Comparative immune responses to candidate arthritogenic bacteria do not confirm a dominant role for Klebsiella pneumoniae in the pathogenesis of familial ankylosing spondylitis

M. A. Stone1,2, U. Payne1, C. Schentag1, P. Rahman3, C. Pacheco-Tena1 and R. D. Inman1,2

Objective. Using humoral immune responses, Klebsiella pneumoniae has been implicated as a candidate microbial trigger in ankylosing spondylitis (AS) by several investigators but refuted by others. The objective of this case–control study was to compare the cellular (T-cell proliferation) and humoral (IgG and IgA, by ELISA) immune responses of affected individuals in multiplex AS families with those of unaffected family members and normal healthy controls in order to find out whether affected individuals exhibit a predominant immune response to K. pneumoniae.

Methods. Twenty-five families with two or more individuals affected with AS and 34 normal healthy controls matched with the affected family members for age, sex and ethnicity were enrolled in the study. All affected (n = 57) and unaffected (n = 37) family members had a detailed clinical evaluation. Peripheral blood was drawn to determine T-lymphocyte proliferation and the IgG and IgA (by ELISA analysis) immune responses to K. pneumoniae, Salmonella typhimurium, Yersinia enterocolitica and Chlamydia trachomatis. Immune responses to each of the four candidate organisms were compared in affected and unaffected individuals. Each individual was classified by the predominant antigenic immune response that they showed when comparison was made among the same concentrations of the four candidate microbial antigens. This stratification was then used (i) to compare immune responses in affected and unaffected family members and (ii) to compare clinical characteristics of affected family members.

Results. There was no difference in mean stimulation indices or antibody responses between affected and unaffected family members for each of the candidate organisms. In terms of predominant cellular immune responses to these organisms, there was no difference between affected and unaffected family members with respect to K. pneumoniae, C. trachomatis or Y. enterocolitica. However, a higher percentage of affected family members (25.9%) exhibited a predominant response to S. typhimurium compared with unaffected family members (5.9%, P < 0.02). In assessing antibody titres, K. pneumoniae was the predominant amongst these four organisms, but there was no difference between affected family members, unaffected family members and normal healthy controls. There was no relationship between immune responses and clinical characteristics.

Conclusion. Our analysis of affected and unaffected family members in familial AS demonstrated no significant differences with respect to cellular or humoral immune responses to K. pneumoniae and three control microbes. In addition, K. pneumoniae did not exhibit a predominant immune response in affected individuals. Thus we find no supportive evidence to implicate a causal role for K. pneumoniae in familial AS.

KEY WORDS: Ankylosing spondylitis, Klebsiella pneumoniae, HLA-B27, Immune responses.

Ankylosing spondylitis (AS), a chronic inflammatory disease that primarily affects the spine, is thought most likely to be due to the interplay of genetic determinants and environmental factors, such as a microbial trigger. The role of infectious triggers in AS, however, is controversial. Several studies in the literature have suggested a role for Klebsiella pneumoniae in the pathogenesis of the disease. Early observations of Ebringer et al. [1] demonstrated increased faecal carriage of K. pneumoniae in active disease and subsequent studies of this question were supportive [2]. Yet other investigators have been unable to confirm a difference in faecal colonization with K. pneumoniae between cases and controls [3, 4].

This potential infectious relationship has led to several theories to explain a possible link between K. pneumoniae and AS. First, the theory of molecular mimicry postulated antigenic cross-reactivity of microbial and host determinants. It was demonstrated, for example, by Schwimmbeck et al. [5] that there was a degree of sequence homology between HLA-B27 and the nitorgenase enzyme of K. pneumoniae. However, molecular mimicry, while a common biochemical reality, has been difficult to prove as a pathogenic mechanism [6, 7]. Secondly, K. pneumoniae antigens may be capable of specifically modifying HLA-B27 or a hypothetical B27-associated receptor, and the resulting tissue damage is caused by
cytolytic attack against the infecting organism [8]. Support for this notion came from the observation that cytotoxic T lymphocytes might recognize certain secreted bacterial antigens in association with HLA-B27, suggesting that this interaction may contribute directly to an immune-mediated inflammation during the initial stages of the disease [9, 10]. Again, these events have proved difficult to prove rigorously as pathogenic mechanisms.

