Molecular mimicry and ankylosing spondylitis: possible role of a novel sequence in pullulanase of *Klebsiella pneumoniae*

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Abstract **Molecular mimicry has been shown between two sequences of *Klebsiella pneumoniae* pulD secretion protein (DRDE) with HLA-B27 (DRED) and pulA (pullulanase) enzyme (Gly-X-Pro) with types I, III and IV collagen respectively. IgG antibody levels in AS patients were elevated against 16mer synthetic peptides of HLA-B27 and pulD by enzyme linked immunosorbent assay (ELISA) compared to controls ($P < 0.001$). ELISA assays against *K. pneumoniae* grown in the absence and presence of pullulan demonstrated significant levels of IgA antibody in AS patients compared to controls ($P < 0.001$). Increased IgA and IgG antibody levels to pulA and types I and IV collagen were observed in AS patients compared to controls ($P < 0.001$). These observations could be relevant in the sequence of molecular events in AS.**

Key words: Ankylosing spondylitis; Pullulanase; Novel sequence in pullulanase; Molecular mimicry; *Klebsiella pneumoniae*

1. Introduction

Ankylosing spondylitis (AS) is chronic inflammatory rheumatic disorder particularly affecting the axial skeleton. A link between AS and the major histocompatibility antigen HLA-B27 has been well established [1]: over 95% of patients with AS are HLA-B27 positive [2,3]. There are seven subtypes of HLA-B27 ranging from B*2701 to B*2707. It has been suggested that B*2705 is the most common HLA-B27 subtype in Caucasian populations, whilst it is proposed that B*2703 is the most common B27 antigen in black populations, where AS is rare. This observation has led to the suggestion that B*2703 is not associated with AS [4]. Subtypes of HLA-B27, other than B*2705, containing different amino acid sequences are also involved in AS. In this paper the HLA-B27 subtype under examination is B*2705. The cause of AS remains controversial, although an environmental agent in the form of the gram negative bacterium, *Klebsiella pneumoniae*, present in the gut, has been suggested as a possible aetiological agent [5] to which antibodies have been shown in several centres [6]. Any aetiological agent implicated in AS must provide an explanation for the link with HLA-B27. Schwimmbeck et al. [7] identified an amino acid homology, QTDRED found in residues 72–77 of B*2705 and residues 188–193 of *K. pneumoniae* nitrogenase enzyme. The validity of this homology, as a cross reactive antigen, has however, been criticised, in both biochemical and immunological terms. For nitrogenase production, an extremely low level of fixed nitrogen is required [8] and this condition is not achieved in the human gut. Additionally, extremely anaerobic conditions are required for nitrogenase production in *Klebsiella* [8], and whilst the human gut has a low level of oxygen, debate persists as to whether this degree of anaerobiosis is sufficient to allow nitrogenase production. Furthermore, HLA-B27 is a self protein and therefore should not trigger an immune response. The part of *K. pneumoniae* nitrogenase that contains the sequence QTDRED should been seen as self by the immune system, and therefore should not evoke an immune response. These criticisms have led to a re-evaluation of the relevance of *K. pneumoniae* nitrogenase in AS and initiated the search for different sequence homologies between *K. pneumoniae* proteins and B*2705. In this study a computer search has revealed a novel homology between B*2705 and another component of *K. pneumoniae* in the form of the secretion protein (pulD) of the inducible, starch debranching enzyme pullulanase. Additionally, amino acid homology has been described between the extracellular starch induced enzyme pullulanase (pulA) and types I, III and IV collagen [9]. In the present study the effect of the induction of the pullulanase system on serum antibody in AS patients has been studied using bacteria grown in the presence or absence of the starch substrate pullulan. Furthermore, the levels of cross reactive antibodies between B*2705 and pulD synthetic peptides, pulA and two types of collagen (I and IV), have been measured in order to demonstrate their relevance in the development of the disease.

2. Materials and methods

2.1. Computer search

The BLAST database searching program was used to search the PIR-Protein database (V.33.0) containing 42,215 sequences. The sequence QTDRED from HLA-B27 and *K. pneumoniae* were compared with all the sequences in the database allowing for mismatches.

2.2. Patients

Sera were collected from 97 active AS patients (New York criteria) (erythrocyte sedimentation rate (ESR) > 15 mm/h, all HLA-B27 positive) attending the AS Research Clinic at the Middlesex Hospital, and 25 active rheumatoid arthritis (RA) patients (ARA criteria) attending the Rheumatology Department at the Lister Hospital, Stevenage.

