

## **The action of deoxyfructose serotonin on intracellular bacilli and on host response in leprosy**

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*Summary* The drug deoxyfructose serotonin has already shown anti-leprosy activity according to *in vitro* tests on bacilliary suspensions, in the mouse footpad and in a pilot clinical trial. In a group of further tests in our Institute, activity against intracellular bacilli has been obtained both in macrophages from LL patients and bacilli within Schwann cells in organized nerve culture. *In vitro* tests also show enhanced lymphocyte macrophage interaction promoted by the drug with infected macrophages suggesting a possible enhancement of cellular immune response. Some protection *in vivo* against sciatic nerve damage by *Mycobacterium leprae* has also been shown in mice.

### **Introduction**

Deoxyfructose serotonin, a derivate of serotonin, was first shown to be active against *Mycobacterium leprae* using a rapid *in vitro* test for drug response.<sup>1</sup> We used the technique in which bacilli were incubated for 9 days in the presence and absence of the drug and subsequently tested for metabolic viability by pulse labelling with <sup>3</sup>H DOPA.<sup>2</sup> Positive results were also obtained in the mouse footpad.<sup>3, 4</sup> Rees has shown that 20 mg/kg body weight and 2 mg/kg by continuous oral administration completely inhibits multiplication of *M. leprae* in the mouse footpad. 0.2 mg/kg is without effect. He has found that the minimum inhibitory dose is 2 mg/kg both for DDS sensitive and DDS resistant strains of *M. leprae*; some toxicity tests indicated extremely low toxicity. General de Medicine Saint André therefore treated 7 patients in Bamako, 4 of these were polar LL cases.<sup>5</sup> A good clinical response was obtained. This was confirmed independently

by two leading leprologists who visited Bamako during the trial. Extensive chronic toxicity tests have now been carried out according to the internationally accepted protocol. Absence of toxic and side effects have been shown up to  $200 \times$  the clinical dose. Further clinical trials are planned.

In view of the possible clinical importance of this drug, some further studies have been carried out at the foundation for Medical Research. A number of techniques are in daily use for basic studies in leprosy. We decided to submit the drug deoxyfructose serotonin to some of these routine tests in the hope that the results might give some leads for the further work in the development of drugs related to molecules found in nerve tissue. In view of the well-known affinity of *M. leprae* for nerve tissue, a unique characteristic amongst the mycobacteria, such drugs may eventually play an important role in leprosy treatment.

## Materials and methods

### MACROPHAGE CULTURES

Macrophages obtained from peripheral blood of lepromatous leprosy patients were cultured in Leighton tubes according to the method of Birdi *et al.*<sup>6</sup> and Salgame *et al.*<sup>7</sup> The macrophages were incubated for 7 days in Eagle's minimum essential medium containing 40% human AB serum, the medium being changed every 48 hr. The high proportion of macrophages was identified by non-specific esterase staining.

Leprosy bacilli were obtained from LL patients' skin biopsies by the method of Ambrose *et al.*<sup>1</sup>  $5 \times 10^6$  Bacilli were added per live culture for 24 hr and ingested bacilli then removed by washing. A suspension of 1% sheep erythrocytes, sensitized with goat anti-sheep erythrocyte antibody, was added for 30 minutes at 37°C under 5% CO<sub>2</sub>. Cultures were washed, fixed in glutaraldehyde and stained with Fite-Neelsen acid fast staining to identify *M. leprae*. Control cultures without *M. leprae* were tested by rosetting. Uninfected cultures were also treated with 15 µg per culture of deoxyfructose serotonin for 24 hr and then tested by rosetting.

Another group was incubated for 24 hr with the drug, infected with *M. leprae* for a further 24 hr, washed with drug containing medium and incubated for a further 24 hr. A further set of infected cultures was maintained for 7 days before rosetting. The infected cultures treated with drug were washed 24 hr after infection and the drug removed before further incubation.

