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Studies on the Interaction of *Mycobacterium microti* and *Mycobacterium lepraemurium* with Mouse Polymorphonuclear Leucocytes

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When polymorphonuclear leucocytes (PMN) elicited in mice were infected with *Mycobacterium microti* or *Mycobacterium lepraemurium*, phagosome–lysosome fusion occurred with both species. This contrasts with the situation in macrophages where phagosome–lysosome fusion is inhibited by *M. microti* but not *M. lepraemurium*. No evidence was found for killing of *M. microti* or *M. lepraemurium* when the bacteria were isolated from PMN and their viability tested in cell-free medium or macrophages.

INTRODUCTION

Intracellular parasites which survive in macrophages interact with the vacuolar system of the host cells in a variety of ways. *Mycobacterium lepraemurium* (Brown *et al.*, 1969) and *Leishmania mexicana* (Alexander & Vickerman, 1975) can survive phagosome–lysosome fusion. *Mycobacterium tuberculosis* (Armstrong & Hart, 1971), *Mycobacterium microti* (Hart *et al.*, 1972) and *Toxoplasma gondii* (Jones & Hirsch, 1972) inhibit the fusion process, while *Mycobacterium leprae* (Evans & Levy, 1972) and *Trypanosoma cruzi* (Kress *et al.*, 1975; Noguiera & Conn, 1976) are thought to traverse the phagosome or plasma membrane and lie unbounded in the cytoplasm.

Inflammatory reactions are characterized by an influx of phagocytic cells. Initially, polymorphonuclear leucocytes (PMN) predominate and in experimental cutaneous infections with mycobacteria they ingest a large proportion of the bacteria present (Closs & Haugan, 1975). Mycobacteria are soon released by PMN, which have a short life-span, but are rephagocytosed either by other PMN or, more likely, by mononuclear phagocytes which are the predominant cell type within 24 h of the development of the inflammatory reaction (Adams, 1975; Closs & Haugan, 1975).

The present study was undertaken to determine whether *M. lepraemurium* or *M. microti*, which show contrasting phagosome–lysosome interactions in macrophages, behave similarly in PMN. The bactericidal potential of PMN alone and in co-operation with macrophages against ingested mycobacteria was assessed.

METHODS

Animals. Twelve-week female outbred albino P strain and inbred C57Bl mice were obtained from the National Institute for Medical Research animal unit.

Bacteria. Mycobacterium lepraemurium, Douglas strain, was isolated from infected livers of P strain mice using the method of Hart & Rees (1960). *Mycobacterium microti*, strain OV254, was grown in a glycerol-free medium (Ungar *et al.*, 1962) for 14 d. Bacilli were collected by centrifugation and resuspended, prior to use, in 0·05 % (w/v) Tween 80 with 0·1 % (w/v) bovine serum albumin in 0·9 % (w/v) NaCl solution.

Preparation of PMN for electron microscopy. Albino P strain mice were inoculated intraperitoneally with 2 ml sterile 1·2 % (w/v) sodium caseinate (Difco) in 0·9 % (w/v) NaCl. The mice were killed by cervical

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dislocation 18 h later and exudates were harvested from the peritoneal cavities with 3 ml cold medium NCTC 109 (Gibco Biocult, Glasgow, Scotland) containing 5 units heparin ml^{-1} and 100 I.U. penicillin ml^{-1}. Exudates from several mice were pooled, samples were distributed to Petri dishes and *M. microti* or *M. lepraemurium* were added to give 10^9 bacilli ml^{-1}. Cultures were incubated at 37 °C for 30 min. The infected PMN were fixed using a modified method of Hirsch & Fedorko (1968). Sections were cut with glass knives on an LKB ultramicrotome, stained with uranyl acetate and Reynolds' lead citrate and examined in an AEI EM801 electron microscope with an accelerating voltage of 60 kV.

Preparation of infected PMN for mycobacterial viability studies. In these experiments a number of techniques were used to harvest and infect PMN with mycobacteria. As all results obtained were similar, only representative experiments are reported. Exudates of PMN were elicited in and harvested from C57Bl mice as already described. In some experiments 10^9 *M. lepraemurium* or 5×10^8 *M. microti* were included in the eliciting inoculum. The exudates from several mice were pooled, concentrated by centrifugation at $300g$ for 5 min and resuspended in medium NCTC 199 containing 25 mM-HEPES, 10 % (v/v) heat-inactivated horse serum (Gibco Biocult) and 100 I.U. penicillin ml^{-1} to give 2×10^6 PMN ml^{-1}.