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Herts. The London Blood Transfusion Service provided 25 healthy control sera. All sera were stored at −20°C until required.

2.3. Peptide synthesis and ELISA study

The synthetic peptides were prepared by solid phase synthesis, purity assays were performed by high performance liquid chromatography. All peptides had a purity of at least 90%. Three 16mer peptides were constructed: the B*2705 sequence (residues 67–83) CKAKAQTDRED-LRTLL, the pulD secretion protein sequence (residues 590–605) RPTVIRDRDEYRQASS, and a control peptide sequence made from a scrambled sequence of the pulD peptide, RPTVRSDIDYRQAESR. Sera were tested against the three peptides by non competitive enzyme linked immunosorbent assay (ELISA). The assay was carried out as follows: polystyrene microtitre plates (Dynatech) were coated with the synthetic peptide (5.0 μg/well) overnight at 4°C. After absorption and washing with PBS-Tween, the plates were saturated with 2% casein-PBS-Tween, incubated for 1 h at 37°C, followed by washing with PBS-Tween. Serum samples (200 μl at 1/50 dilution in PBS-Tween) were added to the plates, incubated for 90 min at room temperature, followed by washing with PBS-Tween and peroxidase conjugated rabbit anti-human class specific IgG, IgA or IgM (Dako. Ltd) diluted 1/500 in PBS-Tween added and plates incubated for 90 min at room temperature. After washing and enzyme reaction with substrate 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) (Sigma Chemical Co. Ltd.) at room temperature for 20 min, the reaction was stopped with sodium fluoride and the OD measured at wavelength of 630 nm.

2.4. Organisms and growth conditions

Proteus mirabilis was obtained from the Department of Microbiology, King's College whilst the *Klebsiella pneumoniae* used was a clinical isolate kindly provided by the Medical Microbiology Department, Princess Royal Hospital, Haywards Heath. The cultures were grown aerobically in 250 ml conical flasks on an orbital shaker for 16–18 h in minimal media supplemented with 0.005% yeast extract (Difco) [10]. If induction of the pullulanase enzyme system was required the starch substrate pullulan (0.3% w/v) (Sigma) was added to the minimal medium. The cells were harvested by centrifugation (MSE 18; 6 × 250 ml rotor) and washed in 0.15 M phosphate buffered saline (PBS). The suspension was then centrifuged as before. This procedure was repeated 3 times. After washing, the cells were resuspended in 20 ml of PBS and a stock solution was prepared to give an OD reading of 0.25 on the Spectrophotometer (Corning Model 258).

2.5. ELISA studies on the effect of antibody binding to bacteria grown in minimal media in the presence of pullulan

Aliquots of 200 μl of the diluted suspension of bacteria grown on minimal media plus 0.005% yeast extract (Difco) were adsorbed onto 96 well ELISA plates (Dynatech) overnight at 4°C. The ELISA procedure was carried out as described previously, with the exception of the application of the HRP-conjugated second antibody. In this assay, when *K. pneumoniae* was used as the antigen the HRP-conjugated second antibody was of the IgA class, whilst when *Proteus mirabilis* was the antigen the HRP-conjugated antibody was of the IgG class. This experiment was repeated with a suspension of bacteria grown on minimal media plus 0.005% yeast extract (Difco) supplemented with pullulan (Sigma) (0.3% w/v).

2.6. ELISA studies on pullulanase (pulA)

Aliquots (200 μl) of a solution of pullulanase (Sigma) (2 μg/well) were adsorbed onto 96-well ELISA plates (Dynatech) overnight at 4°Ç. The ELISA procedure was carried out as described previously, however only IgA and IgG classes of the HRP-conjugated second antibody were used.

2.7. ELISA studies on collagen types I and IV

Solutions of collagen type I or type IV (Sigma) (3 μg/ml) were adsorbed onto 96-well ELISA plates (Dynatech) overnight at 4°C in 200 μl volumes. The ELISA plate was washed and satured with 2% casein-PBS-Tween described previously, however incubation for 1 h was performed at room temperature. Additionally, only IgA and IgG classes of the HRP-conjugated second antibody were used in this assay.

