Interaction of *Salmonella* serotypes with porcine macrophages *in vitro* does not correlate with virulence

Patricia R. Watson, Sue M. Paulin, Philip W. Jones and Timothy S. Wallis

Author for correspondence: Timothy S. Wallis. Tel: +44 1635 577230. Fax: +44 1635 577263.
e-mail: timothy.wallis@bbsrc.ac.uk

Institute for Animal
Health, Compton,
Newbury RG20 7NN, UK

The interaction between *Salmonella* serotypes and macrophages is potentially instrumental in determining the outcome of infection. The nature of this interaction was characterized with respect to virulence and serotype-host specificity using pigs as the infection model. Experimental infection with *Salmonella typhimurium*, *Salmonella choleraesuis* or *Salmonella dublin* resulted in enteric, systemic or asymptomatic infection, respectively, which correlates well with the association of *S. choleraesuis* with systemic disease in pigs in epidemiological studies. Persistence within porcine alveolar macrophages *in vitro* did not directly correlate with virulence since *S. typhimurium* persisted in the highest numbers, and *S. choleraesuis* in the lowest. Comparison to other studies revealed that the relatively high persistence of *S. typhimurium* in macrophages correlates with its virulence in a broad range of animals: this could be a virulence mechanism for broad-host-range serotypes. There were little or no significant differences in the induction of pro-inflammatory cytokines by macrophages infected with the three serotypes. *S. typhimurium* and *S. dublin*, but not *S. choleraesuis*, damaged porcine macrophages, and the mechanism of damage did not resemble apoptosis. In conclusion, the virulence of *Salmonella* serotypes in pigs did not directly correlate with their interaction with porcine macrophages *in vitro*. The interaction of *Salmonella* and macrophages *in vitro* may not accurately model their interaction *in vivo*, and this will form the basis of further study.

Keywords: *Salmonella* serotypes, porcine virulence, macrophages, apoptosis, cytokine bioassays

INTRODUCTION

Salmonellosis is an important zoonosis ranging in severity from subclinical infection, through mild diarrhoea, to severe systemic disease. One of its characteristics is that some serotypes, for example *Salmonella typhimurium*, have a broad host range, whereas several other serotypes, for example, *Salmonella choleraesuis*, *Salmonella typhi*, *Salmonella dublin* and *Salmonella pullorum*, are associated with disease in specific, and usually a very limited, range of host animals (pigs, humans, cattle and poultry, respectively). This has been termed serotype-host specificity, host adaptation or host restriction, although for the sake of clarity, only the first of these terms will be used here. In pigs, *Salmonella*

infections are associated with significant animal suffering and economic losses and pigs may be a reservoir of infection for humans (Berends *et al.*, 1997). Natural infection of pigs with *S. choleraesuis* is usually associated with systemic disease, whereas infection with *S. typhimurium* is associated with enteric disease (reviewed by Wilcock & Schwartz, 1992). This is analogous to *S. typhi* and *S. typhimurium* infections in humans. It is possible to infect pigs experimentally with *S. choleraesuis* and *S. typhimurium*, and the resulting disease is similar to that reported in the field (Reed *et al.*, 1986). Therefore pigs are a good model for studying the pathogenesis of systemic and enteric forms of human salmonellosis and for investigating the mechanisms of serotype-host specificity.

The factors influencing *Salmonella* serotype host-specificity remain poorly defined, although there is some

Abbreviation: LDH, lactate dehydrogenase.

evidence that bacterial survival within macrophages is important. *S. typhimurium* can persist in significantly higher numbers than *S. typhi* in primary murine macrophages *in vitro* (Lissner *et al.*, 1985; Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi & Arai, 1996; Schwan & Kopecko, 1997), which correlates with the virulence of these serotypes for mice. Similarly, *S. typhi* persists better in human compared to murine macrophages *in vitro*, which again correlates with virulence. However, the relative persistence of *S. typhi* and *S. typhimurium* in human macrophages varies considerably between different studies (Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi & Arai, 1996). This variation may be explained by unquantified differences in the magnitude of macrophage lysis, which can have a major effect on the interpretation of results from bacterial persistence studies (Guilloteau *et al.*, 1996; Sizemore *et al.*, 1997).

This study has used *Salmonella* serotypes of defined virulence in pigs to characterize the interaction of *Salmonella* serotypes with porcine macrophages. Bacterial persistence, the magnitude and nature of *Salmonella*-induced macrophage damage, and the production of pro-inflammatory cytokines were assessed and correlated with virulence.

METHODS

Bacterial strains. *S. typhimurium* strains ST4/74 and ST12/75, *S. dublin* strains SD2229 and SD3246 and *S. choleraesuis* var. *kunzendorf* strains SCSA50 and SCS14/74 have been described previously and extensively characterized (Baird *et al.*, 1985; Wallis *et al.*, 1995; Guilloteau *et al.*, 1996; Watson *et al.*, 1998; Bolton *et al.*, 1999). To date, there is no evidence that there are any differences between the two strains of each serotype in a variety of *in vitro* and *in vivo* assays. Strains were stored as cultures in Luria-Bertani broth containing 30% glycerol at 70 °C and were streaked onto MacConkey plates and incubated at 37 °C overnight for use. Inocula for all of the *in vitro* assays were prepared by inoculating 10 ml Luria-Bertani broth with several freshly streaked colonies and incubating overnight at 37 °C with shaking at 150 r.p.m. The cultures were subcultured 1 in 100 into fresh broth and incubated as before for 4 h. The number of bacteria present in each subculture was estimated by spectrophotometry, the subcultures were diluted to the required number of c.f.u. ml⁻¹, and the bacterial concentration was determined by viable count. Heat-inactivated bacteria were prepared by placing the diluted subcultures in a boiling water bath for 10 min.

