*Clin. exp. Immunol.* (1986) **64,** 35–40.

# The influence of killed *Mycobacterium leprae* and other mycobacteria on opsonized yeast phagocytosis

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*(Accepted for publication 7 November 1985)*

## SUMMARY

The influence of killed mycobacteria on the metabolic burst associated with phagocytosis of opsonized zymosan by normal human polymorphonuclear leucocytes (PMNL) or monocytes was studied by chemiluminescence (CL) measurements. *M. leprae* reproducibly reduced the peak and total CL of both types of phagocyte to a small, but highly significant extent. Electron microscopy showed that *M. leprae* were phagocytosed: cells with ingested or adherent *M. leprae* phagocytosed fewer zymosan particles. *M. leprae* did not cause aggregation of the phagocytes or quenching of CL. *M. lepraemurium* did not influence the CL response to zymosan. Addition of *M. tuberculosis* caused an increased response with PMNL but not with monocytes.

**Keywords** chemiluminescence phagocytes mycobacteria leprosy

## INTRODUCTION

Several species of pathogenic mycobacteria are successful parasites of macrophages, despite the potential of these cells to kill them by the production of oxygen radicals (Jackett, Aber & Lowrie, 1978; Klebanoff & Shephard, 1984; Lowrie, 1983). Even in lepromatous leprosy patients, non-parasitized macrophages are able to produce superoxide normally (Sharp & Banerjee, 1985) and parasitized macrophages can kill ingested mycobacteria if exposed to T lymphocyte-conditioned medium (Prasad, Singh & Nath, 1982). Reduction of numbers of cell membrane Fc receptors in parasitized macrophages (Birdi *et al.*, 1983) *in vitro* suggests that *Mycobacterium leprae* may also produce general defect in macrophage function which could contribute to the anergy seen in lepromatous leprosy (Birdi *et al.*, 1983; 1984). The ability of phagocytes exposed to *M. leprae* to respond to a second phagocytic stimulus might be impaired by such changes in the numbers of these or other binding sites on the cell membrane. We have therefore examined the influence of killed mycobacteria on the phagocytosis of opsonized zymosan by normal human PMNL and monocytes.

## MATERIALS AND METHODS

*Phagocyte preparation.* Neutrophil polymorphonuclear leucocytes (PMNL) and mononuclear cells (MNC) were separated by density gradient centrifugation (Ferrante & Thong, 1980) using a commercial Ficoll-Hypaque preparation (Mono-Poly Resolving Medium, Flow Laboratories, Irvine, UK) from heparinized venous blood freshly obtained from healthy Scottish volunteers. PMNL were washed once in Hank's balanced salt solution (HBSS) without phenol red (GIBCO

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Bio-cult, Paisley, UK) and resuspended to $2 \times 10^6$/ml in HBSS with 0·1% BSA (Sigma Chemical Company, Poole, UK). MNC were washed three times in HBSS to remove most of the platelets and resuspended to $4 \times 10^6$/ml in HBSS with 0·1% BSA. Cell purity was assessed using a coulter counter with channelyser (Potts *et al.*, 1980) and by microscopy of Giemsa-stained drop preparations. In the PMNL preparations, lymphocyte contamination was less than 1% and erythrocyte contamination was always less than 5%. Monocytes formed 11·5% to 20% of the mononuclear fraction judged by volume spectroscopy (Potts *et al.*, 1980); microscopic examination showed that no erythrocytes were present.

*Microorganisms.* Mycobacteria of three species killed by γ-irradiation (Armadillo-derived *M. leprae*, batch CD41, purified by Immlep protocol 1/79; *M. lepraemurium*; and *M. tuberculosis*, strain H37Rv), were donated by Dr R.J.W. Rees (Division of Communicable Diseases, Clinical Research Centre, Harrow). Bacilli were diluted to a concentration of $10^8$/ml in HBSS immediately before use. Microscopy of drop preparations showed no evidence of aggregation at this concentration. Zymosan was prepared according to the method described by Lachmann and Hobart (1978) and was suspended to a concentration of $5 \times 10^7$ particles/ml prior to use.

