ANTIBODY ACTIVITY IN ANKYLOSING SPONDYLITIS SERA TO TWO SITES ON HLA B27.1 AT THE MHC GROOVE REGION (WITHIN SEQUENCE 65-85), AND TO A KLEBSIELLA PNEUMONIAE NITROGENASE REDUCTION PEPTIDE (WITHIN SEQUENCE 181-199)

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Ankylosing spondylitis (AS) is an inflammatory disease of unknown etiology, affecting the sacroiliac joints, spine, and peripheral joints. Patients may also develop ocular inflammation, such as acute anterior uveitis. More than 90% of Caucasian patients with AS carry the MHC class I gene, HLA B27, whereas the frequency of HLA B27 in the general Caucasian population is <10%. One of the hypotheses postulated to explain this association and the possible etiology of AS is the concept of molecular mimicry, i.e., that there is immunological crossreactivity between antigens on bacteria and the HLA B27 molecule.

The first indirect evidence of this crossreactivity came from the work of Ebringer and colleagues in 1976 (2), who showed that antisera from rabbits immunized with HLA B27+ human lymphocytes reacted with Klebsiella aerogenes. These results suggested that there was partial crossreactivity between some antigens present on HLA B27 lymphocytes, possibly HLA B27 itself, and antigenic components on Klebsiella. Subsequent studies showed that Klebsiella pneumoniae is carried more frequently in the feces of AS patients with active disease (3, 4), and in patients with acute anterior uveitis (5, 6). Similarly, patients classified as having active disease and erythrocyte sedimentation rates of >15 mm/h were shown to have elevated levels of IgA antibodies to Klebsiella in their sera, compared with sera of patients with inactive disease and sera from healthy control subjects (7, 8).

More recent confirmations of this immunological crossreactivity have been demonstrated by Ogawasara and colleagues (9) with an anti-HLA B27 mAb reactive against 60,000 and 80,000 dalton antigens on Klebsiella pneumoniae and not with other Gram-negative bacteria. The 80,000 dalton molecule was purified and used to immunize guinea pigs. The guinea pig sera showed reduced antibody activity to Klebsiella envelopes when adsorbed with HLA B27+ cells. It has now been shown that there is a
consecutive sequence of six amino acids, Gln-Thr-Asp-Glu-Asp (QTDRED) shared between HLA B27.1 and the nitrogenase reductase enzyme of *Klebsiella pneumoniae* (10). This sequence homology has provided a possible structural basis for cross-reactivity to occur. The resolution of the three-dimensional structure of the HLA class I antigen HLA A2 revealed that the area of sequence homology occurs on the surface of the α domain, in the hypervariable region of MHC class I molecules (11, 12). It has also been shown that sera from patients with AS or Reiter's syndrome contain antibodies that react with synthetic peptides containing the homologous sequence that is shared by HLA B27.1 and *Klebsiella pneumoniae* nitrogenase reductase (10). 29% of sera from AS patients had antibody activity to a peptide of 16 amino acids from HLA B27.1 (residues 69–84), whereas none of the HLA B27* healthy individuals (n = 22) had this antibody activity. Greater than 40% of the AS patient sera had antibodies to a synthetic peptide of 13 amino acids from *Klebsiella pneumoniae* nitrogenase (residues 184–196). One of the 90 healthy controls (non–HLA-matched) had antibody activity to the *Klebsiella* peptide.

In the study reported here, overlapping peptides from the regions of interest were systematically synthesized and tested with a series of HLA-typed sera from AS patients and healthy controls to confirm the presence of antibody activity to *Klebsiella* and HLA B27 peptides in AS patient sera and to delineate the putative crossreactive epitopes with greater precision.

