Pulmonary infection in Wegener’s granulomatosis and idiopathic pulmonary fibrosis.

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Abstract (220)

**Rationale:** Wegener’s Granulomatosis (WG) has previously been associated with increased nasal carriage of Staphylococcus aureus, but no studies have investigated the occurrence of pathogen growth in the lower airways.

**Objectives:** To culture bronchoalveolar lavage fluid (BALF) from WG, idiopathic pulmonary fibrosis (IPF) patients and normal controls.

**Methods:** 33 patients with WG, 22 with IPF and 8 normal controls underwent bronchoscopy and bronchoalveolar lavage. Quantitative culture established bacterial levels in the lower airways. Culture experiments were designed to investigate whether bronchoalveolar lavage fluid (BALF) is a supportive environment for *S. aureus* growth. BALF cytokines were measured by ELISA.

**Results:** Pathogens were commonly grown from both WG and IPF patients BALF. *S. aureus* was particularly associated with WG patients both in relapse and in remission. BAL levels of IL-1ra were statistically significant elevated in those patients who grew a pathogen from lavage fluid. BALF from WG and IPF patients stimulated *S. aureus* growth compared to normal lavage fluid.

**Conclusions:** Pathogens are more commonly isolated from WG BALF than from IPF or normal controls and with a different culture profile. IL-1ra was associated with pathogen growth in WG and IPF. WG BALF is a trophic environment for *S. aureus* growth. Pulmonologists treating patients with acute or relapsing WG should consider bronchoscopic microbiological sampling and consider antibiotics with anti-staphylococcal activity.
Introduction

Wegener’s granulomatosis is a small vessel systemic vasculitis characterised by anti-neutrophil cytoplasmic antibodies (ANCA) directed against proteinase-3 (PR-3). A role for infection in WG was first postulated by Frederick Wegener in 1936 but a more substantial argument was put forward in 1980, when a retrospective trial suggested nearly 50% of relapses were provoked by infection(1). Nasal carriage of Staphylococcus aureus (S. aureus) is increased in WG, and is associated with a higher relapse rate (2). How this relates to relapse and whether a similar process affects the lower respiratory tract is unknown.

It is unclear why patients with WG have increased nasal carriage of S. aureus. A controlled cytokine response is essential for bacterial clearance and resolution of inflammation. However, experiments in acute respiratory distress syndrome have shown that elevated interleukin 6 (IL-6), IL-1 receptor antagonist (IL-1ra) and IL-1β BALF levels are associated with a promotive growth environment for S. aureus (3, 4). These studies suggest that a pro-inflammatory environment may be permissive for S. aureus growth raising the possibility of a cause and effect relationship. Several reports have suggested the importance of cytokines in the inflammatory response seen in WG, but the clinical implications of these measurements have not been fully established.

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease associated with damage to the alveolar capillary barrier. A number of viruses have been implicated in IPF pathogenesis (5-7) but studies have not established whether bacteria in the lower airways are also present or play a pro-inflammatory role in IPF patients. The current study was designed to establish the prevalence of lower airway pathogen growth in WG, IPF and normal controls and then to
determine any relationship with clinical or radiological findings. We went on to establish BALF levels of IL-6, IL-1ra and IL-1β cytokine levels and lastly investigated whether WG BALF promotes S. aureus growth in vitro.
Methods

This manuscript has an online methods supplement.

Patient recruitment: 33 patients with WG meeting the classification criteria according to the Chapel Hill consensus statement were recruited(8). Patients were enrolled consecutively as they presented at University Hospital Birmingham from 2003-2008 with new or relapsing disease and remission patients were consecutively recruited from clinic at routine appointment. Disease onset, relapse and remission definitions were defined according to European League against Rheumatism recommendations(9). Disease activity in WG was monitored using the Birmingham Vasculitis Activity Score (BVAS)(10).

Contraindications to enrollment for WG patients were refractory hypoxia (P: F ratio <300 on 40% oxygen), the need for ventilatory support (CPAP, NIV or IPPV) or continuous renal replacement therapy.