*Chemiluminescence (CL).* The CL measurements were performed using a Luminometer (Model 1250, LKB, Selsdon, UK). To each polystyrene reaction vial (Sarstedt, Leicester, UK) was added 0·5 ml of cell suspension and 0·5 ml of lucigenin (Sigma Chemical Co., Poole, UK) at a concentration of $10^{-4}$ M on HBSS (pH 7·5). The vials were allowed to equilibrate in a water bath at 37°C for 15 min and initial baseline CL measurements were made. Then 0·2 ml of *M. leprae* suspension was added, or an equal volume of HBSS in control experiments. After measurement of the baseline CL levels for a further 10 min, 0·2 ml of either (a) zymosan suspension with fresh homologous serum (pre-incubated for 30 min in a 1:1 ratio), (b) zymosan with an equal volume of HBSS, (c) serum with HBSS or (d) HBSS alone was added to the vial. This resulted in a 7% final concentration of serum, if present, and a PMNL/zymosan particle ratio of 1:10 in experiment with zymosan. This low phagocyte/zymosan ratio produced small, but highly reproducible CL responses and did not obscure responses to the mycobacteria. CL was measured at intervals of approximately 4 min throughout the experiments and the vials were kept in a water bath at 37°C between readings. After each reading the vials were agitated by hand. PMNL experiments were continued for 60 min and MNC experiments for periods of up to 110 min.

*Visual counts.* At the end of each experiment, phagocytosis was stopped by the addition of 1 ml 4% neutral buffered methanal (formaldehyde) to each vial and the cells were washed once in Isoton II (Coulter Electronics, Luton, UK) to remove non-adherent *M. leprae*. Air-dried drop preparations were stained with a modified Ziehl-Neelsen technique in which 0·2% methyl green was substituted for methylene blue. This method gave particularly good contrast between the *M. leprae* and the cell nuclei. Each slide was viewed by oil immersion microscopy for counting the number of zymosan particles ingested by 100 cells, with and without adherent *M. leprae*.

*Electron microscopy.* In separate experiments phagocytes exposed to mycobacteria for varying lengths of time up to 50 min were washed in Isoton II and the cell pellets were embedded in Araldite. Ultra-thin sections were cut using an OMu3 ultramicrotome and stained with lead acetate and uranyl citrate. Sections were viewed in a Jeol 100CX transmission electron microscope.

*Data analysis.* The CL response was measured in millivolts (mV) using the analogue output from the Luminometer to a chart recorder. From these measurements the peak CL, total CL and the rate of initial rise in CL were calculated. Statistical analysis of the CL response was performed by analysis of variance on each set of experiments, the microscopic assays and the results from all individuals were analysed by the chi-squared test.

## RESULTS

The first experiments were performed to investigate the ability of an arbitrary number of *M. leprae* to inhibit the normal phagocytic response of PMNL to osponized zymosan. A total of $2 \times 10^7$ bacilli were added to $1 \times 10^6$ PMNL, giving a PMNL: *M. Leprae* ratio of 1:20. The results of experiments with PMNL and MNC from a healthy donor are shown in Fig. 1; the peak CL and the total CL were

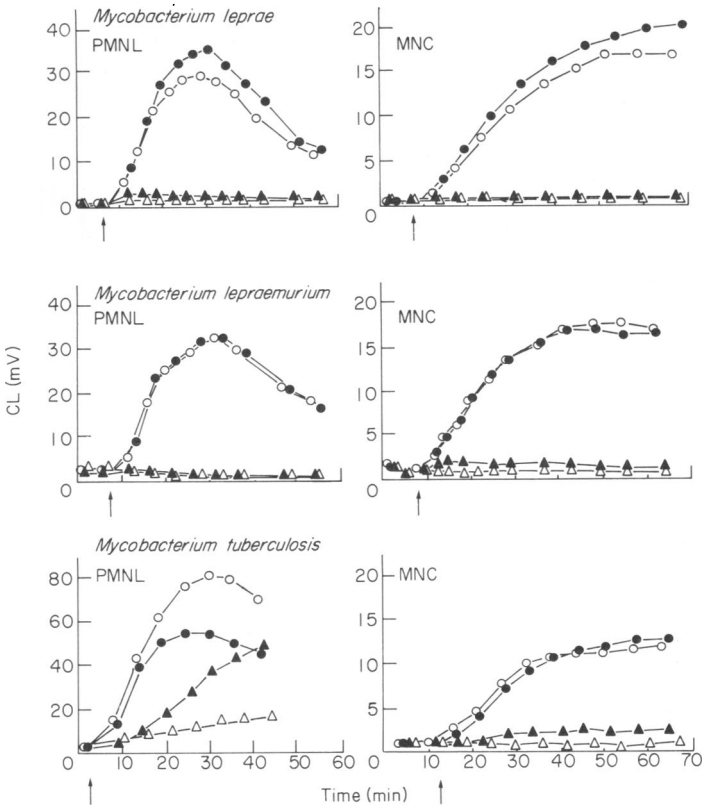


**Fig. 1.** CL responses for PMNL and MNC with each species of mycobacterium: phagocytosis of opsonized zymosan with (○) and without (●) added mycobacteria. The CL responses to each mycobacterium in the presence (▲) and absence of serum (△) are also shown. Serum alone produced no CL response. Each point is the mean of triplicate experiments, all of which were performed simultaneously. Arrows indicate the time of addition of serum, with or without yeast.