Virulence of *Salmonella* serotypes following inoculation of pigs. Camborough pigs (6–7 weeks old) were supplied by the Institute for Animal Health farm. Pigs were weaned at 2 weeks and given a 3 d course of enrofloxacin (Baytril; Bayer), to compensate for their lowered resistance to bacterial infections during weaning and transport to the disease-secure animal unit. Thereafter, pigs were fed on a diet of antibiotic-free, irradiated pellets. None of the pigs excreted salmonellas at 0, 2 or 4 weeks before infection as determined by enrichment of faecal swabs in Rappaport broth (at 37 °C for 18 h) and selenite brilliant green broth (at 42 °C for 18 h) followed by overnight incubation at 37 °C on modified brilliant green agar (Difco).

Bacterial cultures of *S. typhimurium* ST4/74, *S. dublin* SD3246

or *S. choleraesuis* SCS14/74 were prepared by inoculating Bacto-Tryptone broth (Jones, 1975) with several bacterial colonies and incubating statically at 37 °C for 18 h. For oral inoculation, approximately 3×10^8 c.f.u. were suspended in 10 ml ant-acid [5% Mg(SiO₃)₃, 5% NaHCO₃ and 5% MgCO₃ in H₂O] and administered directly into the stomach of each pig immediately before the morning feed using a 10_{FG} catheter (Arnolds Veterinary Products). For intravenous inoculation, approximately 1×10^6 c.f.u. in a volume of 1 ml were injected into the superior vena cava. Rectal temperatures were recorded and animals were monitored for clinical signs of disease twice daily. All animals were humanely killed at 7 d after inoculation. The numbers of bacteria in the lung, spleen, liver, ileum, caecum and colon and in the lymph nodes associated with these sites were determined by viable counts of one sample per site as described by Watson *et al.* (1998). The limit of accurate quantification was $2.0 \log_{10}$ c.f.u. (g tissue)⁻¹ and any samples containing fewer bacteria were enriched in Rappaport and selenite brilliant green broth as described above. Samples that were positive on enrichment were given a value of $2.0 \log_{10}$ c.f.u. g⁻¹ and those that were negative were given a value of 0 c.f.u. g⁻¹.

***Salmonella* infection of porcine alveolar macrophages and preparation of conditioned supernatants for cytokine bio-assay.** Alveolar macrophages were isolated from healthy Camborough pigs by bronchoalveolar lavage as described previously for cattle (Guilloteau *et al.*, 1996). Viability of cells, of which 90–99% are alveolar macrophages (Ganter & Hensel, 1997), was >99% as assessed by trypan blue exclusion. Cells were allowed to adhere to 24-well tissue culture plates at 5×10^5 cells ml⁻¹ in Iscove's modified Dulbecco's medium containing 10% foetal calf serum (FCS), 100 units penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 100 µg gentamicin ml⁻¹ and incubated overnight at 37 °C in 5% CO₂ and 95% humidity. Two hours before infection, non-adherent cells were washed off and the culture medium was changed to Dulbecco's modified Eagle's medium Ham's nutrient mix F-12 without phenol red (DME/F12) containing 5% FCS. Bacterial cultures were prepared as described above and diluted in DME/F12 medium to give a ratio of infection of five bacteria per macrophage. Viable and heat-inactivated bacteria were added to the macrophage monolayers and the monolayers were incubated for 1, 3, 24 or 48 h. Overgrowth of extracellular bacteria in monolayers incubated for 24 or 48 h was prevented by washing the monolayers 1 h after infection and adding DME/F12 containing 5% FCS and 100 µg gentamicin ml⁻¹, followed by washing the monolayers after a further hour and adding DME/F12 containing 5% FCS and 10 µg gentamicin ml⁻¹. Gentamicin was not added to the monolayers incubated for 1 or 3 h, because changing the media would have prevented the measurement of cytokines released immediately after infection. After the appropriate incubation time, the supernatants, termed conditioned macrophage supernatants, were collected and centrifuged (13500 g, 2 min, 4 °C) to reduce the amount of cell debris and bacteria. An aliquot (50 µl) was used to quantify macrophage damage by measuring the amount of lactate dehydrogenase (LDH) released by the monolayers using the cytotoxic 96 kit (Promega). Results obtained using this method correlate with the severity of macrophage damage by microscopic assessment (Guilloteau *et al.*, 1996). The results were expressed as the percentage of LDH released by infected monolayers compared to the LDH released by detergent treatment of control monolayers. The remaining conditioned macrophage supernatants were stored at -70 °C in aliquots. The number of macrophage-associated bacteria was determined by washing monolayers twice with

DME/F12 without supplements and adding 1 ml PBS containing 0.1% sodium deoxycholate. Serial dilutions of the lysates were plated onto MacConkey agar plates and incubated overnight at 37 °C.