IPF: We used IPF as a disease control which has not been associated with S. aureus nasal carriage and as a comparator group that also receive immunosuppression to enable comment about the disease specific nature of lower airway S. aureus infection and the influence of immunosuppression to be addressed. 22 patients diagnosed, with IPF according to current American Thoracic Society (ATS) criteria(11) were recruited sequentially at first presentation to our interstitial lung disease clinic before immunosuppressive treatment.
No IPF patients who had a history or clinical features to suggest active pulmonary infection within the last 4 weeks prior to bronchoscopy were included. Eight normal volunteers free from respiratory disease were recruited as controls. The study was conducted according to the Declaration of Helsinki. All Patients gave informed consent and this study was approved by the local ethics committee (South Birmingham, UK, LREC ref 2003/166).

**Imaging:** WG and IPF patients underwent high resolution computerized tomography scanning (HRCT). Scans were read blinded to conditions by a respiratory specialist (DRT) and a radiologist (PG) and assessed for features previously noted in a review of pulmonary findings in WG (12).

**Pulmonary function testing:** Forced vital capacity (FVC) was measured using the Jaeger Compact system (Viasys Healthcare). Total lung diffusing capacity for carbon monoxide (TLCO) was measured by single-breath technique (Jaeger Compact system). Results are expressed as the percent of predicted values.

**Bronchoalveolar lavage:** All patients underwent bronchoalveolar lavage (BAL), according to national guidelines(13). To prevent nasal contamination patients were intubated through the mouth. A nasal swab was performed for culture to look for matched nasal and lower airway bacteriology.

**Repeat Bronchoscopy:** All WG patients were invited to undergo a repeat bronchoscopy to establish whether there was evidence of persistent colonization – 7 WG patients agreed to have repeat bronchoscopy entirely for research purposes after induction of remission, 4 others agreed
to repeat BAL as they needed bronchoscopy for the assessment of disease relapse. 9 IPF patients agreed to repeat bronchoscopy after at least 3 months treatment with prednisolone, azathioprine and latterly N-acetylcysteine.

**Cytokine measurements:** BALF cytokine levels were measured by ELISA (R&D systems, UK) according to manufacturer’s instructions.

**Quantitative culture (QC):** Serial 10 fold dilutions (10^4-10^8) of the original BALF specimen were made in normal saline. An inoculum of each dilution was plated on chocolate and blood agar. Bacterial numbers at 24 and 48 hours were estimated by two investigators, blinded to conditions, and expressed as colony forming units (CFU)/ml BALF.

**Culture experiments:** To assess whether BALF is a permissive environment for *S. aureus* growth a laboratory *S. aureus* (National Collection of Type Cultures, 6571, Oxford) was incubated with BALF from 10 WG, 10 IPF and 6 normal controls. The *S. aureus* was cultured in brain heart infusion (BHI). An inoculum of 10^-5 bacteria per ml, determined using a haematocytometer, was added to 1ml of BALF or 0.9% saline as a control in a 50:50 mix with 1ml BHI. Following an overnight incubation, 1:10 dilutions of the broth were made and dilutions of 10^-4 – 10^-7 were inoculated onto chocolate agar plates, in triplicate. The plates were read, after 24 hour incubation at 37°C and 5% CO2 by two investigators blinded to the variable conditions. Preliminary experiments revealed that at the dilutions performed, the initial protein content did not effect *S. aureus* growth (data not shown). BALF experiments were repeated following heat inactivating the proteins in the BALF for 10 minutes at 80°C.
Statistics- Non parametric data were assessed by Kolmogorov-Smirnoff test and are presented as median and interquartile range (IQR). Differences in pathogen growth patterns between cohorts and also between activities groups in the WG patients were examined using Fisher’s exact test (FET). Cytokine analysis was performed using a Kruskal-Wallis test followed by a Dunn’s test to look at differences between cohorts and then look at differences within a disease group when a pathogen was present. Culture experiments had a Kruskal-Wallis test applied and Dunn’s test was used to compare groups. A p value of less than or equal to p=0.05 was considered statistically significant. Statistics were performed using SPSS 15.
Results