### LYMPHOCYTE-MACROPHAGE INTERACTION

Peripheral blood was obtained from lepromatous leprosy patients. The leuco-

cytes were separated and passed through a Ficoll-Triosil gradient to obtain peripheral blood monocytes.

$5-10 \times 10^6$  Peripheral blood monocytes were placed in 1 ml volume in Leighton tubes with coverslips. Live *M. leprae* was added as an antigen at a dose of  $5 \times 10^6$  bacilli per tube. Macrophages adhered to the coverslip and the tubes were incubated for 16–18 hr at 37°C in 5% CO<sub>2</sub>.

Cultures were terminated and fixed in glutaraldehyde. They were stained; 100 adhering macrophages were counted on the coverslip and those showing 20 or more lymphocytes attached were recorded.

Control cultures and cultures treated with 15 µg/ml of deoxyfructose serotonin were compared.

## SCHWANN CELL CULTURES

### (a) Culture methods

Sensory ganglia of 1- to 3-day-old Swiss white mice were removed under sterile conditions. The ganglia were treated with 0.25% trypsin in calcium and magnesium free phosphate buffered saline. Adult rat tail collagen in 1% acetic acid was dialysed against distilled water. A few drops were used to coat coverslips and polymerized in ammonia vapour for 5 min. The rinsed ganglia were placed on the collagen coated coverslips; they were incubated in a small volume of 70% Eagle's minimum essential medium containing 0.6% glucose, 20% foetal calf serum, 10% embryo extract and 100 IU/ml of penicillin.<sup>8</sup>

Schwann cells could be identified by several methods. They were closely associated with nerve fibres in the collagen matrix. Neurofibroblasts grew predominantly on the glass below. When unattached to nerve fibres, Schwann cells became, after a short time, bipolar with extremely long and thin pseudopodia, whether in high or low density cultures. Neurofibroblasts exhibited one or more fan-like leading pseudopodia in low density culture. They only became bipolar due to contact inhibition at high density.

Schwann cells when not attached to nerve fibres divided slowly reaching a peak only after 2 weeks in culture. Neurofibroblasts divided rapidly from the commencement of culture. Cytosine arabinoside and fluoro-deoxyuridine at  $10^{-5}$  M concentration much reduced the number of neurofibroblasts.

When Schwann cells associated with nerve fibres, basement membrane could be demonstrated in the electron microscope. After the second week of culture myelin secretion by the Schwann cells could be demonstrated by Sudan black B staining.

### (b) Labelling

2 µCi/ml of <sup>3</sup>H thymidine (specific activity 8.9 Ci/mM) and 2 µCi/ml of <sup>3</sup>H leucine

(specific activity  $7.9 \text{ Ci/mM}$ ) were in each case added for 24 hr. Cultures were washed, fixed and coated with Ilford K5 nuclear emulsion, they were subsequently examined as autoradiographs both under high power transmission microscopy and with a polarizing vertical illuminator to identify the silver grains unambiguously.

*(c) Infection with M. leprae*

Suspensions of *M. leprae* prepared in the same way as for infection of macrophage cultures at  $5-8 \times 10^7$  bacilli per culture were added to 1-2 week old Schwann cell cultures for 72 hr. Cultures were incubated for a total of 28 days. All cultures were fed on alternate days, being of course maintained at 100% humidity in 5%  $\text{CO}_2$ .

In the cultures both DNA and protein synthesis were estimated in separate cultures by thymidine and leucine labelling followed by autoradiography. This test was carried out during the proliferative phase of Schwann cell activity, prior to myelin secretion.

Uninfected cultures treated with  $10 \mu\text{g/ml}$  of deoxyfructose serotonin were also subsequently pulse labelled both with thymidine and with leucine in separate experiments.

Cultures infected with *M. leprae* were set up in parallel sets.

One group after infection for 72 hr and washing was fixed and Fite-Neelson acid fast stained; the other set was maintained for a further 28 days and fixed.<sup>9</sup> A parallel group was treated with  $10 \mu\text{g/ml}$  of deoxyfructose serotonin for 28 days then fixed and stained. A total of 10 sets of experiments with parallel control and drug treated cultures were carried out.

