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Affinity of *Mycobacterium leprae* with Lewis rat Schwannoma cell line (Lewis TC 98)

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Summary The possible affinity of *Mycobacterium leprae* with Lewis TC 98 cell line established from Lewis rat spinal Schwannoma tissue was investigated. Lewis TC 98 cells phagocytosed *M. leprae* well, showing a phagocytic index of over 60% after 5-hr exposure at MC ratio = 25 (explained in the Introduction), higher than C6 cells from rat Glioma and still more higher than cells from human Neuroblastoma. In a comparative study with three kinds of inocula, i.e. live *M. leprae*, heat damaged *M. leprae*, and *M. lepraemurium*, only live *M. leprae* revealed a high affinity with Lewis TC 98 cells. Also, the phagocytic activity to *M. leprae* of Lewis TC 98 cells was not affected by changing the condition of the cell growth with low doses of foetal bovine serum (FBS) in a culture medium. These results may suggest the special affinity of *M. leprae* with Schwann cells and the possible presence of a receptor with reactivity to live *M. leprae*, presumably existing on cell surfaces of Lewis TC 98 cells. However, two rabbit antisera against Lewis TC 98 cell surface antigens could not block the interaction between Lewis TC 98 cells and *M. leprae*.

Introduction

A characteristic feature of *M. leprae* which causes leprosy is their neurotropism. Thus, the possible affinity between *M. leprae* and the Schwann cells as one of the main targets has given one of the basic explanations for the cause of the involvement of the peripheral nervous system in leprosy.[1,2] During the last two decades, therefore, many attempts to clarify this affinity have been made *in vitro*. Primary cultures derived from human acoustic Schwannoma have been used as cell material on account of their vigorous phagocytic activity and their high affinity with *M. leprae*.[3,4] Further studies elucidated the poor affinity of heat damaged *M. leprae* compared with that of live *M. leprae* with Schwann cells from the dorsal root ganglia of newborn mice.[5] *M. lepraemurium* also revealed a poor

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affinity with organized nerve tissue cultures.[6] Moreover, it has been shown that Schwann cells are capable of engulfing any particulate matter, such as hemosiderin, myelin debris and India ink particles.[7] These findings would become a basis for our investigation of the close relationship between *M. leprae* and Schwann cells. However, conventional cell materials always need more complicated techniques and chances of success in the preparation of nerve tissue cultures. In this study, we used Lewis rat Schwannoma cell line (Lewis TC 98) established from Lewis rat spinal Schwannoma tissue. This was thought to be a more adequate cell material for the target of *M. leprae* because of easier maintenance and propagation of the cells for the experimental use. This article reports the characteristics of the affinity of *M. leprae* with Lewis TC 98 cells quantitatively *in vitro*. In this report, the proportion of total number of exposed Mycobacteria to total number of cells in a plate is simply expressed as MC ratio and the term phagocytic index is used as the meaning of a proportion (%) of the number of cells containing ingested bacilli to the number of cells to be inoculated. The series of experiments were conducted in this study on: 1, how changing MC ratio when *M. leprae* are infected influences the phagocytic activity of Lewis TC 98 cells and how low doses of FBS in a medium affects their phagocytic activity; 2, a comparison of the phagocytic activity of Lewis TC 98 cell line on *M. leprae* with two other neural cell line, i.e. rat Glioma cell line (C6) and human Neuroblastoma cell line; 3, the relative affinity of three inocula, i.e. live *M. leprae*, heat damaged *M. leprae* and *M. lepraemurium* that does not infect the nervous tissues,[6] with Lewis TC 98 cells; 4, a detection of a receptor, if any, on Lewis TC 98 cells, based on the presumption that the receptor masked cells by antiserum would show a lower phagocytic index than unmasked cells.

Materials and methods

CELL LINES AND MYCOBACTERIA

Lewis rat Schwannoma cell line (Lewis TC 98) was kindly furnished by Dr B H Liwnicz, University of Cincinnati College of Medicine; human Neuroblastoma cell line by Dr T Ito and Dr H Nomaguchi, Osaka University; and rat Glioma cell line (C6) was purchased from Flow Laboratories Inc., Virginia.