**Demographics**: At presentation WG patients were classified as acute (first ever presentation), relapse or remission based on their BVAS and clinical presentation. Clinical features of the individual activity groups, the 22 IPF patients and 8 normal controls are described in table 1. 3/11 (27%) acute patients underwent bronchoscopy before immunosuppression with steroids and cyclophosphamide and 2/10 (20%) remission patients were on no medication (60% steroids, 30% azathioprine, 20%, 10% cyclophosphamide and methotrexate). All relapse patients were on immunosuppressive medication at the time of bronchoscopy (83% steroids, 25% cyclophosphamide, 33% azathioprine, 16% each mycophenolate, rituximab and infliximab). Patients receiving cyclophosphamide had co-trimoxazole chemoprophylaxis. Pathogen growth was unrelated to individual immunosuppressive therapies in active or remission patients (data not shown). Of the 16 patients who were on co-trimoxazole chemoprophylaxis, 10 grew a pathogen of which 6 were *S. aureus*. No IPF patients had received treatment prior to the first bronchoscopy.
Table 1: Clinical features of patient groups at time of first bronchoscopy

<table>
<thead>
<tr>
<th></th>
<th>Acute</th>
<th>Relapse</th>
<th>Remission</th>
<th>Normal</th>
<th>IPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient numbers</td>
<td>11</td>
<td>12</td>
<td>10</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>Sex: Male</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>52</td>
<td>59</td>
<td>50</td>
<td>45</td>
<td>70</td>
</tr>
<tr>
<td>Smoking: Never/Ex/Current Pack years (mean)</td>
<td>5/6/0</td>
<td>4/7/1</td>
<td>3/7/0</td>
<td>2/5/1</td>
<td>5/12/3</td>
</tr>
<tr>
<td>FVC (mean, SE)</td>
<td>108 (3.7)</td>
<td>109 (4.3)</td>
<td>112 (7.2)</td>
<td>N/A</td>
<td>73 (6.6)</td>
</tr>
<tr>
<td>TLco (mean, SE)</td>
<td>90 (4.7)</td>
<td>87 (4.6)</td>
<td>88 (4.7)</td>
<td>N/A</td>
<td>49 (3.4)</td>
</tr>
<tr>
<td>CRP (mean, SE)</td>
<td>23 (6.0)</td>
<td>45 (26.0)</td>
<td>5.8 (5.3)</td>
<td>&lt;1</td>
<td>6 (10.3)</td>
</tr>
<tr>
<td>ANCA positivity</td>
<td>100%</td>
<td>71%</td>
<td>33%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>PR3 positivity</td>
<td>73%</td>
<td>50%</td>
<td>17%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>MPO positivity</td>
<td>18%</td>
<td>14%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>BVAS (mean, SE)</td>
<td>5.4 (1.4)</td>
<td>5.6 (1.6)</td>
<td>0.5 (0.3)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>VDI (mean, SE)</td>
<td>3.2 (0.7)</td>
<td>4.3 (0.6)</td>
<td>2 (0.4)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>18%</td>
<td>43%</td>
<td>50%</td>
<td>N/A</td>
<td>74%*</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>73%</td>
<td>36%</td>
<td>33%</td>
<td>N/A</td>
<td>100%</td>
</tr>
<tr>
<td>Cavities</td>
<td>18%</td>
<td>50%</td>
<td>17%</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>Nodules</td>
<td>91%</td>
<td>71%</td>
<td>66%</td>
<td>N/A</td>
<td>32%</td>
</tr>
<tr>
<td>Emphysema</td>
<td>18%</td>
<td>18%</td>
<td>22%</td>
<td>N/A</td>
<td>32%</td>
</tr>
<tr>
<td>Haemoptysis</td>
<td>9%</td>
<td>14%</td>
<td>0%</td>
<td>N/A</td>
<td>0%</td>
</tr>
</tbody>
</table>