Cells infected with mycobacteria *in vivo* were incubated at 37 °C for 2 to 20 h in Universal bottles. Non-infected PMN were distributed in 5 ml portions to 25 cm^3 plastic tissue-culture flasks and incubated with 10^8 *M. lepraemurium* or $2{\cdot}5 \times 10^7$ *M. microti* for 1 h at 37 °C. The adherent cells were washed three times to remove extracellular bacilli and the infected PMN were incubated for a further 1 h.

Preparation and infection of macrophage cultures. Mice were killed by cervical dislocation, their femurs were excised and the bone marrow was flushed out with medium NCTC 109 containing 40 % (v/v) in-activated horse serum, 5 units heparin ml^{-1} and 100 I.U. penicillin ml^{-1}. Cell suspensions from several animals were pooled and 1 ml portions were distributed to Leighton tubes and incubated at 37 °C overnight. Non-adherent cells were removed and the monolayers were incubated at 37 °C for 6 to 10 d in medium NCTC 109 containing 40 % (v/v) inactivated horse serum, 2 % (v/v) bovine embryo extract (Difco) and 100 I.U. penicillin ml^{-1}. The established macrophage monolayers were infected by overlaying with 10^8 *M. lepraemurium* or 3×10^5 *M. microti* for 2 h. Excess bacilli were removed and the monolayers were washed once with medium NCTC 109 before continuing incubation in fresh culture medium.

Mycobacterial counts and viability assays. Mycobacteria were released from PMN and macrophages ultrasonically (Ultrasonic generator and transducer, 800 kHz; Luziesa Ltd, 70 Rue J. P. Timbaud, Paris, France) using suitable volumes of Tween/albumin solution for *M. microti* or 0·1 % (w/v) albumin for *M. lepraemurium*. Bacilli were counted by a modified Breed smear technique (Hart & Rees, 1960). The viability of *M. microti* was determined by the method of Sharp (1973). Viability of *M. lepraemurium* was estimated indirectly from length distributions of stained bacilli measured with an eyepiece graticule after culture in elongation medium (Hart & Valentine, 1963).

RESULTS

Electron microscopic studies

Mycobacterium microti and *M. lepraemurium* were rapidly phagocytosed by PMN and phagosome–lysosome fusion occurred with both species. As well as neutrophils, a large number of eosinophils were found in these preparations and these also clearly demonstrated phagosome–lysosome fusion (Fig. 1). The process of fusion was rapid and after 30 min more than 80 % of mycobacteria were in phagolysosomes and a large number of PMN had totally degranulated. Degranulation did not occur in uninfected sodium caseinate-elicited PMN. When macrophages were present in PMN preparations, *M. lepraemurium* were found in association with phagolysosomes, whereas in 19 out of 20 separate observations *M. microti* inhibited phagosome–lysosome fusion.

Mycobacterial counts and viability

Cell exudates elicited in C57Bl mice contained a total of $0{\cdot}7 \times 10^7$ to $2{\cdot}2 \times 10^7$ leucocytes of which more than 75 % were PMN. The average infection rate with *M. microti* was less than 1·5 bacilli per PMN and with *M. lepraemurium*, 8 to 25 bacilli per PMN. The viability of *M. microti* isolated from either *in vitro* or *in vivo* infected PMN ranged from 77 to 103 % and was never significantly different from that of corresponding untreated bacilli. *Mycobacterium lepraemurium* recovered from PMN infected *in vitro* or *in vivo* increased in length to the same extent as untreated *M. lepraemurium* when incubated in elongation medium for