## SCIATIC NERVE OF MICE

The technique previously described to study nerve damage in the radial cutaneous nerve of patients<sup>11</sup> was used for the mouse sciatic nerve investigations.

Ten Swiss mice were inoculated in the mouse footpad with *M. leprae* obtained from a skin biopsy of an LL patient. Footpad harvests were carried out at 6, 7, 8, 10, 11 and 12 months post inoculation.

A similar group of uninfected mice was fed with  $20 \text{ mg/kg}$  body weight daily of deoxyfructose serotonin by oral administration.

A third group of uninfected mice with *M. leprae* was similarly fed with  $20 \text{ mg/kg}$  body weight of the drug.

At harvest, paraffin sections of the sciatic nerve were prepared. Sections were stained with TRIFF and observed under high power in the light microscope. Ultra-thin sections were examined in the transmission electron microscope.

## EXPERIMENTAL RESULTS

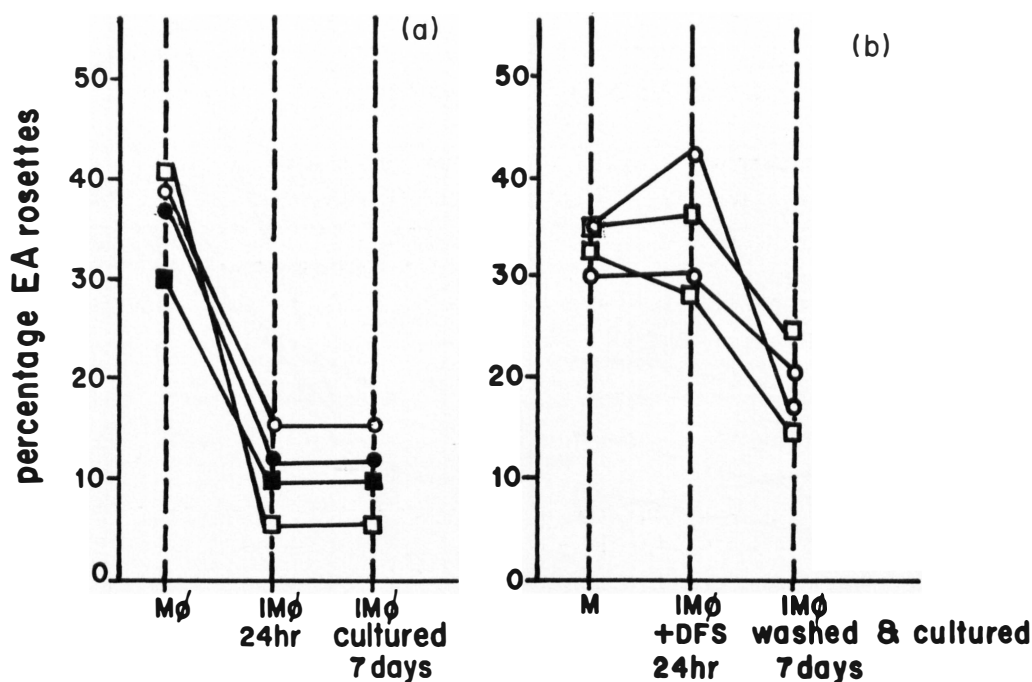
## 1. Macrophage cultures

(a) *Rosetting*. Results are shown in Figure 1 for 4 separate sets of experiments.

On the left of Figure 1(a) and (b) (M0) is shown the percentage of rosetting for macrophages of LL patients before treatment. After 24 hr infection with live *M. leprae* there is a marked fall (IM0).

Uninfected cultures treated for 24 hr with 15  $\mu$ g per culture of deoxyfructose serotonin maintain *no alteration* in percentage rosetting. (Heat killed *M. leprae* also produced no fall in rosetting.) *M. leprae* infected cultures treated with 15  $\mu$ g per culture of deoxyfructose serotonin show a return to the percentage level of rosetting for uninfected cultures, Figure 1(b) (IM0 + DFS 24 h).