Indirect evidence implicating K. pneumoniae in AS has come largely from studies of host immune response [11–16]. Such an approach has in some studies shown increased serum antibody responses to K. pneumoniae [17, 18]. However, there remains significant controversy in the literature over the specificity of antibodies against K. pneumoniae. A recent study found no evidence for AS-specific serological responses to multiple serotypes of K. pneumoniae in a cohort of 187 AS patients compared with 195 controls [19]. In addition, the role of Klebsiella has proved difficult to integrate into a testable hypothesis of microbial pathogenesis. Attempts to identify Klebsiella DNA by the polymerase chain reaction in saccroiliac biopsies of AS patients have not been successful, so there is little to support localization of the organism in target joints [20]. In addition, no improvement in disease activity has been demonstrated with antibiotic therapy [21], which further weakens the argument for the role of at least an active infection in AS.

The objective of this case–control study was to compare the immune profile of AS patients in multiplex families with that of unaffected family members and normal healthy controls in order to find out whether there was evidence of a predominant immune response to K. pneumoniae in AS.

Methods

Patient ascertainment

Twenty-five multiplex AS families with two or more affected individuals were recruited from Eastern Canada. Cases were defined by the modified New York criteria [22]. All subjects had a detailed assessment including physical examination of axial and peripheral joints. All assessments were carried out by the same examiner (MAS). In particular, the following items were recorded: age, sex, current medications, disease duration, history of extra-articular features [i.e. psoriasis, inflammatory bowel disease (IBD), uveitis], X-rays of sacroiliac joints were read by an expert musculoskeletal radiologist blinded to patient details. Disease activity in affected individuals was recorded using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [23], functional impairment using the Bath Ankylosing Spondylitis Functional Activity Index (BASFI) [24] and quality of life by the Health Assessment Questionnaire (HAQ) [25]. Patients were typed for HLA-B27. The study was approved by the ethics committee of the University Health Network and all subjects signed written informed consent.

There were two control groups. Control group A consisted of 39 unaffected family members and control group B consisted of 34 normal healthy individuals. Group A was defined as unaffected by the following criteria: no history of inflammatory back pain, and no radiographic changes consistent with sacroiliitis for which a history suggestive of inflammatory back pain was elicited. Unaffected family members were chosen as controls for shared environmental influences in the respective families. Group B was a cohort of 34 individuals who were matched for age, sex and ethnicity with the family members. These normal subjects were not subjected to a physical examination, but a detailed history was taken, and any individual with a personal or family history of arthritis was excluded.

Lymphocyte stimulation. For each patient, peripheral blood was collected into sterile heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient (Pharmacia, Uppsala, Sweden), washed three times in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (Gibco-BRL, Rockville, MD, USA) containing 10% heat-inactivated fetal calf serum (Gibco-BRL). The number of viable lymphoid cells used for the stimulation studies was 0.5 × 10⁶ cells/ml. Lymphocytes were cultured in 96-well flat-bottom tissue culture plates. The candidate microbe in question, K. pneumoniae serotype K-43, was obtained from Dr A. Geczy (Melbourne, Australia). As control microbes, Chlamydia trachomatis (L2 strain), Salmonella typhimurium and Yersinia enterocolitica were used. The last two were clinical isolates from patients with reactive arthritis. Bacteria were cultured under routine conditions then heat-killed, and the protein concentration was determined. The microbial antigens were added at concentrations of 1, 5 and 10 μg/ml for each respective organism. Phytohaemagglutinin (PHA) was included as a mitogen control at 1 μg/ml. All determinations were performed in triplicate. The plates were incubated at 37°C in 5% CO₂ for 4 days, then the lymphocytes were pulsed with ³H-thymidine (Amersham, Amersham, UK) at 1 μCi/well. After 18 h of incubation the cells were harvested onto glass-fibre filters and the incorporated radioactivity was determined in a liquid scintillation counter (Beckman LS-800). Results are expressed in counts per minute (c.p.m.) and presented using the stimulation index (SI), which is defined as the ratio of c.p.m. in response to candidate microbes to c.p.m. induced with medium alone.