Bioassay for IL-1 activity. Cytokine release was quantified using biological assays, which have the following characteristics: they measure cytokine release rather than expression; they are very sensitive; and they are unlikely to be affected by unrecognized inhibitors often present in serum, when using monocultures of cells. In addition, ELISAs for porcine cytokines are not readily available. IL-1 activity was measured using the A375 cell line. The growth of A375 cells is inhibited in the presence of human and porcine IL-1 (Nakai *et al.*, 1988; Cendan *et al.*, 1994). The cells were maintained in Eagle's minimal essential medium with Earle's salts containing 10% FCS, 100 units penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 100 µg gentamicin ml⁻¹. Aliquots of the conditioned macrophage supernatants were thawed and serially diluted 1 in 2 in the above media to give a final volume of 50 µl per well, in 96-well plates. Medium alone was used as a negative control. Fifty microlitres of A375 at 1×10^5 cells ml⁻¹ in Eagle's minimal essential medium with supplements was added to each well and incubated for 96 h at 37 °C in 5% CO₂ and 95% humidity. Following incubation, the culture medium was removed and the wells were washed once with PBS. The remaining cells were stained with freshly prepared crystal violet stain (0.1% crystal violet, 1% acetic acid, 5% formaldehyde, 4.25 g NaCl l⁻¹, 2.25 g NaH₂PO₄ anhydrous l⁻¹, 2.75 g Na₂HPO₄ anhydrous l⁻¹) for 2 h at room temperature and then the excess stain was removed by washing with PBS. The retained crystal violet was dissolved in 100% methanol and quantified by spectrophotometry (OD₅₉₅). The percentage growth inhibition was calculated using the formula: $[(OD_{595} \text{ negative control} - OD_{595} \text{ test sample}) / OD_{595} \text{ negative control}] \times 100$. The range of detection of the assay was 0.1 units per well (resulting in 7% growth inhibition) to >10 units per well (resulting in 75% growth inhibition) as estimated from a dose response curve to recombinant human IL-1α (Genzyme Diagnostics) which was determined in parallel with each assay.

Bioassay for IL-6 activity. IL-6 activity was measured using the 7TD1 cell line (kindly supplied by P. Kaiser, IAH, Compton, UK), whose growth is dependent on the presence of IL-6 (Van Snick *et al.*, 1986). The suitability of this cell line in a bioassay for porcine IL-6 has been reported previously (Frank *et al.*, 1996). The cells were maintained in RPMI 1640 containing 10% FCS, 100 units penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 100 µg gentamicin ml⁻¹, 0.05 mM 2-mercaptoethanol. Recombinant murine IL-6 (R&D Systems) was added at a concentration of 4 ng ml⁻¹ for routine growth of the cells. For use in the bioassay, cells were washed three times to remove IL-6 and were resuspended in RPMI 1640 with supplements, but without IL-6. Aliquots of the conditioned macrophage supernatants were thawed and serially diluted 1 in 5 in RPMI 1640 with supplements to give a final volume of 50 µl per well, in 96-well plates. Fifty microlitres of 7TD1 cell suspension at 1×10^5 cells ml⁻¹ in RPMI with supplements was added to each well. The assay plates were incubated for 72 h at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. Growth of the 7TD1 cells was estimated by measuring DNA synthesis. An aliquot of [³H]thymidine containing 0.015 MBq radioactivity was added to each well 18 h before the end of the incubation period. The cells were harvested onto glass fibre filters and incorporation of [³H]thymidine into the cells was measured using a betaplate liquid scintillation counter. The range of detection was 0.001 pg ml⁻¹ (5000 c.p.m. incorporated triti-

ated thymidine) to greater than 8 pg ml⁻¹ (45000 c.p.m.) as estimated from a dose response curve to murine IL-6 determined in parallel to each assay.

Bioassay for TNF-α activity. TNF-α activity was measured using the WEHI 164 clone 13 cell line (kindly supplied by G. Entrican, Moredun Research Institute, Edinburgh, UK). This cell line is sensitive to the cytotoxic activity of human TNF-α (Espevik & Nissen-Meyer, 1986) and is suitable as a bioassay for porcine TNF-α (Kim *et al.*, 1991). Cells were maintained in Dulbecco's modified essential medium containing 10% FCS, 100 units penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 100 µg gentamicin ml⁻¹. Semi-confluent monolayers were prepared by adding 3×10^4 WEHI cells into wells of a 96-well plate and incubating overnight at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. The medium overlying the monolayers was replaced with 50 µl fresh medium. Aliquots of the conditioned macrophage supernatants were thawed and serially diluted 1 in 2 into the medium overlying the monolayers to give a final volume of 50 µl. Medium alone was used as a negative control. Finally, 50 µl media containing 0.5 µg actinomycin D-mannitol ml⁻¹ (Sigma) was added to each well. The monolayers were incubated for 18 h as above. Following incubation, the remaining cells were stained with crystal violet stain as described for the IL-1 bioassay. The percentage cytotoxicity was calculated according to the formula: $[(OD_{595} \text{ negative control} - OD_{595} \text{ test sample}) / OD_{595} \text{ negative control}] \times 100$. The range of detection was 0.001 units per well (3% cytotoxicity) to greater than 0.1 units per well (85% cytotoxicity), as estimated from a dose response curve to recombinant human TNF-α (Genzyme Diagnostics) determined in parallel to each assay.