Infected cultures maintained for a further 7 days and rosetted on the 7th day maintain a low level of rosetting, Figure 1(a) IM0 cultured 7 days.



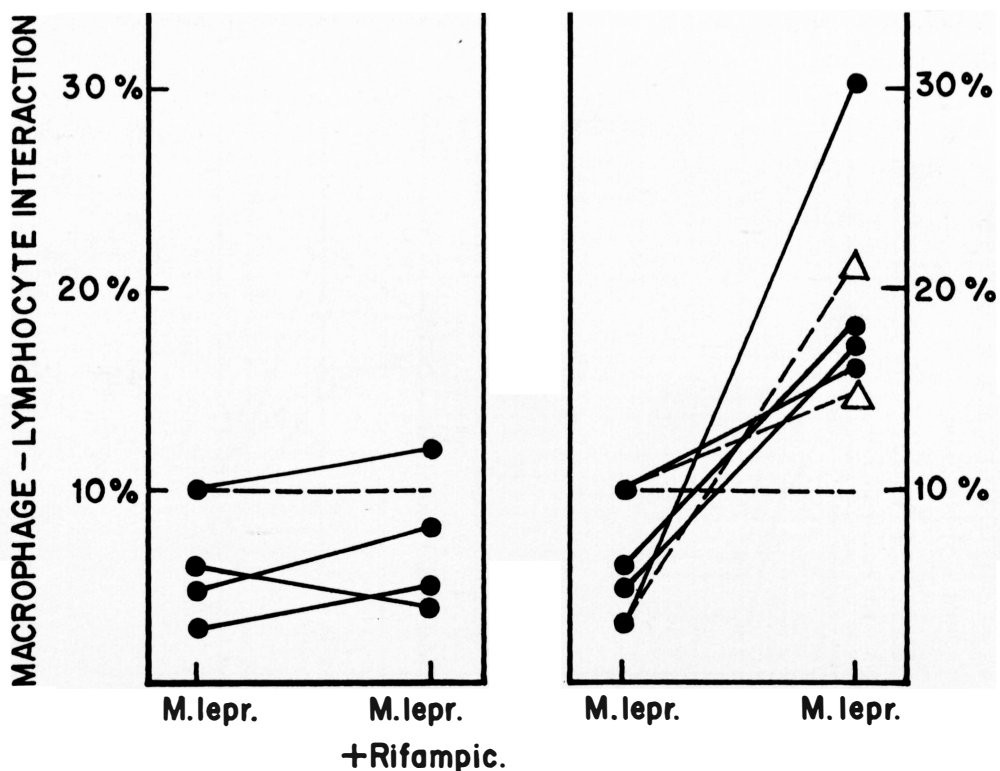
**Figure 1.** (a) Effect of infection with *M. leprae* on percentage of EA rosetting of macrophages. M0, level of rosetting before infection with *M. leprae*; IM0 24 hr, level of rosetting after infection of macrophages with *M. leprae*; IM0 7 day, level of rosetting after further culture for 7 days. (b) Effect of DFS on rosetting of macrophages infected with *M. leprae*. M0, level of rosetting of control macrophages treated with DFS (20  $\mu$ g/ml); IM0 & DFS 24 hr, level of rosetting of macrophages infected with *M. leprae* and treated with DFS; IM0 washed 7 days, level of rosetting after washing to remove DFS and culture for a further 7 days.

With drug treated cultures washed after 24 hr drug treatment and maintained and rosetted after a further 7 days the percentage rosetting falls as shown in Figure 1(b) (M0 washed and cultured 7 days).

The experiments therefore show that drug treatment causes the live bacilli to behave like dead bacilli, although this condition is not maintained after washing away the drug.

(b) *Lymphocyte-macrophage interaction.* In the case of LL macrophages infected with *M. leprae*, the level of rosetting is extremely low, Figure 2. Both fresh and heat killed bacilli give about the same percentage rosetting. This is not appreciably changed by rifampicin treatment.

