Original Research Paper

In Vitro infection of human dura-mater fibroblasts with *Staphylococcus aureus*: colonization and reactive production of IL-1beta

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Objective: Post-operative meningitis, caused mainly by *Staphylococcus aureus* and Gram-negative rods, is a life-threatening complication after neurosurgery, and its pathogenesis is far from clear. The purpose of this work was to study the experimental infection of human dura-mater fibroblasts and whole human dura by *S. aureus*.

Methods: *In vitro* cultures of human dura-mater fibroblasts and organotypic cultures of small pieces of human dura mater were inoculated with a human-derived *S. aureus* strain. The pattern of bacterial infection as well as cytokines secretion by the infected fibroblasts was studied.

Results: Our results suggest that colonisation of human dura-mater fibroblasts in culture and whole dura-mater tissue by *S. aureus* includes bacterial growth on the cell surface, fibroblast intracellular invasion by bacteria and a significant synthesis of interleukin 1beta (IL-1beta) by the infected cells.

Conclusion: This is the first report of human dura-mater fibroblast infection by *S. aureus*. Hopefully, these results can lead to a better understanding of the pathogenesis of meningitis caused by this bacterial species and to a more rational therapeutic approach.

Keywords: Human, Dura-mater, Fibroblasts, Culture, *Staphylococcus aureus*

Introduction

Bacterial meningitis is one of the most common life-threatening infections of the central nervous system, and can lead to sequelae after the acute phase of the disease has been controlled.[1] It can be classified as community-acquired meningitis and post-operative meningitis. Community-acquired meningitis is a consequence of bacterial dissemination towards the central nervous system after primary colonisation of the upper respiratory tract due to human transmission.[2] In this kind of meningitis, the brain and its coverings are intact. By contrast, post-operative meningitis develops after the skull has been opened in cranial surgery.[3] The aetiology of each kind of meningitis is also different. While community-acquired meningitis is mainly caused in children by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenza*,[1,2] post-operative meningitis is more frequently caused by *Staphylococcus aureus and* Gram-negative rods at any age.[3] Although community-acquired meningitis is severe, its incidence has been markedly decreased by mass vaccination. However, although careful prevention of post-operative meningitis is regularly performed during neurosurgical procedures, infections can occur once the cranial vault is open. In this form of infection, the biology and resistance to antibiotics shown by *S. aureus*, as well as the surgical techniques employed, are major determinants of the outcome of the disease. External ventricular drains and the use of prosthetic devices such as shunts are real microbiological challenges, leading to a higher risk of infection, which can eventually provoke chronic diseases.[4,5] The pathogenesis of post-operative meningitis due to *S. aureus* is far from clear, given that the mechanisms used by this bacterium to cause damage are unknown, particularly in the dura mater.

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Moreover, very little information is available on the pathogenesis of acute or chronic inflammation after bacterial infection of the meninges.[6,7]

Most, if not all, bacteria colonise through the production of biofilms composed of bacterial microcolonies, water channels and an exo-polysaccharide matrix (reviewed in Refs. [8,9]). Biofilms can develop under aerobic as well as microaerobic[10] conditions and may also be the mechanism of bacterial colonisation of animal keratinised epithelia.[11] *S. aureus* produces biofilms with an extracellular matrix composed of polysaccharides, proteins, extracellular DNA, toxins, and immune-modulatory peptides.[12,13] These biofilms manipulate the host's immune response[14–16] and resistance to most antibiotics[17] through a complex genetic control mechanism.[5,18] The production of biofilms is a critical step for *S. aureus* in colonising inert materials as well as the surface of animal tissues. This suggests the hypothesis that there exist biofilms due to *S. aureus* in human meningitis. Also, an eventual bacterial migration inside dura-mater cells could be consistent with mechanisms of escape for antibiotic treatment, making meningitis even harder to treat. Moreover, the synthesis of pro-inflammatory cytokines in the dura mater after bacterial infection could induce fibrosis by blocking cerebrospinal fluid circulation, causing hydrocephalus. A better understanding of these phenomena – the eventual production of biofilms, bacterial internalisation, and the synthesis of pro-inflammatory cytokines – after *S. aureus* infection of human dura mater, could enable the development of more rational strategies to treat and prevent the damage caused by this pathogen. However, no experimental model (neither *in vitro* nor in animals) has been reported so far, to our knowledge.