Characteristics relate to the time of patients’ first bronchoscopy. Data is presented as mean (SE). FVC - forced vital capacity (% predicted). TLco - transfer factor for carbon monoxide (% predicted). VDI - vasculitis damage index. BVAS - Birmingham vasculitis activity score. PR-3 = proteinase-3 antibody (serum), MPO - myeloperoxidase antibody (serum). Only relevant medication was included. Normal CRP at our institution is <10. In WG pulmonary fibrosis was limited focal disease in all but 1 case. * Bronchiectasis in IPF patients describes traction bronchiectasis.
Microbiology of first bronchoscopy after enrolment

Pathogens were grown from 64% (21/33) of WG BALF compared with 36% (8/22) of IPF (FET, p=0.058) and 0% (0/8) of normal controls (FET, p=0.001). *S. aureus* grew in 40% (13/33) of all WG patients compared with 0% (0/22) of IPF patients (FET, p=0.001). Other bacteria were identified equally in the two disease groups. No organisms were grown in the BALF from normal control subjects.

Table 2: Frequency of pathogens occurring in WG, IPF and normal controls

<table>
<thead>
<tr>
<th>Organism</th>
<th>WG*</th>
<th>IPF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of BALs</td>
<td>CFU/ml</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>13</td>
<td>3x10^5</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>3</td>
<td>5x10^6</td>
</tr>
<tr>
<td>Haemophilus parainfluenzae</td>
<td>3</td>
<td>2x10^4</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>1</td>
<td>5x10^7</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>2</td>
<td>1x10^5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1</td>
<td>1x10^6</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>1</td>
<td>4x10^5</td>
</tr>
</tbody>
</table>

* Three patients grew two pathogens. Bacterial growth presented as mean CFU/ml. No normal controls grew pathogenic bacteria in their BALF.
**BALF pathogen growth in the different WG activity groups**

Pathogen were more commonly grown in WG disease relapse (83% (10/12)) and remission (70% (7/10)) than at first acute presentation (36% (4/11)) although this did not reach statistical significance (FET p=0.076). *S. aureus* was statistically more likely to be grown in the relapse (50% (6/12)) and remission group (60% (6/10)) than in the first acute presentation with WG (8.3% (1/12)) (FET p=0.031).

Five of 13 (38%) WG patients who grew *S. aureus* in the BALF had only mixed normal flora (MNF) grown from their nasal swab. Two of 12 (17%) patients who grew *S. aureus* from their nasal swab had a sterile BALF. The remaining samples were concordant and all *S. aureus* were methicillin sensitive.

**Sequential BAL microbiology**

There were no differences in the bacterial growth patterns from the first or second WG BALF. 3/11 sequential WG patients grew pathogens in both their BALF cultures, although the bacteria grown were different giving no evidence of colonisation. Three patients grew a pathogen in the first culture but not the second. Four patients grew a pathogen only in the second culture. A pathogen was more commonly grown on the second bronchoscopy although this was not statistically significant (FET, p=0.076).

5/9 IPF patients who underwent sequential bronchoscopy had two sequential sterile cultures. Two patients grew pathogens only on the first occasion and 2 grew pathogens only on the repeat BAL. There was no increased likelihood of growing a pathogen on the second BAL culture (p=0.376).
Pathogen growth related to clinical-radiological findings in WG

Most WG patients (91%) had radiographic pulmonary abnormalities (table 1) with cavities more likely to occur in the disease relapse group (p=0.031). The presence of a pathogen in WG BALF was not associated with bronchiectasis (FET, p=0.761), cavities (p=0.399), fibrosis (p=0.724) or nodules (p=0.555). There were no statistically significant differences in blood CRP or WCC in patients with WG and IPF who had evidence of bacterial infection (data not shown).

BALF cytokine levels and the relationship with pathogen growth

BALF levels of IL-1α (Dunn’s test, p=0.048), IL-1β (p=0.003), IL-1ra (p<0.0001) and IL-6 (p=0.008) were elevated in WG patients compared to normal controls. IL-1ra (p<0.001) and IL-6 (p=0.009) levels were elevated in IPF patients compared with controls. IL-1α (p=0.05) and IL-1β (p=0.001) levels were higher in WG patients compared with IPF disease controls. Levels of IL-1ra were elevated in WG (p=0.039) and IPF (p=0.015) patients who grew a pathogen compared with those who didn’t. The other cytokine levels measured did not relate to the presence or absence of a pathogen (table 3).
Table 3: BALF Cytokine levels

