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Original Article

Characterisation of a refined rat model of respiratory infection with *Pseudomonas aeruginosa* and the effect of ciprofloxacin

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Abstract

Background: We sought to characterise a refined rat model of respiratory infection with *P. aeruginosa* over an acute time course and test the antibiotic ciprofloxacin.
Methods: Agar beads were prepared±SPAN®80. Rats were inoculated with sterile agar beads or those containing 10^5 colony forming units (cfu) *P. aeruginosa* via intra-tracheal dosing. Bacterial load and inflammatory parameters were measured.
Results: Differing concentrations of SPAN® 80 modified median agar bead diameter and reduced particle size distribution. Beads prepared with 0.01% v/v SPAN®80 were evaluated *in vivo*. A stable lung infection up to 7 days post infection was achieved and induced BALF neutrophilia 2 and 5 days post infection. Ciprofloxacin (50 mg/kg) significantly attenuated infection without affecting the inflammatory parameters measured.
Conclusion: SPAN® 80 can control the particle size and lung distribution of agar beads and *P. aeruginosa*-embedded beads prepared with 0.01%v/v SPAN®80 can induce infection and inflammation over 7 days.

Keywords: *Pseudomonas aeruginosa*; Agar beads; Ciprofloxacin

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is the major colonising pathogen in cystic fibrosis (CF). Morbidity and mortality in CF is primarily caused by an excessive inflammatory response and subsequent progressive lung damage associated with the chronic bacterial infection of the airways [1]. The inflammatory response associated with chronic infection is dominated by a neutrophilic infiltrate, where the excessive release of oxidants and proteases play a central role in tissue damage [2]. Increased levels of pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokines are also present [3]. The rat model of chronic respiratory infection with *P. aeruginosa* was first described by Cash et al. in 1979 [4] and similar models in which mice are infected with *P. aeruginosa* embedded in agar beads are extensively used [5–8]. While these models mimic aspects of CF, a key challenge for these models, where an infectious agent is embedded in beads, has been the ability to routinely produce beads of a standard size and particle distribution, as marked heterogeneity in size and distribution is typically observed.

The primary aim of this study was thus to develop a method to control the particle size and distribution of agar beads embedded with bacteria using the non-ionic surfactant SPAN® 80. Using standardized beads we then sought to characterise an acute time course of the infection and inflammatory changes together with evaluating the effect of the clinically used antibiotic ciprofloxacin.

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2. Methods

2.1. Preparation of agar beads embedded with P. aeruginosa

Using a modified version of the method described by Cash et al. [4], an overnight culture of *P. aeruginosa* PAO1V strain (courtesy of Mike Vasil, University of Colorado) was prepared in tryptic soy agar broth (TSB) (Beckton & Dickenson, UK). PAO1V, a partially mucoid strain, is a derivation of the PAO1 strain which itself is widely used in this model. The bacteria were embedded into agar beads by mixing the overnight culture with molten 2% w/v agar noble (Beckton & Dickenson, UK) which was then spun into warmed mineral oil (Sigma Aldrich, UK)±0.1% v/v or 0.01% v/v the non-ionic surfactant SPAN® 80 (Sorbitan monooleate, Sigma Aldrich, UK). The mineral oil was cooled, the beads centrifuged at 10,000 g for 20 min and then washed with phosphate buffered saline (PBS) pH 7.2 (Invitrogen, UK). A cfu determination was performed on the bead slurry by growing *P. aeruginosa* colonies on tryptic soy agar (TSA) plates with 5% sheep blood (Beckton & Dickenson, UK). The slurry was diluted with PBS pH 7.2 to obtain the desired cfus. Sterile beads were prepared using PBS. Bead preparations were sized using a Mastersizer 2000™ (Scirocco 2000, Malver Instruments).

2.2. Inoculation of male Sprague–Dawley rats with P. aeruginosa

Animal work was performed under the UK Animal (Scientific Procedures) Act, 1986. On day 0, male Sprague–Dawley rats (250–320 g, n=16/group, Charles River, UK) were inoculated with 10^5 cfu of *P. aeruginosa* embedded into agar beads into their left lung via intra-tracheal dosing under isoflurane anaesthesia (Virbac Ltd, UK). A sham group were inoculated with sterile agar beads. Body weight was measured daily, and animals were observed for signs of pain and distress. The model was characterised over time by euthanizing sham and infected animals 2, 5 or 7 days post inoculation. Additionally, the antibiotic ciprofloxacin was tested to determine if the lung infection could be attenuated. On day 1, animals were subcutaneously dosed with either 50 mg/kg ciprofloxacin (Hospira Inc, USA) or vehicle (saline). Sham animals were treated with vehicle. A further dose was administered in the evening of day 1. Animals were then euthanized on the morning of day 2. The dosing schedule was selected based on previous experience with this antibiotic and its efficacy in rodent infection models.

