Effect of growth condition on in-vitro susceptibility of *Shigella dysenteriae* type 1 to killing by murine peritoneal macrophages

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The intracellular fate of *Shigella dysenteriae* type 1 strains grown in casamino acid-yeast extract (CYE) broth and nutrient broth (NB) was studied in casein-elicited mouse peritoneal macrophages. Virulent strains 14731 and W30864 cultured in NB and opsonised with normal mouse serum were susceptible to killing by peritoneal macrophages (66 SEM 1.7% killing by 2 h). In contrast, both strains grown in CYE broth and opsonised with normal mouse serum showed resistance to killing by peritoneal macrophages (76 SEM 1.4% survival by 2 h). Electronmicroscopy demonstrated that the bacteria escaped from the phagosome compartment by lysing the phagocytic vacuole and remained within the cytoplasm. Lipopolysaccharide (LPS) stimulated peritoneal macrophages to kill the opsonised strains 14731 and W30864 grown in CYE broth (85.4 SEM 1.6% killing by 2 h). Recombinant murine gamma interferon (rIFN- γ) also stimulated macrophages to kill CYE-grown bacteria (52.1 SEM 1.3% killing by 2 h). However, an avirulent rough mutant strain W30864-22 grown in either NB or CYE broth showed marked susceptibility to killing by peritoneal macrophages, which was similar to that of NB-grown strain 14731 or W30864. The results of the present study suggest that in-vitro growth conditions may modulate the susceptibility of *S. dysenteriae* type 1 to killing by phagocytes.

Introduction

Shigella dysenteriae type 1 causes bloody, purulent diarrhoea by invading and destroying colonic epithelial cells and inducing an inflammatory response in the mucosal tissues [1]. The organism enters epithelial cells by induced phagocytosis and rapidly lyses the phagocytic vacuole leading to its release into the cytoplasm [2, 3]. Strains of *S. dysenteriae* type 1 produce high levels of a potent cytotoxin called Shiga toxin. Recent studies have suggested that Shiga toxin may exacerbate the disease by specifically damaging capillaries in the colonic mucosa and by mediating the influx of inflammatory cells into the intestinal compartment [4]. It is well known that intracellular multiplication of the invading pathogen is a prerequisite for virulence. The correlation between the rate of intracellular multiplication and the level of Shiga toxin production has been proposed as one of the means by which invasive shigellae preferentially incorporate amino acids through the shut off of host cell protein synthesis [5]. Studies have demonstrated that invasive strains of *S. dysenteriae* type 1 induce early cell damage even in the toxin-resistant cell line Henle-407 [5, 6]. It has been shown recently that *S. dysenteriae*

type 1 produces an extracellular slime layer under certain growth conditions that mediates resistance to killing by serum and neutrophils [7, 8]. Many studies of phagocyte interactions with facultative intracellular bacteria have investigated the mechanisms by which these pathogens frustrate the host's microbicidal capabilities and how phagocytes bind and phagocytose these pathogens.

Strains of *S. dysenteriae* type 1 are generally non-fimbriated [9–11] and, unless opsonised, are unable to bind to phagocytic cells or to induce an oxidative burst in these cells. Lipopolysaccharide (LPS) activates macrophages to produce and release several cytokines [12, 13]. The mechanisms involved in the interaction of LPS with surface receptors, thereby activating secondary signals and ultimately activating the macrophage, are currently being investigated by many groups [14–16]. Interferon-gamma (IFN- γ) also stimulates macrophages [17], although its efficacy has not been tested against *S. dysenteriae* type 1.

The aim of this study was to understand the pathogenesis of *S. dysenteriae* type 1 infection by investigating its interactions with murine peritoneal macrophages. Specific objectives of the study were: to test the ability of casein-elicited peritoneal macrophages to engulf and kill strains of *S. dysenteriae* type

1 in the presence of normal serum; to compare the effect of bacterial culture conditions on the sensitivity to killing by peritoneal macrophages; and to evaluate the enhanced phagocytic activities of peritoneal macrophages stimulated by rIFN- γ and LPS.

Materials and methods

Bacterial strains

S. dysenteriae type 1 strain W30864 and its O-antigen-negative derivative W30864-22 [18, 19] were obtained from the National Institute of Health (NIH), Japan. Strain 14731 [11] was obtained from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), Dhaka. Strains W30864 and 14731 were virulent as judged by Congo red binding [20] and positive Sereny test results [21].