**Materials and Methods**

**Patients and Control Sera.** Fifty patients fulfilling the New York criteria for AS were examined and bled in the Rheumatology Clinic of the Department of Medicine, University of Melbourne, Austin Hospital. The patients were tissue typed at the Royal Melbourne Hospital tissue typing laboratory, by Dr. Brian Tait. Sera were aliquoted and stored at −20°C. Family studies of AS patients had previously identified healthy first-degree relatives with no history or clinical signs of AS. These relatives were tissue typed and their sera stored at −20°C. Twenty-two HLA B27* and 22 HLA B27+ sera were randomly chosen from these first-degree relatives as control sera.

**Peptide Synthesis.** Peptides were synthesized at Coscela Mimotopes Pty. Ltd., Parkville, Australia. Peptides were synthesized on solid polyethylene rods using the method of Geysen (13).

**Measurement of Antibody Activity to Peptides by ELISA.** Assemblies of 96 polyethylene rods with the peptides covalently attached were arranged on supports in a configuration that allowed the rods to be inserted into the wells of a microtiter tray. The rod-coupled peptides were precoated with 2% BSA (Commonwealth Serum Laboratories, Parkville, Australia) and 0.1% Tween 20 (vol/vol) in PBS, pH 7.4, for 1 h at 20°C. Dilutions of sera at 1/2,000 in PBS containing 2% BSA (wt/vol), 0.1% Tween 20 (vol/vol), and 0.2% sodium azide (wt/vol) were prepared, and 175 μl was added to appropriate wells of microtiter plates. The precoated peptides on the rods were incubated with the diluted sera overnight at 4°C. The rods were then washed four times for 10 min by immersing the rods in a shaking bath containing PBS. The rods were then incubated with enzyme conjugate, goat anti-human IgG + IgA + IgM (heavy + light chains), coupled to horse radish peroxidase (Kirkgaard and Perry Laboratories, Gaithersburg, MD), diluted to 1/2,000 in PBS containing 0.1% (wt/vol) sodium caseinate (United States Biochemical Co., Cleveland, OH) and 1% (vol/vol) normal sheep serum for 1 h at 20°C. After washing, the rods were incubated with the substrate H₂O₂ (0.01% vol/vol) and 2,2'-azino-di-(3-ethyl-benzothiazolesulfonic acid) 0.09 mM (Boehringer-Mannheim, Federal Republic of Germany) in 80 mM citric acid/100 mM phosphate buffer (pH 4.0). The absorbance at 405 nm was read after 45 min in a Titertek Multiskan MC (Flow Laboratories, McLean, VA).
Results

Testing of Amino Acid Octamers from Klebsiella and HLA B27. All possible overlapping peptides of eight amino acids from Klebsiella pneumoniae nitrogenase reductase (residues 181-199) and HLA B27.1 (residues 65-85) were synthesized onto polyethylene rods, with eight rods of each peptide being made. AS patient sera selected on the basis of elevated erythrocyte sedimentation rate (ESR >15 mm/h), and sera from HLA B27+ healthy individuals, were tested. Each serum was tested in duplicate. Fig. 1 shows the activity of one AS patient serum and two control sera against overlapping Klebsiella peptides of eight amino acids. Patient 237 serum had maximal antibody activity against peptides ICNSRQTD (residues 183-190) and CNSRQTDR (residues 184-191). By contrast, the two B27+ control sera showed minimal binding activity against all peptides. Fig. 2 shows the same patient serum and control sera reacting with overlapping peptides of eight amino acids synthesized from the HLA B27.1 molecule. The patient serum showed antibody activity against two regions in the sequence spanning residues 65-85. The most reactive peptides were CKAQTD (residues 67-74), KAKAQI'DR (residues 68-75), and REDLRTLLL (residues 75-82). The control sera showed minimal binding to the peptides. It is of note that octapeptides containing the entire homologous sequence (QTDRED) did not have a high reactivity against the patient serum. Adding further amino acids of the sequence to QTDR in the direction of the COOH terminus (or removal of amino acids from the NH2 terminus) reduced the reactivity of the serum against the peptide markedly. Figs. 3 and 4 show a further set of four patient sera reacting with the same Klebsiella and HLA B27.1 amino acid octamers, as shown in Figs. 1 and 2. All five AS sera selected gave similar antibody binding profiles against the peptides, and confirmed the presence of two reactive sites within the HLA B27.1 sequence tested. Both these regions included part, but not all, of the homologous sequence QTDRED. Similarly, the most reactive Klebsiella peptides contained the amino acids QTDR or QTDR from the homologous sequence.