ELISA studies

The enzyme-linked immunosorbent assay (ELISA) studies were carried out in duplicate as published by our laboratory [26]. The heat-killed organisms outlined above were coated onto ELISA plates (Costar-Fisher Scientific, Nepean, Ontario, Canada) at 1, 5 and 10 μg/ml and kept at 4°C with 5% CO₂. The plates were then washed six times with PBS, 0.2 ml of blocking solution (PBS and 1% bovine serum albumin) was added and the plates were incubated at room temperature for 1 h. The blocking solution was then discarded. Test serum (0.2 ml of 1:100 dilution) was added to each well in triplicate and the plates were incubated at 37°C for 1.5 h. The plates were then washed six times with PBS containing 0.05% Tween 20. Horseradish peroxidase-conjugated secondary antibody (Fab₂, Serotec, Raleigh, NC, USA) was added at a dilution of 1:10000 (0.2 ml per well) and the plates were incubated at 37°C for 1.5 h. They were then washed six times and 0.2 ml of the substrate o-phenylenediamine was added to develop the plates. The optical density (OD) at 490 nm was determined with an ELISA reader.

Statistical analysis

Comparison of immune responses in affected and unaffected individuals. The cellular immune responses of affected and unaffected family members for each of the candidate organisms at each concentration (1, 5 and 10 μg/dl) were compared using a two-sample t-test. In the primary analysis, no threshold was set for the SI, so that small differences were not overlooked, but for secondary analysis a threshold was set at an SI of ≥3, as has been used in other studies, so that small differences might not be overinterpreted [27]. A similar analysis was performed for the humoral immune responses, comparing affected and unaffected family members and normal healthy controls.

Defining the predominant immune response. Next, each patient was classified by the predominant antigenic immune response elicited by one of the four candidate microbial antigens. For the proliferation analysis, this was obtained by comparing the SI for each patient in response to the same concentration of each of the four organisms (10 μg/dl). The predominant response was thus defined as the microbial antigen (of the four examined) that induced the maximal stimulation index. Thus, the predominant microbial response for each sample was mutually exclusive.
There was an *a priori* proviso that the maximal stimulation index must exceed the next highest by ≥5%. In cases where there was < 5% difference between the stimulation indices, a predominant microbial response was not assigned to that cell sample and the immune profile was defined as indeterminate. The analysis was repeated, as described above, after a threshold had been set for the SI of >3, so that small differences might not be overinterpreted. The predominant immune response was determined similarly for each sample for both T-cell proliferation analysis and antibody studies. However, in the case of the predominant antibody response, the maximal OD was selected from amongst the four microbial antigens (concentration 10 μg/dl). The percentage of affected and unaffected individuals who exhibited a predominant immune response for each of the four candidate organisms was recorded and the analysis described below was based on this.

**Analysis based on the predominant immune response.** The χ² test was used to detect differences between the percentages of affected individuals who exhibited a predominant T-cell immune response to *K. pneumoniae* and the percentage of unaffected individuals who exhibited a predominant T-cell immune response to this microbe. Fisher’s exact test was used where the frequency in a particular cell was < 5. A similar analysis was performed to detect a difference in predominant T-cell response between affected and unaffected individuals for each of the control microbes (*C. trachomatis*, *Y. enterocolitica* and *S. typhimurium*). The odds ratio (adjusted for family) with 95% confidence intervals (CI) was reported for the likelihood of affected status according to predominant selection for one of the candidate microbes.

