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# An in-vitro model of intracellular bacterial infection using the murine macrophage cell line J774·2

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A simple model of intracellular bacterial infection based on the ability of the macrophage cell line J774·2 to phagocytose *Escherichia coli* in a reproducible manner is described. Viable counting of intracellular bacteria and microscopic examination of infected macrophage cultures after treatment with fluorescent *E. coli* antibody showed that the bacteria multiplied within the J774·2 cells. Viable intracellular bacteria may be used to study the activity of bactericidal and bacteriostatic drugs within the macrophage. The low apparent intracellular bactericidal activity of streptomycin, which was time- and concentration-dependent, accords with a low permeability of the J774·2 macrophages to this antibiotic. An exclusively intracellular infection could be achieved by inactivation of non-phagocytosed extracellular bacteria by streptomycin treatment of infected macrophage cultures. Under appropriate conditions intracellular bacterial viability was unaffected.

We are interested in the activity of antimicrobial agents against bacteria within mammalian cells and how drug-carrier systems may be used to modify drug activity against these intracellular organisms. In-vivo, antibiotics vary in their ability to inactivate intracellular bacteria and this lack of activity within cells may reflect the inability of antimicrobial agents to penetrate to the bacteria or inactivation of the agent by the intracellular environment.

We describe a model system based on the murine macrophage cell line J774 described by Ralph et al (1975) which was maintained as monolayer culture, and exhibited several macrophage characteristics, most importantly the non-specific phagocytosis of latex beads and of micro-organisms. Reproducible levels of infection with *Escherichia coli*, which can survive within the non-activated macrophage, exhibited minimal toxicity to the host cell and so provided an intracellular target for the study of antimicrobial agents.

## MATERIALS AND METHODS

*Materials.* Streptomycin sesquisulphate (Sigma) was used as received and antibiotic concentrations are expressed in terms of this salt. Cytochalasin B (Sigma) an inhibitor of phagocytosis (Axline & Reaven 1974) from *Helminthosporium dematoideium* was dissolved in dimethylsulphoxide (Fisons). The J774·2 cell line was obtained from the Sir William Dunn School of Pathology, Oxford. *Escherichia coli* (NCTC 9001) and *E. coli* (NCTC 8603, Aberdeen β) serotype 055:k59 (B5):H6 (National Collection of Type Cultures, London), fluorescent antibody (Bacto FA *E. coli* Poly A), Giemsa stain, (Michrome), and trypan blue (Flow Laboratories) were also used.

*Methods*

*Macrophage cultivation.* The J774·2 murine macrophage cell line was maintained in tissue culture medium (TCM) comprising RPMI 1640 (Moore et al 1967) supplemented with 5% v/v foetal calf serum and 2 mM L-glutamine and buffered to pH 7·2 with 20 mM (final concentration) HEPES/$NaHCO_3$.

Cells were grown to confluence (1 week at 37 °C with daily changes of TCM) in 50 ml flat-bottomed culture flasks (Nunclon) then harvested by addition of 20 ml ice cold TCM with shaking. Volumes (2 ml) of pooled cell suspension were added to the 20 mm square wells of tissue culture petri plates (Flow Laboratories), each well containing a 17 mm square coverslip. After 60 min at 37 °C, the number of adherent cells per coverslip was determined by direct counting. A mean value of $10^6$ macrophages per coverslip was obtained and cell viability, as determined by trypan blue exclusion, was better than 98%.

E. coli *cultures. E. coli* cells grown for 18 h statically in nutrient broth (Oxoid No. 2) at 37 °C were suspended in TCM at different concentrations and the viable count determined by surface plating on Oxoid No. 2 nutrient agar. Within 15 min of their preparation these bacterial cell suspensions in TCM were added to macrophage coverslip cultures.