Testing AS Sera with Different Length Peptides from Klebsiella and HLA B27. In seeking to delineate the putative crossreactive epitopes further, overlapping peptides ranging
from 4- and 8-amino acids, derived from *K. pneumoniae* nitrogenase (183–193) and HLA B27.1 (67–77) were synthesized on polyethylene rods and tested with four AS patient sera. Table I shows the strategy of the peptides synthesized.

This strategy of synthesis allowed us to determine the optimum and minimum sequence to which antibodies in AS sera can bind. Fig. 5 shows the cumulative data from the testing of these peptides (not all data shown). The three most reactive peptides were RQTDR (*Klebsiella*), NSRQTDR (*Klebsiella*), and KAKAQTDR (HLA B27.1). Thus RQTDR, a 5-mer derived from *Klebsiella* is able to form a sequential epitope. The 7-mer NSRQTDR was comparably reactive against AS patient sera. It is likely that this epitope identified on *Klebsiella* nitrogenase comprises more than one overlapping epitope, with the boundaries of the epitope being 183ICNSRQ-TDR193. The sequence for optimum antibody binding in the HLA B27.1 set of peptides was KAKAQTDR. Shorter peptides about this region gave comparable binding against AS sera. This region is likely to comprise more than one overlapping epitope, the boundaries of this epitope being 67CKAKAQ-TDR75.

The above data showed that QTDR appears to be an important sequence for antibody binding, but this peptide of four amino acids alone had little reactivity against
AS patient sera. Also, the peptide QTDRED was not highly reactive against patient sera.

Based on the above results of testing peptides of different lengths, the three most reactive peptides were synthesized on polyethylene rods. A bank of sera from AS patients and sera from healthy B27* and B27- individuals who were first-degree relatives of AS patients were tested simultaneously by ELISA against these peptides. Each serum was tested with each peptide in quadruplicate.

Figs. 6–8 show the data for 50 AS patient sera, 22 HLA B27* control sera, and 22 HLA B27- control sera, binding to peptides RQTDR, NSRQTDR, and KAK-AQTDR, respectively. The overlap in antibody activity in patient sera and control

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FIGURE 4. Four AS patient sera reacting with overlapping synthetic peptides of eight amino acids from HLA B27.1 (sequence 65-85) by ELISA. Peptides tested are shown along the horizontal axis. The y-axis shows absorbance at 405 nm. Sera were tested at dilutions of 1/2,000. Dotted squares represent patient 68, filled diamonds represent patient 436, filled squares represent patient 224, and open diamonds represent patient 209.
sera was considerable, but for reactivity against KAKAQTDR, the mean absorbance value of 50 patient sera was statistically significantly greater than the mean absorbance of either control group sera, using the Student's t-test ($p < 0.001$). For binding to NSRQTDR, the mean absorbance value for 50 patient sera was significantly greater.
than that of the B27- control group (p < 0.002). There was a less significant difference between the patient group and the B27+ control group (p < 0.02). Although differences were observed between patient and control groups with binding to RQTDK, these were not statistically significant (p < 0.07). In all these ELISA assays there were no significant differences between the mean absorbance values of the B27+ and B27- control group sera.

**Comparison of Antibody Activity Against NSRQTDR and KAKAQTDK in AS Patient Sera.** Antibody activity against the HLA B27.1 peptide KAKAQTDK was plotted against antibody activity to the *Klebsiella* peptide NSRQTDR for the 50 patient sera tested, shown in Fig. 9. AS patient sera that showed high antibody binding activity against NSRQTDR also showed high binding against KAKAQTDK (r = 0.90, p < 0.001).

