Adherence of *Mycobacterium leprae* to Schwann cells *in vitro*

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Summary. Adherence of *Mycobacterium leprae* was studied *in vitro* in monolayer cultures of purified mouse Schwann cells. The optimum temperature and time for adherence were determined. Pretreatment of Schwann cells with lipase reduced adherence, but pretreatment with trypsin enhanced it and with four monosaccharides—L-arabinose, L-galactose, L-rhamnose and D-glucose—there was no significant effect, indicating that the receptors involved in adherence may be lipid.

Introduction

Mycobacterium leprae, the causative agent of leprosy, has a selective affinity for peripheral sensory nerves (Khanolkar, 1951; Iyer, 1965; Job, 1970), specifically for Schwann cells. *M. leprae* multiplies within Schwann cells and can survive in them for long periods as 'persistor' or 'resistor' organisms (Kahn and Scott, 1974; Waters *et al.*, 1980). Apart from macrophages, Schwann cells are therefore considered the main natural host cells of *M. leprae* (Stoner, 1979). However, very little is known about the mechanism of entry of *M. leprae* into Schwann cells.

In this study we have examined adherence of *M. leprae* to the Schwann cell surface and we present data about the nature of the adherence.

Materials and methods

Schwann cell culture

Sciatic and brachial nerves from 2–3-day-old Swiss white mice were dissected, chopped into fine pieces and incubated with Collagenase-Type II (Sigma, St Louis, USA) 0.05% w/v in a water bath at 37°C for 2 h. The nerve pieces were allowed to settle and the supernate containing the enzyme aspirated and replaced by feeding medium (see below). The nerve pieces were then passed twice through 21-gauge and twice through 23-gauge needles. The density of the single-cell suspension thus obtained was assessed by microscopy in a Neubauer chamber and plated on 13-mm coverslips coated with rat-tail collagen at a density of 2×10^3 cells/coverslip. The feeding mixture consisted of Dulbecco's Minimum Essential Medium (Gibco, Grand Island, NY, USA) with fetal calf serum (Gibco) 10% and antibiotics (penicillin 100 IU/ml, streptomycin 50 µg/ml and mycostatin 50 U/ml); cultures were incubated at 37°C. Fibroblast contamination in the culture was minimised by adding cytosine arabinoside to the feeding medium (Mukherjee *et al.*, 1980). Confluent

monolayer cultures of 95% pure Schwann cells were obtained within 4 days. The purity of the Schwann cells in cultures was determined by positive staining for non-specific esterase by the α -naphthyl acetate method (Bancroft, 1975) and by an indirect immunofluorescence test with anti-laminin antibody (Cornbrooks *et al.*, 1983).

Inoculation with *M. leprae*

Schwann-cell cultures 4–5 days old were inoculated with *M. leprae* suspensions, freshly prepared from armadillo-liver tissue by homogenisation, at final concentrations of $(5-50) \times 10^6$ /ml of culture medium. The inoculated cultures were incubated at 37°C.

Adherence test

Cultures infected with *M. leprae* were washed thoroughly with Hanks's Balanced Salts Solution (HBSS) (Centron Laboratories, Bombay) and examined for adherence as follows:

Scanning Electronmicroscopy (SEM). Cultures were fixed with glutaraldehyde 3% v/v at 4°C overnight, washed in three changes of cacodylate buffer, fixed in osmium tetroxide 1% w/v prepared in the same buffer, washed again and then dehydrated in graded concentrations of ethanol. The cultures were dried in a critical-point drier, coated with carbon and gold palladium, and scanned under the JEOL Scanning Electron Microscope.

Indirect immunofluorescence. Antibody to phenolic glycolipid-I (PGL), provided by Professor B. R. Bloom, was used to stain *M. leprae* adherent to Schwann cells. Coverslips were incubated with anti-PGL antibody, at a dilution of 1 in 40 in HBSS containing horse serum 10% (HBSS-HS), for 40 min in a humidified chamber at room temperature. Antibody was washed off and cultures were stained with goat anti-mouse IgG-conjugated to fluorescein isothiocyanate, diluted 1 in 15 in HBSS-HS, and incubated at room temperature for 30 min. Cultures were washed, fixed in 4% formol saline for 10 min, mounted with glycerol-phosphate-buffered saline, sealed with nail varnish and observed under the $\times 63$ oil-immersion

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objective of a Zeiss Standard WL Microscope equipped with epifluorescence.