2.3. Lung harvest for microbiological and histological assessment

At the appropriate time point post infection, 8 animals from each group were euthanized by administering an overdose of sodium pentobarbitone (Merial, UK). cfus were determined for homogenised lungs by serially plating onto TSA blood agar plates. Additionally, samples of kidney, spleen and blood were taken to monitor for the presence of systemic infection. The remaining 8 animals in each group were euthanized, the trachea cannulated and a bronchoalveloar lavage (BAL) performed using PBS pH 7.4 (Invitrogen, UK), to determine total and differential BAL cell counts. The left lungs were inflated with 10% (v/v) buffered formalin (VWR, UK) and immersion fixed for 24–48 h. The fixed lung was sliced tangentially into 6 slices and the 4 central slices processed as one block. After processing, de-waxed sections were treated with 1% (v/v) aqueous acetic acid for 2 min and subsequently with 1% (w/v) aqueous alcian blue (Fluka, Sigma Aldrich, UK) for 30 min before the application of a routine haematoxylin and eosin stain (AB-H&E) to permit histological assessment. Additional sections were cut and stained for CD3 (monoclonal rabbit CD3, Sigma Aldrich, UK); de-waxed sections were treated with primary CD3 antibody for 60 min.

2.4. Total and differential cell counts from bronchoalveolar lavage fluid (BALF)

The collected BALF was mixed vigorously by inverting the tube and an aliquot was removed for differential cell counting: cytospin slides were prepared by loading sample into the corresponding cytospin funnel and centrifuged at 700 rpm for 5 min with a slow acceleration (Shandon Cytospin 4, Thermoscientific). The slides were then stained with Wright's stain. Under a light microscope the number of macrophages, neutrophils, lymphocytes and eosinophils in a total of 200 cells were determined at 40× magnification. The remaining BALF was centrifuged at 320 g for 10 min at 4 °C. Aliquots were removed and stored at −80 °C. The cell pellet was re-suspended in 1 ml of methyl violet fixative (0.01% methyl violet, 1.5% acetic acid Sigma Aldrich, UK) and stored at 4 °C. A total cell count was performed using a multi-chamber haemocytometer. Total cell count was subsequently used to extrapolate back the number of macrophages, neutrophils, lymphocytes and eosinophils in the sample. IL-1β, CINC-3 and IFNγ were measured in BALF supernatant using ELISA kits (R&D Systems), according to manufacturer's instructions.

2.5. Histopathological scoring

A scoring system was developed to assess the histopathological changes in the lung tissue on four criteria: bronchopneumonia, interstitial inflammation, epithelial changes and goblet cell hyperplasia. The lung sections were then blindly scored on a scale of 0–5, with 0 being no change and 5 being the greatest change (i.e. "severe", meaning>80% of lung parenchyma being affected), for each criterion. $CD3^+$ staining of sections was analysed using Aperio ImageScope v 9.1.19.1569.

2.6. Statistical analysis

All data are presented as the mean±standard error of mean (SEM), except for bead size which was expressed as median± SEM. All data were analyzed using GraphPad Prism 4.0 (GraphPad software, San Diego, CA). Comparisons were made using Kruskall Wallis one-way analysis of variance followed by a Dunn's multiple comparison test. A value of $P<0.05$ was taken to be statistically significant.

3. Results

3.1. The use of SPAN® 80 to standardise agar bead particle size distribution

Initial experiments ascertained the effect of 0.01% v/v and 0.1% v/v SPAN® 80. Without SPAN® 80 the median particle size for the beads formed was 539±57 μm (distribution at 50%, d0.5) (Fig. 1). In addition the bead population was heterogeneous. 0.01% v/v and 0.1% v/v SPAN® 80 reduced the median particle size of the beads (Fig. 1) as well as producing more homogeneous bead populations by reducing the particle size distribution (Fig. 1). In order to ascertain the effect of bead size on lung distribution, a comparison was made between the largest (0% SPAN 80®) and the smallest (0.1% v/v SPAN 80®) bead populations. The beads were administered intra-tracheally and two days post infection beads prepared with 0% SPAN 80® were found to have a wide distribution in the lungs, reflecting