Media and growth conditions

Bacteria were grown in Casamino acid-Yeast extract Broth [11], pH 7.4 (Difco Laboratories) supplemented with 1 mM CaCl_2 (CYE) in screw-capped test tubes containing 5 ml of medium, for 18 h with shaking (100 rpm) at 37°C. Bacteria were also grown in Nutrient Broth (NB) (Eiken Chemical Co., Tokyo, Japan), for 18 h with shaking (100 rpm) at 37°C.

Sera

Normal sera from 20 BALB/c mice were pooled and stored in 1-ml portions at -70°C. To opsonise bacteria with fresh sera, a portion was thawed immediately before being used in the experiment.

Macrophages

Macrophages were elicited from BALB/c mice by intraperitoneal injection of sodium caseinate (Wako Pure Chemicals Industries Ltd, Osaka, Japan) 5% in NaCl 0.9%, 4 days before harvesting as described previously [22]. Briefly, peritoneal cells were collected by peritoneal lavage with 3 ml of PBS. Cells were then washed in Ca^{2+} , Mg^{2+} -free Hanks's Balanced Salts Solution (HBSS) and resuspended in RPMI 1640 medium (Flow Laboratories Inc., McLean, VA, USA) plus fetal calf serum (Gibco Laboratories, Grand Island, NY, USA) 10%. After washing, macrophages were incubated at 37°C in CO_2 5% in 35-mm plastic tissue culture dishes and allowed to adhere for 4 h, after which time the non-adherent cells were removed by washing with HBSS.

Opsonisation

To opsonise the strains of *S. dysenteriae* type 1, bacterial suspensions (2×10^6 cfu/ml) were incubated with normal mouse serum 10% v/v at 37°C for 30 min. Strains were also incubated at 37°C for 30 min with

complement-inactivated mouse serum. Bacterial suspensions were then washed twice with PBS and suspended in serum-free RPMI.

In-vitro assay for survival of bacteria within macrophages

Macrophages were incubated in individual wells of 24-well plates (Coster, Cambridge, MA, USA) at a concentration of 2×10^5 cells/well in a total volume of 1 ml of RPMI 1640 supplemented with complement-inactivated fetal calf serum (Gibco Laboratories) and allowed to adhere for 3 h at 37°C. After incubation, non-adherent cells were washed off and the medium was replaced by 1 ml of pre-opsonised bacterial suspension (2×10^6 cfu/ml) in RPMI without serum. Plates were centrifuged for 10 min at 2200 *g* and incubated at 37°C for 15 min to allow entry of bacteria into the cells. Plates were washed twice with HBSS and covered with 1 ml of RPMI supplemented with 2 mM glutamine and gentamicin (25 $\mu\text{g/ml}$). This step was considered as time zero. Plates were then sampled at 0, 30, 60, 120 and 180 min. After infection, triplicate macrophage samples from each well were lysed with Triton X-100 (0.1%), plated and the number of shigellae was counted.

Peritoneal macrophages were also plated in triplicate in 24-well tissue culture plates at 2×10^5 cells/well and were incubated overnight at 37°C with either rIFN- γ 100 U or LPS 10 $\mu\text{g/ml}$. After treatment, plates were washed with HBSS and bacterial suspensions were added, and the assays performed as described above.

Scanning electronmicroscopy

At selected intervals after infection (between time zero and 15 min of incubation), cells were fixed for 1 h at room temperature with glutaraldehyde 2.5% in 0.1 M cacodylate buffer (pH 7.2) (Wako Pure Chemicals). They were washed with the same buffer and the cells were fixed for 1 h at room temperature with osmium tetroxide 1% in 0.1 M cacodylate buffer. The fixed samples were dehydrated with ethanol and critical point-dried. The samples were then coated with gold-palladium and analysed by scanning electron microscopy (Hitachi, S-700).

Transmission electronmicroscopy

To identify more precisely the intracellular behaviour of *S. dysenteriae* type 1, transmission electronmicroscopy of strain 14731-infected macrophages was performed as described previously [3]. Briefly, infected macrophages were fixed at selected intervals for 1 h at room temperature with glutaraldehyde 2.5% in 0.1 M cacodylate buffer. They were then washed and incubated overnight at 4°C in this buffer. Further fixation was performed in osmium tetroxide 1% in the same

buffer. The cells were then scraped off the culture dishes with a "rubber policeman" diagonal (Wakenyaku Co., Ltd, Japan), concentrated in agar, and treated for 1 h with uranyl acetate 1%. Samples were then dehydrated and embedded in Epon®. Thin sections were stained with uranyl acetate 2% and lead citrate.