<table>
<thead>
<tr>
<th></th>
<th>IL-1α</th>
<th>IL-1β</th>
<th>IL-1ra</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Not detected</td>
<td>3.8 (2.2-4.0)</td>
<td>755 (165-1212)</td>
<td>0.3 (0-0.5)</td>
</tr>
<tr>
<td>WG all patients</td>
<td>58.9 (0-84.9) #</td>
<td>13.21 (4.0-41.5)#</td>
<td>4,957 (1,453-36,819)#</td>
<td>2.3 (1.0-16.8) #</td>
</tr>
<tr>
<td>WG pathogen present</td>
<td>28.1 (0-77.7)</td>
<td>5.0 (4.0-38.5)</td>
<td>5,824 (4085-45,175)*</td>
<td>3.5 (2.1-15.7)</td>
</tr>
<tr>
<td>WG no pathogen</td>
<td>84.6 (38.11)</td>
<td>4.0 (4.0-50.6)</td>
<td>3,307 (1,112-11,275)</td>
<td>1.8 (1.3-16.8)</td>
</tr>
<tr>
<td>IPF all patients</td>
<td>33.7 (0-50.0)</td>
<td>0.5 (0.5-23.9)</td>
<td>8061 (1450-22,104) #</td>
<td>8.4 (3.5-17.4)#</td>
</tr>
<tr>
<td>IPF pathogen present</td>
<td>36.5 (0-74.5)</td>
<td>10.3 (0.5-36.9)</td>
<td>22,104 (2890-33978)*</td>
<td>13.4 (6.0-22.1)</td>
</tr>
<tr>
<td>IPF no pathogen</td>
<td>18.9 (0-21.6)</td>
<td>1.0 (0.5-5.0)</td>
<td>3,008 (628-8,002)</td>
<td>3.5 (2.7-12.9)</td>
</tr>
</tbody>
</table>

Measurements are expressed at pg/ml concentration. Levels expressed as median (interquartile range). * indicates statistically significant difference between pathogen present and pathogen not present. # signifies statistically significant difference between disease group and normal controls.

**Soluble factors within BALF promote S. aureus growth.**

Incubations with WG BALF promoted statistically significantly more *S. aureus* growth [Dunn’s test, 168.4 cfu/ml (IQR 95-268), p=0.025] than BALF from IPF patients [75.0 cfu/ml (IQR 37-122), p=0.041] and normal controls [23 cfu/ml, (IQR 19-42)]. Heat inactivation resulted in a statistically significant reduction in *S. aureus* growth with WG BALF [23.5 cfu/ml (IQR 3-63), (p=0.0008)(figure 1).
**Discussion**

This study has shown that pathogens were more commonly isolated from WG and IPF BALF than from normal controls. *S. aureus* was most frequently found in the WG remission and relapse groups rather than at disease presentation. Serial bronchoscopy suggests that pathogen presence in BALF was due to transient infection rather than persistent lower airway colonisation. There were no significant associations between pathogen growth and radiological findings, pulmonary function tests, immunosuppression, or antibiotic treatment. Culture experiments revealed that WG BALF was a permissive environment for *S. aureus* growth and that this effect was heat sensitive. Also, cytokines, previously reported to be promotive of *S. aureus* growth *in vitro*, were elevated in WG.

Previous studies looking at nasal carriage of *S. aureus* have suggested that persistent nasal *S. aureus* carriage is associated with disease relapse in WG (2). We believe that ours is the first study to demonstrate that lower airway bacterial infection, especially with *S. aureus*, is also common in patients with WG. Chronic lung damage is a recognised feature of the WG lung (14) and this may contribute to pathogen growth in conjunction with immunosuppressant therapy. Previous studies have shown that with progressive epithelial cell damage, adherence and internalization of *S. aureus* is increased (3). In WG this may be important since ANCA itself also induces IL-1, IL-8 release from neutrophils (15) potentially amplifying local inflammatory tissue damage and thereby promoting *S. aureus* growth.
The mechanisms by which *S. aureus* may have a predilection for the lower airways of patients with WG especially in chronic disease are likely to be multifactorial- related to structural changes, functional immunodeficiency, defective host innate immunity or variability in the virulence of *S. aureus* strains. There was a high incidence of bronchiectasis and cavities in the WG relapse and remission patients but this did not relate to the presence of a pathogen. Equally most but not all patients who had lower airway *S. aureus* growth also had *S. aureus* in the nose suggesting a link with nasal carriage as part of the general respiratory tract response.