Recently, one of us (Ezequiel Goldschmidt) successfully cultured normal human fibroblasts obtained from dura mater *in vitro*.[19] This allowed us to use this new procedure in a straightforward experimental approach to examine this hypothesis. The purpose of this study was to investigate the mechanism by which *S. aureus* infects human fibroblasts of the dura mater in organotypic cultures and in cell cultures, and how these cells respond to bacterial injury.

Materials and Methods

Bacteria strain and culture conditions

A clinically derived strain of *S. aureus*, (ATCC 25923) regularly used for the production of biofilms, was grown in 2xYT broth (16 g tryptone, 8 g yeast extract, 5 g NaCl, 1000 ml distilled water, pH 7.4) or trypticase-soy broth Difco® at 37°C. When semi-solid media was needed, 15 g/l of agar (Difco®) was added to the 2xYT broth or to trypticase soy broth before sterilising by autoclave 20 minutes at 121°C. Media were distributed into sterile plastic Petri dishes or sterile, capped glass tubes. Further characterisation of the strain was performed by culture in Chapman medium (Difco®) to determine the bacterial ability to grow in a high concentration of NaCl and mannitol fermentation, as described elsewhere. Traditional catalase and coagulase bacteriological tests were also conducted. The original bacterial strain behaved as expected for *S. aureus* after all tests were performed.

Organotypic cultures and cell cultures

Small pieces (2 mm wide) of normal human dura mater obtained from craniotomies performed in patients with subdural haematoma were cultured on sterile sponge strips (Spongostan®) into 60-mm Petri dishes with Dulbecco's modified Eagle's medium (DMEM) (Gibco®) containing 20% foetal calf serum (Gibco®) in the liquid–air interphase at 37°C with 5% CO_2. Fibroblasts obtained from primary cultures of human dura mater and characterised as described previously[19] were employed after seven passages in 60-mm plastic Petri dishes containing DMEM with 20% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C and an atmosphere of 5% CO_2 until used. Although a continuous (not a primary) human cell line was used in this work, experiments were evaluated and approved by the Ethics Committee of Hospital Italiano de Buenos Aires, Argentina, where the surgical interventions were performed.

Cell staining and histology

In all the experiments, cells were cultured on glass coverslips and, at the required time-points, fixed for 20 minutes with methanol at room temperature, then stained with Giemsa or with periodic acid-Schiff slightly counter-stained with haematoxylin as described elsewhere. Dura-mater tissue was fixed in Bouin's fluid, dehydrated in ethanol from 70 to 100%, clarified in xylene and routinely embedded in paraffin. Slides were stained with haematoxylin and eosin (H–E) and Gram techniques.

Scanning Electron Microscopy

Infected and uninfected fibroblasts cultured on glass coverslips were gently washed three times with phosphate-buffered saline (PBS) at pH 7.4 and immediately fixed with 4% formaldehyde freshly prepared from paraformaldehyde, and 1% glutaraldehyde in PBS, for 2 hours at 4°C. After washing with PBS, samples were post-fixed in 1% osmium tetroxide in PBS for 2 hours, washed with the same buffer and progressively dehydrated in ethanol until 100% ethanol was added. Then, the critical point drying process was performed and cells were completely dried. Pieces of infected or uninfected dura mater

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0.5–1 mm thick were treated with the same protocol. Each specimen was mounted on an aluminium stub and coated with gold-palladium. The SEM observations were conducted at 10 kV in a Philips XL 30 TMP.

Cytokine quantification

Commercially available enzyme-linked immune sorbent assay kits were employed to measure the concentration of human interleukin 1beta (IL-1beta) (R&D Systems), human interleukin 8 (IL-8) (BD Biosciences), and human tumour necrosis factor alpha (TNF-alpha) (R&D Systems) in culture supernatants.

Statistics

Data from samples with normal distribution were analysed using the Student's *t*-test from Graph Pad Prism software version 4.0. A P value <0.05 was considered as statistically significant. Data were collected from at least two independent experiments performed in triplicate.

Experimental design

Colonization

S. aureus was grown overnight in a shaker at 37°C, then spun down, washed three times with DMEM and finally resuspended in fresh DMEM without serum or antibiotics to a standardised optical density of about 10^7 bacteria/ml. This suspension was used as the inoculum. After washing with PBS, organotypic dura mater cultures were incubated for 1 hour with approximately 2×10^7 colony-forming units of *S. aureus* contained in 5 ml of DMEM without serum or antibiotics. In duplicate, some other cultures were mock-infected with DMEM without serum or antibiotics as a negative control. After gently washing twice with PBS, 5 ml of fresh DMEM was added. At 24, 48 and 96 hours post-infection (pi), cultures were washed thoroughly with sterile PBS, fixed for 6 hours at 4°C and processed for SEM and traditional histology in paraffin-embedded slides.