Mycobacteria used in this study were *M. leprae* serially passaged in nude mice, and *M. lepraemurium* (Hawaiian strain) developed in BALB/C mice. These mycobacteria were kindly supplied by Dr M Matsuoka, National Institute for Leprosy Research, Tokyo. After storage of the footpad of nude mouse and BALB/C mouse spleen at $-80°C$ for 3 weeks, the respective suspensions were prepared in Hank's balanced salt solutions containing 0·1% bovine albumin and stored at 0°C (wet ice) until use. All inoculations described in this report were completed within 48 hr after preparation of mycobacterial suspensions.[8,9] Counts

of mycobacteria were taken by the method of Shepard[10] and the bacterial suspensions with appropriate MC ratio for the experiments were prepared by making a suitable dilution with the medium. The heat damage of *M. leprae* was done by autoclaving.

PREPARATION OF HOST CELLS AND PROCEDURES OF INOCULATION AND OBSERVATION

Three cell lines were propagated in RPMI-1640 medium supplemented with 10% heat inactivated FBS with 100 IU of penicillin. First, 5 ml of 1×10^5 cells in 10% FBS medium were cultured on 60-mm diameter Falcon plastic plates with 6 glass slips in a 5% CO^2 chamber at 37°C for 24 hr. After washing with FBS free medium, cells on glass slips were infected with mycobacteria supplemented in 5 ml of 10% FBS medium and cultured under the same conditions. In the series of experiments, low percentages of FBS in the medium, i.e. 5% and 2·5% were used in the only experiment to elucidate how changing the conditions of cell growth influenced the phagocytic activity, during all stages of experiment. The cultures were terminated at desired intervals of cultivation, specifically at 1, 3 and 5 hr, respectively. The terminated one pair of cultures on each glass slip were washed with 1/100 M phosphate buffered saline, pH 7·2 (PBS), fixed in 10% buffered formalin for 24 hr, and stained with Ziehl–Neelsen for acid-fast bacilli. More than 200 cells were randomly examined microscopically and the phagocytic indexes were scored.

PREPARATION OF ANTISERA TO LEWIS TC 98 CELL MEMBRANE ANTIGENS

Antisera were prepared as follows: Lewis TC 98 cells in vigorous condition were harvested by rubber policemen, washed twice with cold physiologic saline and prepared in 2×10^6 cells ml^{-1}. Then inoculations of 0·5 ml each of cell suspension were respectively made into 2 rabbits intravenously. Two weeks later, bloods were collected and separated sera were stored at −20°C until use. The titres of these two antisera against antigenic determinants on the surface of Lewis TC 98 cells were titrated as 1:640 (antiserum-1) and 1:40 (antiserum-2), respectively by the indirect membrane immunofluorescence test. Two sera before inoculations (serum-1 and serum-2) did not contain any detectable antibodies. Before experiment, antisera and sera before inoculations as controls were inactivated by heat at 56°C for 30 min and filtered through a membrane filter (0·45 μ pore size).

IMMUNOFLUORESCENCE

The indirect membrane immunofluorescence test[11] was used. The living cells on glass slips were washed twice with PBS, allowed to stand to react with diluted antiserum at 37°C for 30 min and washed with PBS. The cells were then reacted

with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Cappel Laboratories, West Chester, PA) for 30 min at 37°C. After washing with PBS, glass slips were placed on a slide. Fluorescent cells were examined under a fluorescence microscope, Chiyoda-Fluorophoto.