The lack of association with pulmonary features investigated in this study may reflect the relatively low numbers recruited in this trial, due to rarity of WG rather than a true lack of effect. However, if the lack of relationship is true it is likely that, in contrast to cavitating *staphylococcal* pneumonia(16), the underlying disease process rather than infection is driving the cavitation process, especially since several cases of cavitating disease had completely sterile BALF. There was also no clear effect of immunosuppression or chemoprophylaxis on bacterial growth although the data were difficult to interpret because of the relatively small numbers in each group. Bacteria were isolated in active and remission patients despite the prophylactic co-trimoxazole prescribed, demonstrating this dose was inadequate to control pathogen growth.

The microbiology pattern in WG was different between our three defined activity groups with *S. aureus* more commonly isolated in disease relapse and remission patients rather than at acute presentation suggesting that *S. aureus* may have a role in disease relapse. The predilection of WG for *S. aureus* infection appears to be disease related as these changes were not seen in IPF patients who we used as a pulmonary disease control. Due to the intense inflammatory response associated with WG activity, we were unable to associate the presence of a pathogen with the
intensity of the local cellular inflammatory response to confirm that this represented an infection rather than colonization. However, BALF IL1-Ra levels were increased in association with the presence of a pathogen in both WG and IPF patients.

IL-1Ra is an acute phase proteins and Staphylococci are highly efficient in mediating the upregulation of both IL-1α, IL-1β and IL-1Ra(17). These data suggest that the presence of Staphylococci may induce or relate to a local alveolar inflammation. In addition since IL-1Ra has previously been shown to be a trophic factor for S. aureus(4), we performed relevant but simple BALF growth experiments in vitro. The observation that BALF from WG patients stimulated growth of SA supports a role for a trophic interaction between the host inflammatory response and the bacteria within the lung. In this regard, WG BALF appears to have similar properties to acute respiratory distress syndrome(4).

There are a number of potential mechanisms whereby S. aureus infection might initiate relapse in WG; S. aureus is able to directly stimulate the tumour necrosis factor alpha receptor (17), superantigen release(18), generation of immune complexes(19), expansion of memory effector T cells(20) and may induce glucocorticoid resistance(21). Further recruitment, in a longitudinally studied cohort, will be required to assess the importance of our findings, and to address whether the prevalence of S. aureus in the lower airway is associated with an increased risk of subsequent disease relapse, thus making intervention relevant.

This study has also demonstrated that a significant number of patients with IPF grow pathogenic bacteria from their BALF with a different spectrum of pathogens to WG. We specifically excluded any patient thought clinically to have had evidence of infection within the 4 weeks
prior to bronchoscopy and on their first bronchoscopy they were not immnosuppressed. To our knowledge no study has previously looked at the issue of bacterial colonization in IPF. Given the variable presence of cellular neutrophilic inflammation in IPF patients, it is possible therefore that unrecognized airway colonization is a determinant of this phenomenon. The clinical relevance of this observation warrants further evaluation especially in view of the recent publication of a pilot study demonstrating improved exercise tolerance and lung function with continuous co-trimoxazole usage in IPF patients (22).

This study has limitations. Firstly, during intubation oral-pharyngeal contamination of the bronchoscope could have contributed to BALF pathogen growth. To counter this potential problem we used quantitative culture, a microbiological culture technique that has evolved as a sensitive and specific technique for the diagnosis of bacterial infection(23). Serial dilutions help to overcome contamination of the bronchoscope suction channel by oropharyngeal bacteria(24) and results were greater than would be expected from contamination alone. Secondly, we are unable to demonstrate in our BALF growth experiments that IL1-Ra is directly responsible for the growth promoting potential within the BALF as there is no effective inhibitor of IL-1ra bioactivity. Finally, this was an observational study to ascertain the prevalence of pathogenic bacteria in the lungs of WG patients and our results need validation in another WG patient cohort.