**Discussion**

Molecular mimicry is defined as the sharing of epitopes from disparate proteins, and sharing of epitopes may be an important phenomenon in the pathogenesis of ankylosing spondylitis. Antibodies made to bacteria like *Klebsiella* may crossreact with HLA B27, thereby mimicking an autoimmune reaction.

Our studies have shown that there are antibodies in AS sera which bind to *K. pneumoniae* nitrogenase reductase and HLA B27.1 peptides. In the initial studies with overlapping octapeptides, five AS sera showed maximal antibody binding to ICNS-
FIGURE 8. 50 HLA B27⁺ AS patient sera (●), 22 HLA B27⁺ healthy control sera (○), and 22 HLA B27⁻ healthy control sera (□) reacting with HLA B27.1 synthetic peptide KAKAQTDR (residues 68-75) by ELISA. The y-axis shows absorbance at 405 nm. Sera were tested at dilutions of 1/2,000. SEM is shown next to each group of sera.

RQTD (residues 183-190) and CNSRQTDR (residues 184-191). The sera showed relatively low antibody activity against octapeptides that included the entire homologous sequence QTDRED. In fact, the sera showed markedly diminished activity against octapeptides with ED (Glu-Asp) added on in the direction of the COOH terminus. Our data show that QTDR appear to be the important residues (of the homologous sequence) of K. pneumoniae nitrogenase for antibody binding. In our studies with overlapping peptides of varying lengths from four to eight amino acids derived from K. pneumoniae nitrogenase, AS sera showed the highest antibody activity against

FIGURE 9. Simple regression plot of 50 AS sera reacting with HLA B27.1 8-mer KAKAQTDR versus 50 AS patient sera reacting with K. pneumoniae 7-mer NSRQTDR. Values plotted on both the y- and x-axes are absorbances at 405 nm. Correlation coefficient = 0.90, p < 0.001.
Although we had identified QTDR as being important residues, 4-mers of QTDR showed little reactivity against AS sera. Our conclusion is that there are overlapping epitopes identified by AS sera on K. pneumoniae of five to eight amino acids contained within the sequence $^{183}$ICNSRQTDR$^{191}$. The boundary of this epitope in the COOH-terminal direction is arginine (residue 191).

In studies with overlapping octapeptides from HLA B27.1, two distinct regions within the sequence 65–85 were identified as being reactive against AS sera. The region that we have named epitope I is represented by KAKAQTDR (residues 68–75). The second region, which similarly included part, but not all, of the homologous sequence was the peptide REDLRRTL. We have provisionally named this region epitope II. Octapeptides from HLA B27.1 that included the entire homologous sequence were again weakly reactive. Synthesis and systematic testing of overlapping peptides from four to eight amino acids in the region of epitope I demonstrated that KAKAQTDR was the most reactive peptide. Epitope I is likely to comprise several overlapping peptides, the boundaries of the epitope being $^{67}$CKAKAQTDR$^{75}$. Other studies have shown that an epitope recognized by polyclonal antisera is likely to be comprised of several overlapping epitopes (14). Epitope II on HLA B27.1 has not yet been mapped in detail to determine the minimal and optimal peptide to which antibodies in AS sera bind. However, it also corresponds to an epitope recognized by mouse (anti-Klebsiella) serum (Ewing, C.M., et al., manuscript in preparation).