BLOCKING TEST OF A RECEPTOR ON LEWIS TC 98 CELLS BY ANTISERA

Lewis TC 98 cells were cultured on glass slips in four plates and each plate was used for the cell treatments by four different sera, i.e. antiserum-1, antiserum-2, serum-1 and serum-2, respectively. Cells on glass slips were washed with FBS free medium and then treated with 10-fold diluted antiserum for 30 min in the same chamber. After washing with FBS free medium, antibody treated cells were immediately infected with fresh medium supplemented with *M. leprae* prepared

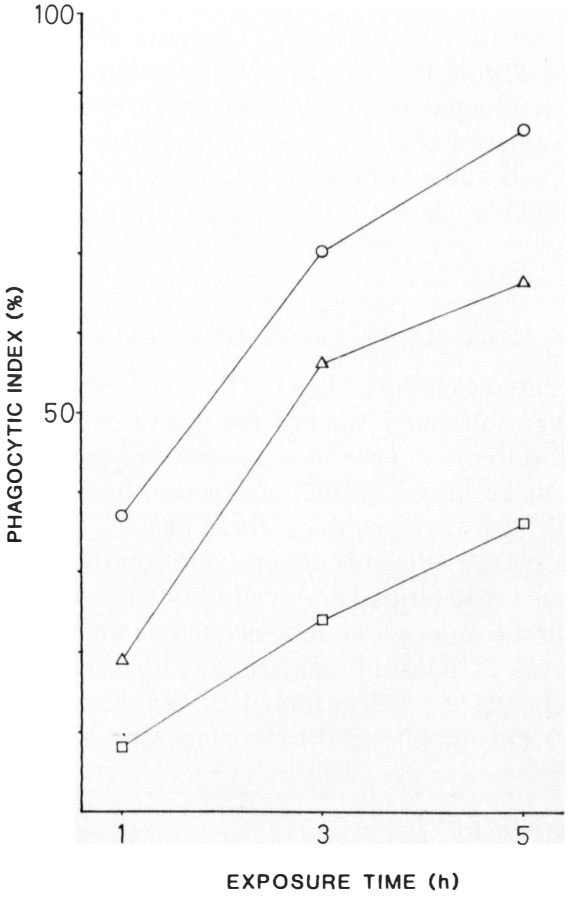

Figure 1. Relationship between MC ratio and phagocytosis by Lewis TC 98 cell line of *M. leprae*. (○——○) MC ratio = 50, (△——△) MC ratio = 25, (□——□) MC ratio = 12·5.

at MC ratio = 25. The terminated one pair of glass slip cultures were fixed, stained and then the phagocytic indexes were scored as described above. The presence of antibodies reacted with cell surface antigens were also examined by the indirect membrane immunofluorescence test, at 0, 1 and 3 hr after infection.

Results

INFLUENCES ON THE PHAGOCYTIC ACTIVITY OF CHANGING MC RATIO AND THE CELL GROWTH

The influence of MC ratio on the phagocytic index is shown in Figure 1. The phagocytic indexes at MC ratio = 50 and MC ratio = 25 after only 1 hr inoculation were already 37 and 19%, respectively, which continued to rise sharply to reach over 80 and 60% within 5 hr. In the case of MC ratio = 12·5, 8% shown on the phagocytic index after 1 hr went up gradually and reached 36% after 5 hr.

It is worth considering that the phagocytic activity would be activated in vigorous cells. Proceeding from this observation, the growth of Lewis TC 98 cells