Human dura mater-derived fibroblasts were grown on glass coverslips into 60 mm plastic Petri dishes with DMEM containing 20% foetal calf serum and antibiotics until 80–90% confluency was observed. The culture medium was removed 24 hours before the beginning of the experiment, cells were thoroughly washed three times with DMEM without serum or antibiotics and fresh DMEM was added; 5 ml on each plate. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for further 24 hours in these conditions. Aliquots of the bacterial suspension were used to infect fibroblasts at a multiplicity of infection of 20 bacteria/cell. Cultures were incubated for 1 hour. Then, at 2, 5 and 8 hours pi, samples were gently washed three times with DMEM to eliminate nonadherent bacteria and fixed in methanol at room temperature for 20 minutes. Duplicates were fixed in parallel and processed for SEM. The same procedure was applied in mock-infected control cultures.

Invasion

Fibroblasts grown in DMEM as indicated previously were harvested by trypsinization and seeded at a density of approximately 2×10^5 cells/ml into 12 well-tissue culture plates. Fibroblasts were infected with the bacterial suspension, as before, at a multiplicity of infection of 20 bacteria/cell. At 2 and 4 hours pi, nonadherent bacteria were eliminated by washing three times with PBS. At each time-point, half of the samples were treated for 10 minutes with 1 ml of 0.1% Triton X-100 in deionised sterile water. Serial dilutions of these lysates were made in PBS and then plated on trypticase soy broth to determine the number of viable associated (both adherent and internalised) bacteria. A gentamicin protection assay was performed on the other half of the samples to eliminate extracellular bacteria, as described elsewhere.[20] Briefly, infected cells were incubated in DMEM and 100 μg/ml of gentamicin for 1 hour. Then, cells were washed and treated with Triton X-100 as described, to determine the number of viable intracellular bacteria.

Cytokine production

To establish the inflammatory potential of dura mater fibroblasts, cells grown in confluent cultures were stimulated with *S. aureus* under the same conditions and cytokine/chemokine production was assessed in the culture supernatant at different time-points.

Results

S. aureus colonises dura-mater organotypic cultures and in vitro cultures of dura-mater fibroblasts

Bacteria were observed from 24 hours pi on the organotypic cultures growing on both faces of the dura mater but more intensely on the side facing the pia mater. Their number increased as time progressed, until a uniform layer of bacteria was observed on the dura mater surface by Gram and H–E staining (results not shown). Scanning electron microscopy (SEM) allowed a more detailed observation of the same phenomenon, including the beginning of cellular internalisation (Fig. 1). Bacteria were initially observed as isolated cocci on cultured fibroblasts, and then their density augmented and micro-colonies were observed by optical microscopy. These data were further confirmed by SEM (Fig. 2). Periodic acid-Schiff staining was very slight and limited to the cell surface (results not shown).

Bacteria are internalised by dura-mater fibroblasts

Viable intracellular bacteria were recovered at 2 and 4 hours pi. There were $3.6 \pm 1.2 \times 10^5$ colony-forming