**Associations between clinical characteristics and immune responses.** To explore any possible associations with clinical variables and a particular antimicrobial immune response, two separate analyses were performed. The first analysis included all AS patients and the second included only patients having primary AS. Primary AS was defined as AS in the absence of coexisting extra-articular features, such as IBD, psoriasis or reactive arthritis. Parametric (two-sample t-test) and non-parametric tests (Wilcoxon rank sum test and Kruskal–Wallis test) were used to compare clinical variables in patients stratified by the predominant antigenic T-cell proliferative immune response. Finally, multiple logistic regression was performed for each of the four candidate microbes to determine if affected status could be predicted by the predominant antigenic T-cell proliferative immune response. The regression model was adjusted for age, sex and HLA-B27 status. The model was fitted manually.

**Results**

**Patient demographics**

There were 25 multiplex families, with a total of 96 individuals: 57 affected and 39 unaffected. The mean number of individuals studied per family was 4 (range 2–12). In the affected individuals, the mean age was 39.4 ± 14.65 yr compared with 49.6 ± 20.5 in the unaffected individuals (*P* = 0.004). As expected, the majority was male; the male:female ratio was 38:19, in contrast to 16:23 in the unaffected group (*P* = 0.01). The mean disease duration was 12.3 ± 11.74 yr. Ninety-three per cent of affected individuals were positive for HLA-B27 compared with 60% of unaffected individuals (*P* = 0.0004). Affected individuals as a group had relatively mild disease activity, functional impairment and disability, as shown by a mean BASDAI of 4.2 ± 2.3, a mean BASFI of 3.88 ± 2.54 and a mean HAQ of 0.74 ± 0.64 respectively. Forty-one AS patients were being treated with non-steroidal anti-inflammatory drugs (NSAIDs) and 14 with disease-modifying anti-rheumatic drugs (DMARDs). On physical examination, 14 patients had extensive axial involvement and 11 had no clinical evidence of axial involvement (apart from sacroiliitis). The remaining 32 patients had a limited range of motion in the back. Forty-five patients had primary AS, whereas 12 had evidence of a coexisting condition.

**Cellular immune response**

Eight patients had an indeterminate T-cell response; therefore in the final analysis results are presented on 54 affected and 34 unaffected family members. There was no difference between affected and unaffected individuals either in mean baseline proliferation (affected, 2051 ± 2682 c.p.m.; unaffected 2315 ± 5748 c.p.m.; *P* = 0.3) or in proliferation on stimulation with PHA (affected, 86 792 ± 49 824 c.p.m.; unaffected, 99 097 ± 68 604 c.p.m.; *P* = 0.6). There was no difference between affected and unaffected individuals when actual stimulation indices were compared for any of the candidate microbial antigens (Table 1, Fig. 1).

### Table 1. Comparison of antigenic T-cell proliferative responses of affected and unaffected family members for each concentration of the candidate organisms with and without a set threshold of >3 for the stimulation index

