative, and up to 14th January negative.

**Case 11.** H.G., white male of 35 years, husband of above case No. 10. On 24th December was inoculated with 0.2 ml. in left thigh. On 26th December at 48 hours, doubtful positive. On 14th January, +. After 3 weeks became Mitsuda -positive.

### Comments

Five out of 10 leprosy cases,  $N_2$  and  $N_3$  type, gave a positive + test. Two  $L$  cases in frank regression gave one +, suspicious. Three  $L_2$  and  $L_3$  cases gave completely negative test and 1 adult contact of active leprosy gave mild positive + test.

### Conclusion

The boiled leprotic antigen derived from the black mouse gave results not different from the classical Mitsuda Test. The patients will be observed further for some time in order to allow of definite assessment.

### References

1. FUMIO HAYASHI, "Mitsuda's Skin Reaction in Leprosy", *Internat. J. Lep.*, I, 1, 1933, pp. 33-38.