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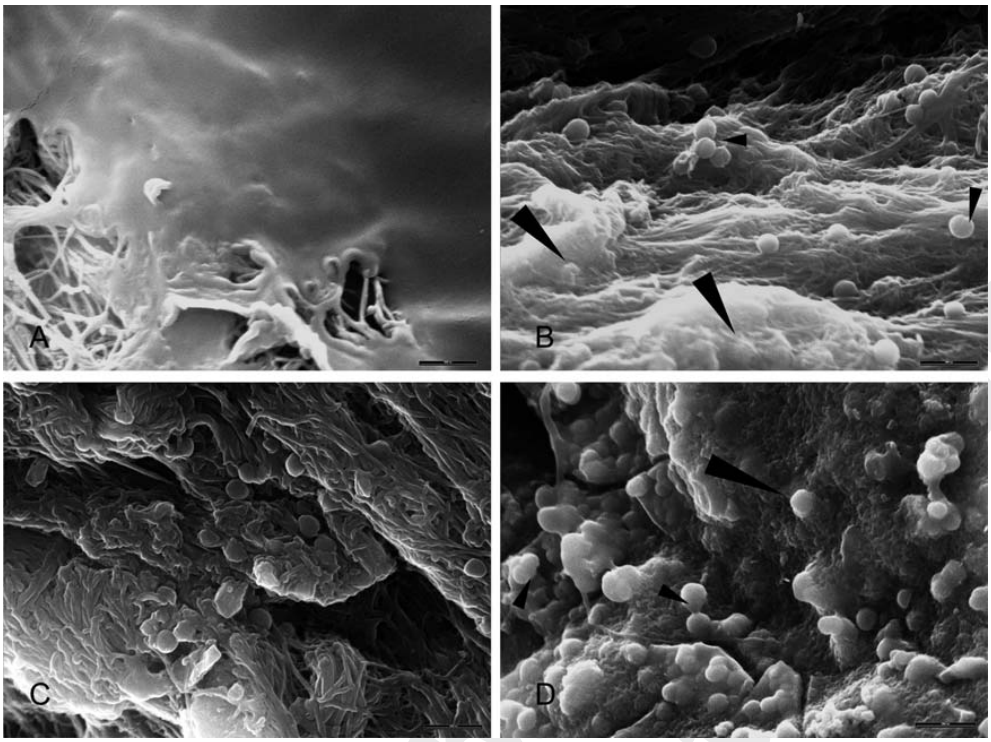

Figure 1 Scanning electron microscopy (SEM) of human dura mater, mock-infected (A) and infected with *Staphylococcus aureus* at 24 hours post-infection (pi) (B), 48 hours pi (C) and 96 hours pi (D). Note the increase in the number of bacteria from 24 to 96 h ours pi. Bacteria are firmly attached to the dura mater surface although they were thoroughly washed before fixation. In (B) and (D), bacteria can be observed at different levels of dura mater invasion. Short arrowheads point to extracellular bacteria. Large arrowheads indicate bacteria in the process of internalisation. Bar, 2 μm.

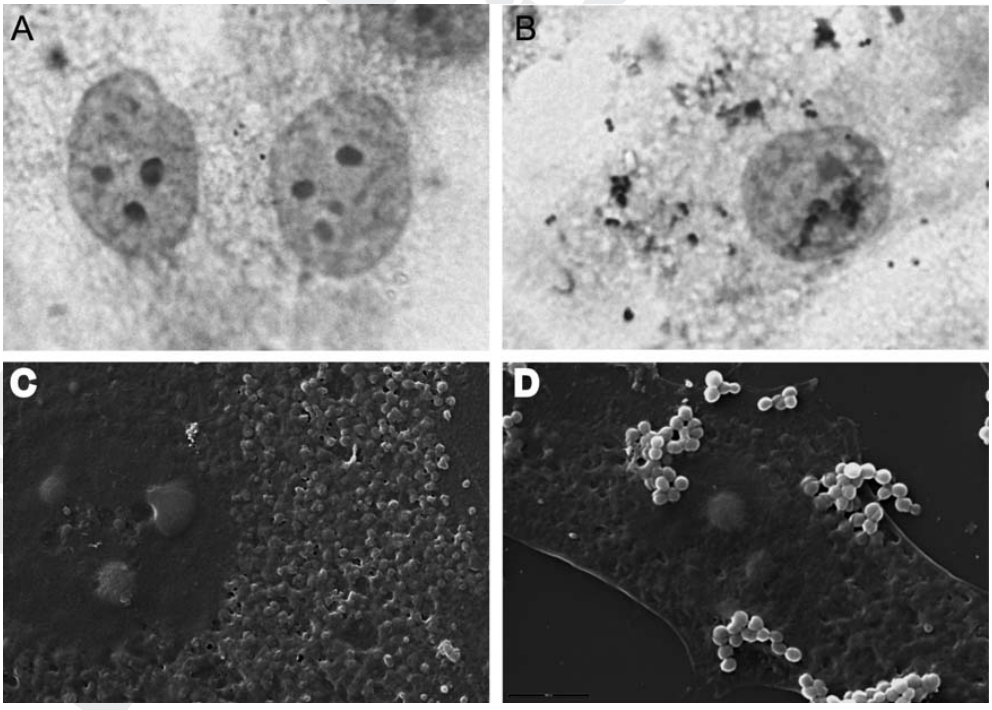

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Figure 2 Giemsa staining and scanning electron microscopy (SEM) of uninfected (A, C) and infected (B, D) human dura mater fibroblasts cultures. Note the development of micro colonies exclusively on the surface of cells at 5 hours post-infection (B, D). Normal "blebs" observed in the surface of uninfected cells (C) are diminished in infected cells (D), probably due to cytoskeleton damage. Bar, 5 μm. Magnification of Giemsa-stained cells: 280 × .