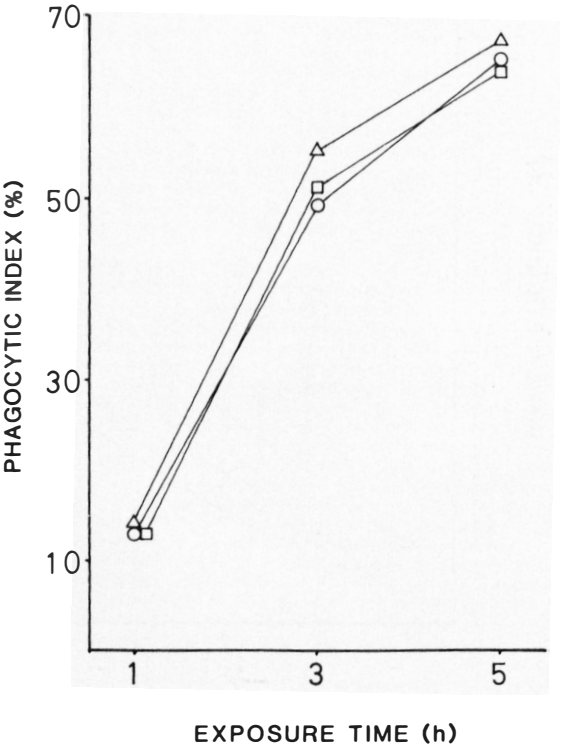

Figure 2. No reduction of phagocytosis by Lewis TC 98 cell line cultured in the medium with low percentages of FBS. (○——○) 10% FBS, (△——△) 5% FBS, (□——□) 2·5% FBS.

was controlled by low doses, i.e. 5 and 2·5%, of FBS in the medium, and its influences on the phagocytic index was investigated at MC ratio = 25 (Figure 2).

Contrary to expectation, there was no definite relationship between the percentage of FBS and the phagocytic index. This result indicates that Lewis TC 98 cells under controlled growth conditions are capable of engulfing *M. leprae* as many as cells in the growth medium, and might be an evidence of some special affinity between *M. leprae* and Schwann cells.

COMPARATIVE EXPERIMENT WITH OTHER NEURAL CELL LINES

Lewis TC 98 cells were compared with two other neural cell lines, i.e. human Neuroblastoma cells and rat Glioma cells (C6), to clarify any differences between their affinities with *M. leprae* at MC ratio = 25. As shown in Figure 3, Lewis TC 98 cells showed an affinity significantly higher than those of C6 and Neuroblastoma cells. It is of interest to note that the phagocytic index of C6 cells was lower than that of Lewis TC 98 cells but higher than that of Neuroblastoma cells. It seems

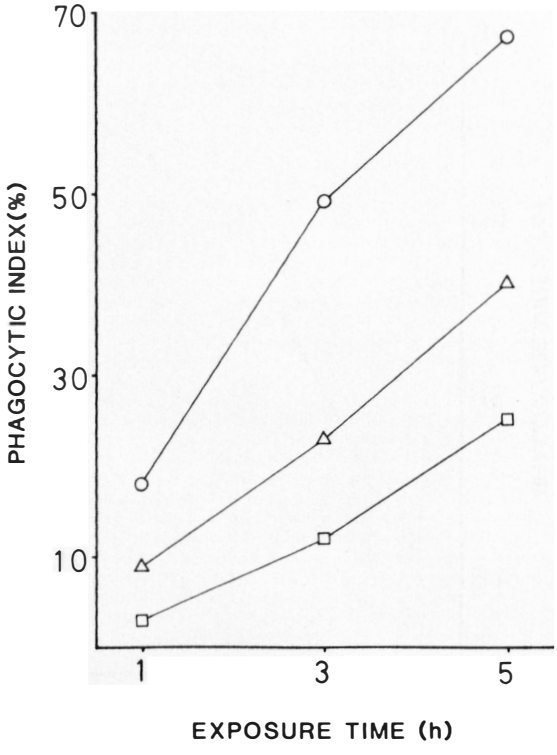

Figure 3. Comparison of phagocytosis by three neural cell lines of *M. leprae*. (○——○) Lewis rat Schwannoma cell line (Lewis TC 98), (△——△) rat Glioma cell line (C6), (□——□) human Neuroblastoma cell line.

this phenomenon should be discussed in relation to the styles of the nerve damage in leprosy.

COMPARATIVE EXPERIMENT WITH HEAT DAMAGED *M. LEPRAE* AND *M. LEPRAEMURIUM*

The poor affinities of *M. lepraemurium* with organized nerve tissue cultures and heat damaged *M. leprae* with Schwann cells from dorsal root ganglia *in vitro* were respectively described by Fildes[6] and Mukherjee *et al.*[5] In order to find out whether Lewis TC 98 cells show the same property, live *M. leprae*, heat damaged *M. leprae* and *M. lepraemurium* were inoculated to Lewis TC 98 cells at MC ratio = 25. The results in Figure 4 show that the uptake of heat damaged *M. leprae* was very poor, reduced to almost the same level as *M. lepraemurium* which did not show any affinity. Overall, the lack of affinities that heat damaged *M. leprae* and *M. lepraemurium* had with Lewis TC 98 cells coincided with the data described by the above two investigators.