*Phagocytosis assay.* The TCM of the macrophage

* Correspondence.

coverslip cultures in the Petri plate wells was replaced with 2 ml of TCM containing *E. coli* cell suspension. Phagocytosis of bacteria was allowed to proceed at 37 °C for 50 min with gentle agitation (0·5 Hz approximately) on a platform shaker (New Brunswick Scientific) after which time the coverslips were removed to the wells of a second Petri plate containing 2 ml TCM and 500 μg $ml^{-1}$ streptomycin sulphate to inactivate extracellular bacteria. After 20 min each coverslip was dip-washed through several changes of sterile 0·9% NaCl (saline) at 37 °C to remove the streptomycin. The number of ingested bacteria was determined either immediately or after incubation in TCM as described below.

*Determination of viable intracellular bacteria.* The coverslips with adherent macrophages were vortexed for 5 min in 10 ml sterile distilled water to effect hypotonic lysis and then, for each coverslip, two serial ten fold dilutions of the hypotonic lysate were made in distilled water for surface counting.

In all experiments, coverslips were set up in triplicate and phagocytosis expressed as the mean number of colony forming units recovered per macrophage.

*Streptomycin pretreatment of macrophages.* Macrophage coverslip cultures were incubated at 37 °C in TCM-streptomycin mixtures for 7, 5, 3, 2 and 1 h before infection with *E. coli* suspended in TCM. The streptomycin concentrations used were 500, 100, 50 and 10 μg $ml^{-1}$. After the incubation period, the antibiotic-treated macrophage coverslip cultures were dip-washed in saline then used in the phagocytosis assay as described above. However, in these experiments there was a 3 h incubation period in antibiotic-free TCM between inactivation of non-ingested bacteria and hypotonic lysis of the macrophages which allowed the bactericidal activity of any intracellular antibiotic to become apparent relative to the controls.

*Cytochalasin B-pretreatment of macrophages.* Coverslip cultures were incubated for 60 min at 37 °C in either TCM containing 20 μg $ml^{-1}$ Cytochalsin B in 0·5% v/v dimethylsulphoxide (final concentration) or in TCM containing 0·5% v/v dimethylsulphoxide as a control. The coverslip cultures were then saline rinsed and incubated for 15, 30 and 50 min in *E. coli* TCM suspension before hypotonic lysis of the macrophages and surface plating of intracellular bacteria. However, before lysis, only half of the coverslip cultures were treated with streptomycin (500 μg $ml^{-1}$ for 20 min) to inactivate non-ingested bacteria while the remainder were used to quantify cell associated bacteria ingested and adsorbed.

*Fluorescent staining.* Macrophage coverslip cultures infected with *E. coli* (NCTC 8603) were dip-rinsed in saline and immersed in Giemsa stain. After 10 min the coverslips were saline rinsed, placed on moist filter-paper in a Petri dish then flooded with a 1 in 5 dilution of fluorescent *E. coli* antibody in 13 mM pH 7·2 phosphate buffer containing 0·18% w/v NaCl. After 30 min the coverslips were saline rinsed then mounted in glycerol containing 5% w/v propyl gallate to prevent photobleaching (Giloh & Sedat 1982).

## RESULTS AND DISCUSSION

Simple rinsing of the macrophage monolayer cultures without streptomycin treatment did not remove all non-phagocytosed bacteria (Table 1) and on subsequent incubation an exponential increase in extracellular bacterial numbers was found. Of the non-phagocytosed bacteria those adsorbed are most likely to remain after washing, and without streptomycin inactivation of these the number taken up is over-estimated. The failure of simple washing to remove all extracellular bacteria has been found with other macrophages (Mackaness 1960) necessitating use of a bacterial inactivator such as lysostaphin for extracellular *Staphylococcus aureus* (Hirt & Bonventre 1973) or an antibiotic normally excluded from the macrophage interior such as streptomycin (Bonventre et al 1967).