In summary, this study has found a high incidence of pathogen carriage in the lower airways of patients with WG and IPF. The predilection for S. aureus in WG is likely to be multifactorial but may relate to a dynamic relationship between alveolar inflammation and bacterial growth. Determining the role and mechanisms of lung S. aureus carriage in disease and relapse may
provide strategies for future treatments in WG. Physicians treating patients with acute or relapsing WG who wish to treat potential pulmonary infection should consider bronchoscopic microbiological sampling and prescribing antibiotics with anti-staphylococcal activity.

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Figure 1 *S. aureus* growth in culture with BALF. BALF or normal saline (50:50 mix) with brain heart infusion were incubated with an inoculum of $10^5$ bacteria per ml. After 24 hours of incubation, quantitative culture was performed to determine the number of *S. aureus* colony forming units. BALF experiments were repeated following heat inactivating the proteins in the BALF for 10 minutes at $80^\circ$C.
Patient recruitment: 33 patients with WG were studied, meeting the classification criteria according to the Chapel Hill consensus statement[1]. Patients were enrolled consecutively into the study as they presented with new or relapsing disease and remission patients were consecutively recruited from clinic at routine appointment. Disease onset, relapse and remission definitions were defined according to European League against Rheumatism recommendations [2]. New onset or relapse was defined as the new appearance or recurrence of disease activity attributable to active vasculitis. For the lung this includes clinical, radiological or bronchoscopic evidence of pulmonary haemorrhage or granulomata, significant subglottic or bronchial stenosis. Major relapse was defined as the re-occurrence or new onset of potentially organ or life threatening disease that required full escalation of treatment. All other relapses were classed as minor. All the patients with acute and relapsing disease in this study met the criteria for a major pulmonary relapse. Active pulmonary vasculitis was proven histologically in cases (6 endobronchial biopsy, 3 CT guided chest biopsy, 1 surgical lung biopsy). In addition, 3 patients had BAL cytological evidence of pulmonary haemorrhage). Remission was defined as the complete absence of active clinical disease.

Disease activity in WG was monitored using the Birmingham Vasculitis Activity Score (BVAS)[3]. BVAS is a validated clinical index measurement of disease activity based on the signs and symptoms in nine separate organ systems and is weighted to reflect the clinical importance of specific organ involvement. By measuring abnormalities that have developed within the 4 weeks that precede each scoring, it is devised to be a reflection of disease activity, without regard to the degree of cumulative damage (whether from the disease itself or its treatment). It is weighted
toward patients with renal disease, so patients with pulmonary relapse tend to have lower scores.

Contraindications to enrollment for WG patients were refractory hypoxia (P:F ratio <300 on 40% oxygen), the need for ventilatory support (CPAP, NIV or IPPV) or continuous renal replacement therapy such as haemofiltration due to physician / ethical committee concerns. Where bacteria were cultured in significant numbers, appropriate antibiotic therapy was prescribed to the WG patients according to local hospital guidelines.

**IPF:** 22 patients diagnosed, with IPF according to current American Thoracic Society (ATS) criteria[4] were recruited sequentially at first presentation to the interstitial lung disease clinic. Diagnosis was supported by open lung biopsy in five cases, histology post lung transplant in one case and high-resolution computed tomography (HRCT) evidence in all cases. Where open lung biopsy was performed, tissue was obtained from at least two sites, the upper and lower lobes of the same lung. Baseline bronchoscopy was performed during investigation, before immunosuppressive treatment.

No IPF patients who had a history or clinical features to suggest active pulmonary infection within the last 4 weeks prior to bronchoscopy were included. Eight normal volunteers free from respiratory disease were recruited as controls from hospital volunteers. The study was conducted according to the Declaration of Helsinki. All Patients gave informed consent and this study was approved by the local ethics committee (South Birmingham, UK, LREC ref 2003/166).
**Imaging:** WG and IPF patients underwent high resolution computerized tomography scanning (HRCT). Scans were read blinded to conditions by a respiratory specialist (DRT) and a radiologist (PG) with an interest in interstitial lung disease and were assessed for the presence or absence of bronchiectasis, fibrosis, cavities, nodules and centrilobular emphysema; features previous noted in a review of pulmonary findings in WG[5].