Ziehl-Neelsen staining. Cultures were washed with HBSS, fixed in 10% formal saline overnight, stained by the Ziehl-Neelsen method and observed with a $\times 100$ oil-immersion objective by light microscopy. The bacilli adherent to Schwann cells were counted for each of several microscopic fields of each culture.

Effect of different agents on adherence

The effect of each of the following agents (in aqueous solution) was investigated: (i) Lipase-type 1 (wheat germ), tested at 25, 50, 75, 100, 150 and 200 $\mu\text{g/ml}$. (ii) Trypsin (Difco, Detroit, MI, USA), tested at 0.1%, 0.25% and 0.5% w/v. (iii) L-Galactose, tested at 5, 10 and 20 mM. (iv) L-Arabinose, tested at 5, 10 and 20 mM. (v) L-Rhamnose, tested at 5, 10 and 20 mM. (vi) D-Glucose, tested at 5 and 20 mM. All the chemicals except trypsin were from Sigma, St Louis, USA.

Results

Schwann cells in culture were identified by their

positive staining with non-specific esterase (fig. 1A) and anti-laminin antibody (fig. 1B) and *M. leprae* organisms by their intense staining in indirect immunofluorescence tests with anti-PGL antibody. Adherence of *M. leprae* was observed by light microscopy and by SEM (figs. 2A and 2B). Generally, there were 5–6 bacilli adherent to each of 70–80% of the Schwann cells.

Optimal conditions for adherence

Time of exposure. Schwann cells were exposed to 50×10^6 *M. leprae* organisms/ml of culture medium at 37°C for periods of 2, 4, 6, 8 and 12 h. Optimum adherence, determined by observation of 200 Schwann cells by light microscopy, was obtained 8 h after inoculation (fig. 3).

Bacillary concentrations. *M. leprae* was added to Schwann-cell cultures at concentrations of 5, 10, 20, 40 and 50×10^6 bacilli/ml of feeding medium. The cultures were then incubated for 8 h at 37°C, washed and fixed, and 200 Schwann cells were examined by light microscopy. There was an increase in the number of adherent bacilli from 50 to 580/100 cells as the inoculum was increased from 5×10^6 to 50×10^6 organisms/ml (fig. 4a).

Fig. 1. Histochemical staining of cultivated Schwann cells (S): (A) stained with non-specific esterase; (B) stained by indirect immunofluorescence with anti-laminin antibody.

Fig. 2. Adherence of *M. leprae* to cultivated Schwann cells demonstrated by: (A) indirect immunofluorescence; brightly fluorescent organisms (arrowed) adhering to Schwann cells (S) can be seen; (B) scanning electronmicroscopy; adherent organisms (arrowed) and Schwann cells (S).

Fig. 3. Kinetics of adherence; each point in the graph is the mean ($-S.D.$) of results from three separate experiments.

Fig. 4. Histograms showing the relationship between adherence of *M. leprae* to Schwann cells and (a) bacillary concentration of inoculum; (b) incubation temperature of inoculated culture; (c) use of live, heat-killed or formalin-fixed inoculum (mean value ($-S.D.$) of three separate experiments for each test).

Fig. 5. Effect on adherence of *M. leprae* after treatment of Schwann cells with: (A) lipase; (B) trypsin; (C) monosaccharides (galactose, arabinose, rhamnose and glucose). Data plotted as mean values ($\pm$ S.D.) of three separate experiments for each test. Percentage inhibition of adherence was calculated from the difference between the control and test values divided by control value and multiplied by 100 $\frac{(\text{control} - \text{test})}{\text{control}} \times 100$.