<table>
<thead>
<tr>
<th>Candidate organism and concentration (μg/dl)</th>
<th>Affected: mean ± s.d. (s.e.m.)</th>
<th>Unaffected: mean ± s.d. (s.e.m.)</th>
<th>Affected (SI &gt; 3): mean ± s.d. (s.e.m.)</th>
<th>Unaffected (SI &gt; 3): mean ± s.d. (s.e.m.)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td></td>
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<tr>
<td>1</td>
<td>5.7 ± 5.9 (0.8)</td>
<td>8.0 ± 12.9 (2.2)</td>
<td>0.35</td>
<td>9.3 ± 7.7 (1.4)</td>
<td>12.7 ± 12.7 (3.1)</td>
</tr>
<tr>
<td>5</td>
<td>5.3 ± 5.6 (0.7)</td>
<td>7.6 ± 11.1 (1.1)</td>
<td>0.27</td>
<td>8.4 ± 6.0 (1.1)</td>
<td>11.5 ± 12.6 (2.8)</td>
</tr>
<tr>
<td>10</td>
<td>5.4 ± 6.7 (0.9)</td>
<td>7.2 ± 10.7 (1.9)</td>
<td>0.41</td>
<td>8.9 ± 5.9 (1.0)</td>
<td>11.8 ± 15.1 (3.3)</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
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</tr>
<tr>
<td>1</td>
<td>4.5 ± 5.2 (0.7)</td>
<td>4.9 ± 5.8 (1.0)</td>
<td>0.73</td>
<td>7.6 ± 6.2 (1.2)</td>
<td>8.1 ± 5.6 (1.4)</td>
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<tr>
<td>5</td>
<td>4.9 ± 5.4 (0.7)</td>
<td>6.1 ± 6.5 (1.1)</td>
<td>0.33</td>
<td>8.4 ± 5.8 (1.1)</td>
<td>8.81 ± 6.5 (1.6)</td>
</tr>
<tr>
<td>10</td>
<td>4.8 ± 5.4 (0.7)</td>
<td>6.2 ± 6.5 (0.9)</td>
<td>0.33</td>
<td>8.5 ± 5.7 (1.0)</td>
<td>10.6 ± 6.9 (1.7)</td>
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<tr>
<td><em>C. trachomatis</em></td>
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</tr>
<tr>
<td>1</td>
<td>6.4 ± 5.2 (1.1)</td>
<td>5.2 ± 7.2 (1.2)</td>
<td>0.50</td>
<td>11.03 ± 8.7 (2.2)</td>
<td>9.6 ± 8.7 (2.3)</td>
</tr>
<tr>
<td>5</td>
<td>5.2 ± 7.9 (1.0)</td>
<td>5.2 ± 6.7 (1.1)</td>
<td>0.99</td>
<td>10.01 ± 9.8 (1.9)</td>
<td>9.3 ± 7.8 (1.9)</td>
</tr>
<tr>
<td>10</td>
<td>5.5 ± 7.7 (1.0)</td>
<td>6.0 ± 7.7 (1.4)</td>
<td>0.88</td>
<td>10.2 ± 8.9 (1.6)</td>
<td>9.7 ± 8.7 (2.0)</td>
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<tr>
<td><em>Y. enterocolitica</em></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.7 ± 9.9 (1.3)</td>
<td>5.2 ± 7.2 (1.2)</td>
<td>0.50</td>
<td>10.5 ± 14.9 (3.2)</td>
<td>10.5 ± 9.5 (2.6)</td>
</tr>
<tr>
<td>5</td>
<td>3.9 ± 5.0 (0.7)</td>
<td>5.7 ± 8.7 (1.6)</td>
<td>0.21</td>
<td>6.7 ± 5.9 (1.1)</td>
<td>10.3 ± 10.7 (2.6)</td>
</tr>
<tr>
<td>10</td>
<td>4.1 ± 5.2 (0.7)</td>
<td>5.8 ± 7.5 (1.3)</td>
<td>0.21</td>
<td>7.6 ± 6.12 (1.1)</td>
<td>10.3 ± 8.5 (2.1)</td>
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</tbody>
</table>
With respect to the predominant T-cell immune response, the percentages of patients responding maximally to the respective microbial antigens were as follows. Among affected individuals, 25.9% responded maximally to *S. typhimurium*, 11.1% to *Y. enterocolitica*, 38.9% to *C. trachomatis*, and 24.1% to *K. pneumoniae*. Corresponding figures for unaffected individuals were 5.9, 23.5, 29.4, and 41.2%. There was no difference between affected and unaffected individuals in terms of the predominant response with respect to *K. pneumoniae*, *C. trachomatis* or *Y. enterocolitica*. However, there was a significant difference between affected and unaffected family members, indicating a predominant response to *S. typhimurium* in affected individuals \( (P < 0.02) \) (Table 2A). Using a threshold of 3 in the stimulation index for the analysis, the difference between affected and unaffected individuals was even more pronounced with respect to *S. typhimurium* \( (P < 0.002) \) (Table 2B), but no difference was noted between affected and unaffected individuals for any of the other organisms.