units per ml at 2 hours pi and $4.9 \pm 0.3 \times 10^5$ colony-forming units per ml at 4 hours pi. These quantities represent the $1.3 \pm 0.8\%$ and $3.2 \pm 0.7\%$ of the total number of viable bacteria associated (adherent to the surface and internalised) with the fibroblasts at each time-point.

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In vitro infection of dura-mater fibroblasts with S. aureus induces IL-1beta secretion

Dura-mater fibroblasts secreted IL-1beta, starting at 2 hours after stimulation with *S. aureus.* A significant increase in IL-1beta levels was observed at 4 hours, compared with uninfected cells (Fig. 3). IL-1beta continued to be detectable by 8 hours after stimulation with *S. aureus.* Endogenous accumulation of IL-8 (150 pg/ml) was detected after 4 hours in the supernatant of uninfected cells. No significant IL-8 induction was observed in response to *S. aureus*. Similar results were obtained for TNF-alpha quantification, with accumulation of 15–30 pg/ml in 4 hours with or without *S. aureus* infection (results not shown).

Discussion

We studied experimental *in vitro* infection of whole human dura-mater and human dura mater fibroblasts by *S. aureus*. When small pieces of freshly obtained dura mater were infected with *S. aureus* the attachment of bacteria to both surfaces of this membrane could be demonstrated by Gram staining on paraffin-embedded slides, though bacterial colonisation was stronger in the surface facing the subdural space. *S. aureus* colonised dura mater organotypic cultures in a time-dependent manner, and a single layer of bacteria was observed regularly distributed on dura mater cells by Gram staining and SEM. This culture method does not indicate whether all cells covering the dura mater were alive after several days of incubation and after bacterial colonisation. Nevertheless, the pattern of bacterial adhesion to the cell surface demonstrated by SEM suggested that cells were viable from the beginning to the end of the experiment, since bacteria could be observed not only attached to the cell surface but also at different levels of penetration.

In a second experiment, dura mater fibroblasts obtained from the seventh passage after a primary *in vitro* culture and already established as a normal, continuous cell line, were infected with *S. aureus.* Several preliminary approaches were taken to determine the optimal multiplicity of infection of *S. aureus*, and to establish the best time-point to support bacterial growth and fibroblast indemnity. Giemsa staining revealed a progression of colonisation of the fibroblasts by *S. aureus*, until cell monolayers were destroyed by the effect of the infection, some 8 hours pi. Finally, multiplicity of infection of 20 bacteria/cell was used on confluent fibroblast monolayers. One possible result could have been that bacteria led to fibroblast lysis simply through the secretion of toxins. Instead, at 2 hours pi only a small amount of bacteria had developed in the cell culture without any noticeable morphological change of cell shape, but then the bacterial population increased until there was complete development, and a uniform layer covering the whole fibroblast culture was observed. This proliferation also included discrete points of bacterial overgrowth, as observed by Giemsa staining and SEM. The bacterial adhesion involved the development of microcolonies exclusively on fibroblast cell membranes, resembling biofilm-like structures, but not on the glass coverslip, which was washed gently before fixation. The production of an exopolysaccharide matrix could not be determined by the periodic acid-Schiff method, given that normal glycocalix present on fibroblasts is also periodic acid-Schiff positive, but its existence is probable, since this would provide a reasonable explanation as to why bacteria were not simply lying on the fibroblast surfaces but were tightly attached to them.

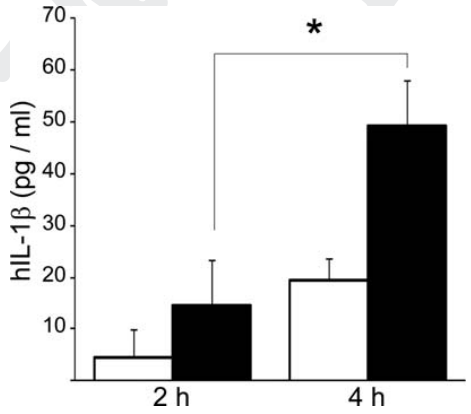

Figure 3 Secretion of IL-1beta by human dura mater fibroblast cultures, uninfected (white bars) and inoculated with *Staphylococcus aureus* (*S. aureus*) (black bars), at 2 and 4 hours pi. Bars show data from two experiments, each done in triplicate. *$P = 0.01$.