Characterization of DNA from macrophages. Porcine alveolar macrophages were isolated and maintained in 24-well plates as described above. Bacterial cultures were prepared as described above and, where required, were opsonized in 10% autologous serum for 40 min on a rolling platform. The monolayers were infected and subsequently incubated as described above: the overgrowth of bacteria in the culture medium of infected monolayers incubated for 24 and 48 h was prevented with the use of gentamicin. Actinomycin D-mannitol was added to three control monolayers to give a final concentration of 1 µg ml⁻¹. DNA was extracted from macrophages by the method of Zychlinsky *et al.* (1992) with minor modifications as follows. Monolayers were washed twice with PBS and 200 µl lysis buffer (10 mM Tris; 5 mM EDTA; 0.5% SDS) was added and incubated at room temperature for 15 min. The lysate was pooled from triplicate wells, 1 µl 20 mg proteinase K ml⁻¹ was added and the mixture was incubated for 2 h at 56 °C. An equal volume of Tris-saturated phenol/chloroform (1/1, v/v) was added and mixed gently for 5 min. The mixture was centrifuged (13500 g, room temperature, 15 min) and the upper aqueous layer was recovered. DNA was precipitated overnight at -20 °C by the addition of 1/10 volume 3 M sodium acetate and 2 vols ethanol and collected by centrifugation (13500 g, room temperature, 20 min). The DNA was washed once in 70% ethanol, air-dried and dissolved in 30 µl TE buffer containing 10 µg RNase ml⁻¹. Samples (10 µl) were separated through a 1.5% agarose gel and examined on a UV transilluminator.

Electron microscopy of macrophages. Porcine alveolar macrophages were isolated and maintained (at 30 ml of 1×10^5 cells ml⁻¹ in 250 ml flasks) as described above. Monolayers were infected with bacteria with or without opsonization as described above. Actinomycin D-mannitol was added to control monolayers to give a final concentration of 1 µg ml⁻¹.

At 24 h after infection, the monolayers were washed once with 0.1 M cacodylate-buffered 2.5% glutaraldehyde and incubated overnight. The fixed macrophages were washed twice in 0.1 M phosphate buffer for 30 min and then post-fixed in 1% 0.1 M phosphate-buffered osmium tetroxide for 90 min. The macrophages were washed five times in double-distilled water and resuspended in 1% aqueous uranyl acetate for 1 h in the dark. After a further wash in water, the macrophages were embedded in 0.5 ml 1% agar. The solidified agar blocks were cut into 2 mm³ cubes, dehydrated through a methanol gradient and incubated for 2 × 15 min in propylene oxide. The blocks were embedded in araldite resin and incubated at 60 °C for 48 h to allow polymerization to occur. Blocks were sectioned at a thickness of approximately 90 nm and the sections were collected on 3-mm-diameter nickel Athens grids and stained with lead citrate for 10 min. The sections were examined using a Philips EM 300 transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

S. typhimurium is moderately virulent, *S. dublin* is of low virulence and *S. choleraesuis* is highly virulent for pigs

The relative virulence of *S. typhimurium*, *S. dublin* and *S. choleraesuis* in pigs was assessed following oral inoculation with approximately 3×10^8 c.f.u. Clinical symptoms were monitored for 7 d after inoculation and the number of salmonellas present in several intestinal and systemic sites was determined on the seventh day at post-mortem. *S. typhimurium* induced a rapid, but short-lived, increase in rectal temperature (Fig. 1), and during this time the pigs were diarrhoeic, vomiting and appeared depressed. Two to three days after inoculation, clinical symptoms ceased and temperatures returned to normal. At post-mortem, salmonellas were recovered at between 2.5 and 4.0 log₁₀c.f.u. g⁻¹ from all three regions of intestinal wall and associated nodes, but were not recovered from the systemic sites in quantifiable numbers (Fig. 2a). Pigs inoculated with *S. dublin* had no clinical symptoms and only a mild and short-lived

Fig. 1. Rectal temperatures of pigs following oral inoculation with *S. typhimurium* (■), *S. dublin* (○) or *S. choleraesuis* (▲). Each datum point is the mean of three pigs and is presented with the standard error of the mean.

pyrexia, intermittent diarrhoea and appeared depressed and lethargic for the duration of the experiment. *S. choleraesuis* was recovered in significantly higher numbers ($P < 0.05$) than either *S. typhimurium* or *S. dublin* from the intestinal sites and was the only serotype recovered from the systemic sites in quantifiable numbers (approximately 4.0 log₁₀c.f.u. g⁻¹).

Similar results were obtained following intravenous inoculation of pigs with 1×10^6 c.f.u. *S. choleraesuis* was the only serotype to induce disease symptoms, with a pyrexia response from day 4 and onwards, and was the only serotype recovered in quantifiable numbers from systemic sites (approximately 5.0 log₁₀c.f.u. g⁻¹) (Fig. 2b).

S. typhimurium and *S. dublin* persist within, and induce damage to, porcine alveolar macrophages at significantly greater magnitude than *S. choleraesuis*

The relative recovery of *S. typhimurium*, *S. dublin* and *S. choleraesuis* was assessed up to 48 h after infection of porcine alveolar macrophages. Gentamicin was added after 1 h of infection to macrophages incubated for 24 or 48 h to prevent overgrowth of extracellular bacteria. Non-adherent bacteria and gentamicin were removed by washing the macrophages immediately before lysing the macrophages for bacterial recovery. Therefore, the bacterial recovery at 1 and 3 h is obtained from adherent and intracellular bacteria, and at 24 and 48 h from only intracellular bacteria. The number of bacteria associated with the macrophages at 1 h after infection is significantly greater for *S. typhimurium* compared to either *S. dublin* or *S. choleraesuis* ($P < 0.05$) (Table 1). Between 1 and 3 h, the recovery of *S. typhimurium* and *S. dublin* decreased slightly (0.1–0.3 log₁₀c.f.u. ml⁻¹) whereas the recovery of *S. choleraesuis* decreased by almost 1.0 log₁₀c.f.u. ml⁻¹, and it was recovered in significantly lower numbers than either *S. typhimurium* or *S. dublin* ($P < 0.05$). Between 24 and 48 h, the recovery of *S. typhimurium* and *S. dublin* remained constant, whereas the recovery of *S. choleraesuis* continued to decrease. At 48 h after infection, *S. choleraesuis* was recovered in significantly lower numbers than either *S. typhimurium* or *S. dublin* ($P < 0.02$).