3. Results

*3.1. Amino acid sequence homology between B*2705 and* Klebsiella pneumoniae

The computer search revealed a number of sequence homologies between B*2705 and various microorganisms including the nitrogenase protein of *K. pneumoniae* as had been reported previously by Schwimmbeck et al. [7]. The mismatch function revealed the presence of another sequence in *K. pneumoniae* that showed homology with the QTDRED region in B*2705. This sequence was a tetramer present in the pullulanase secretion protein pulD (residues 596–599) of a starch debranching enzyme present in *K. pneumoniae* (Table 1). The pulD sequence was the second amino acid homology to be identified between the hypervariable domain of B*2705 and the gram negative bacteria *K. pneumoniae*.

3.2. Peptide ELISA study

ELISA assays performed on the B*2705 synthetic peptide showed significant antibody elevation in AS patients in IgG ($P < 0.001$) antibody class, when compared to control subjects but no significant elevation in IgA or IgM antibodies. Furthermore, in the RA patients no significant elevation of IgA, IgM or IgG classes were detected when compared to controls (Fig. 1A).

Assays performed on the pulD peptide demonstrated signif-

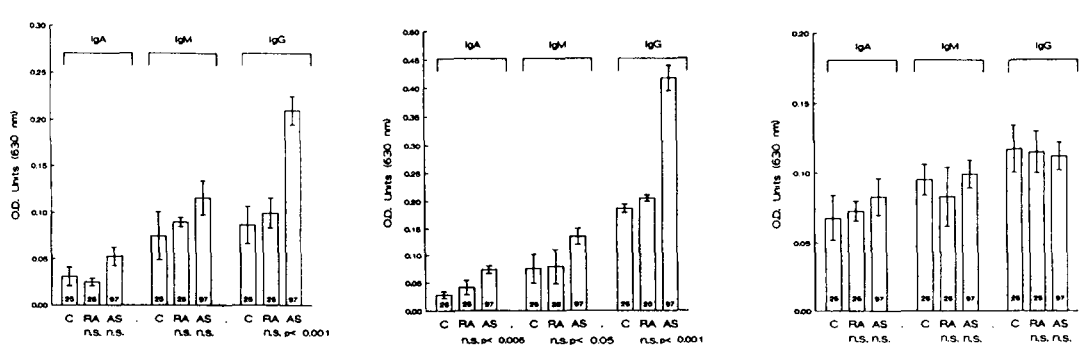

Fig. 1. Antibody titres (mean ± S.E.M.) for IgA, IgM and IgG in 25 controls (C), 25 rheumatoid arthritis (RA) patients and 97 ankylosing spondylitis (AS) patients when tested by ELISA against 16mer peptides of HLA-B27 (A), pulD (B) and control peptide (scrambled pulD) (C). OD = optical density; n.s. = not significant.

