

## Mechanism of Phagocytosis of Mycobacteria by Schwann Cells and Their Comparison with Macrophages<sup>1</sup>

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Schwann cells, the predominant neural target cells of *Mycobacterium leprae*, lack a mechanism for the specific uptake of *M. leprae*<sup>(?)</sup>. Rat sciatic nerve-derived Schwann cells as well as the rat Schwannoma cells (33B cell line) phagocytosed all the mycobacteria tested, as well as inert latex particles. In this property they resemble macrophages which are professional phagocytic cells. However, it is not known whether the mechanism of phagocytosis by Schwann cells is similar to that of macrophages. This report describes the influence of a number of factors on the phagocytosis of mycobacteria by these two cell types.

### MATERIALS AND METHODS

The 33B rat Schwannoma cell culture, the harvesting and culture of rat peritoneal macrophages, and the culture and enumeration of mycobacteria were as described<sup>(?)</sup>.

**Radiolabeling of "*Mycobacterium w*" with <sup>14</sup>C-acetate.** "*Mycobacterium w*" was incubated in Middlebrook 7H9 medium (Difco Laboratories, Detroit, Michigan, U.S.A.) containing 10  $\mu$ Ci/ml of (U-<sup>14</sup>C)-sodium acetate (56.7 mCi/mM; Bhabha Atomic Research Centre, Bombay, India) for 4 hr at 37°C. The bacteria were washed 3 times and centrifuged at 2500 rpm for 30 min at 4°C in Dulbecco's modified Eagle's medium (DME) without antibiotics. The pellet was resuspended in DME equivalent to the original volume and an aliquot (5–10  $\mu$ l) was

counted in a liquid scintillation counter. Labeled bacteria were stored frozen (–20°C) in aliquots until used.

**Radiometric phagocytosis assay using <sup>14</sup>C-labeled "*Mycobacterium w*."** For the phagocytosis assay,  $5 \times 10^4$  33B cells or  $2 \times 10^5$  peritoneal exudate cells were plated in 0.5 ml medium (DME + 10% fetal calf serum) in wells of 24-well plates. After 24 hr, nonadherent cells were removed by gentle washing; 20,000 to 40,000 cpm of <sup>14</sup>C-acetate-labeled "*Mycobacterium w*," equivalent to about  $2.5 \times 10^6$  bacilli, were then added per well and the cells were incubated at 37°C. At the end of the incubation period, the medium containing the unphagocytosed bacteria was removed. The cells were washed 3 to 4 times with warm (37°C) DME, and then 0.5 ml of distilled water was added to lyse the cells. After 15 min, the lysates were transferred to scintillation vials. The wells were further washed twice with 0.5 ml volumes of distilled water and the washes were also added to the vials. The vials were then dried in an oven at 110°C, and the radioactivity was counted in a Packard model 3320 liquid scintillation counter. The background counts, obtained by incubating the same amount <sup>14</sup>C-labeled "*Mycobacterium w*" in wells without cells, were subtracted from the experimental values.

The scintillation mixture consisted of 5 g PPO (2,4-diphenyloxazole; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and 0.1 g of POPOP (1,4-bis(2-(5-phenyloxazolyl) benzene); Sigma) per liter of sulfur-free toluene (Sarabhai Chemicals Ltd., India). The efficiency of counting was 89%.

The sodium azide, colchicine, cytochalasin B, and dibutyryl cyclic AMP used in our study were obtained from Sigma. Cytochalasin B was dissolved at 2.5 mg/ml in dimethyl sulfoxide (DMSO); other modulators were made in DME. The controls received the solvent only.