On the other hand treatment of infected cultures with levamisole leads to an enhanced lymphocyte-macrophage interaction. Treatment with 15  $\mu$ g per culture of deoxyfructose serotonin has a similar effect.



**Figure 2.** Interaction between lymphocytes sensitized to *M. leprae* antigen and autologous macrophages infected with *M. leprae*. (a) Ordinate indicates the percentage of macrophages showing adhering lymphocytes. *M. leprae*, percentage of interaction with macrophages infected with *M. leprae*; *M. leprae* and rifampicin, the same after treatment with 2  $\mu$ g/ml rifampicin. (b) Effects of deoxyfructose serotonin and levamisole on interaction. *M. leprae*, percentage of interaction with macrophages with *M. leprae*; ●—, *M. leprae* infected + 15  $\mu$ g/ml DFS; △---, *M. leprae* infected + levamisole.

## 2. Schwann cell cultures

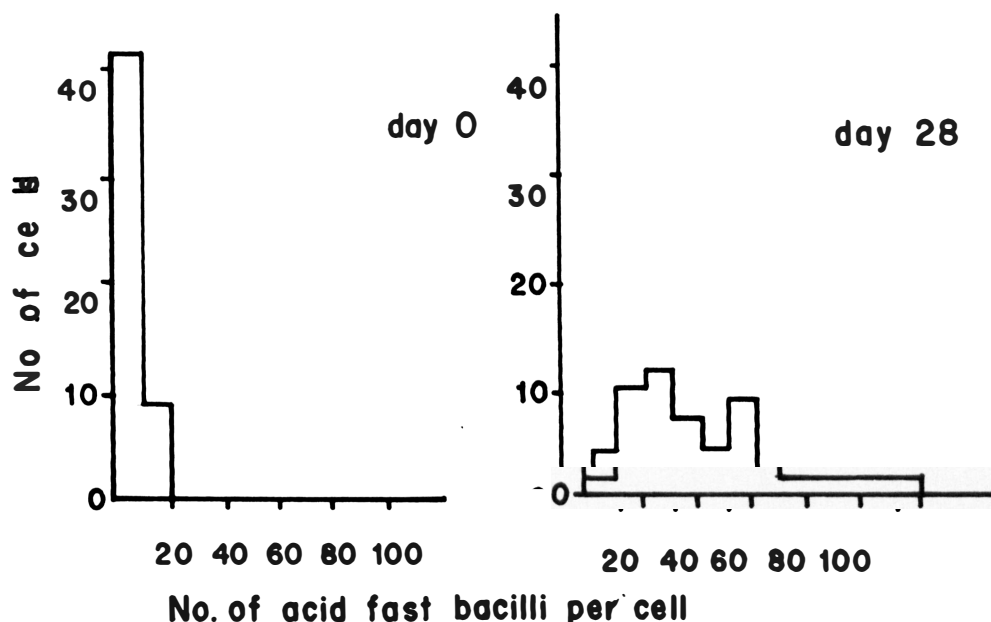
In Figure 3 are shown histograms for counts before and after 28 days in culture of numbers of bacilli per Schwann cell in 1 out of 10 similar experiments. Only cells containing 1 or more bacilli were counted at day 0 because only such cells can exhibit multiplication of *M. leprae*. The histograms show cells grouped in the range 1–10, 10–20, etc., bacilli per cell. Counts for day 0 and day 28 are shown.

In Figure 4 are shown two similar sets of infected cultures incubated for 28 days and two sets of these cultures treated with 10  $\mu\text{g/ml}$  of deoxyfructose serotonin throughout the 28 days of incubation. Set (a) and Set (b) are out of 10 of the separate experiments giving similar results. Set (a) and Set (b) were counted by different observers.