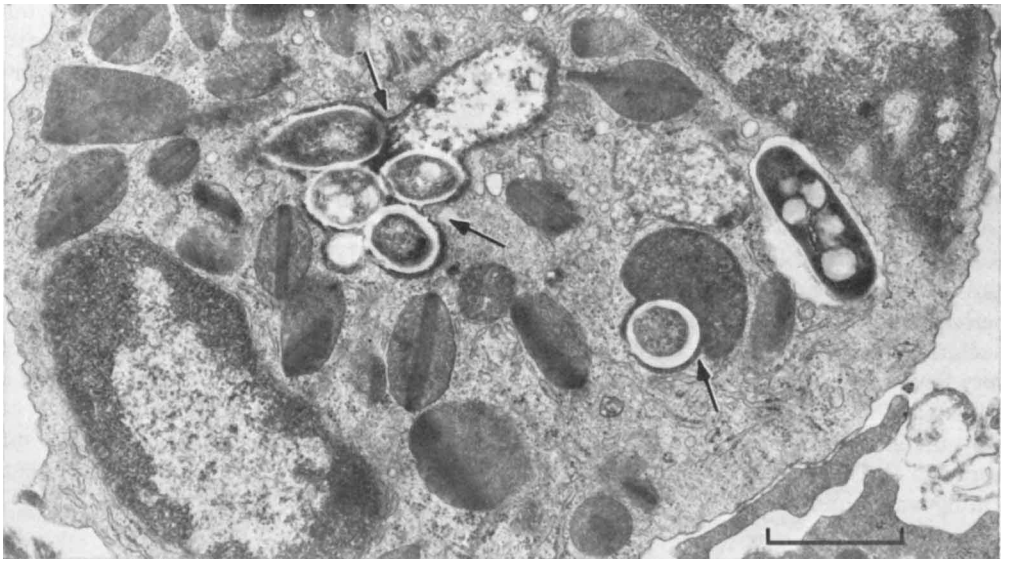

Fig. 1. An eosinophil fixed 30 min after infection with *M. microti*. Phagosome–lysosome fusion has occurred (arrows) in two of three phagosomes containing mycobacteria. Bar marker represents 1 μm.

up to 20 d. Killed bacilli in control incubations containing 1 % (v/v) formalin showed no increase in length.

Growth in macrophage cultures

Mycobacterium microti recovered from PMN elicited in C57Bl mice with 1·2 % (w/v) sodium caseinate and infected *in vivo* multiplied in C57Bl bone marrow macrophage cultures at the same rate as untreated *M. microti*. In a representative experiment, intracellular counts increased from $0{\cdot}7 \times 10^5$ bacilli per monolayer at the time of infection with PMN-derived *M. microti* to $2{\cdot}86 \times 10^5$ bacilli per monolayer 6 d later, equivalent to a mean generation time of 2·95 d. The corresponding counts for macrophage monolayers infected with untreated bacilli were $0{\cdot}68 \times 10^5$ and $2{\cdot}5 \times 10^5$ bacilli per monolayer, representing a mean generation time of 3·13 d. The increase in bacterial counts was significant in each case ($P < 0{\cdot}01$) but the differences in generation times between the groups were not significant. Less than 1 % of bacilli present were extracellular and the generation time of *M. microti* in either fresh or macrophage-conditioned culture medium was 10 d. Multiplication of *M. microti* in macrophage culture medium was not significant. In similar experiments, *M. lepraemurium* recovered from PMN recorded a 2·5-fold increase in numbers in macrophage monolayers incubated for 20 d compared with a 2·2-fold increase for untreated bacilli. The difference was not significant.

DISCUSSION

Although *M. microti* (Hart *et al.*, 1972), unlike *M. lepraemurium* (Brown *et al.*, 1969), inhibits phagosome–lysosome fusion in macrophages this did not happen in PMN. It seems unlikely that sodium caseinate elicitation of PMN promoted this fusion as degranulation did not occur in uninfected PMN, and in those *M. microti* infected macrophages found in the preparation phagosome–lysosome fusion had been inhibited. Little is known of the factors which induce or inhibit phagosome–lysosome fusion but the fact that *M. microti* behaves differently in PMN and macrophages suggests different mechanisms are involved.

The evidence that *in vitro* infected PMN were unable to kill *M. microti* directly is consistent with the findings of Hanks & Evans (1940). The studies were extended by following the

growth of mycobacteria recovered from PMN in cultured macrophages as this would reproduce the sequence of events *in vivo*. Damage of mycobacteria while in PMN could possibly make them more susceptible to killing and degradation by macrophages. However, no difference in growth in macrophages was noted between untreated mycobacteria and those recovered from PMN.

The mechanisms by which pathogenic mycobacteria survive or are killed within phagocytic cells remain largely unknown. Although *M. tuberculosis* and *M. microti* can inhibit macrophage phagosome–lysosome fusion, they survive and grow equally well when coated with antisera and phagosome–lysosome fusion occurs (Armstrong & Hart, 1975; R. M. Barr, unpublished results). Under our experimental conditions, *M. lepraemurium* and *M. microti* appear to withstand exposure to PMN lysosomal enzymes. This would perhaps suggest that other mechanisms, as yet unknown, may be involved in killing mycobacteria. Infection of PMN *in vivo* resulted in even distributions of intracellular bacilli with very few extracellular bacteria, which ensured accurate assessment of viability. Godal *et al.* (1970) and R. M. Barr (unpublished results) have demonstrated that macrophages can limit the growth of small, but not large, infections with *M. microti*. Similar limitations may apply to PMN. Infection rates in these experiments were reduced to a minimum but no killing was found.

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