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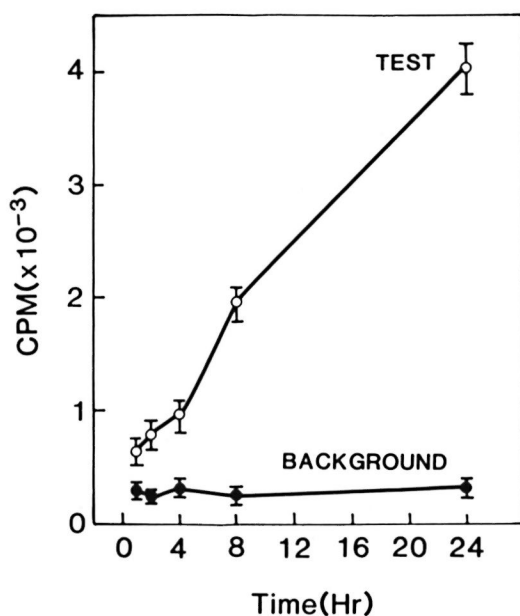


FIG. 1. Time course of the uptake of <sup>14</sup>C-labeled "Mycobacterium w" by 33B cells; 20,000 cpm of labeled mycobacteria were added per well. Background = counts in wells without 33B cells. Each point is the mean  $\pm$  S.D. of triplicates.

The statistical significance of the various treatments was determined by Student's *t* test. All values have been presented as mean  $\pm$  standard deviation (S.D.) of triplicates.

## RESULTS

It was necessary to utilize a rapid and simple assay for measuring quantitatively the uptake of mycobacteria. It was also important to avoid the fallacies in morphological counting of mycobacteria due to loss of acidfastness by some mycobacteria, as encountered earlier with *M. smegmatis* (2). A radiometric phagocytosis assay was, therefore, set up.

"Mycobacterium w" (8, 20) was used as a model mycobacterium for these experiments because it is nonpathogenic (20) but antigenically crossreactive with *M. leprae* (15-17) and one of the candidates for an antileprosy vaccine (23). Moreover, it grows as an evenly dispersed suspension in Middlebrook medium and is easy to label with <sup>14</sup>C-acetate (3).

**Phagocytosis of <sup>14</sup>C-labeled "Mycobacterium w" by 33B cells and macrophages.**

TABLE 1. Uptake of <sup>14</sup>C-labeled "Mycobacterium w" by 33B cells and macrophages.

Cell type	Uptake (mean cpm $\pm$ S.D.) <sup>a</sup>	
	Background	Test
33B cells	211 $\pm$ 38	2541 $\pm$ 213
Macrophages	198 $\pm$ 33	1811 $\pm$ 165

<sup>a</sup> Cells were incubated with labeled mycobacteria (20,000 cpm/well) for 6 hr. Background represents counts in control wells without cells. Each value is mean  $\pm$  S.D. of triplicates.

A time course study showed that the amount of labeled "Mycobacterium w" phagocytosed by 33B cells increased with time (Fig. 1). The background counts remained low throughout the time course. The assay could detect significant phagocytosis as early as 4 to 6 hr. A similar time-related increase in the uptake of labeled bacteria by peritoneal macrophages was observed, as expected (data not shown). Phagocytosis at 6 hr was used for subsequent experiments. At this time point, considerable uptake of labeled mycobacteria with very low backgrounds was observed in both 33B cells and macrophages (Table 1).

**Effect of various modulators on phagocytosis by 33B cells and macrophages.** To study the effect of various modulators, the cells were preincubated with respective agents for 1 hr at 37°C (except for the effect of incubation at low temperature) before adding labeled mycobacteria. Where stock solutions were made in DMSO, the controls were treated with the highest concentration of DMSO used in the experiment. In the concentrations used, DMSO by itself did not significantly affect phagocytosis.

Uptake of <sup>14</sup>C-labeled "Mycobacterium w" by 33B cells as well as macrophages was significantly decreased by incubation at 4°C, by treatment with sodium azide, and by Formalin-fixation of the host cells (Table 2). These experiments indicate that the phagocytosis of mycobacteria by both of these cell types requires live and actively metabolizing host cells.