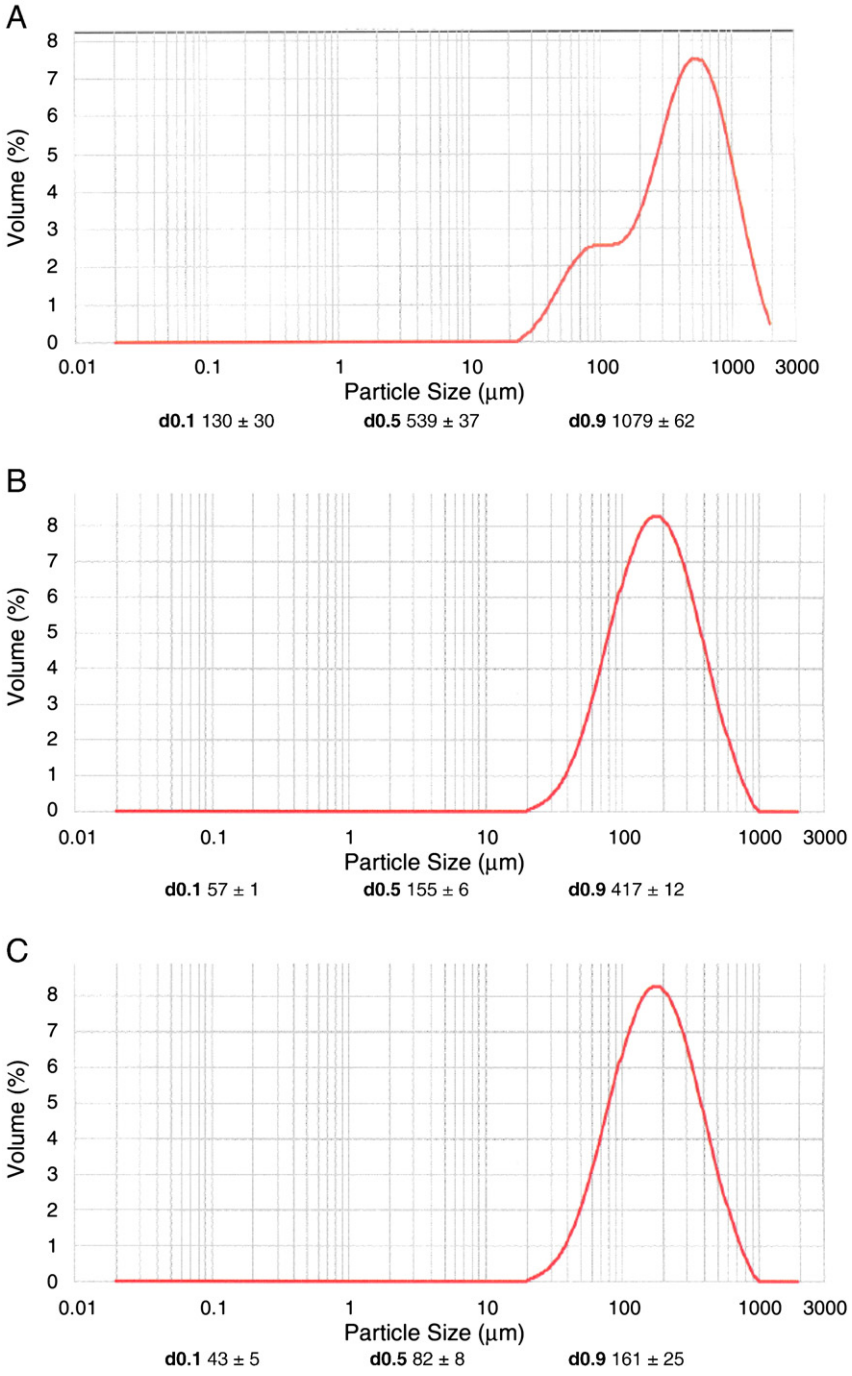

Fig. 1. The effect of SPAN®80 on agar bead particle size (μm) distribution as measured using a Mastersizer 2000. Agar beads were prepared with either A, no SPAN® 80, B, 0.01% SPAN 80 or C 0.1% SPAN® 80. Images are representative of n=3 separate preparation. d0.1 — distribution at 10%, d0.5 — distribution at 50%, d0.9 — distribution at 90%. Data are presented as mean±SEM of n=3 separate preparations.

their wide particle size distribution. Smaller beads were located in the alveolar spaces while large beads also were lodged in the larger airways (Fig. 2A). In contrast, beads prepared with 0.1% SPAN 80® were found primarily in the alveolar spaces (Fig. 2B). The lung bacterial load from the 0% and 0.1% SPAN 80® beads was found to be similar resulting in 5.9±0.9, 0.1% SPAN 80 6.1±0.3 log cfu respectively. When compared, the two bead populations elicited similar total cell and neutrophil numbers present in the BALF on both infected and sham animals (Fig. 2C–D) indicating that bead size does not appear to affect lung bacterial load or *P. aeruginosa*-induced inflammation.

Beads produced with 0.01% v/v SPAN® 80 were selected for further characterisation *in vivo*. Although these beads had a greater particle size distribution than those produced with 0.1% v/v SPAN® 80 it was felt that the median bead size would enable the beads to lodge in the small airways and thus be more reflective of CF.

3.2. Time course of infection and inflammation using agar beads prepared with 0.01% v/v SPAN®80

The lung bacterial load was found to be 5.97±0.17, 6.19±0.15, 5.95±0.15 log cfu 2, 5 and 7 days post inoculation indicating a stable infection. No evidence of systemic infection was observed (data not shown) and no bacteria were present in the sham groups either in the lungs or systemically.

P. aeruginosa induced a 3–4 fold increase in the total number of cells present in the BAL fluid compared to sham ($7.23\pm1.26\times10^6$ cells versus $1.95\pm0.13\times10^6$ cells respectively, n=7–8) 2 days post infection and an approximate 2-fold increase in BAL fluid cells at day 5 ($2.25\pm0.39\times10^6$ cells compared to $1.20\pm0.13\times10^6$ cells respectively, $P<0.05$, n=7–8) (Fig. 3A). The increased BALF cells reflected an increase in neutrophil number ($5.63\pm1.20\times10^6$ compared to sham $0.23\pm0.05\times10^6$ cells, $P<0.05$ at day 2 and $1.00\pm0.38\times10^6$ compared to sham $0.03\pm0.01\times10^6$ cells, $P<0.01$ at day 5) (Fig. 3B). No significant changes in macrophages, eosinophils or lymphocytes were observed (data not shown). No significant changes in BALF cell numbers were observed at day 7.