Table 1. Effect of 60 min cytochalasin B (CCB) treatment of J774·2 macrophage cultures on uptake of *E. coli* (NCTC 9001) and on the bactericidal activity of streptomycin in the infected culture

| Macrophage treatment and Uptake period (min) | Viable macrophage-associated bacteria* recovered after 20 min treatment with | |
|---|---|---|
| | 500 μg $ml^{-1}$ streptomycin | 0·9% Saline |
| 0·5% v/v DMSO (control) | | |
| 15 | 1·20 ± 0·06 | 3·14 ± 0·22 |
| 30 | 4·98 ± 0·18 | 9·97 ± 0·38 |
| 50 | 5·10 ± 0·22 | 9·94 ± 0·56 |
| 20 μg $ml^{-1}$ CCB in 0·5% v/v DMSO | | |
| 15 | 0·02 ± 0·01 | 3·14 ± 0·13 |
| 30 | 0·56 ± 0·04 | 9·60 ± 0·31 |
| 50 | 2·48 ± 0·15 | 8·90 ± 0·67 |

* Mean of 5 determination ± s.e.m.

Streptomycin's ability to permeate mammalian cells has been the subject of a debate complicated by the variety of methods used and in some cases whether the findings truly represented intracellular events.

With *E. coli* (NCTC 9001) a minimal inhibitory concentration (MIC) of 7·5 μg ml$^{-1}$ was found for streptomycin. This was determined in nutrient broth or TCM, and the time taken to inactivate a bacterial suspension of $10^6$ organisms ml$^{-1}$ was about 10 min at 500 μg ml$^{-1}$ and about 20 min at 30 μg ml$^{-1}$ streptomycin.

The effectiveness of the 20 min 500 μg ml$^{-1}$ streptomycin treatment against extracellular bacteria was best demonstrated with cytochalasin B-pretreated macrophage cultures (Table 1) in which few bacteria were phagocytosed. The use of fluorescent antibody, which may be assumed not to reach intracellular bacteria, allowed discrimination between intracellular and extracellular bacteria. For the 15 min uptake period, >98% of the cell associated bacteria were inactivated which provides good evidence that bacterial cells adsorbed to the macrophage surface are susceptible to streptomycin inactivation and are not protected by any surface microenvironment effect. The inhibitory effects of cytochalasin B on phagocytosis were reversible and the apparent decrease in streptomycin activity shown in Table 1 for the 30 and 50 min uptake periods represents recovery of phagocytic ability.

In uninhibited macrophage cultures, the number of bacteria ingested per macrophage has been shown to be proportional to the number of bacteria presented to these phagocytic cells (Stevenson et al 1982) and about 30% of the inoculum was recovered as intracellular bacteria at all infection ratios studied up to the highest inoculum of 32 bacteria per macrophage. This was for uptake periods in excess of 40 min which appeared to mark the start of a plateau such that incubation of bacteria with macrophages for shorter periods (Fig. 1) resulted in <30% take-up of the inoculum.

If it is assumed that the bactericidal activity of streptomycin is not significantly impaired in the macrophage intracellular environment, then the results shown in Fig. 2 illustrate the penetration of streptomycin into macrophages. Thus at a streptomycin concentration of 500 μg ml$^{-1}$, only at 3 h incubation of macrophages in antibiotic was there apparent penetration of antibiotic to the macrophage interior, as evidenced by a reduction in viable intracellular bacteria. A 7 h incubation produced an 80% reduction in the number of viable intracellular bacteria recovered from the lysed macrophages, for periods of up to 5 h, streptomycin concentrations of 10, 50 and 100 μg ml$^{-1}$ had no significant effect on intracellular bacterial numbers (results not shown).