Results

Effects of serum opsonins on binding and phagocytosis of S. dysenteriae type 1

Strains of *S. dysenteriae* type 1 grown in either NB or CYE broth and opsonised with normal mouse serum were bound and taken up equally by mouse peritoneal macrophages. Opsonisation of both virulent and avirulent strains with complement-inactivated serum reduced the binding and uptake of bacteria compared with opsonisation with normal serum (Table 1). Opsonisation of a rough mutant strain W30864-22 with either normal serum or complement-inactivated serum showed an increased uptake of bacteria compared with that of virulent strains. However, bacterial culture condition did not affect the uptake of either virulent or avirulent strains of *S. dysenteriae* type 1 (Table 1). Under non-opsonic conditions, there was no binding or uptake of bacteria by peritoneal macrophages (data not shown).

Survival of S. dysenteriae type 1 within peritoneal macrophages

Strains 14731 and W30864 grown in CYE broth and opsonised with normal mouse serum survived within the peritoneal macrophages (76 SEM 1.4% bacterial survival) (Fig. 1). In contrast, an avirulent rough mutant strain W30864-22 grown in CYE broth showed increased susceptibility to killing by peritoneal macrophages (Fig. 1). The numbers of the rough mutant had decreased by 63% at 60 min and 85% after incubation for 120 min.

Intracellular killing of strains 14731, W30864 and W30864-22 by peritoneal macrophages

Opsonised strains 14731 and W30864 grown in NB

Fig. 1. Intracellular survival of *S. dysenteriae* type 1 in murine peritoneal macrophages. Each value is the mean and SEM for three different determinations at each time point; —○—, 14731; —●—, W30864; —■—, W30864-22.

were killed (66 SEM 1.7%) by peritoneal macrophages within 120 min of incubation (Fig. 2). The intracellular killing of strain W30864-22 was enhanced relative to that of strain 14731 or W30864, and the kinetics of killing of both virulent and avirulent strains was almost the same after incubation for 30 and 60 min.

Effect of rIFN-γ and LPS on macrophage killing activity

Macrophages exhibited significantly enhanced ability to kill (52.1 SEM 1.3% killing by 2 h) *S. dysenteriae* type 1 when they were activated with rIFN-γ (Fig. 3A). The intracellular killing of *S. dysenteriae* type 1 by LPS-activated macrophages was increased (85.4 SEM 1.6% killing by 2 h) (Fig. 3B) compared to that by the rIFN-γ activation.

Table 1. Binding and uptake of *S. dysenteriae* type 1 by murine peritoneal macrophages

Strain no.	Uptake of bacteria after incubation for 15 min*			
	CYE broth-grown bacteria in		NB-grown bacteria in	
	normal serum	complement-inactivated serum	normal serum	complement-inactivated serum
14731	90.25	48.75	87.00	51.75
W30864	94.75	50.25	90.00	47.00
W30864-22	152.00	72.50	151.00	69.50

*Bacteria were opsonised with mouse serum 10% as indicated and then mixed 10:1 with casein-elicited peritoneal macrophages for 15 min at 37°C. At time zero, macrophages were lysed and samples were plated and counted. Results from three experiments are presented as the mean bacterial counts (10^4); SEM were <10%.

Fig. 2. Intracellular killing of *S. dysenteriae* type 1 in murine peritoneal macrophages. Each value is the mean and SEM for three different determinations at each time point; —○—, 14731; —●—, W30864; —■—, W30864-22.

Electronmicroscopy of strain 14731-infected peritoneal macrophages

Scanning electronmicroscopy of CYE broth-grown strain 14731-infected macrophages showed the binding

of bacteria to mouse peritoneal macrophages at 5 min (Fig. 4A). Strain 14731 grown in CYE medium was also used in the transmission electronmicroscopy study for a better understanding of intracellular survival of *S. dysenteriae* type 1. These electronmicroscopic observations demonstrated that some bacteria were located inside the phagosomes (Fig. 4B.1), and some bacteria exhibited lysis of the membrane-bound vacuole (Fig. 4B.2) after 30 min. Electronmicroscopy of strain 14731-infected macrophages also demonstrated some bacteria in the cytoplasm (Fig. 4C.3), and the remaining bacteria were degraded (Fig. 4C.4) after incubation for 120 min.