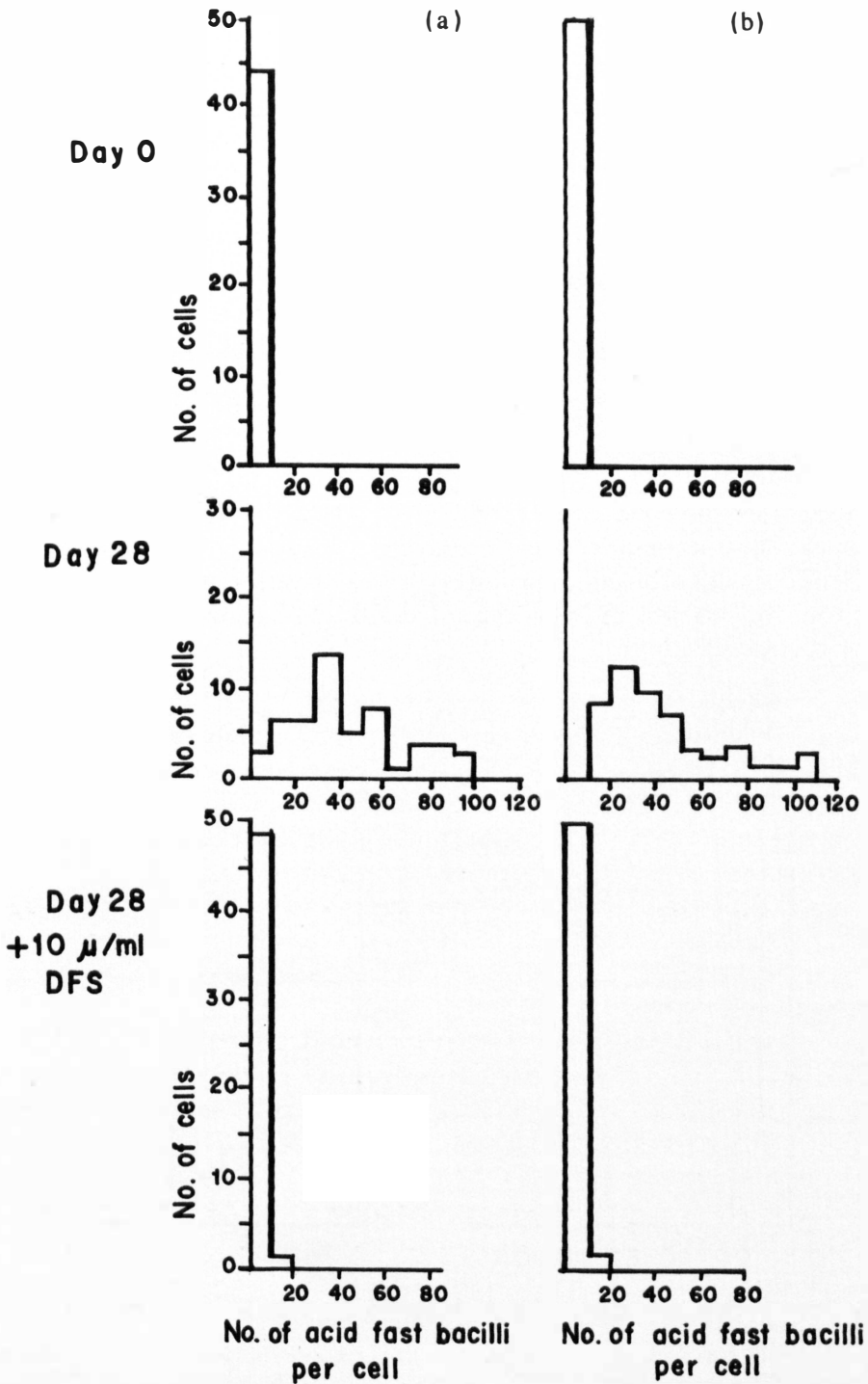
## 3. Mouse sciatic nerve

The results are shown in Table 1. DFS alone was without effect on mouse sciatic nerve histology, as compared with controls.

Considerable protection against nerve damage due to mouse footpad infection was shown by the mice fed 20 mg/kg body weight orally of deoxyfructose serotonin.