Using the predominant microbial response, we determined the odds ratio and 95% CI for each of the candidate antigens being associated with AS (Table 2A, B). The odds ratios for this analysis were 5.6 for *S. typhimurium* (95% CI 1.1, 53.5), 0.41 for *Y. enterocolitica* (95% CI 0.1, 1.5), 1.15 for *C. trachomatis* (95% CI 0.6, 4.3) and 0.5 for *K. pneumoniae* (95% CI 0.2, 1.3). Applying the cut-off of 3 for the stimulation index, the odds ratio for *K. pneumoniae* declined further to 0.3 (95% CI 0.1, 1.1).

**Humoral immune response**

There was no difference in humoral immune responses between affected and unaffected family members for any of the four candidate antigens. Neither was there a difference in responses between unaffected family members and healthy controls. However, affected family members exhibited a greater immunoglobulin (Ig) G immune response to all four microbes compared with normal healthy controls (Fig. 2).

There was no difference between affected and unaffected individuals with respect to the predominant antibody response to the four candidate microbes. This was true for both IgG and IgA (Table 3A). Interestingly, the majority of individuals in both groups responded predominantly to *K. pneumoniae*, in both the IgG ELISA and the IgA ELISA. In the IgA ELISA, 66.7% of affected and 70.2% of unaffected individuals responded predominantly to *K. pneumoniae* \( (P = 0.82) \). The odds ratio for affected status depending on predominant pathogen reactivity was lowest for *K. pneumoniae* for IgG (0.4; 95% CI 0.1, 1.1).
The maximal SI was defined as the microbial antigen (of the four examined) that induced a predominant immune response to one of the three control organisms. Furthermore, when the affected individuals were analysed, there was no difference in predominant cellular or humoral immune responses between primary AS and secondary AS (data not shown). Finally, there was no difference in immune responses in HLA-B27-positive affected and unaffected individuals.

### Discussion

In this study of familial AS we have demonstrated that there is no significant difference between affected and unaffected individuals with respect to either cellular or humoral immune responses to *K. pneumoniae*. In addition, when comparison was made with three other candidate microbial organisms, *K. pneumoniae* did not exhibit a predominant cellular or humoral immune response in affected individuals. Finally, we were unable to demonstrate any associations between clinical characteristics and immune responses. While this is the largest study to date to provide a systematic analysis of familial AS, it is possible that our sample size may not have been large enough to have the power to detect a difference.

As outlined above there has been much debate in the literature over the possible aetiopathogenic link between AS and *K. pneumoniae* [1, 8, 9, 11, 12, 28, 29]. Controversy persists as investigators have continued to report elevated concentrations of antibodies to this organism in the serum of patients with AS [11, 15, 30]. Most studies addressing microbial pathogenesis in AS have used only serum antibody profiles to implicate potential pathogens. There are far fewer studies evaluating T-cell responses to candidate organisms in AS, yet this may represent a more comprehensive approach to detecting an immune fingerprint of a recent or remote infection in an individual with AS.