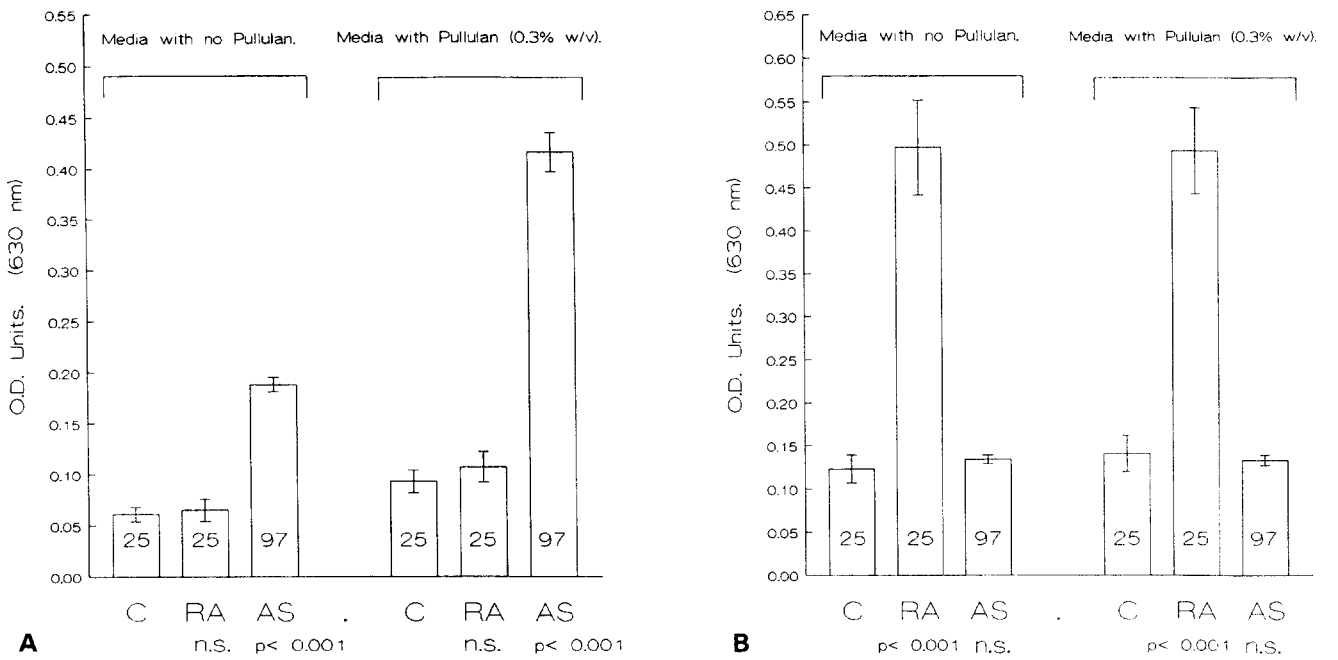

Fig. 2. Antibody titres (mean ± S.E.M.) for IgA in 25 controls (C), 25 rheumatoid arthritis (RA) patients and 97 ankylosing spondylitis (AS) patients when tested by ELISA against a clinical isolate of *Klebsiella pneumoniae* (A) and of *Proteus mirabilis* (B) grown in the absence and the presence of pullulan. OD = optical density; n.s. = not significant.

icant antibody elevations in AS patients, in all three antibody classes, IgA ($P < 0.005$), IgM ($P < 0.050$) and IgG ($P < 0.001$) when compared to controls. The RA patients showed no significant antibody elevations in IgA, IgM or IgG classes with respect to control subjects (Fig. 1B).

ELISA studies on the control peptide demonstrated no significant elevations with IgA, IgM or IgG antibody classes in RA or AS patients when compared to control subjects (Fig. 1C).

3.3. *Effect of bacterial growth conditions with respect to antibody binding*

Determination of antibody levels in AS patients to *K. pneumoniae* grown in minimal media with yeast extract showed elevation in IgA antibody titres ($P < 0.001$) when compared to controls (Fig. 2A). This observation confirms previous reports of elevation of the IgA response to *K. pneumoniae* in AS patients [6]. When the experiment is repeated with the addition of the starch component, pullulan (Sigma) to the medium (0.3% w/v) the level of IgA binding in AS patients was again elevated ($P < 0.001$) when compared to control subjects (Fig. 2A). This increase in the level of antibody binding is likely to be due to the development of capsular polysaccharide on the cell surface of the bacteria.

The assay was repeated using another gram negative enteric organism, *Proteus mirabilis*, grown under exactly the same conditions as *K. pneumoniae* on minimal media in the absence and presence of pullulan. Measurement of the IgG response was performed by the use of an ELISA assay. Significant and specific antibody elevations were observed in the RA patients, confirming previous observations [11]. The antibody elevations were observed in the RA patients regardless of the presence ($P < 0.001$) (Fig. 2B) or absence ($P < 0.001$) (Fig. 2B) of pullulan when compared to controls. Furthermore the levels of antibody titres in the AS, RA or control group did not appear to be affected by the presence or absence of pullulan in the growth media of *P. mirabilis*.

Table 1
Comparison of amino acid sequence homologies between HLA-B27 and proteins from *Klebsiella pneumoniae*

Protein	Residues	Amino acid sequence
HLA-B27	67–82	CKAKAQT**DRED**LRTLL
Klebsiella pneumoniae nitrogenase	184–199	CNSRQT**DRED**ELIGGC
Klebsiella pneumoniae pulD secretion protein	590–605	RPTVIR**DRDE**YRQASS

Regions of homology between HLA-B27, *K. pneumoniae* nitrogenase enzyme and *K. pneumoniae* pulD secretion protein are underlined.

3.4. *Determination of the level of serum antibodies to the pullulanase (pulA) enzyme*

Titres of both IgA and IgG antibody classes were determined by ELISA to a pullulanase target. AS patients showed titres of IgA ($P < 0.001$) in comparison to control subjects. The level of the IgG response showed similar elevations ($P < 0.001$) when compared to controls. However, the RA patients showed no significant elevation in IgA or IgG response with respect to the control group (Fig. 3).