**Pulmonary function testing:** Forced vital capacity (FVC) was measured using the Jaeger Compact system (Viasys Healthcare). Total lung diffusing capacity for carbon monoxide (TLCO) was measured by single-breath technique (Jaeger Compact system). Results are expressed as the percent of predicted values.

**Bronchoalveolar lavage:** All patients underwent bronchoalveolar lavage (BAL), according to national guidelines[6]. BAL was performed in the right middle lobe in all cases. To prevent nasal contamination patients were intubated through the mouth. A nasal swab was performed for culture and sensitivity to look for matched nasal and lower airway bacteriology.

**Repeat Bronchoscopy:** All WG patients were invited to undergo a repeat bronchoscopy to establish whether there was evidence of persistent colonization – 7 agreed to have repeat bronchoscopy entirely for research purposes after induction of remission, 4 others agreed to repeat BAL as they needed bronchoscopy for the assessment of disease relapse. Remission WG patients underwent repeat bronchoscopy at least 4 weeks after any clinical features suggestive of pulmonary
infection (fever, increased sputum volume/purulence or dyspnoea). 9 IPF patients agreed to repeat bronchoscopy after at least 3 months treatment with prednisolone, azathioprine and latterly N-acetylcysteine.

**Cytokine measurements:** BALF cytokine levels were measured by ELISA (R&D systems, UK) according to manufacturer’s instructions.

**Quantitative culture (QC):** Serial 10 fold dilutions ($10^4$-$10^8$) of the original BALF specimen were made in normal saline. An inoculum of each dilution was plated on chocolate and blood agar. Plates were evaluated for growth at 24 and 48 hours following incubation at $37^0$C and 5% CO$_2$. Bacterial numbers were estimated by two investigators, blinded to conditions, and expressed as colony forming units (CFU)/ml BALF.

**Culture experiments:** To assess whether BALF is a permissive environment for *S. aureus* growth a laboratory *S. aureus* (National Collection of Type Cultures, 6571, Oxford) was incubated with BALF from 10 WG, 10 IPF and 6 normal controls. The *S. aureus* was cultured in brain heart infusion (BHI). An inoculum of $10^{-5}$ bacteria per ml, determined using a haematocytometer, was added to 1ml of BALF or 0.9% saline as a control and 1ml BHI. Following an overnight incubation, 1:10 dilutions of the broth were made and dilutions of $10^{-4}$ – $10^{-7}$ were inoculated onto chocolate agar plates, in triplicate. The plates were read, after 24 hour incubation at $37^0$C and 5% CO$_2$ by two investigators blinded to the variable conditions. Preliminary experiments revealed that at the dilutions performed, the initial protein content did not effect *S.
*aureus* growth (data not shown). BALF experiments were repeated following heat inactivating the proteins in the BALF for 10 minutes at 80°C.

**Statistics** - Non parametric data were assessed by Kolmogorov-Smirnoff test and are presented as median and interquartile range (IQR). Differences in pathogen growth patterns between cohorts and also between activity groups in the WG patients, were examined using Fisher’s exact test. Cytokine analysis was performed using a Kruskal-Wallis test followed by a Dunn’s test to look at differences between cohorts and then look at differences within a disease group when a pathogen was present. Culture experiments had a Kruskal-Wallis test applied and Dunn’s test was used to compare groups. The experiment was designed to see if there were differences between disease groups and whether heating the BALF had an affect on growth in both disease cohorts. A p value of less than or equal to p=0.05 was considered statistically significant. Statistics were performed using SPSS 15.

S. aureus Colony forming units x 10^6/ml

- WG
- WG heated
- Normal
- IPF
- IPF heated

p-values:
- p=0.002
- p=0.001
- p=0.002
- p=0.011
- p=0.031