This is the first study that compares affected with unaffected family members to address these issues in evaluating the role of *K. pneumoniae* in the pathogenesis of AS. Since one of the unique features of our study was the familial case-control design, we addressed whether there was any clustering of specific antimicrobial immune responses within families, but we saw none. This analysis was somewhat limited by the sample size. With that caveat, adjusting our analysis for family did not alter our results. It is interesting to note that our study showed that the majority of affected and unaffected family members responded predominantly to *K. pneumoniae* in the humoral studies. However, there was no difference between affected and unaffected individuals, which suggests that this may be an in vitro laboratory phenomenon related to the affinity of the antigen with the test plate. However, affected individuals exhibited a greater IgG response compared with normal healthy controls. This highlights the importance of careful selection of environmental controls, such as the unaffected family members used in this study. In addition, this observation may shed some light on the elevated serum anti-*Klebsiella* antibodies reported in previous studies in which affected individuals were compared with normal healthy controls as opposed to family members. Thus, while we confirm the results of prior investigations showing that there appears to be a heightened serum antibody pattern against *K. pneumoniae*, we find no specificity for AS. This speaks against a pathogenic role for this organism in AS.

The finding that *S. typhimurium* exhibited a predominant cellular immune response in affected individuals when compared with the remaining candidate microbes warrants further mention. It is unlikely that this finding provides substantive evidence of a causal role for *S. typhimurium* as no difference was observed for actual stimulation indices between affected and unaffected family members ($P = 0.80$). Furthermore, the results of the logistic
regression analysis suggest that age or sex may have been confounders in the relationship between S. typhimurium and outcome (affected status), and this may have accounted for the difference observed between affected and unaffected individuals for the cellular immune responses. This also highlights the importance of careful consideration of clinical covariates in the interpretation of the cellular immune responses in these patients. Whether a disease process that began as a post-Salmonella reactive

**FIG. 2.** Box plots illustrating the comparison of IgG (a) and IgA (b) humoral immune response in affected (A) and unaffected (U) family members and normal healthy controls (C) to K. pneumoniae (10μg/dl). OD, optical density units; NS, not significant.

**TABLE 3A.** Comparison of predominant humoral (IgG and IgA) immune responses between affected and unaffected family members

<table>
<thead>
<tr>
<th>Candidate microbe</th>
<th>Unaffected (n=39)</th>
<th>Affected (n=57)</th>
<th>2×2 table; Fisher’s exact test</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgG</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>S. typhimurium</td>
<td>5 (12.8)</td>
<td>9 (15.8)</td>
<td>0.77</td>
<td>1.3 (0.4, 5.3)</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>2 (5.1)</td>
<td>8 (14.0)</td>
<td>0.19</td>
<td>3.0 (0.6, 30.5)</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>1 (2.6)</td>
<td>5 (8.8)</td>
<td>0.39</td>
<td>3.6 (0.4, 177.3)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>31 (79.5)</td>
<td>35 (61.4)</td>
<td>0.07</td>
<td>0.4 (0.1, 1.1)</td>
</tr>
<tr>
<td><strong>IgA</strong></td>
<td></td>
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</tr>
<tr>
<td>S. typhimurium</td>
<td>5 (12.8)</td>
<td>9 (15.8)</td>
<td>0.26</td>
<td>0.4 (0.1, 2.2)</td>
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<td>Y. enterocolitica</td>
<td>3 (7.7)</td>
<td>10 (17.5)</td>
<td>0.23</td>
<td>2.6 (0.6, 15.7)</td>
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<tr>
<td>C. trachomatis</td>
<td>5 (12.8)</td>
<td>4 (7.0)</td>
<td>0.48</td>
<td>0.5 (0.1, 2.6)</td>
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<td>K. pneumoniae</td>
<td>26 (66.7)</td>
<td>40 (70.2)</td>
<td>0.82</td>
<td>1.2 (0.4, 3.1)</td>
</tr>
</tbody>
</table>

Overall 4×2 table P=0.22 for IgG, P=0.25 for IgA.