Moreover, a fraction of the infecting *S. aureus* penetrated fibroblasts, and survived intracellularly, as determined by the gentamicin assay. Also, as observed by SEM, a diminished expression of surface membrane processes (blebs) was detected in infected cells as compared with normal, uninfected cells, which suggests a compromise in the integrity of the cytoskeleton. However, a study of the mechanisms employed by this microbe to penetrate dura mater fibroblasts, and its dynamic is beyond the scope of this work, and will be the subject of future experiments.

After 2 hours pi, dura mater fibroblasts infected with *S. aureus* secreted IL-1beta into the culture medium in a time-dependent pattern while mock-infected cells only showed basal levels of this cytokine. However, TNFalpha and IL-8 levels were not increased. Fibroblasts produced IL-1beta in small amounts compared with those usually shown after infection of macrophages. Whereas the action of this cytokine is necessary to clear *S. aureus*,[21] and it has a very potent inflammatory activity, which

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might be harmful to the host. In fact, high levels of this cytokine are significantly associated with fatal outcomes in pneumococcal meningitis,[22] and other cytokines have been reported to be produced by human keratinocytes treated with the supernatant of biofilm-producing *S. aureus* cultures.[23] The increased synthesis of IL-1beta could eventually be related to the development of subarachnoid fibrosis, thus being involved in the pathogenesis of hydrocephalus.

To our knowledge, this is the first report of *in vitro* infection of human dura mater fibroblasts by *S. aureus.* Cell lysis produced by the action of bacterial toxins cannot be ruled out in this experimental model. Moreover, it is possible that the morphological alterations in cell shape and their detachment from the plastic support by the end of the experiments were indeed due to these toxins. However, the most striking finding was the observation of a particular colonisation pattern on the fibroblast surface, involving first the formation of a layer composed of single bacterial cells, and then the development of microcolonies. It would be bold to affirm that, in this model, *S. aureus* grew as biofilms, as the presence of an extracellular matrix could not be demonstrated. However, it is clear that bacteria selectively grew on the outer side of the fibroblast cell membrane and, later on, entered the fibroblasts and survived inside them.

Taken together, the results reported here strongly suggest that *S. aureus*, a major cause of post-surgical infections, produces cell damage to human dura mater fibroblasts, not only by releasing toxins but also by colonising cells and later on by invading them, leading the fibroblast to up-regulate the synthesis of the pro-inflammatory cytokine IL-1beta. It is possible that *in vivo*, these facts are responsible for the strong inflammatory reaction observed in acute post-traumatic or post-operative meningitis. The pattern of *S. aureus* colonisation on the fibroblast surface described and the detection of live intracellular bacteria could also be involved in the pathogenesis of chronic meningitis and antibiotic resistance. Hopefully, these results will lead to a better design of therapeutic strategies for treating this kind of central nervous system infection.

Conclusions

We demonstrated for the first time that *S. aureus* can infect *in vitro* organotypic cultures of human dura mater, and cultures of fibroblasts derived from human dura mater, producing a biofilm-like colonisation pattern and invading the cells. As a consequence of the infection, human dura mater fibroblasts increased the synthesis of IL-1beta, a powerful pro-inflammatory cytokine. These results could explain some aspects of the pathogenesis of acute and chronic meningitis caused by *S. aureus* and lead to a more rational approach in the treatment of this disease.

Disclaimer Statements

Contributors Conception and design: NAS. Acquisition of data: all authors. Drafting the article: MAA, NAS. Critically revising the article: all authors.

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Ethics approval None.