Treatment with increasing concentrations of colchicine caused a dose-related inhibition of the phagocytosis by both 33B cells and macrophages (Fig. 2). The inhibition of phagocytosis by 33B cells was sig-

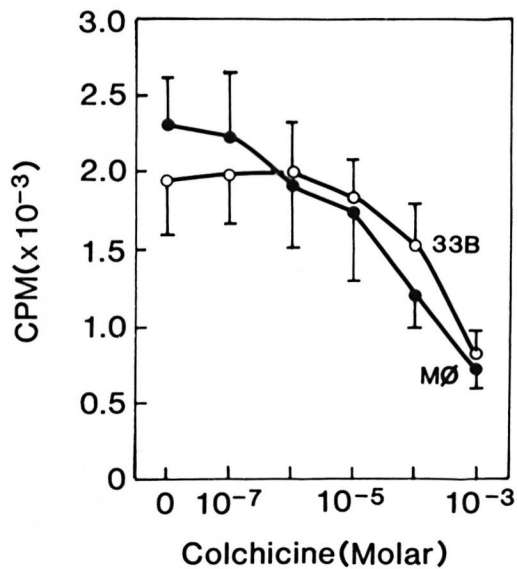


FIG. 2. Effect of colchicine on the uptake of labeled "Mycobacterium w" by 33B cells and macrophages (MØ). Each point is the mean  $\pm$  S.D. of triplicates.

nificant at concentrations of  $10^{-3}$  M ( $p < 0.025$ ), and that by macrophages was significant at  $10^{-4}$  M ( $p < 0.005$ ) and  $10^{-3}$  M ( $p < 0.025$ ) concentrations. However, the inhibition was less than 100%, even at  $10^{-3}$  M concentration. Higher concentrations were avoided due to cytotoxicity.

Cytochalasin B produced a concentration-related inhibition of the phagocytosis

by 33B cells as well as by macrophages (Fig. 3). The effect on both of these cell types was significant at  $5 \mu\text{g/ml}$  and at  $25 \mu\text{g/ml}$  concentrations ( $p < 0.005$ ). Inhibition was complete at the higher concentrations.

Dibutyryl cyclic AMP had a small inhibitory effect on the phagocytosis by both 33B cells and macrophages at  $10^{-3}$  M concentrations (Fig. 4). The effect on 33B cells was significant ( $p < 0.05$ ).

The above experiments suggest a dependence of phagocytosis on microtubule and microfilament systems. Moreover, these experiments demonstrate a close parallel between the phagocytic mechanisms of 33B cells and macrophages regarding the uptake of mycobacteria.

#### DISCUSSION

In order to study the mechanism of mycobacteria-Schwann cell interaction, 33B cells were compared with macrophages regarding their phagocytosis of  $^{14}\text{C}$ -labeled "Mycobacterium w" under different conditions. Phagocytosis requires live and actively metabolizing host cells, as demonstrated by a drastic inhibition of the uptake of mycobacteria by incubation at  $4^\circ\text{C}$  or with sodium azide, and fixation of host cells with Formalin. This is consonant with the reported inhibition of macrophage phagocytosis with such treatments (4). Uptake of latex particles by 33B Schwannoma cells was

TABLE 2. Modulation of uptake of  $^{14}\text{C}$ -labeled "Mycobacterium w."

Cell type	Treatment <sup>a</sup>	Uptake (mean cpm $\pm$ S.D.) <sup>b</sup>	p value
33B	37°C	3721 $\pm$ 468	
	4°C	715 $\pm$ 223	<0.005
Macrophage	37°C	2246 $\pm$ 355	
	4°C	457 $\pm$ 154	<0.005
33B	Sodium azide 0%	1950 $\pm$ 185	
	Sodium azide 0.2%	1256 $\pm$ 180	<0.05
	Sodium azide 1.0%	56 $\pm$ 64	<0.005
Macrophage	Sodium azide 0%	2315 $\pm$ 303	
	Sodium azide 0.2%	1364 $\pm$ 210	<0.025
	Sodium azide 1.0%	121 $\pm$ 95	<0.001
33B	- Formalin	4520 $\pm$ 715	
	+ Formalin	346 $\pm$ 194	<0.005
Macrophage	- Formalin	2171 $\pm$ 307	
	+ Formalin	128 $\pm$ 102	<0.001

<sup>a</sup> Treatments were started 1 hr before adding labeled mycobacteria. For Formalin fixation, cells were treated with 10% Formalin in phosphate buffered saline for 30 min followed by washing twice with distilled water.