The amount of macrophage damage induced during infection with the different serotypes was quantified by measuring the release of LDH. Macrophages infected with *S. choleraesuis* did not release significant amounts of LDH compared to the uninfected macrophages ($P > 0.1$) at either 3, 24 or 48 h (Table 2). Macrophages infected with *S. typhimurium* and *S. dublin* released significantly more LDH than *S. choleraesuis* for several, but not all, of the possible combinations of strains at 24 and 48 h ($P > 0.001$).

Fig. 2. Recovery of *S. typhimurium* (filled bars), *S. dublin* (open bars) and *S. choleraesuis* (hatched bars) from intestinal and systemic sites following (a) oral inoculation and (b) intravenous inoculation. Each bar is the mean of three pigs, except that for the intravenous inoculation with *S. typhimurium*, which is from only two pigs, and is presented with the standard error of the mean. The limit of accurate quantification was $2.0 \log_{10} \text{c.f.u. g}^{-1}$, and samples that do contain salmonellas, but in numbers too low to quantify, are indicated with an asterisk. HLN, hepatic lymph node; BLN, bronchial lymph node.

Table 1. Recovery of *S. typhimurium* (ST4/74 and ST12/75), *S. dublin* (SD2229 and SD3246) and *S. choleraesuis* (SCSA50 and SCS14/74) from porcine alveolar macrophages

Macrophages were infected with five bacteria per macrophage and the numbers of associated bacteria were determined after incubation for 1, 3, 24 or 48 h. Gentamicin was added at 1 h after infection to the monolayers used for the 24 and 48 h time points to prevent the overgrowth of extracellular bacteria. The results are the means of three experiments, each performed in triplicate, and are presented with the standard error of the mean.

Strain	Inocula ($\log_{10} \text{c.f.u. ml}^{-1}$)	Bacterial recovery ($\log_{10} \text{c.f.u. ml}^{-1}$)			
		1 h	3 h	24 h	48 h
ST4/74	6.5 ± 0.0	5.7 ± 0.2	5.6 ± 0.3	4.9 ± 0.4	4.7 ± 0.6
ST12/75	6.5 ± 0.0	5.8 ± 0.2	5.6 ± 0.3	4.9 ± 0.5	5.1 ± 0.3
SD2229	6.5 ± 0.1	5.4 ± 0.1	5.3 ± 0.2	4.5 ± 0.6	4.3 ± 0.5
SD3246	6.5 ± 0.0	5.3 ± 0.0	5.0 ± 0.2	4.5 ± 0.7	4.4 ± 0.3
SCSA50	6.6 ± 0.0	5.3 ± 0.2	4.4 ± 0.3	3.7 ± 0.3	3.1 ± 0.7
SCS14/74	6.4 ± 0.1	5.2 ± 0.1	4.6 ± 0.2	4.0 ± 0.2	3.1 ± 0.3

Release of pro-inflammatory cytokines during infection of porcine alveolar macrophages with different *Salmonella* serotypes does not correlate with virulence

The release of the pro-inflammatory cytokines IL-1, TNF- α and IL-6 from porcine alveolar macrophages infected with different *Salmonella* serotypes was assessed by biological assays using the same infected macrophages as used in the persistence and lysis assay. Uninfected macrophage monolayers released little IL-1, TNF- α or IL-6 over the time course of the assay (Table 3). Macrophages infected with viable bacteria released IL-1 and TNF- α at all three time points and increasing

amounts of IL-6 over the three time points. There were no significant differences between any of the strains ($P > 0.05$) with the exception of the release of TNF- α by ST4/74 and ST12/75 at 48 h after infection ($P > 0.02$). The general lack of difference between the serotypes was not due to the amount of cytokines being outside the range of detection of the assay as assessed by testing serial dilutions of all of the samples (data not shown). Macrophages infected with heat-inactivated bacteria released low or undetectable levels of all three cytokines at 24 and 48 h after infection. At 3 h after infection, IL-1 and TNF- α were released, but in lower amounts than from macrophages infected with viable bacteria ($P < 0.01$), with the exception of TNF- α release by SCSA50

Table 2. Damage to porcine alveolar macrophages during infection with *S. typhimurium* (ST4/74 and ST12/75), *S. dublin* (SD2229 and SD3246) or *S. choleraesuis* (SCSA50 and SCS14/74)

Damage was estimated by measuring the amount of LDH released by the infected macrophages into the overlying tissue culture medium and is expressed as a percentage compared to the LDH released following detergent treatment of macrophage monolayers. Each result represents the mean of three separate experiments, with each experiment performed in triplicate, and is presented with the standard error of the mean.

Strain	Macrophage damage (%)		
	3 h	24 h	48 h
ST4/74	18.6 ± 2.8	39.6 ± 3.7	75.2 ± 6.2
ST12/75	11.8 ± 0.8	25.7 ± 4.4	58.7 ± 6.3
SD2229	12.4 ± 1.1	24.8 ± 5.0	58.8 ± 5.8
SD3246	12.7 ± 0.8	28.5 ± 7.1	47.0 ± 3.3
SCSA50	13.0 ± 3.0	11.7 ± 3.2	37.1 ± 7.7
SCS14/74	8.6 ± 1.0	11.7 ± 2.5	39.3 ± 7.0
Uninfected	9.3 ± 1.7	8.1 ± 0.9	39.7 ± 4.3

($P > 0.05$). Heat-inactivated ST4/74 and SD2229 released more IL-1 than heat-inactivated SCSA50 ($P < 0.001$), and there was no difference in the amount of

TNF- α released by the different heat-inactivated serotypes.