Discussion

S. dysenteriae type 1 strains 14731 and W30864 grown in NB were killed effectively by casein-elicited peritoneal macrophages, whereas the same strains grown in CYE broth were resistant even in the presence of normal serum. It has been demonstrated previously that *S. dysenteriae* type 1 produces an extracellular slime layer when grown in CYE broth, but not in NB, and that the bacteria become resistant to killing by human polymorphonuclear leucocytes [7, 8]. Taken together, these results indicate that the culture conditions affect the susceptibility of *S. dysenteriae* strains to killing by phagocytes. Electronmicroscopy demonstrated that a small number of bacteria escape from the phagosome compartment (Fig. 4B.2 and Fig. 4C.3). After evasion, bacteria start to multiply

Fig. 3. Effect of A, murine recombinant IFN- γ and B, lipopolysaccharide on macrophage killing activity. Peritoneal macrophages were incubated with either rIFN- γ 100 U/ml or LPS 10 μ g/ml for 24 h. Macrophages were then infected with CYE broth-grown 14731 (—○—), W30864 (—●—) and W30864-22 (—■—). The results are expressed as mean and SEM of three experiments.

Fig. 4. Electronmicrographs showing the phagocytosis of *S. dysenteriae* type 1 strain 14731 by mouse peritoneal macrophages. Bacteria grown in CYE broth were opsonised with normal mouse serum, and were incubated with peritoneal macrophages. **A**, Scanning electronmicrograph demonstrating the binding of 14731 to the macrophage at 5 min. **B** and **C**, Transmission electronmicrographs showing the various stages of phagocytosis and evasion — bacteria within the phagosome (1), and evasion of bacteria by lysing the phagosome membrane after incubation for 30 min (2); bacteria within the cytoplasm (3), and degranulation of 14731 (4) after incubation for 120 min.

intracellularly, and this was confirmed by colony counting after incubation for 120 min. Because the rough mutant strain W30864-22 showed an increased susceptibility to peritoneal macrophages, the possible role of the O-antigenic side chain in the evasion process cannot be excluded. It seems that the strains of *S. dysenteriae* type 1 produced longer O-antigenic side chains when grown in CYE broth, and became more virulent. In this capacity, the *S. dysenteriae* type 1 virulence plasmid may also encode a product that renders bacteria resistant to killing by macrophages under certain growth conditions.

Macrophages stimulated by IFN- γ killed strains 14731

and W30864 grown in CYE broth effectively. The reduction in the percentage of viable bacteria was more in the LPS-activated macrophages than those observed in rIFN- γ -activated macrophages, possibly by the increased release of toxic oxygen intermediates, hydrogen peroxide and nitric oxide [23–26].

Transmission electronmicroscopy of macrophages infected with strain 14731 demonstrated that evasion of killing in *S. dysenteriae* strains was the result of early and efficient lysis of the phagosome membrane. The *Shigella* virulence plasmid may play a role in this process. After lysing the phagocytic vacuole, bacteria remained within the cytoplasm as observed by

transmission electronmicroscopy after incubation for 120 min. Intracellular multiplication, ability to lyse phagocytic vacuoles, and contact haemolysis have been shown to be virulence factors for *S. flexneri* [3]. Haemolytic activity has also been demonstrated in strains of *S. dysenteriae* type 1 [27], and the bacteria also creates tissue damage through their highly efficient invasiveness and intracellular multiplication. Secretion of Shiga toxin would certainly represent an additional advantage by precipitating cell death [5]. Studies on the role of Shiga toxin in pathogenesis have focused primarily on its potent cytotoxic activity, and its role in colonic ulceration [28–30]. Shiga toxin also induces expression of pro-inflammatory cytokines from murine peritoneal macrophages [31].

These electronmicroscopic studies suggest that *S. dysenteriae* type 1 may be a facultative intracellular micro-organism which escapes from the phagosome (Fig. 4B.2 and 4C.3), and multiplies intracellularly. However, during natural infection, macrophages may produce a higher bactericidal activity and phagocytose *S. dysenteriae* more efficiently than peritoneal macrophages *in vitro*.

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