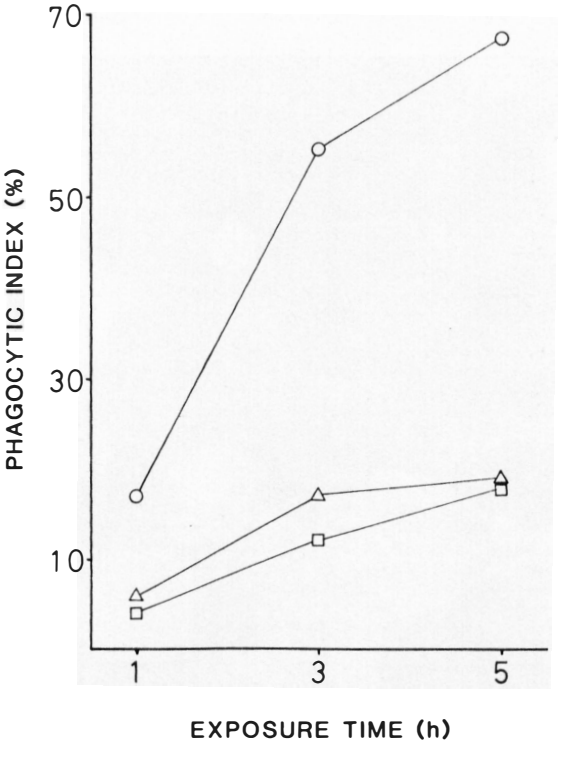

Figure 4. High affinity of live *M. leprae* different from other inocula with Lewis TC 98 cell line. (○——○) live *M. leprae*, (△——△) heat damaged *M. leprae*, (□——□) *M. lepraemurium*.

BLOCKING TEST OF A RECEPTOR ON LEWIS TC 98 CELLS BY ANTISERA

The indirect membrane immunofluorescence was concomitantly carried out to know if both antibodies reacted were sufficiently kept on cell surfaces, i.e. at 0 hr, namely immediately after treatment with antibodies, 1 and 3 hr after infection respectively. Lewis TC 98 cell surfaces at 0 hr were intensely stained with 100% of positive cells. Even cells after 3-hr exposure, 100% of cell surfaces were positively stained with a great intensity as well (Figure 5).

The result of phagocytic indexes was shown in Figure 6. The data represent that phagocytic indexes of antibody treated Lewis TC 98 cells were not inhibited and they paralleled with those of control cells treated with sera before inoculations.

Discussion

For an experimental attempt to cultivate *M. leprae in vitro*, primary cultures derived from human acoustic Schwannoma were used by Lumsden.[3] In his experiments with Schwannoma cells and *M. leprae*, he demonstrated the vigorous