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References

1 Kasanmoentalib ES, Brouwer MC, van de Beek D. Update on bacterial meningitis: epidemiology, trials and genetic association studies. Curr Opin Neurol. 2013;26:282–8.
2 Agrawal S, Nadel S. Acute bacterial meningitis in infants and children: epidemiology and management. Paediatr Drugs. 2011;13:385–400.
3 Dashti SR, Baharvahdat H, Spetzler RF, Sauvageau E, Chang SW, Stiefel MF, *et al.* Operative intracranial infection following craniotomy. Neurosurg Focus. 2008;24:E10.
4 Zunt JR, Baldwin KJ. Chronic and subacute meningitis. Continuum (Minneap Minn). 2012;18:1290–318.
5 Snowden JN, Beaver M, Beenken K, Smeltzer M, Horswill AR, Kielian T. *Staphylococcus aureus sarA* regulates inflammation and colonization during central nervous system biofilm formation. PloS One. 2013;8:e84089.
6 Koedel U, Klein M, Pfister HW. New understandings on the pathophysiology of bacterial meningitis. Curr Opin Infect Dis. 2010;23:217–23.
7 Wippel C, Maurer J, Förtsch C, Hupp S, Bohl A, Ma J, *et al.* Bacterial cytolysin during meningitis disrupts the regulation of glutamate in the brain, leading to synaptic damage. PLoS Pathog. 2013;9:e1003380.
8 Kolter R. Biofilms in lab and nature: a molecular geneticist's voyage to microbial ecology. Int Microbiol. 2010;13:1–7.
9 Lopez D, Vlamakis H, Kolter R. Biofilms. Cold Spring Harb Perspect Biol. 2010;2:a000398.
10 Almiron MA, Roset MS, Sanjuan N. The aggregation of *Brucella abortus* occurs under microaerobic conditions and promotes desiccation tolerance and biofilm formation. Open Microbiol J. 2013;7:87–91.
11 Almirón M, Traglia G, Rubio A, Sanjuan N. Colonization of the mouse upper gastrointestinal tract by *Lactobacillus murinus*: a histological, immunocytochemical, and ultrastructural study. Curr Microbiol. 2013;67:395–8.
12 Peterson BW, van der Mei HC, Sjollema J, Busscher HJ, Sharma PK. A distinguishable role of eDNA in the viscoelastic relaxation of biofilms. MBio. 2013;4:e497–413.
13 Gil C, Solano C, Burgui S, Latasa C, García B, Toledo-Arana A, *et al.* Biofilm matrix exoproteins induce a protective immune response against *Staphylococcus aureus* biofilm infection. Infect Immun. 2014;82:1017–29.

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14 Scherr TD, Heim CE, Morrison JM, Kielian T. Hiding in plain sight: interplay between staphylococcal biofilms and host immunity. Front Immunol. 2014;5:37.
15 Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, *et al.* *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. J Immunol. 2011;186:6585–96.
16 Hanke ML, Kielian T. Deciphering mechanisms of staphylococcal biofilm evasion of host immunity. Front Cell Infect Microbiol. 2012;2:62.
17 Atshan SS, Nor Shamsudin M, Lung LT, Sekawi Z, Pei Pei C, Karunanidhi A, *et al.* Genotypically different clones of *Staphylococcus aureus* are diverse in the antimicrobial susceptibility patterns and biofilm formations. Biomed Res Int. 2013;2013:515712.
18 Garofalo A, Giai C, Lattar S, Gardella N, Mollerach M, Kahl BC, *et al.* The length of the *Staphylococcus aureus* protein A polymorphic region regulates inflammation: impact on acute and chronic infection. J Infect Dis. 2012;206:81–90.
19 Goldschmidt E, Hem S, Ajler P, Ielpi M, Loresi M, Giunta D, *et al.* A new model for dura mater healing: human dural fibroblast culture. Neurol Res. 2013;35:300–7.
20 Almirón M, Martínez M, Sanjuan N, Ugalde RA. Ferrochelatase is present in *Brucella abortus* and is critical for its intracellular survival and virulence. Infect Immun. 2001;69:6225–30.
21 Miller LS, Pietras EM, Uricchio LH, Hirano K, Rao S, Lin H, *et al.* Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo. J Immunol. 2007;179:6933–42.
22 Grandgirard D, Gäumann R, Coulibaly B, Dangy JP, Sie A, Junghanss T, *et al.* The causative pathogen determines the inflammatory profile in cerebrospinal fluid and outcome in patients with bacterial meningitis. Mediators Inflamm. 2013;2013:312476.
23 Tankersley A, Frank MB, Bebak M, Brennan R. Early effects of *Staphylococcus aureus* biofilm secreted products on inflammatory responses of human epithelial keratinocytes. J Inflamm (Lond). 2014;11:17.

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