reduced to a small but highly significant extent in the presence of *M. leprae* ($P < 0{\cdot}001$). Similar results were seen in experiments with PMNL from four volunteers and in a repeat sample from the first subject ($P < 0{\cdot}01$). Exposure to *M. leprae* did not alter the initial rise in CL, indicating that the rate of recruitment of PMNL to the response was similar (Allen, 1977; Robinson *et al.*, 1984). Experiments performed with MNC from three volunteers also showed inhibition of the CL response to a comparable degree. Control experiments with zymosan alone and serum alone showed no significant CL response over the same time period.

It was not possible to distinguish adherence and endocytosis of acid-fast bacilli in drop preparations. Electron microscopic studies showed unequivocal evidence of *M. leprae* phagocytosis (Fig. 2). Considerable variation in the extent of yeast phagocytosis from cell to cell was seen in visual assays of drop preparations and the results are therefore expressed as respective percentages of cells containing one to eight zymosan particles. It was found that cells with adherent or ingested *M. leprae* phagocytosed fewer particles than those showing no adherence or ingestion of leprosy bacilli (Fig. 3).

Experiments performed in the same manner with $\gamma$-irradiated *M. lepraemurium* showed no inhibition of the CL response to yeast phagocytosis and, like *M. leprae*, *M. lepraemurium* produced no CL response with serum alone (Fig. 1). When *M. tuberculosis* was substituted for *M. leprae*, the CL response was enhanced when both zymosan and *M. tuberculosis* were present and inhibition did not occur. A steadily increasing CL response occurred when PMNL were exposed to *M. tuberculosis*

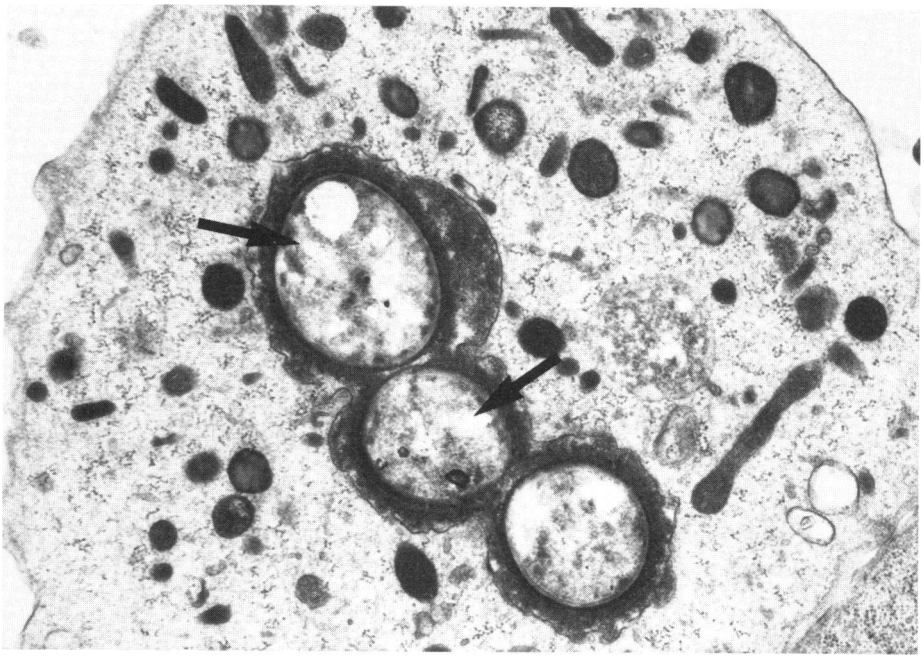

**Fig. 2.** Electron micrograph of *M. leprae* (Arrows) within polymorphonuclear leucocytes 40 minutes after the addition of homologous serum. × 16,000.