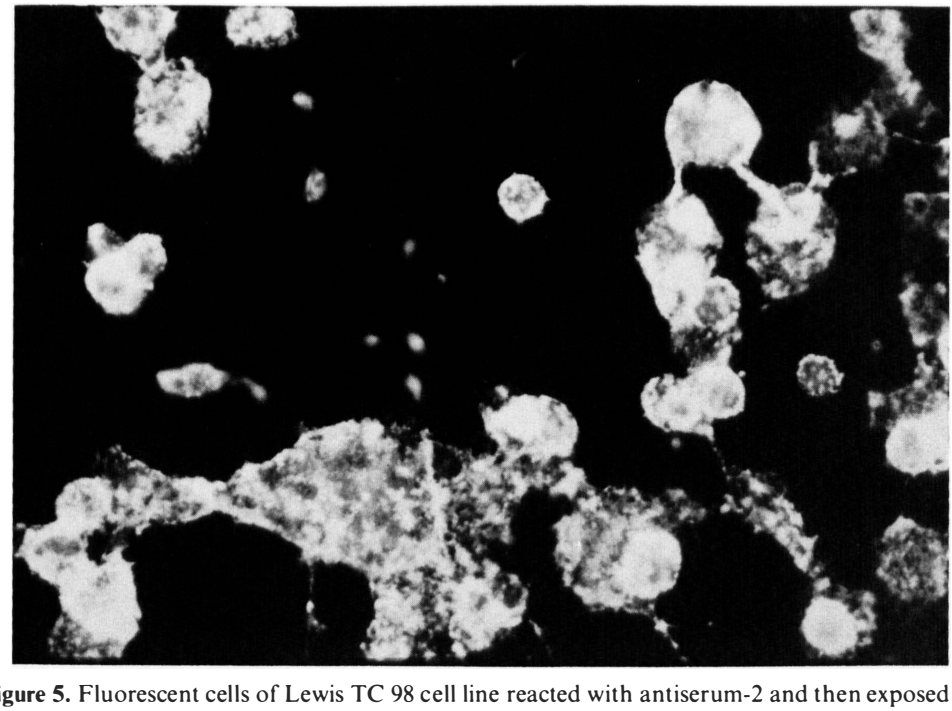

Figure 5. Fluorescent cells of Lewis TC 98 cell line reacted with antiserum-2 and then exposed *M. leprae* for 3 hr in an indirect membrane immunofluorescence. Photograph was taken after keeping the stained cells at 4°C overnight.

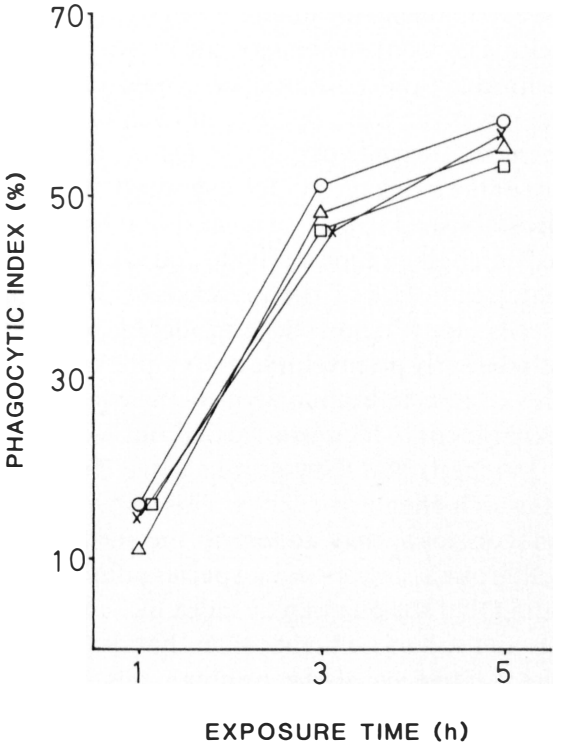

Figure 6. No reduction of phagocytosis by antisera treated Lewis TC 98 cell line. (○——○) antiserum-1, (△——△) serum-1, (□——□) antiserum-2, (X——X) serum-2.

phagocytic activity of Schwannoma cells. Lalitha *et al.*[4] reported that the phagocytic index of Schwannoma cells *in vitro* was over 90% 2 hr after the inoculation of *M. leprae*, although MC ratio was unknown. As shown in Figure 1, the phagocytic index was largely influenced by MC ratio when Schwannoma cells were used as a host. This discrepancy might be due to the difference in the multiplicity of contacts between *M. leprae* and Schwannoma cells on plates. They might have exposed more highly concentrated *M. leprae* suspensions to Schwannoma cells than we did with our materials. However, our data on the phagocytic index seem to be similar to the findings of Lalitha *et al.* While on the other hand, normal Schwann cells from dorsal root ganglia of newborn mice *in vitro* phagocytosed *M. leprae* from 15·9% for the phagocytic index at 24 hr to 67·2% at 72 hr after inoculation and that failed to sufficiently phagocytose heat damaged *M. leprae*, suggesting the importance of the viability of the bacilli infected.[5] In our cultures, although our results coincided with their data on the unsusceptibility of heat damaged *M. leprae*, the uptake of live *M. leprae* by Schwannoma cells was greater; in other words, the phagocytic index was about 20% within only 1 hr and over 60% after 5 hr exposure at MC ratio = 25. The difference in the phagocytic index between newborn Schwann cells and Schwan-