The histopathological findings are illustrated in Fig. 4. Lesions were primarily observed in the lower half of the left lung. Two days post infection, *P. aeruginosa*-treated animals had an increase in the inflammatory infiltrate in the airway lumen and alveolar spaces (bronchopneumonia). This was primarily neutrophilic in nature compared to the predominance of alveolar macrophages observed in sham animals. This

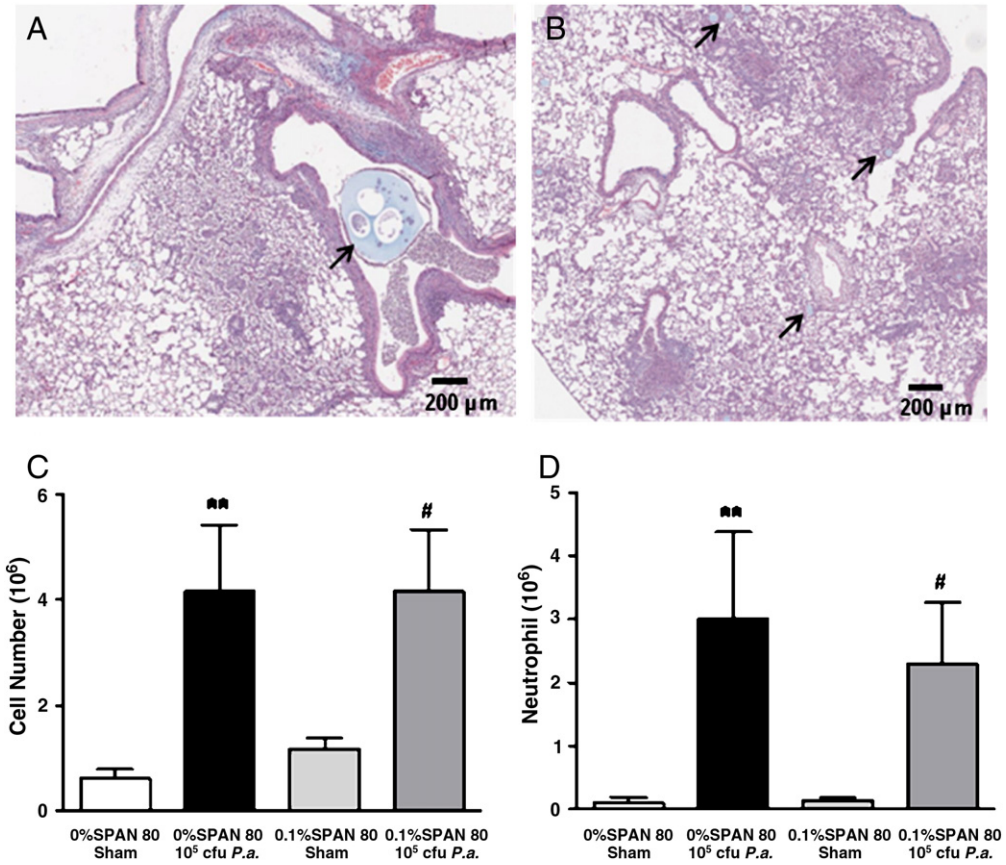

Fig. 2. Representative images from *P. aeruginosa* treated lung sections inoculated with agar beads prepared with 0% SPAN 80® (A) and 0.1% SPAN 80® (B) 2 days post infection and stained with alcian blue and haematoxylin and eosin (arrow pointing to agar beads). The number of C, total cells and D, neutrophils present in BAL fluid 2 days post infection. Data are expressed as the mean±SEM of 5–6 animals. **, $P<0.01$ versus 0% SPAN 80® sham; #, $P<0.05$ versus 0.1% v/v SPAN 80® sham.

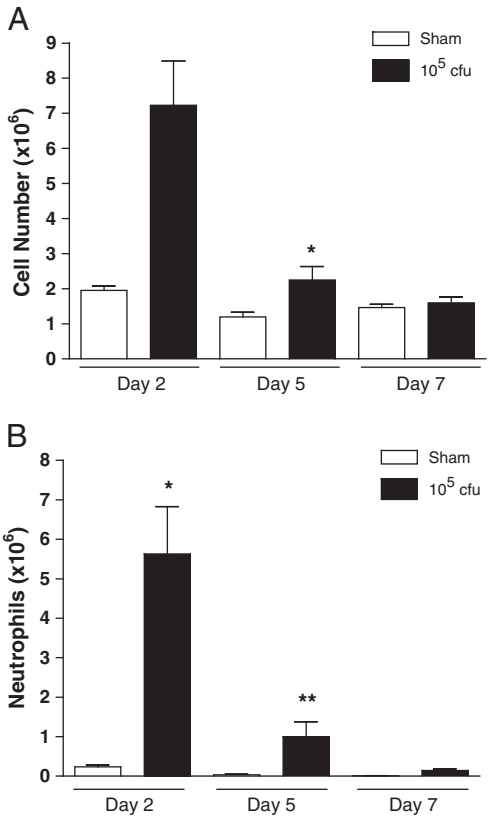

Fig. 3. The number of A, total cells and B, neutrophils present in BAL fluid 2, 5 and 7 days post infection. Data are expressed as the mean±SEM of 7–8 animals. *, $P<0.05$ versus sham; **, $P<0.01$ versus sham.