Salmonella serotypes do not induce porcine alveolar macrophage lysis by apoptosis

The mechanism of *Salmonella*-induced macrophage lysis was investigated by examining macrophage DNA by electrophoresis for the characteristic laddering pattern associated with apoptosis. Macrophage DNA was isolated at 3, 24 and 48 h after infection. There was no increase in DNA fragmentation following infection with any *Salmonella* serotype, at any time point compared to the uninfected control (Fig. 3). Opsonization of the bacteria with autologous sera before infection did not affect the DNA morphology. DNA from macrophages incubated for 24 h with the apoptosis-inducing agent actinomycin D-mannitol exhibited the laddering pattern associated with apoptosis.

The ultrastructure of porcine alveolar macrophages 24 h after infection with *Salmonella* serotypes was examined by transmission electron microscopy. In uninfected monolayers, the large majority of macrophages appeared healthy (Fig. 4a). Infection with *S. dublin* and *S. typhimurium* resulted in loss of pseudopodia and an increase in cell size (Fig. 4b). Opsonization of bacteria before infection had little or no effect on the appearance of the macrophages. Incubation with actinomycin D-mannitol for 24 h resulted in cell shrinkage, formation

Table 3. Release of pro-inflammatory cytokines from porcine alveolar macrophages during infection with viable or heat-inactivated *Salmonella* serotypes

Results are the mean of duplicate bioassays of conditioned supernatants. Supernatants were prepared in triplicate from three independent macrophage infection experiments and are presented with the standard error of the mean.

	IL-1*			TNF- α †			IL-6‡		
	3 h§	24 h	48 h	3 h	24 h	48 h	3 h	24 h	48 h
Viable bacteria									
ST4/74	69.9 ± 2.8	62.1 ± 6.0	79.1 ± 6.1	78.3 ± 1.1	63.7 ± 7.0	41.1 ± 3.4	6.3 ± 0.5	14.7 ± 1.1	22.5 ± 2.0
ST12/75	69.8 ± 3.4	57.0 ± 6.0	70.7 ± 7.5	77.0 ± 2.8	63.3 ± 6.4	40.8 ± 5.7	5.6 ± 0.2	15.3 ± 1.5	18.3 ± 1.3
SD2229	71.6 ± 3.0	60.4 ± 4.8	80.1 ± 5.2	82.4 ± 3.6	74.0 ± 5.3	58.2 ± 2.4	6.2 ± 0.4	14.8 ± 2.0	24.1 ± 1.9
SD3246	72.4 ± 3.3	62.0 ± 4.8	76.3 ± 8.7	73.4 ± 4.9	74.6 ± 5.0	55.3 ± 2.1	5.4 ± 0.3	15.9 ± 2.0	17.2 ± 1.7
SCSA50	71.5 ± 2.0	60.1 ± 5.5	77.0 ± 4.8	73.8 ± 4.9	70.8 ± 5.6	53.9 ± 2.2	8.5 ± 0.8	18.4 ± 1.6	26.7 ± 2.3
SCS14/74	66.0 ± 2.5	57.4 ± 6.6	68.0 ± 7.6	71.3 ± 7.7	64.4 ± 5.8	59.9 ± 2.6	7.3 ± 0.5	17.2 ± 1.3	23.6 ± 2.1
Heat-inactivated bacteria									
ST4/74	52.4 ± 5.8	19.2 ± 4.5	28.8 ± 3.5	51.3 ± 8.0	16.3 ± 5.9	1.7 ± 1.1	5.0 ± 0.3	8.1 ± 0.6	10.4 ± 1.7
SD2229	50.2 ± 5.2	14.7 ± 3.0	29.0 ± 5.7	40.9 ± 9.5	4.5 ± 2.6	3.4 ± 3.9	7.1 ± 0.5	8.6 ± 0.8	10.4 ± 2.3
SCSA50	35.7 ± 4.9	18.4 ± 6.2	28.3 ± 4.0	65.6 ± 7.1	24.0 ± 8.8	3.5 ± 2.3	5.8 ± 0.3	8.1 ± 1.0	6.8 ± 0.9
Uninfected	10.1 ± 2.7	18.1 ± 6.7	16.5 ± 3.7	−1.7 ± 5.4	20.4 ± 11.7	24.1 ± 13.3	4.2 ± 0.3	7.6 ± 0.8	7.5 ± 1.6

* IL-1 bio-activity (% growth inhibition) of conditioned macrophage supernatant on A375 cells.

† TNF- α bio-activity (% cytotoxicity) of conditioned supernatant on WEHI 164 clone 13 cells.

‡ IL-6 bio-activity (cell growth measured by uptake of tritiated thymidine; $\times 1000$ c.p.m.) of conditioned supernatant on 7TD1 cells.

§ Macrophages were infected for 3 h and supernatants were collected.

|| Macrophages were infected for 24 or 48 h, with the addition of gentamicin 1 h after infection to prevent bacterial overgrowth in the media, and supernatants were collected.