<sup>b</sup> Background counts were subtracted from the experimental values. Values represent mean  $\pm$  S.D. of triplicates.

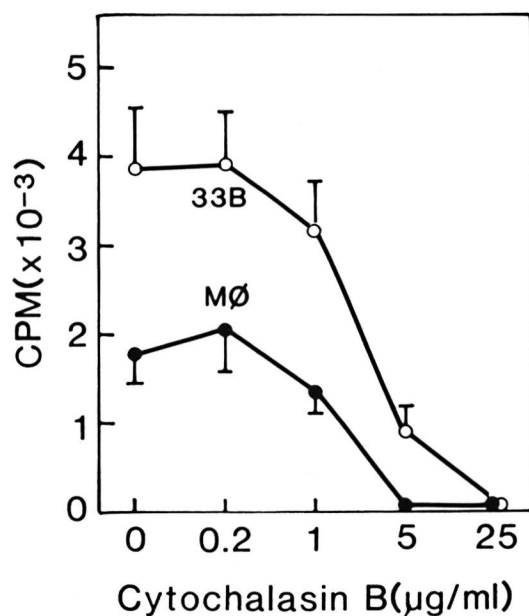


FIG. 3. Effect of cytochalasin B on the uptake of labeled "*Mycobacterium w*" by 33B cells and macrophages (MØ). Each point is the mean  $\pm$  S.D. of triplicates.

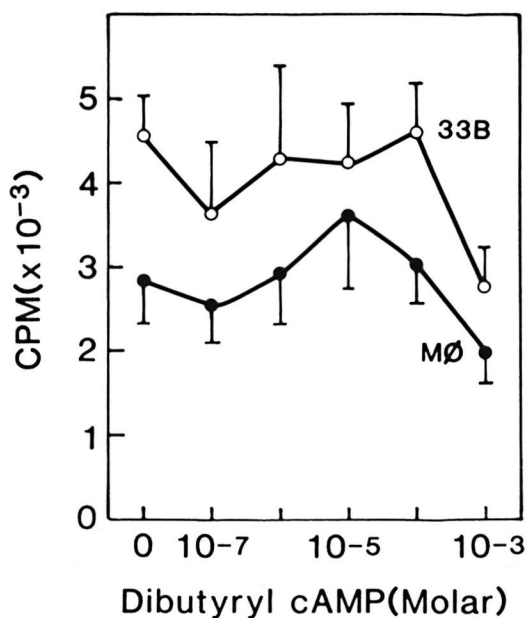


FIG. 4. Effect of dibutyryl cyclic AMP on the uptake of labeled "*Mycobacterium w*" by 33B cells and macrophages (MØ). Each point is the mean  $\pm$  S.D. of triplicates.

earlier shown to be similarly affected by the above treatments (Band, Bhattacharya and Talwar, unpublished observations), indicating a similar requirement for energy production for both mycobacteria and latex phagocytosis in these cells.

The mechanism of phagocytosis is thought to involve elements of the cytoskeleton, a complex intracellular network comprised of three major interacting fiber systems, i.e., the microtubules, microfilaments, and intermediate filaments. The microfilaments provide the motive force for the engulfment of particles, while the microtubules are thought to mediate the intracellular transport of phagosomes and lysosomes (9, 13, 18, 21). Therefore, inhibitors of the contractile system were used to further characterize the mechanism of phagocytosis. Colchicine is known to depolymerize the microtubules of the cell and thus reduce phagocytosis by macrophages (10). Inhibition of phagocytosis by 33B cells and macrophages with colchicine, therefore, indicates the involvement of microtubules in the phagocytic process. On the other hand, as observed earlier, latex phagocytosis by 33B cells is relatively refractory to colchi-

cine (unpublished observations). A similar difference in susceptibility to the colchicine effect has been observed in macrophages depending on the size and, possibly, physical properties of the particles being phagocytosed (19).