neutrophilic infiltrate declined over the seven day time frame when it was replaced by macrophages and fibroblasts (chronic inflammatory changes) (Fig. 5A). In both sham and infected animals, at all three time points, peri-vascular and peri-bronchiolar eosinophils were observed, but this was more marked in the infected animals, where interstitial oedema was also present (Fig. 5B). Infected rats had marked changes in the epithelium at days 2, 5 and 7 post inoculation, characterised by hyperplasia and hypertrophy as well as disruption of the basement membrane at earlier time points (Fig. 5C). Changes in mucus were also observed as measured by alcian blue staining. Alcian blue staining was increased in the infected group at day 2 and was more marked at days 5 and 7 (Fig. 5D). Indeed there appeared to be evidence of mucus plugging in the airway lumen at day 5. A significant increase in the number of $CD3^+$ stained cells as a ratio of the total staining was observed at day 5 ($P<0.05$, n=8) and day 7 ($P<0.001$, n=8) in the lung tissue of infected animals compared to corresponding sham rats suggesting an increase in lymphocytic infiltrate (Fig. 5E).

CINC-3 levels were below the detection limit in the BAL supernatant of sham animals however, CINC-3 was significantly increased in the infected animals at day 2 (104.8± 37.0 pg/ml, $P<0.01$, n=7–8) and day 5 (168.0±68.8 pg/ml, $P<0.01$, n=7–8) post infection. IL-1β was also significantly elevated in the infected animals compared to the sham groups (also below the limit of detection) at day 2 (263.7±50.7 pg/ml, $P<0.001$, n=7–8), day 5 (140.7±18.8 pg/ml, $P<0.01$, n=7–8) and day 7 post infection (119.3±17.0 pg/ml, $P<0.05$, n=7–8). IFN-γ was only significantly elevated in the infected animals at

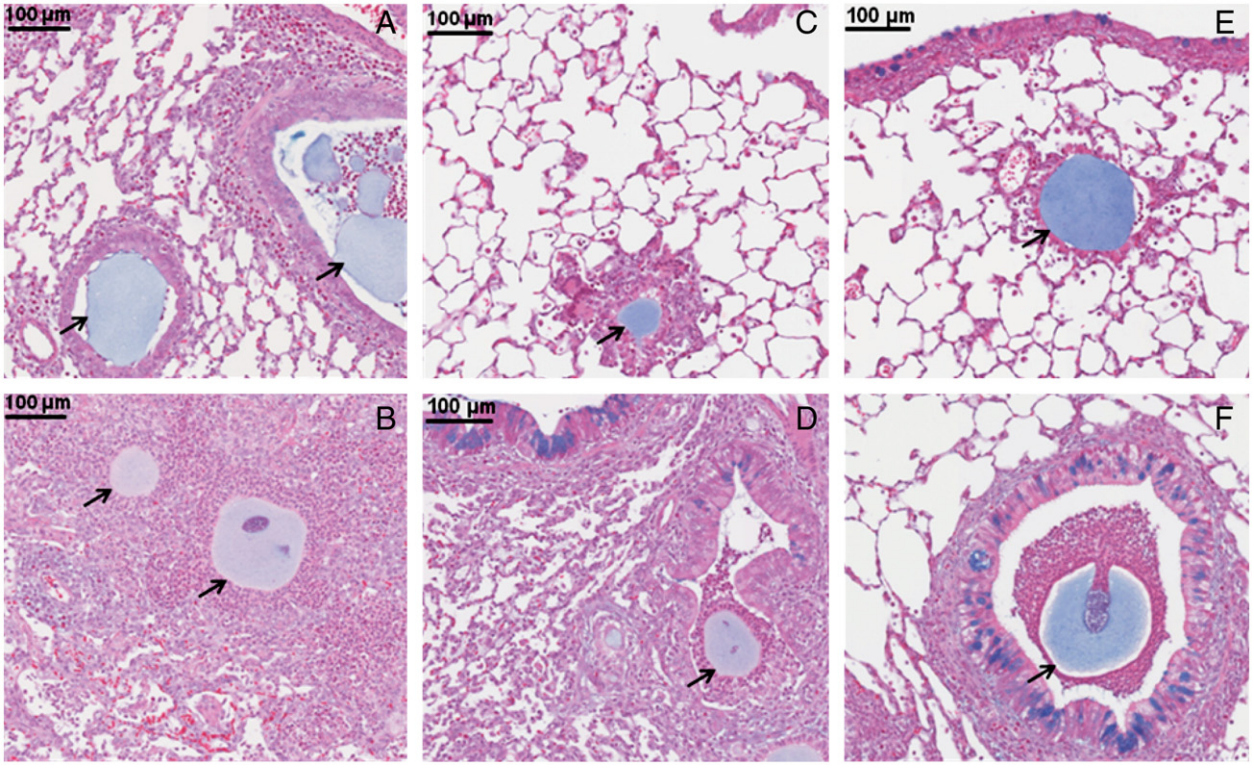

Fig. 4. Representative images from sham and infected lung sections stained with alcian blue and haematoxylin and eosin. A, sham day 2; B, 10^5 cfu *P. aeruginosa* day 2; C, sham day 5; D, 10^5 cfu *P.aeruginosa* day 5; E, sham day 7 and F, 10^5 *P. aeruginosa* cfu day 7. Arrow pointing to agar bead.