Thus those bacteria located within macrophages

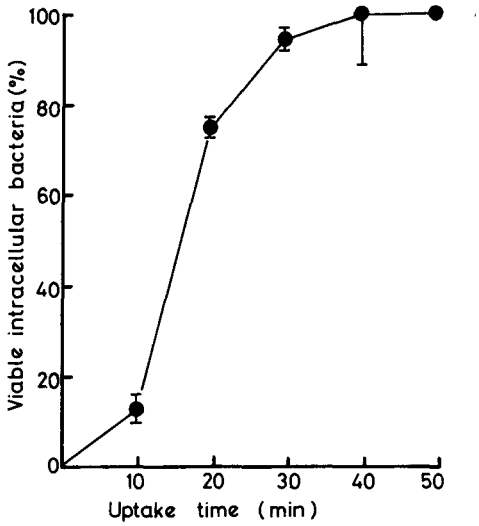


FIG. 1. Time course of phagocytosis of *E. coli* (NCTC 9001) by J774·2 macrophages. Results expressed as % of the 50 min uptake period. Infection ratio, bacteria per macrophage, 1·6, Vertical bars show s.e.m.

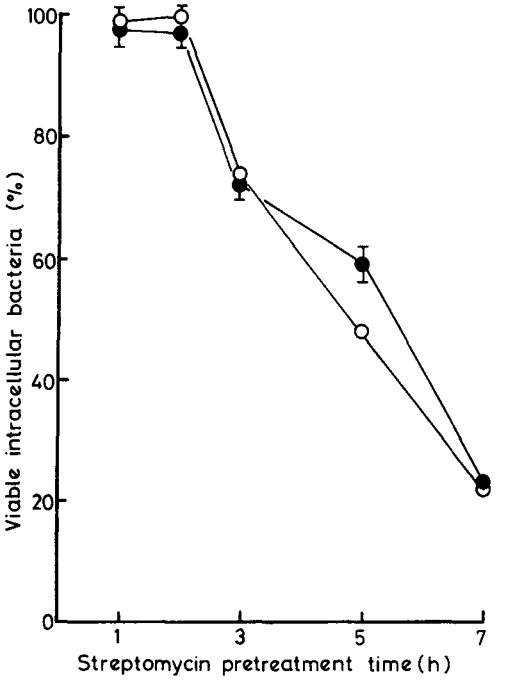


FIG. 2. 500 μg ml$^{-1}$ streptomycin pretreatment of J774·2 macrophages and the viability of subsequently phagocytosed *E. coli* (NCTC 9001). Results, viable bacteria per macrophage, are expressed as % of the appropriate control value. Infection ratios, bacteria per macrophage, were 20 (○) and 4 (●). Vertical bars show s.e.m. (n = 4).

are protected against extracellular streptomycin concentrations which are many times greater than the MIC. This is to be expected since the high water solubility of this antibiotic gives a partition coefficient, log P = −0·45 in octanol-water (Leo et al 1971), unfavourable to diffusion across the macrophage cell membrane. A 20 min exposure to 500 μg ml$^{-1}$ streptomycin may therefore be expected to inactivate extracellular bacteria without significantly affecting phagocytosed bacteria. It can be shown (Fig. 3) that intracellular *E. coli* will multiply within the J774·2 cell, albeit at a slower rate than those growing in nutrient broth or TCM, after such antibiotic treatment of infected macrophages. These results were confirmed by microscopic examination of fluorescent antibody-treated cultures which allowed observation of the increase in intracellular bacterial numbers with time.

Dietz & Cole (1982) have shown that avirulent *Listeria monocytogenes* cells are phagocytosed by the J774 line and that in the unstimulated state the macrophages effect a listericidal action of 75% reduction in intracellular bacterial numbers over a 4 h period. This bactericidal effect was markedly increased after stimulation of the J774 cells by BCG or *Mycoplasma arthriditis* bacteria or culture supernatants.