Fig. 3. Characterization of DNA extracted from porcine alveolar macrophages infected for 24 h with *S. typhimurium* ST4/74, *S. dublin* SD2229 or *S. choleraesuis* SCSA50. Uninfected macrophages were used as the negative control (–ve) and macrophages incubated with actinomycin D-mannitol for 24 h were used as the positive control (+ve). This is a representative result from a total of three separate experiments. The original photograph of the gel was scanned using a Kodak DCS420 digital camera and the contrast of the image was adjusted using Adobe Photoshop 3.0.

of apoptotic bodies and marginalization of condensed chromatin (Fig. 4c).

DISCUSSION

The apparent specificity of a given *Salmonella* serotype for a particular host will be influenced by both the likelihood of natural exposure and the degree of bacterial virulence for the host. Therefore, virulence following experimental infection may not necessarily correlate with epidemiological data on serotype–host specificity. For example, *S. choleraesuis* can infect pigs, cattle, mice, rats, guinea pigs and rabbits after experimental inoculation (Williams Smith & Halls, 1966, 1968; Reed *et al.*, 1986; Nnalue, 1991; Nnalue *et al.*, 1992; Barrow *et al.*, 1994), but is generally only associated with natural infection of pigs, and infrequently of man. In addition, natural infection may occur with a ‘non-specific’ combination of serotype and host as happened during an *S. dublin* epidemic in cattle in England and Wales during the late 1960s, in which

S. dublin was also associated with 19% of porcine *Salmonella* incidents, compared to a mean of only 0–1% at other times (Sojka & Field, 1970; Anonymous, 1978–1998). In contrast, with some combinations of serotype and host there is a strong correlation between virulence following experimental infection and serotype–host specificity. For example, the inability to infect several laboratory animal species with *S. typhi* and *Salmonella gallinarum* correlates with their very restricted host range in nature. Therefore, studies of host specificity need to consider the virulence of each serotype–host combination, and extrapolation of results from one combination to another is not possible.

In the present study, the relative virulence of *S. choleraesuis*, *S. typhimurium* and *S. dublin* for pigs was determined. Pigs can be experimentally infected with *S. typhimurium* and *S. choleraesuis* by both oral and intranasal inoculation (Reed *et al.*, 1986; Fedorka-Cray *et al.*, 1995; Gray *et al.*, 1995), and infection via other routes, independent of the gastro-intestinal tract, may also be possible. The relative importance of different routes of natural infection in pigs is not known, and therefore the virulence of the three serotypes was assessed following both oral and intravenous inoculation. *S. choleraesuis* induced severe symptoms and infected systemic tissues in high numbers, *S. typhimurium* induced an acute, self-limiting enteritis and *S. dublin* was of low virulence. All three strains used to infect pigs are highly virulent for mice (Baird *et al.*, 1985; Plested, 1995; Guilloteau *et al.*, 1996; A. V. Gautier & T. S. Wallis, unpublished data). These results confirm the association of *S. choleraesuis* with systemic disease in pigs, and the general lack of association of *S. dublin* with porcine salmonellosis. They also suggest that the mechanism of serotype–host specificity in pigs does not depend on intestinal colonization and translocation, since *S. choleraesuis* was the most virulent serotype by both routes of inoculation. This supports the recent observation that serotype–host specificity of *S. dublin* and *S. choleraesuis* does not correlate with intestinal invasion of bovine or porcine intestinal mucosa (Bolton *et al.*, 1999).

For many years it has been widely accepted that a key stage in the pathogenesis of *Salmonella* is its persistence within cells of the reticuloendothelial system. For example, susceptibility of mice to salmonellosis correlates with net growth of *S. typhimurium* within isolated macrophages (reviewed by Dunlap *et al.*, 1994) and *Salmonella* mutants that are unable to survive in murine macrophages have reduced virulence in mice (Fields *et al.*, 1986; Libby *et al.*, 1994; Hensel *et al.*, 1998). However, several independent groups have recently reported that *Salmonella* is highly cytolytic for macrophages (Guilloteau *et al.*, 1996; Chen *et al.*, 1996; Lindgren *et al.*, 1996), suggesting that the contribution of macrophage infection to pathogenesis is more complex than previously believed. We have therefore investigated several parameters of the interaction of our strains of defined porcine virulence with primary porcine alveolar macrophages. This macro-

Fig. 4. Transmission electron microscopy of porcine alveolar macrophages either (a) without treatment, (b) 24 h after infection with *S. typhimurium* ST4/74 or (c) 24 h after incubation with actinomycin D-mannitol. See Results for description of morphological changes. This is a representative result from three separate experiments. Micrograph negatives were scanned using a Linotype Saphir flatbed scanner and the image was converted to positive and the contrast was adjusted using Adobe Photoshop 3.0. Bar, 4 µm.

phage population has been relatively well characterized and is phagocytic, bactericidal, produces pro-inflammatory mediators, expresses several surface receptors typical of other macrophages and is easy to isolate in high numbers and purity (reviewed by Van Reeth & Adair, 1997). In addition, in pigs the lungs are an important site for clearance of bacteria and particulates from blood (Winkler, 1988), and pneumonia is a common feature of *S. choleraesuis* infection of pigs and therefore it is probable that *S. choleraesuis* interacts with alveolar macrophages during pathogenesis. However, porcine alveolar macrophages may differ in some of their properties to tissue macrophages in other systemic organs, and therefore may not necessarily accurately model the interaction of *Salmonella* with macrophages in the porcine liver and spleen. Macrophage-derived cell lines were not used because they are dramatically more permissive for uptake and/or intracellular replication of *Salmonella* serotypes compared to primary macrophages (Buchmeier & Heffron, 1989; Vladoianu *et al.*, 1990) and would appear to be inherently unsuitable for such studies. For example, in murine macrophage-like cells, some studies find no difference between *S. typhimurium* and *S. typhi* (Vladoianu *et al.*, 1990; Hirose *et al.*, 1997), and others report differences in either uptake, but not persistence (Pascopella *et al.*, 1995), or persistence, but not uptake (Schwan & Kopecko, 1997). In the present study, the majority of macrophage experiments were performed using non-opsonized bacteria, since preliminary experiments showed that opsonization was not a prerequisite for efficient phagocytosis and did not influence subsequent persistence, as has sometimes been reported for other serotype–host combinations (Ishibashi & Arai, 1996).