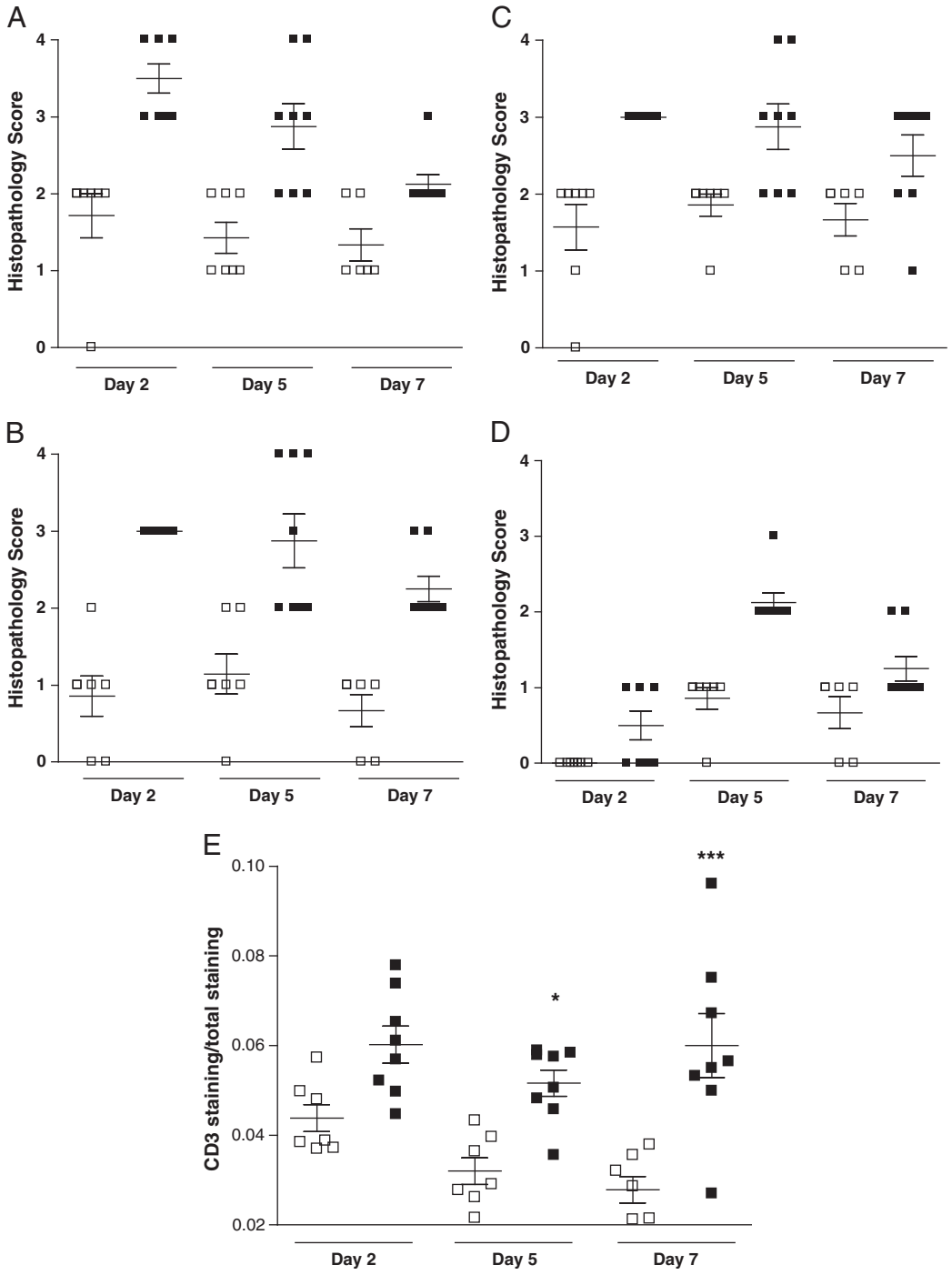

Fig. 5. Histological scoring for sham (open squares) and infected animals (closed squares) 2, 5 and 7 days post infection. Sections were scored on 4 criteria: A, bronchopneumonia; B, interstitial changes; C, epithelial changes and D, changes in mucus staining. Data are presented as the individual score for each animal with the mean of the group. E, CD3 specific staining for lymphocytes in sham (open squares) and infected (closed squares) 2, 5 and 7 days post infection. Sections were analysed using Aperio ImageScope v 9.1.19.1569. Data are presented as the mean±SEM of $CD3^+$ staining as a ratio of total staining. *, $P<0.05$ versus sham; ***, $P<0.001$ versus sham.

day 7 compared to sham animals (66.4±5.9 pg/ml compared to 13.1±8.5 pg/ml respectively, $P<0.05$, n=7–8 animals).

3.3. The effect of ciprofloxacin on infection and inflammation

The effect of the antibiotic ciprofloxacin was tested using the refined bead preparation as part of the model validation. In sham animals, lung infection was absent compared to the infected vehicle-treated group which had a significant lung infection of 5.57±0.16 log cfu. Treatment with 50 mg/kg ciprofloxacin significantly attenuated infection to 1.65±0.15 log cfu, $P<0.01$, n=7–8).