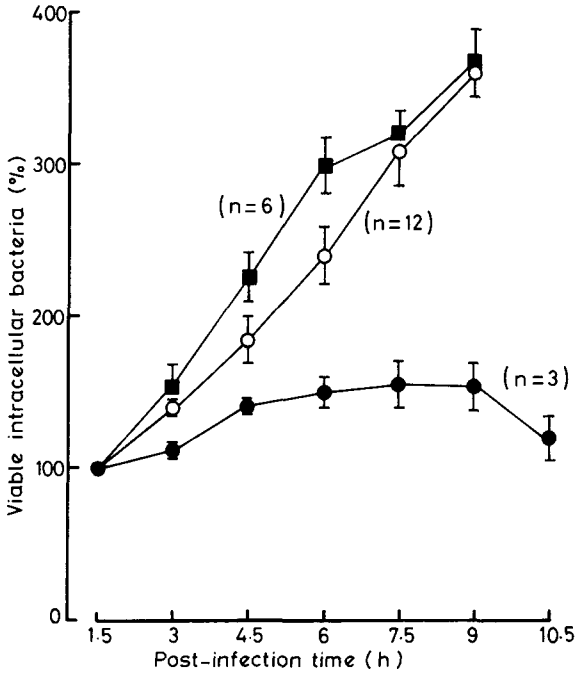


FIG. 3. Intracellular multiplication of *E. coli* after phagocytosis by J774·2 macrophages. Results are expressed as % of the value, bacteria per macrophage, found at 90 min after infection of the culture. Infection ratios, bacteria per macrophage, were 3·8 (○) and 17 (●) for *E. coli* (NCTC 9001) and 5·0 (■) for *E. coli* (NCTC 8603). Vertical bars show s.e.m.

No such bactericidal effect for phagocytosed *E. coli* cells was observed in our system (Fig. 3) during incubation times of up to 10½ h; intracellular multiplication of *E. coli* was so marked, especially at high infection rates (>11 bacteria per macrophage) that after 9–10 h incubation, macrophage viability was impaired by about 60% as determined by trypan blue exclusion. Reduced macrophage viability was accompanied by an observed apparent decrease in colony forming units recovered per macrophage (Fig. 3), representing streptomycin (30 μg ml$^{-1}$) penetration and bacterial inactivation within the trypan blue positive macrophages.

High infection rates in conjunction with prolonged incubation periods were the only conditions found to significantly affect macrophage viability. The marked *E. coli* endotoxin-induced decrease in J774 viability described by Morland & Kaplan (1978) may be discounted here since *E. coli* NCTC 9001 does not produce endotoxin.

The intracellular *E. coli* population increase over 4–5 h (Fig. 3) was comparable to that described for virulent *L. monocytogenes* (Dietz & Cole 1982). The apparent resistance of the avirulent *E. coli* to intracellular inactivation observed here contrasts with the results of Dietz & Cole (1982) for an avirulent *L. monocytogenes* strain, the difference presumably reflecting the relative susceptibility of the species to bactericidal mechanisms. For example, the Gram positive *Listeria* may be at greater risk from lysozyme activity than *E. coli*.

The ability of *E. coli* to multiply within these macrophages (Fig. 3) indicates that the intracellular bactericidal activity of these unstimulated cells is either absent or is present at such a low level that it cannot keep pace with the rate of division of ingested bacteria. The highest division rate observed for intracellular bacteria was two doublings in a 9 h period. This is much lower than the division rate in TCM at 37 °C. Whether this difference represents a bactericidal or bacteriostatic property of the macrophage interior cannot be determined from the present results. No attempt has been made to activate these macrophages and no data has been obtained on the bactericidal activity of these cells in the activated state against *E. coli*.

Prolonged incubation of intracellularly infected macrophage cultures was accompanied by an increasing number of viable *E. coli* recoverable from

the TCM which probably represents outgrowth of bacteria from host cells. This outgrowth was controlled by the presence of 30 μg $ml^{-1}$ streptomycin added to the TCM.

This study shows that, in addition to the advantages inherent in the use of established cell lines (Howard & Glynn 1971), a system comprising unstimulated host cells and essentially avirulent bacteria provides an easily manipulated model of intracellular infection. The bacterial cells grow readily intraphagocytically and the apparently low inherent bactericidal activity of the unstimulated J774 host for *E. coli* may be discounted in the study of antibacterial drugs within the macrophage, making it possible to study not so much the uptake of antibacterial agents into the infected host cell, by, for example the use of labelled compounds, but rather the antibacterial activity of these agents in the intracellular milieu.

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