The relative persistence of *S. typhimurium*, *S. dublin*

and *S. choleraesuis* in porcine alveolar macrophages did not directly correlate with their virulence in pigs. *S. typhimurium* and *S. dublin*, but not *S. choleraesuis*, induced damage to the macrophage plasma membrane. This may allow gentamicin to enter the macrophage and kill intracellular bacteria, and therefore the difference in recovery of *S. typhimurium* and *S. dublin* compared to *S. choleraesuis* is probably greater than that measured by viable counts. These persistence data appear to be in contrast to those obtained with murine macrophages, from which it was concluded that the persistence of *S. typhimurium* and *S. typhi* directly correlated with their host specificity (Lissner *et al.*, 1985; Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi & Arai, 1996; Schwan & Kopecko, 1997). However, *S. typhimurium* can persist relatively well not only in murine macrophages, but also in human-monocyte-derived macrophages, chicken peritoneal exudate cells and porcine alveolar macrophages (Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi & Arai, 1996; M. S. Chadfield & J. E. Olsen, personal communication; this study). Taking these results together, there is a direct correlation between *S. typhimurium* persistence within macrophages and its ability to cause disease in a wide range of animals.

Macrophages are important in the regulation of the host's immune response to infection through the release of pro-inflammatory cytokines and other mediators of inflammation. This contributes to the mobilization of host defences and recruitment of other immune cells required for control of an infection, but may also exacerbate some aspects of disease (reviewed by Murtaugh *et al.*, 1996). The release of three pro-inflammatory cytokines by macrophages infected with different serotypes was quantified to determine whether this correlates with the different disease syndromes.

Infection of porcine macrophages with all three serotypes resulted in a rapid and sustained release of IL-1 and TNF- α activity and a more gradual increase in the release of IL-6 activity. This is despite differences in the numbers of associated bacteria and the magnitude of macrophage damage. Therefore, the ability of different *Salmonella* serotypes to induce the release of pro-inflammatory cytokines from macrophages *in vitro* does not correlate with serotype-host specificity. Heat-inactivated bacteria were included in the cytokine assays to determine if any differences between the *Salmonella* serotypes were related to differences in surface antigens such as LPS. Although it turned out that this control was not necessary, it did reveal a difference in cytokine release induced by viable and killed bacteria. This supports a previous report showing differences in IL-12 induction in murine macrophages by viable and killed *S. dublin* (Chong *et al.*, 1996) and is relevant to the design of vaccines for salmonellosis.

Salmonella-induced macrophage damage has been reported to occur *in vivo* in both mice and pigs (Baskerville *et al.*, 1972; Richter-Dahlfors *et al.*, 1997). However, in the present study there was no direct correlation between *Salmonella*-induced macrophage damage *in vitro* and virulence of the same strains in pigs. Furthermore, macrophage damage induced by *S. typhimurium* and *S. dublin* did not have any of the typical characteristics of apoptosis (reviewed by Allen *et al.*, 1997; McConkey, 1998), which is in contrast to the conclusions of several other studies on *Salmonella*-induced macrophage damage (Chen *et al.*, 1996; Monack *et al.*, 1996; Hersh *et al.*, 1999). This is unlikely to be due to the use of different cell types or bacterial strains, since we have previously shown that *Salmonella* can damage macrophages by a non-apoptotic mechanism using both an immortalized macrophage-like cell line and alveolar macrophages isolated from cattle following infection with several commonly studied *S. typhimurium* strains (Watson *et al.*, 2000). In addition, opsonization of the bacteria did not alter the mechanism of macrophage damage. The induction of macrophage damage by a non-apoptotic mechanism is consistent with several features of *Salmonella* pathogenesis. For example, *Salmonella* infection of the intestines is associated with a large inflammatory response whereas apoptosis is generally believed to limit inflammation (reviewed by Savill, 1997). In addition, there seems little benefit to an intracellular pathogen in inducing apoptosis in its host cell, since the pathogen may then become trapped within the apoptotic cell. This is analogous to many viral infections, in which apoptosis of the host cell is blocked to facilitate viral replication and spread (reviewed by Tschopp *et al.*, 1998). Conversely, the induction of apoptosis by predominately extracellular pathogens, such as *Yersinia* spp., may prevent phagocytosis and subsequent bacterial killing (Monack *et al.*, 1997; Ruckdeschel *et al.*, 1997).

This study reports on several *in vitro* assays which model the interaction of *Salmonella* and macrophages during pathogenesis with respect to host specificity.

Although persistence within macrophages correlated with the broad host specificity of *S. typhimurium*, there were no direct correlations between persistence within macrophages, induction of cytokine release by macrophages or the severity or nature of macrophage damage with *Salmonella* serotype specificity for pigs. There is relatively little known about the interaction of *Salmonella* and macrophages *in vivo* and it is therefore possible that such *in vitro* assays do not accurately model the interaction *in vivo*. Further work will address this problem by characterizing the interaction between *Salmonella* and host cells in intestinal and systemic tissues following experimental infection of natural host animal species.

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