3.5. *ELISA studies on type I and type IV collagen*

IgA elevations to both type I ($P < 0.001$) and type IV ($P < 0.001$) collagen was observed in AS patients in comparison to controls (Fig. 4A). IgG responses to collagen type I

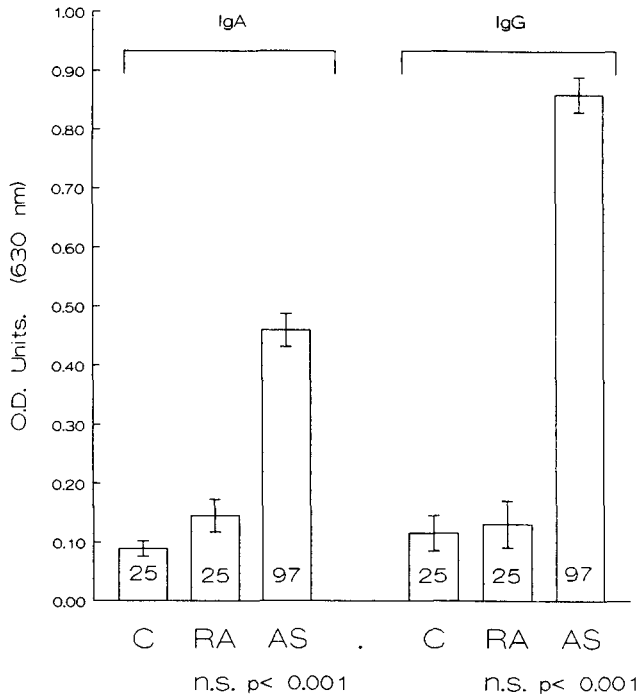

Fig. 3. Titres of IgA and IgG antibody (mean ± S.E.M.) in 25 controls (C), 25 rheumatoid arthritis (RA) patients and 97 ankylosing spondylitis (AS) patients when tested by ELISA against pullulanase enzyme (pulA). OD = optical density; n.s. = not significant.

($P < 0.001$) and type IV ($P < 0.001$) were elevated with respect to controls (Fig. 4B). The RA patients showed no significant IgA or IgG antibody elevations to type I or type IV collagen when compared to controls (Fig. 4A and B).

4. Discussion

Antibody elevations to *Klebsiella pneumoniae* in AS patients were first reported in 1983 [5] and those original observations have since been confirmed in various centres [6]. Furthermore, antibodies to other gram negative organisms such as *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella* spp, *Shigella* spp, and *Yersinia enterolitica* have not been found to be elevated in AS patients [5]. Additionally, a strong association between HLA-B27 and AS has been known for over 20 years [1]. These two observations were linked in 1987 [7] in the form of an amino acid homology between B*2705 and *K. pneumoniae* nitrogenase. The data presented in this paper suggests a new sequence homology between B*2705 and another protein present in *K. pneumoniae*, the pulD secretion protein of the starch debranching pullulanase enzyme system (Table 1). Whilst the pulD sequence does not display the 100% homology shown with *K. pneumoniae* nitrogenase a similarity is nonetheless present in the form of a tetramer, (residues 596–599) (DRDE) and B*2705 (DRED). The pulD sequence (residues 596–599) only differs from the DRED sequence in B*2705 (74–77) and *K. pneumoniae* nitrogenase (190–193) by two conservative substitutions, one, a D (aspartic acid) for an E (glutamic acid) at position 598 and the second, of an E (glutamic acid) for a D (aspartic acid) at position 599. It is clear to see that this sequence (underlined) falls within the region of homology previously published by Schwimmbeck et al. [7] (Table1).

Various other proteins from enteric bacteria have been shown to have sequence homology with HLA-B27 [12]. These include sequences from common gram negative organisms such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas* and *Klebsiella*. It has been suggested that the mechanism for the apparent autoimmune condition in AS occurs from the breakdown in tolerance of the host to its own HLA-B27 molecule and the endogenous peptides bound therein. Once the host is exposed to enteric peptides of similar sequence to that of HLA-B27 an immune response may be evoked. It is this immune response that may breach tolerance and so initiate a chronic inflammatory condition such as that seen in AS [12]. However, antibody levels against various organisms, (such as those shown to have similar sequences to HLA-B27) in AS patients revealed no specific elevation in immunoglobulin levels with respect to controls with the exception of *K. pneumoniae* [5].