Cytochalasin B is a microfilament depolymerizing agent and is known to inhibit macrophage phagocytosis (1, 5). Dose-related and complete inhibition of phagocytosis of mycobacteria by both 33B cells and macrophages on treatment with cytochalasin B, therefore, indicated the crucial dependence of the phagocytic process on intact microfilaments. In this, characteristic phagocytosis of mycobacteria by 33B cells is similar to that of latex, which is also completely inhibited by cytochalasin B (unpublished observations).

Cyclic AMP and agents which stimulate its intracellular accumulation have been shown to either decrease (6, 7, 12, 22) or increase (10, 14) phagocytosis by macrophages and macrophage-like cell lines. This compound had a small inhibitory effect on the phagocytosis by both of the cell types. Cyclic AMP, on the other hand, had no effect on the phagocytosis of latex particles by 33B

cells at comparable concentrations (unpublished observations).

The foregoing results demonstrate a close parallel between Schwannoma cell and macrophage phagocytosis. However, an inhibitory effect of rabbit antimycobacterial antiserum on the phagocytosis of "*Mycobacterium w*" by Schwannoma cells but not by macrophages (data not shown) suggests marked differences between the two cell types regarding the recognition of mycobacteria.

### SUMMARY

Factors influencing the phagocytosis of mycobacteria by 33B rat Schwannoma cells and rat peritoneal macrophages were studied. Uptake of  $^{14}\text{C}$ -acetate-labeled *Mycobacterium w* by these cells was used to set up a radiometric phagocytosis assay. Incubation at  $4^\circ\text{C}$  and treatment with sodium azide (0.2% to 1%), colchicine ( $10^{-7}$  to  $10^{-3}$  M), cytochalasin B (0.2  $\mu\text{g}/\text{ml}$  to 25  $\mu\text{g}/\text{ml}$ ), and dibutyryl cyclic AMP ( $10^{-7}$  to  $10^{-3}$  M) inhibited the phagocytosis by both cell types in a similar manner. These experiments demonstrate similarities in the mechanism of phagocytosis of mycobacteria by Schwann cells and macrophages.

### RESUMEN

Se estudiaron los factores que influyen en la fagocitosis de micobacterias por las células del Schwannoma 33B y por los macrófagos peritoneales de la rata. Se estableció un ensayo radiométrico para medir el consumo de *Mycobacterium w* marcado con acetato  $\text{C}^{14}$ .

La incubación a  $4^\circ\text{C}$  y el tratamiento con azida de sodio (0.2 al 1%), colchicina ( $10^{-7}$  a  $10^{-3}$  M), citocalasina B (0.2 a 0.25  $\mu\text{g}/\text{ml}$ ), y dibutiril AMP cíclico ( $10^{-7}$  a  $10^{-3}$  M) inhibieron, todos, la fagocitosis por ambos tipos celulares de manera similar. Estos experimentos demuestran analogías en los mecanismos de fagocitosis de micobacterias por las células de Schwann y por los macrófagos peritoneales de la rata.

### RÉSUMÉ

On a étudié les facteurs influençant la phagocytose des mycobactéries par les cellules de schwannome de rats 33B et par les macrophages péritonéaux du rat. La capture de *Mycobacterium w* marqué au  $^{14}\text{C}$ -acétate par ces cellules a servi pour mettre au point une épreuve radiométrique d'étude de la phagocytose. La phagocytose par l'une et l'autre espèce de cellules a été inhibée de la même manière par une incubation à  $4^\circ\text{C}$  et par le traitement par l'azide de sodium (0,2% à 1%), la

colchicine ( $10^{-7}$  à  $10^{-3}$  M), la cytochalasine B (0,2  $\mu\text{g}/\text{ml}$  à 25  $\mu\text{g}/\text{ml}$ ), et l'AMP cyclique dibutyryle ( $10^{-7}$  à  $10^{-3}$  M). Ces expériences démontrent des similitudes dans le mécanisme de phagocytose des mycobactéries par les cellules de Schwann et par les macrophages.

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