noma cells would be ascribable to the different cell situations, i.e. nontransformed and transformed cells. The vigorous phagocytic activity of human Schwannoma cells reported by Lumsden[3] and Lalitha *et al.*[4] could just explain these different phenomena. In fact, it was reported that normal Schwann cells from dorsal root ganglia of newborn mice, after phagocyting *M. leprae*, failed to incorporate DNA precursor, indicative of the blockage of DNA synthesis.[12] Meanwhile, since Lewis TC 98 cells, though established from Lewis rat spinal Schwannoma tissue, which is generally regarded as a benign tumor, divide and grow by doubling about every 24 hr, and propagate regardless of the presence of *M. leprae* in cells just like malignant tumor cells, they might be considered to be in more activated conditions than both the early premyelin secretory phase of normal Schwann cells and primary cultures of benign human acoustic Schwannoma.

In the second experiment, which was carried out with low doses of FBS, the data shown in Fig. 2 suggests that the vigorous Lewis TC 98 cell growth does not necessarily lead to the high phagocytic index. This also indicates that *M. leprae*, in the first stage of phagocytosis, may adhere to the surface of Lewis TC 98 cells regardless of the cell growth, suggesting a special affinity between them.

The data indicating that the phagocytic index of Lewis TC 98 cells was higher than that of C6 cells and still more higher than that of Neuroblastoma cells may be explained by the differences of the original cells of these tumors and the involvement of the peripheral nervous system, extending to and ceasing abruptly at the dorsal root ganglion, in leprosy (Fig. 3).[3] 1, Lewis TC 98 cell line originated in Schwann cell, the myelin forming cell of the peripheral nervous system; 2, C6 cell line originated in Glia cell of central nervous system, though undistinguishable whether it is oligodendroglia that is myelin forming cell of central nervous system or not;[13] 3, Neuroblastoma cell line originated in Sympathoblast. The fact that Lewis TC 98 cells were the most phagocytically active, especially compared to C6 cells, would suggest that leprosy was a disease of the peripheral nervous tissue.

The comparative experiment with three kinds of inocula, as shown in Figure 4, showed that only live *M. leprae* was highly phagocytosed. The other two inocula, i.e. heat damaged *M. leprae* and *M. lepraemurium*, were slightly phagocytosed at about the same level. At first, corresponding to the data described by Mukherjee *et al*,[5] heat damaged *M. leprae* were not engulfed well, probably suggestive of the destruction or the loss of some component on *M. leprae* that adheres to the host cell surfaces by autoclaving. The apparently poor affinity of *M. lepraemurium* tends to confirm the data reported by Fildes.[6] She observed large numbers of *M. leprae* and isolated *M. lepraemurium* predominantly in macrophages and fibroblasts in tissues of foetal rat and mouse dorsal root ganglia and whole cross-sections of mice containing somite, cord and ganglia, but unfortunately failed to detect an obvious affinity between *M. leprae* and Schwann cells. In fact, corresponding with her data, our cultures inoculated with *M. leprae* also showed high phagocytic indexes and large numbers of bacilli in the cells