Further increases in the concentration of bacilli in the inoculum increased the numbers of bacilli in the background, to the extent that estimation of adherence became difficult.

Temperature. Adherence studies were performed at temperatures from 10°C to 37°C. Adherence was least at 10°C and greatest at 37°C (fig. 4b).

Effect of heat and formalin. The *M. leprae* inoculum was either (a) killed by autoclaving at 120°C for 20 min, or (b) fixed with 8% formaldehyde for 24 h. The treated organisms were washed thoroughly, then added, at a concentration of 50×10^6 organisms/ml of culture medium, to the Schwann-cell cultures, which were further incubated at 37°C for 8 h. The adherence of both killed *M. leprae* preparations was very markedly reduced (by 96%) in comparison with freshly prepared *M. leprae* suspensions (fig. 4c) (Student's *t*-test, $p \leq 0.05$).

In the light of the above results, an incubation period of 8 h at 37°C, with an inoculum of 50×10^6 freshly prepared *M. leprae* bacilli/ml of culture medium, was used as the standard procedure for all further studies.

Biological characteristics of adherence

Lipase. Pretreatment of Schwann cells at concentrations of 25, 50, 75 and 100 µg/ml reduced adherence and at 150 and 200 µg/ml blocked it (fig. 5A) (Student's paired two tail *t* test $p \leq 0.05$ for 150 and 200 µg/ml concentrations).

Trypsin pretreatment significantly enhanced bacillary adherence at concentrations of 0.1% and 0.25% (fig. 5B); 0.5% had no effect. (Student's *t* test, $p \leq 0.02$ for 0.1% and 0.25%). Concentrations higher than these were toxic for Schwann cells.

Monosaccharides. Galactose and arabinose each caused a slight enhancement of adherence (<10%; Student's *t* test, not significant). Rhamnose and glucose caused some inhibition, but this was not significant (fig. 5C).

Discussion

These results show that *M. leprae* binds to Schwann cells and that the binding is mainly at the polar ends and long processes of the cell. Adherence was greatest at 8 h after inoculation with 50×10^6 bacilli/ml and with incuba-

tion at 37°C. Adherence depended upon the viability of the inoculum. Viable *M. leprae* freshly prepared from armadillo tissue readily adhered to Schwann cells, but killing by heat or formalin markedly reduced adherence. Adherence may involve recognition of adhesins on the *M. leprae* cell wall by complementary receptors on the Schwann-cell membrane. The chemistry of the cell wall of *M. leprae* is not yet fully known (Draper, 1982) but it is very rich in lipids (Ratledge, 1982) and contains a phenolic glycolipid with a trisaccharide moiety on its surface (Hunter and Brennan, 1981). We therefore pretreated the Schwann cells with lipase and trypsin, and with monosaccharides, with a view to broadly characterising the receptor. The finding that adherence was most sensitive to lipase pretreatment suggests that the receptor may be a lipid. The adherence-enhancing effect of trypsin may be due to unmasking of cell surface receptors. Rhamnose and glucose, which form the trisaccharide moiety of phenolic glycolipid (Hunter and Brennan, 1981), caused slight (though statistically insignificant) inhibition of adherence, but galactose and arabinose did not. These preliminary results should be expanded to include the other sugars, lipids and amino acids that form the cell wall of *M. leprae* (Ratledge, 1982; Draper, 1984).

If the inference that adherence of *M. leprae* to Schwann cells is a pre-requisite for phagocytosis, *in vitro* can be extrapolated to *in-vivo* conditions, binding may be the first step in the pathogenesis of nerve damage in leprosy. Such a sequence has been reported in men and mice for systemic bacterial disease in general (Gibbons, 1977). Schwann cells, the supportive and myelin-synthesising cells of the peripheral nervous system (Varon and Manthorpe, 1982), are not involved in any other microbial infections and may possess specific receptors for *M. leprae*. Furthermore, these cells may lack Fc-receptors (Mirsky, 1980), indicating that phagocytosis of *M. leprae* must be mediated by some other surface receptor.

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