The pulD is the terminal secretion protein of the pullulanase system and is known to have regions said to form strongly amphiphatic β sheets which confer membrane spanning properties common to outer membrane proteins [13]. This suggests that the protein should be on the surface of the organism and freely accessible to the surveillance of the immune system. The data obtained from the ELISA studies with synthetic peptides demonstrate that the AS patients have antibodies to the 16 mer B*2705 and pulD peptides. The AS patients showed significant elevations in both IgA and IgG antibody classes to the B*2705 peptide. This binding to a peptide of a self protein demonstrates the presence of autoantibodies in the sera of AS patients. It is, therefore, possible that these antibodies may bind HLA-B27 positive cells in patients, fix complement and thereby initiate inflammation. The microbial source, evoking these antibodies could be in the form of the pulD protein of *K. pneumoniae*. The ELISA data with the pulD synthetic peptide yielded similar data to that obtained with B*2705. The pulD peptide antibody elevations were seen in all three antibody classes whilst, with the control peptide (scrambled pulD peptide), no antibody elevations were observed in any patient group to any antibody class. The data derived from the B*2705 and pulD peptide binding suggests that there is some degree of molecular mimicry or cross reactivity between the two sequences, one self and one microbial.

The presence of the pullulanase system in *K. pneumoniae* allows the organism to cleave $\alpha(1–6)$ bonds in $\alpha(1–4)$ and $\alpha(1–6)$ branched starches such as glycogen and amylopectin. The effect of this property is that the organism gains access to another carbon source, which can alter the type of antigens presented on the surface of the bacteria leading to the development of the polysaccharide capsule layer and so alters the level of patients serum antibody binding. This can be seen in Fig. 2A, where *K. pneumoniae* has been grown in a minimal medium with 0.005% yeast extract. When organisms grown in this media were exposed to sera from AS patients and healthy and disease (RA patients) controls an elevation in antibody titre is observed. However, if the organism is grown on the same media supplemented with pullulan (0.3% w/v) as the carbon source the degree of antibody binding in AS patients is greatly increased. This increase also shows that the capsule is highly antigenic and is one of the predominant antigens recognised by AS patients, an observation also made by other workers [14]. In the control experiment, where *P. mirabilis* is grown in the same conditions, with and without pullulan, antibody elevations are only seen in