The effect of ciprofloxacin on *P. aeruginosa*-induced inflammation was also investigated. BALF from vehicle-treated

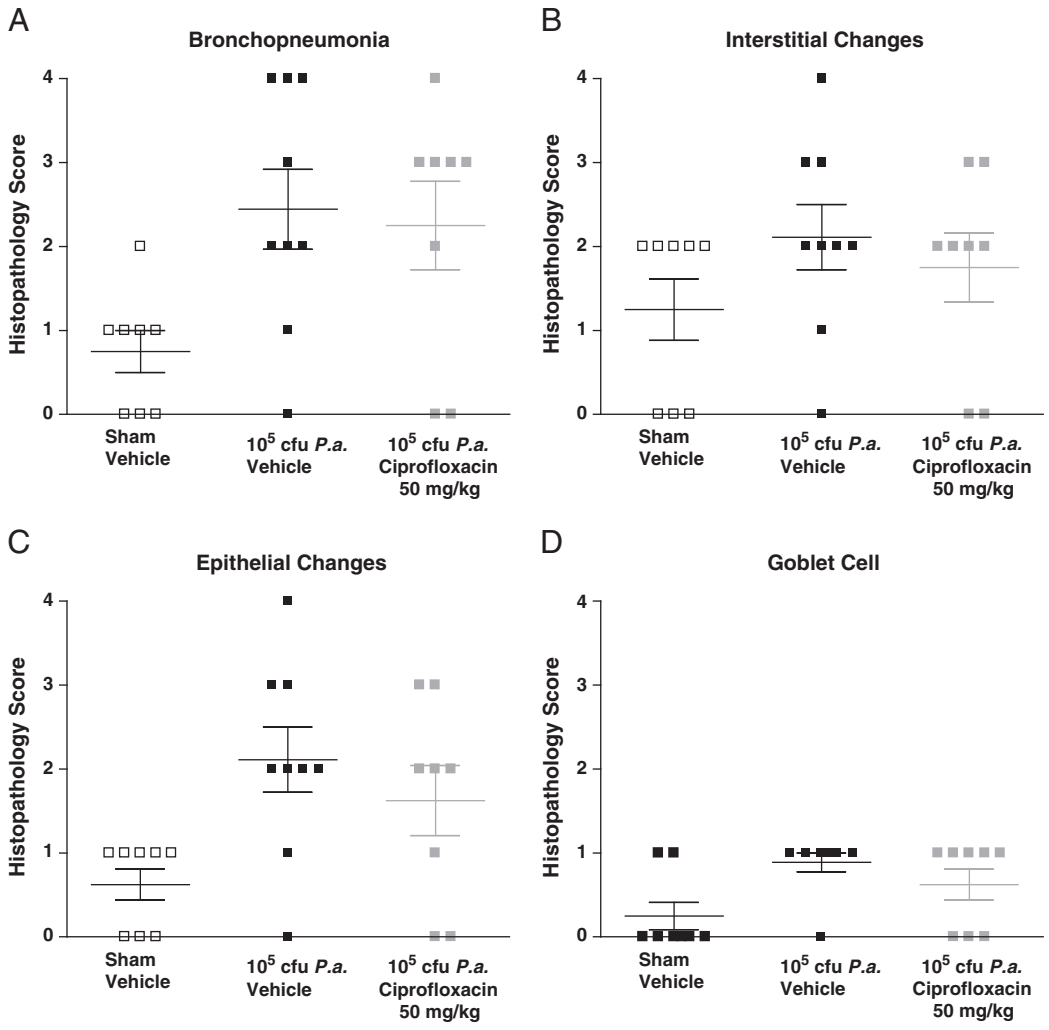

Fig. 6. Histological scoring for vehicle-treated sham (open squares), vehicle-treated infected (closed squares) or 50 mg/kg ciprofloxacin-treated infected animals (grey squares). Sections were scored on 4 criteria: A, bronchopneumonia; B, interstitial changes; C, epithelial changes and D, changes in mucus staining. Data are presented as the individual score for each animal with the mean of the group.

infected rats had a significantly greater number of total cells present versus sham treated rats ($5.03 \pm 0.92 \times 10^6$ and $1.59 \pm 0.18 \times 10^6$ cells respectively, $P < 0.001$, n = 8–9), reflected by a significant increase in neutrophils ($3.53 \pm 0.76 \times 10^6$ and $0.21 \pm 0.05 \times 10^6$ cells, $P < 0.001$, n = 8–9). This was not significantly altered by ciprofloxacin ($2.67 \pm 0.70 \times 10^6$ neutrophils). Ciprofloxacin had no effect on the histopathological findings (Fig. 6).

BALF levels of CINC-3 were significantly elevated in infected vehicle-treated animals (36.32 ± 9.54 pg/ml, $P < 0.05$) compared to sham animals (undetectable). There were no significant changes in the levels of IL-1β in infected vehicle-treated animals compared to sham animals (105.60 ± 30.50 pg/ml versus 21.54 ± 14.37 pg/ml). IL-1β and CINC-3 levels were not affected by ciprofloxacin treatment (72.19 ± 27.60 pg/ml and 26.56 ± 10.51 pg/ml respectively).

4. Discussion

The addition of the non-ionic surfactant SPAN® 80 during the preparation of agar beads embedded with bacteria controlled the median agar bead diameter (with some evidence of concentration dependence) and particle size distribution, allowing the reproducible production of a more homogeneous bead population of a defined size.