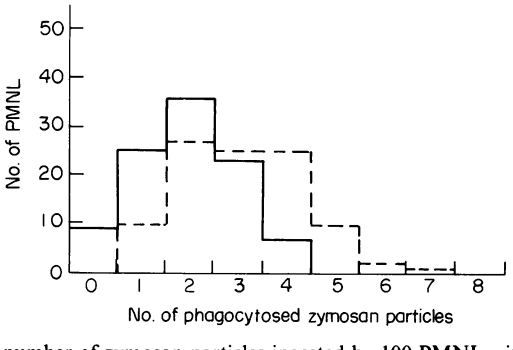


**Fig. 3.** Distribution of the number of zymosan particles ingested by 100 PMNL with (——) and 100 PMNL without (– – –) adherent or ingested *M.* leprae ($P < 0{\cdot}001$).

with serum alone, but the response with MNC was much less impressive (Fig. 1). Visual counts of PMNL exposed to mycobacteria with serum showed that many fewer cells had adherent and/or phagocytosed *M. lepraemurium* (7% of the cells) compared with *M. leprae* (51%) or *M. tuberculosis* (57%).

In order to eliminate the possibility the *M. leprae* was inducing aggregation of the phagocytes and thereby reducing their capacity for phagocytosis, the electronic volume of the cells was measured in a Coulter counter and the size distribution recorded in a multi-channel analyser: duplets, triplets or larger aggregates were never observed and cell numbers were not depleted.

## DISCUSSION

The results show that exposure of PMNL or monocytes to *M. leprae* induces a small but significant

diminution of the peak CL response to opsonized zymosan and of their phagocytic activity. The visual and CL assays indicate that CL commences normally in the presence of adherent *M. leprae*, since the initial rate of CL is unchanged. If quenching had occurred due to the addition of *M. leprae*, the initial rate of CL would have been reduced with the suppression of the peak CL response (Glette, Solberg & Lehmann, 1982; Hastings *et al.*, 1982). Therefore it appears that the cells become prematurely satiated, since there is depression of the peak CL in the presence of *M. leprae*, despite the normal initial rate of CL, and the microscopic assay shows that fewer zymosan particles are ingested. These findings suggest that *M. leprae* influences other phagocyte functions in addition to the prevention of a bactericidal response. In contrast to the reduction of Fc receptors observed by Birdi *et al.* (1983), inhibition of the CL response was induced by killed organisms. However these authors used heat to kill their mycobacteria, rather than $\gamma$-irradiation, which produces less damage to the organisms.

The mechanism behind these effects on macrophage function remains a matter for speculation. However, adherence mediated by binding of a particle to one type of phagocyte receptor may induce a change in function of other types of receptor (Wright *et al.*, 1984) and it seems likely that the reduction of phagocytic activity is dependent upon modulation of cell membrane receptors. While this could be accomplished by a heat-labile substance produced by the parasitized phagocyte as postulated by Birdi *et al.* (1983), it seems more likely that it is produced by the bacterium itself, perhaps as a surface constituent. *M. lepraemurium* was not taken up by the phagocytes to the same extent as the *M. leprae* and it therefore seems likely that the observed depressive effect of *M. leprae* on CL depends upon ingestion.

Phagocytosis of opsonized particles can occur in some instances without producing a CL response (Wright & Silverstein, 1983). This is certainly true of *M. leprae*, as reported by Holzer *et al.* (1984), who noted that mouse macrophages showed no CL response to *M. leprae* opsonized by normal human serum. We are able to confirm that this observation also applies to normal human phagocytes. *M. lepraemurium* does not elicit a CL response from human PMNL and monocytes, but the significance of this observation is less clear since it was taken up poorly by the phagocytes.

A serum-enhanced CL response from PMNL occurs with *M. tuberculosis*, but monocytes appear less able to produce a CL response to the same stimulus. The reason for this difference is not clear, but this effect parallels the psssession of peroxidase activity, which is much less in monocytes than in PMNL. This enzyme may be necessary for effective killing of mycobacteria (Lowrie, 1983) and alveolar macrophages, which lack peroxidase (Klebanoff & Shepherd, 1984), show no serum enhancement of superoxide release in response to *M. bovis* BCG (Jackett, Andrew & Lowrie, 1982).

These observations lend further support to the hypothesis that *M. tuberculosis* and *M. leprae* evade the phagocytes bactericidal response by different mechanisms (Wheeler & Gregory, 1980; Lowrie, 1982) and confirm that parasitism of phagocytes by *M. leprae* alters cell function. If ingestion of *M. leprae* induces similar defects of phagocyte function *in vivo*, this might interfere with the effectiveness of the host immune response and so contribute to the chronicity of leprosy.

We wish to thank Dr G. G. Nicholls for giving us access to the luminometer. We are most grateful to Dr R. J. W. Rees who supplied the mycobacteria. We also wish to thank Mr R. Fawkes for preparation of the diagrams.

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