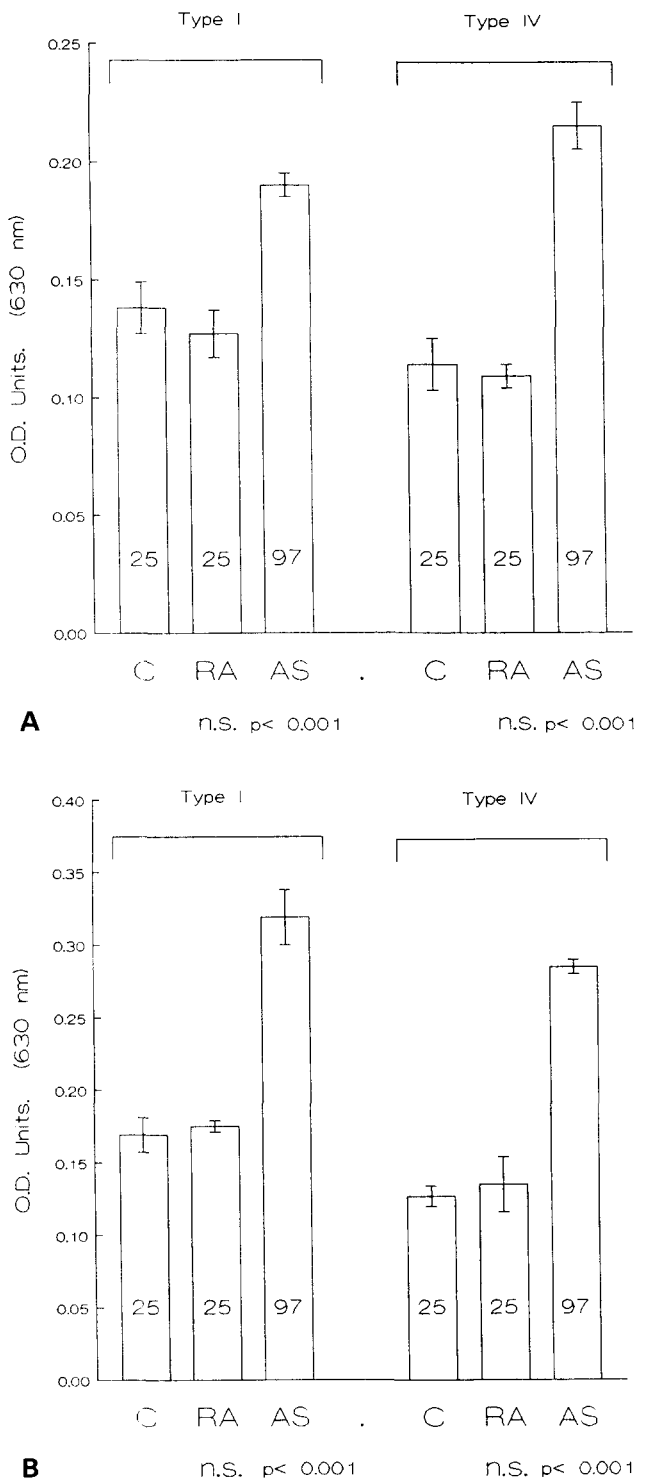

Fig. 4. IgA (A) and IgG (B) antibody titres (mean ± S.E.M.) in 25 controls (C), 25 rheumatoid arthritis (RA) patients and 97 ankylosing spondylitis (AS) patients when tested by ELISA against type I and type IV collagen. OD = optical density; n.s. = not significant.

RA patients not AS or healthy controls. Importantly, there is no significant difference in the antibody binding capacity of *P. mirabilis* when grown in the presence or the absence of pullulan, regardless of whether the sera is from patients or healthy controls (Fig. 2B).

IgA and IgG antibody titres were also found to be elevated against the extracellular enzyme pullulanase (pulA) in AS patients when compared to controls. The pulA enzyme would appear to have a very important property in relation to arthritic disease. At the N-terminal end of the pulA protein in some strains of *K. pneumoniae* there are repeats of the tripeptide Gly-X-Pro, characteristic of collagens type I, type III and type IV [9]. IgA and IgG antibody elevations to both type I and type IV collagens have been demonstrated in AS patients compared to controls. It is interesting to note that type I collagen is present predominantly in tendons and bone [15]. The possibility of antibodies cross reacting between pulA and type I collagen may be important in the deposition of fibrous tissue in the axial skeleton and the development of inflammation at the entheses (enthesitis).

Histological changes in thigh muscle biopsies of AS patients have previously been reported [16] and type III collagen is reported to be present in muscle tissue [15] and type IV collagen is present in basement membranes, basal lamina, retina and cornea. AS patients often suffer from acute anterior uveitis (AAU), which could be triggered by some of these anti-collagen antibodies. The antibody binding to pulA, type I and type IV collagen demonstrated here, as well as the suggested cross reactivity between pulA and collagen type III may explain some of the pathological features of AS.

The potential role of B*2705 and the other subtypes of HLA-B27 in the initiation of an immune response, in tissue typed patients and tissue typed controls, with an intestinal infection with a gram negative bacteria such as *K. pneumoniae* needs to be investigated further. However, studies performed on tissue typed AS patients against a homologous MHC background in tissue typed control subjects, have shown elevations in antibody to *K. pneumoniae* when compared to the control patients [17]. The fact that both the disease and control populations were tissue typed for HLA-B27 precluded the potentiality for the responses seen in the test groups to be due to the humoral immune response against *K. pneumoniae* to be influenced by the MHC class.

AS patients are usually treated by the administration of anti-inflammatory drugs. However, other treatments such as special diets have been proposed. Patients treated with a low starch diet in an open study over a 9-month period demonstrated a decrease in acute phase reactants (ESR and C reactive protein levels) and serum IgA levels [18]. It is possible that this low starch diet may affect the bowel flora by substrate depletion. This may decrease or even prevent the induction of substrate dependent enzymes and proteins such as those in the pullulanase system thereby possibly modifying the disease outcome. The involvement of inducible enzymes and proteins in bacteria related to rheumatic disorders such as AS, may explain the observed patterns of exacerbations and remissions.

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