The ability to control agar bead particle size and distribution could have a number of significant advantages, for example, in addition to rat lung infection models, this novel methodology could impact on mouse lung infection models, where the production of small beads is critical due to the relatively small size of mouse airways. In particular, infection of cystic fibrosis transmembrane conductance regulator protein knock out (*cftr*$^{-/-}$) mice (or mice which model specific mutations in *cftr*) with *P. aeruginosa* embedded in agar beads is a fairly widely used model system [5–9]. Standardisation of agar bead size is expected to improve animal welfare, as large beads have the potential to block the airways, leading to respiratory distress.

Using beads standardized with 0.01% v/v SPAN 80®, *P. aeruginosa* colonies were recoverable up to 7 days post infection, whilst no systemic infection was observed. The log

cfu values achieved at days 2 and 7 post infection were comparable to similar time points reported in the literature using PAO1 [10,11]. A clear pro-inflammatory effect of *P. aeruginosa* infection as measured by a significant increase in neutrophils and cytokines in the BAL fluid and lung tissue of infected versus sham animals was observed. The increase in neutrophils observed in BAL fluid 2 days post infection is consistent with data published by Cantin et al. [10] 3 days post infection. However, unlike Cantin et al., where the neutrophils present in BAL fluid remained constant up to 9 days post infection, in these studies a drop in the neutrophil number in BAL fluid was observed at day 5, and by day 7 no clear difference between sham or infected animals was seen. The neutrophil cell counts presented here, are however consistent with the observations of Boyer et al. [12] over a similar time scale (2–8 days) and also consistent with those reported by Amano et al. [13]. Whilst the mechanisms underlying neutrophil recruitment are unclear, a reduction in the number of neutrophils observed in BAL fluid is associated with lower levels of the neutrophil chemoattractant CINC-3 as well as IL-1β in the BAL fluid. The elevation of IFN-γ in the BAL fluid at day 7 could suggest the initiation of a T cell response and perhaps therefore a shift from the innate to the adaptive immune response. This is supported by the histological analysis of tissue sections for $CD3^+$ staining where a significant increase in $CD3^+$ staining was observed at days 5 and 7. Amano et al. [13] also observed an increased number of lymphocytes albeit in BAL fluid rather than lung tissue 7 days post infection. Therefore, it is suggested that the nature of the inflammation in this model is changing over time, so that by day 7 a more chronic inflammation is present, as evidenced by a significant increase in the number of CD3+ cells present in the tissue.

The histopathology observed acutely in this model resembles that seen by Cash et al. [4] and has similar characteristics to the pathology observed in CF (neutrophilic infiltrate and remodelling/thickening of the airway epithelium, mucus plugging). In agreement with the findings of Cash et al. we have observed increases in mucus using an alcian blue stain. The lung histopathology is also reflective of the BALF data in that after day 2, the acute inflammatory infiltrate (i.e. the neutrophils) begins to decline. At later time points, macrophage and fibroblastic components are (chronic inflammatory components) present in the tissue, as well as structural changes such as epithelial hyperplasia.

A number of compounds used clinically to treat CF for example tobramycin (anti-microbial) [14] and ibuprofen (anti-inflammatory) [15] have shown efficacy in this model system as has ciprofloxacin, a fluoroquinolone antibiotic used clinically as an early eradication therapy for *P. aeruginosa* in CF patients [16–18]. In a study to investigate resistance selection, rats inoculated with 10^6 cfu PAO1 and then treated with ciprofloxacin (12.5 mg/kg i.p.) every 5 h showed a 2 log reduction in cfu 34 h post infection [18]. We observed a more dramatic decrease in lung bacterial load (4 log reduction 2 days post infection) using a higher dose of ciprofloxacin administered s.c. BID.

We also sought to investigate the effect of ciprofloxacin on inflammatory parameters as there is evidence that some fluroquinolones containing a cyclopropyl moiety such as ciprofloxacin can have an immunomodulatory effect (reviewed in Dalhoff and Shalit [19]). For example, in a model of acute lung injury with lipopolysaccharide in mice, prophylactic ciprofloxacin (200 mg/kg) treatment reduced the levels of cytokines (including IL-1β) in BALF 3 and 6 h post challenge [20]. Sub-inhibitory anti-microbial treatment with ciprofloxacin also reduced *P. aeruginosa* tissue injury in the rat model of respiratory infection in which bacteria were embedded in agar beads [21]. However, no immunomodulatory effects of ciprofloxacin were observed in our study. There are a number of potential reasons for our different findings for example, route of compound administration, inflammatory stimulus used (LPS or live bacteria), bacterial dose used and time points evaluated. Future studies will also include investigating additional antibiotics and dosing strategies to investigate the relationship between infection and inflammation.

In conclusion, the addition of the non-ionic surfactant SPAN® 80 can control the particle size and has the potential to enable a controlled distribution of agar beads embedded with bacteria. This represents advancement for this model in that we now have the capability to generate a more homogenous agar bead population of a specific size distribution. Furthermore, using the anti-microbial ciprofloxacin, we have shown a dissociation between infection and inflammation at the 2 day time point in this model i.e. ciprofloxacin attenuated infection whist having no effect on lung inflammatory parameters measured here. In theory this novel method using defined concentrations of SPAN® 80 could enable specific targeting of the inoculums to different regions of the airways within the lung, and facilitate the assessment of novel anti-infectious and anti-inflammatory agents in this refined model system.

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