**TABLE 3B.** Comparison of predominant humoral (IgG and IgA) immune responses for unaffected family members and normal healthy controls

<table>
<thead>
<tr>
<th>Candidate microbe</th>
<th>Unaffected (n=39)</th>
<th>Controls (n=33)</th>
<th>2×2 table; Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgG</strong></td>
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<tr>
<td>S. typhimurium</td>
<td>5 (12.8)</td>
<td>5 (15.2)</td>
<td>1</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>2 (5.1)</td>
<td>3 (9.1)</td>
<td>0.66</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>1 (2.6)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>31 (79.5)</td>
<td>25 (75.8)</td>
<td>0.21</td>
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<td><strong>IgA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>5 (12.8)</td>
<td>3 (9.1)</td>
<td>0.72</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>3 (7.7)</td>
<td>5 (15.1)</td>
<td>0.46</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td>5 (12.8)</td>
<td>1 (3.0)</td>
<td>0.21</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>26 (66.7)</td>
<td>24 (72.7)</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Overall 4×2 table P=0.72 for IgG and P=0.35 for IgA.

Klebsiella and ankylosing spondylitis
arthriti
can evolve over time into a clinical picture that meets the defini
tion of AS is an issue that has been raised in the literature
before, but the frequency of this event is unknown.

There are relatively few studies addressing T-cell responses to
Klebsiella antigens in the literature. Dominguez-Lopez et al. [27]
evaluated T-cell responses in a small cohort of 13 AS patients. Using
PBMC, seven of 13 patients with AS and some patients with
RA, but no healthy controls, demonstrated a significant cellular
immune response, defined in this study as SI > 3, to a GroEL-like
protein (HSP60) of K. pneumoniae. However, numbers were small
in this study and the antigen preparation used was different from
that used in our study. Furthermore, the key control group was
rheumatoid arthritis, whereas in our study the unaffected family
members served as controls. Interestingly, these same authors also
showed that HLA-B27-positive individuals recognized not only
the GroEL-like HSP protein from K. pneumoniae but GroEL-like
proteins from other organisms, such as Mycobacterium [12]. This
suggests that the humoral immune response they have detected in
AS may be related to HSP from a range of microbes, but with no
specificity for K. pneumoniae.

In the only prior study addressing cellular immune responses in
familial AS, Hohler et al. [31] reported that there was no difference
in T-cell responses between affected and unaffected twins in a
study of 11 monozygotic twins. Affected twins, however, had a
decrease in the calculated frequency of K. pneumoniae-responsive
T cells in peripheral blood lymphocytes (PBL) compared with
healthy B27+ subjects. It was speculated that this quantitative
reduction in specific T cells might reflect a defective host defence
against Klebsiella, thereby allowing bacterial antigens to reach the
joint. In this study also, various antigen preparations of K.

pneumoniae were used for ELISA antibody studies; there were no
differences between affected and unaffected for K21, but there was
a lower response to K43 in affected subjects. In our study we used
K43 as the antigen preparation as it has been the antigen used
most consistently in prior studies implicating K. pneumoniae in
the pathogenesis of AS [9, 13]. Interestingly, the cellular immune
responses were lower in affected than in unaffected family
members, though the difference was not significant.

Hermann et al. [32] also reported a quantitative reduction in K.

pneumoniae-responsive T cells in the PBL of AS patients compared
with healthy controls. Because of the suggestion in this study that
PBMC responses to K. pneumoniae may be depressed in comparison
with concurrent synovial fluid mononuclear cell responses (SFMC), we analysed paired samples PBMC and SFMC in four patients with AS. We found there was no significant
difference in the pattern of peripheral blood and synovial fluid lymphocyte responses to K. pneumoniae in these patients (data not shown). However, the methods used in the studies by

Hermann et al. differed from those used in our studies and this
may account in part for the some of the differences in results.

In conclusion, our study is the largest to date to examine
cellular and humoral immune responses to K. pneumoniae in
affected vs unaffected individuals in familial AS. We did not find
evidence that affected individuals exhibit a dominant immune
response to K. pneumoniae in familial AS. Thus we find no
supportive evidence supporting a causal role for K. pneumoniae
in familial AS.

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**Conflict of interest**

The authors have declared no conflicts of interest.

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