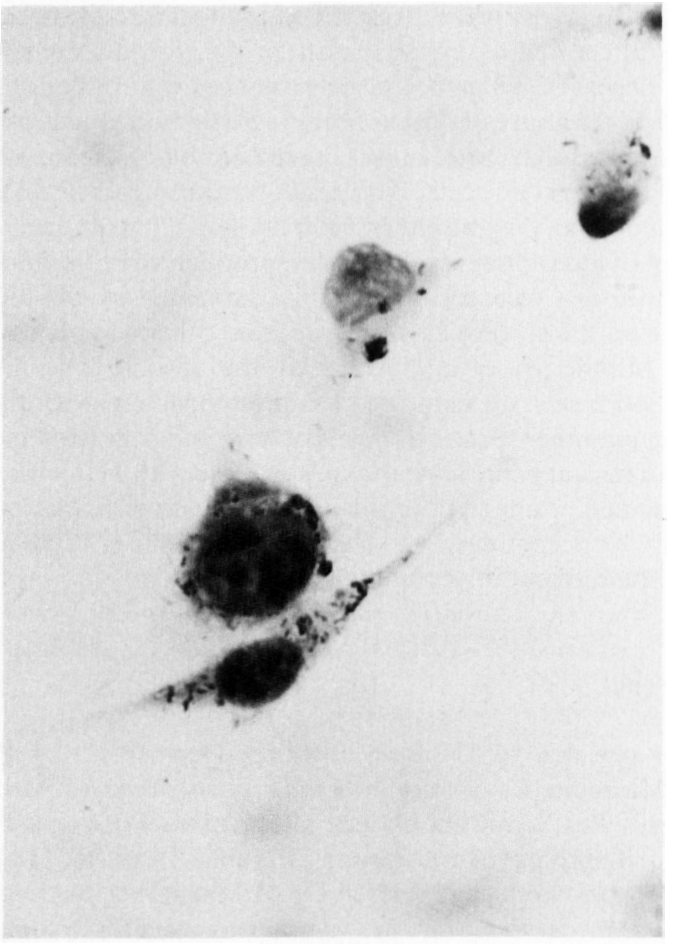

Figure 7. Lewis TC 98 cells engulfing large numbers of *M. leprae*, stained with Ziehl–Neelsen's method.

(Figure 7), whereas those inoculated with *M. lepraemurium* showed low phagocytic indexes and only isolated bacilli in the cells, indicating a poor affinity of *M. lepraemurium* with Schwann cells.

In conclusion, from the joint findings presented here, the following hypothesis could be accepted: that is, Lewis TC 98 cells and nontransformed Schwann cells as well may have two functional features that help to phagocytose live *M. leprae*. One is their general phagocytic activity, low levels of which may be explained by the poor affinities of heat damaged *M. leprae* and *M. lepraemurium*. The other is the specific adherence of live *M. leprae* to the surface of cell membranes in the first stages of phagocytosis, presumably explained by the presence of receptors on the cell surfaces specifically reactive to live *M. leprae*. From this hypothesis, the blocking test by two rabbit antisera against Lewis TC 98 cell surface antigens,

titrated as 1:640 and 1:40 respectively by the indirect membrane immunofluorescence test, was attempted, assuming that the phagocytic index would be reduced if receptors on Lewis TC 98 cells had been masked by antibodies. As shown in Figure 6, however, cells treated by antisera revealed no reductions on phagocytic index. This indicates that these antisera could not block the interaction between *M. leprae* and Schwannoma cells. This result further suggests two possibilities on this phenomenon: one may be either the weakness of the antigenicity or the too scanty amount of a receptor, resulting in no production of the antibody; another may be the lack of a receptor, presumably explained by the difference of the electric charge on the surface of *M. leprae* from other mycobacteria.

Recently Mukherjee *et al.*[14] reported the specific phenomenon of the adherence of *M. leprae* to Schwann cells in comparison with the insignificant adherence of eight other species of mycobacteria and suggested the possibility of the existence of receptors on Schwann cells reactive with *M. leprae*. Their and our observations would be one step for further investigations of the special affinity of live *M. leprae* with Schwann cells to clarify the mechanism of *M. leprae* infection and also the involvement of peripheral nervous system.

Acknowledgments

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