**Figure 3.** Histogram showing the numbers of acid fast bacilli per Schwann cell in Schwann cell culture infected with *M. leprae* and after further culture for 28 days. The number of bacilli per Schwann cell are grouped within the ranges 0–10, 10–20 etc. The ordinate shows the number of cells counted with bacilli numbers in each range.



**Figure 4** (a) As for Figure 3 except that cultures treated with 10 µg/ml of DFS were compared with control cultures at 28 days, showing complete arrest of proliferation of acid fast bacilli within Schwann cells at 28 days. (b) A similar experiment counted by a different observer. (A total of 10 reproducible experiments were carried out.)

**Table 1.** Foot-pad counts/the sciatic nerve damage in DFS treated mice

| Months<br>—post | <i>M. leprae</i> only |   | <i>M. leprae</i> + DFS |   | DFS only     |
|-----------------|-----------------------|---|------------------------|---|--------------|
|                 | AFB/FP                | Nerve damage  | AFB/FP                 | Nerve damage  | Nerve damage |
| 6th             | $2 \times 10^4$       | —   | —                      | —   | Nil          |
| 7th             | $7.3 \times 10^4$     | —   | —                      | —   | —            |
| 10th            | $1.53 \times 10^5$    | Unmyelinated fibre changes (++)<br>Demyelination (±)  | $5.33 \times 10^4$     | Unmyelinated fibre changes (+)<br>Demyelination (±) | Nil          |
| 11th            | $5.9 \times 10^5$     | Not done  | $2.67 \times 10^4$     | Not done  | Nil          |
| 12th            | $2.35 \times 10^6$    | Unmyelinated fibre changes (+++)<br>Demyelination (+) | $2.57 \times 10^4$     | Unmyelinated fibre (+)<br>Demyelination             | Nil          |

## Discussion

The results obtained both with rosetting of macrophages and culture within Schwann cells indicate that deoxyfructose serotonin has an effect on intracellular bacilli. In the case of the macrophage cultures, 24 hr treatment brings the rosetting level up to the value for heat killed bacilli. The fall which follows after washing may be due to the drug having a bacteriostatic action, but 24 hr is a short period of exposure and long term cytotoxicity cannot be excluded. Lymphocyte-macrophage interaction, as described in these experiments is expected to arise from the presentation of leprosy antigen at the macrophage surface. The result showing a similar type of enhancement as that obtained with levamisole suggests that deoxyfructose serotonin also enhances the capacity of the macrophage to process leprosy antigen and liberate it at the macrophage surface, i.e. it possibly enhances the cellular immune response.

There is complete inhibition of replication of *M. leprae* within the cytoplasm of the Schwann cells by 10 µg/ml of deoxyfructose serotonin, whereas the same concentration is without effect either on DNA synthesis or protein synthesis by Schwann cells in the proliferative phase. The fact that the bacilli do not disappear from Schwann cells after 28 days exposure to drugs, although proliferation is arrested, may also be due to a bacteriostatic effect. But Schwann cells do not have a normal phagocytic function unlike macrophages. They may not possess the apparatus to rid themselves completely of moribund bacilli. The protective action of the drug against damage to the sciatic nerve in mice infected with *M. leprae* is also encouraging.

It has already been shown that the suppression of DNA synthesis in proliferative Schwann cells by live *M. leprae* is not shown by heat killed *M. leprae* or by fresh related mycobacteria.<sup>10</sup> These include ICRC-C44, and a group of cultivable acid fast strains provided by Professor L. Kato. This again brings home

the importance of looking for specific features of *M. leprae* which lead to nerve damage. So far drug design for leprosy, in the absence of a suitable culture technique has been based on drug series already effective against other mycobacteria. With deoxyfructose serotonin we have a drug which is specific in its action against *M. leprae*, being inactive against other mycobacteria tested. Specificity of uptake by *M. leprae* is also shown with  $^3\text{H}$  DOPA.<sup>12</sup> In the search for new and more effective drugs for leprosy treatment, it should surely be in the direction of compounds related to nerve tissue products and metabolites that efforts need to be directed.

## Acknowledgments

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