The results of these systematic studies with Klebsiella and HLA B27.1 peptides have given specific definition to the peptides that may form the putative crossreactive epitopes. The data suggest that QTDR itself is not the crossreactive antigenic determinant, but that peptides containing part of this sequence (QTDR) are responsible for the initial antibody response to Klebsiella and subsequent crossreactivity with HLA B27. Data obtained by Schwimmbeck and Oldstone (15) showed that antisera raised in rabbits and rats to peptides derived from HLA B27.1 (69–84) reacted with the peptide used for immunization but also with a K. pneumoniae nitrogenase peptide (185–196). A shorter peptide derived from HLA B27.1 (69–78) containing the homologous sequence also reacted with the antisera. They concluded that the antigenic determinant is identical to the linear six amino acid sequence shared by HLA B27.1 and K. pneumoniae nitrogenase. Our data have more precisely identified the epitopes using human AS sera, and moreover we have shown that there are at least two distinct autoantigenic determinants on HLA B27.1. Our results, however, do not preclude that antibodies in AS patient sera can bind to longer peptide sequences that include one or both of these HLA B27 epitopes. Crossreaction of antigenic determinants in vivo means that antibodies to short Klebsiella peptides must necessarily bind to the native HLA B27 molecule to induce “autoimmunity.” Indeed, it has also recently been shown that antibodies to peptide sequences derived from K. pneumoniae and HLA B27.1 that contain the homologous sequence bind to synovial tissue from patients with AS and Reiter’s syndrome (16).

Not all subtypes of HLA B27 contain the sequences QTDR. Substitutions occur at position 77. It has been shown that antisera raised in rabbits to HLA B27.1 peptide 69–84, which includes the homologous sequence, can discriminate between the subtypes of HLA B27 in cytotoxicity assays (17). Further data obtained by Schwimmbeck and Oldstone (18) also demonstrated that antibodies raised to HLA B27.1 peptides showed reduced or abrogated binding to peptides representing other B27 sub-
types, i.e., where the aspartic acid at position 77 had been substituted. QTDRE (residues 72–76) are common to all subtypes (except HLA B27f where the aspartic acid at position 74 is substituted with a tyrosine), and this homology or a shorter homologous sequence (QTDRE) may be sufficient to explain antibody reactivity with other subtypes of HLA B27, since it has not been shown that one subtype is more closely associated with AS (19).

The importance of QTDRE has been confirmed by data obtained from protein sequences in other arthritogenic bacteria. Stieglitz et al. (20) showed that a plasmid pHS-2 from several arthritogenic Shigella strains contained a DNA sequence for an inferred pentapeptide AQTDR, which is common to four subtypes of HLA B27 (residues 71–75). Antisera raised to an inferred decapetide including AQTDR from the plasmid sequence, and to a 24-amino acid peptide from HLA B27.1 (residues 61–84) were tested with a panel of bacteria by Western blot. The antisera recognized one band that was unique to arthritogenic bacteria. While the HLA B27.1 peptide includes the homologous sequence, the entire homologous sequence is not coded for within the Shigella plasmid, and therefore QTDRED does not appear to be the prerequisite sequence for crossreactivity to occur. We have also demonstrated that AS patient sera contained antibodies that bind to AQTDR (data not shown).

It has been reported that as few as six or seven amino acids can form an antigenic determinant, and as few as four or five homologous amino acids can constitute a crossreacting antigenic determinant (18). The peptides we have identified as sequential epitopes are of this order of size.

The two distinct antigenic regions on HLA B27.1 that we have identified share one positional amino acid. Residue 75, an arginine, is a surface residue on one of the α-helices that flanks the deep groove on MHC class I molecules, which is proposed to be the binding site for small peptides that are presented to cytotoxic T cells. This arginine points away from the groove, and is predicted to be in a position to be contacted by the TCR (11, 12). It is the overlapping residue of epitopes I and II, and its position and orientation on the MHC groove means that it could act as a "post" or common residue for two autoantigenic determinants.

The other main finding of this study was that AS patient sera showed, on average, significantly higher binding activity against KAKAQTD (from HLA B27.1 and to NSRQTD from K. pneumoniae nitrogenase than HLA B27+ and HLA B27− control sera. There was a considerable overlap of individual serum activity between the patients and controls against these peptides. These results are consistent with other studies that showed that not all AS sera contain antibodies to peptides derived from HLA B27.1 (10). In our studies, some of the healthy control sera showed high antibody activity to the peptides identified as epitopes. It may be that such antibody activity could precede the onset of AS or spondylarthritis in these HLA B27− relatives. Much wider testing of sera from the normal population and from patients with other rheumatic diseases is necessary to determine the distribution and specificity of these antibodies. The presence of antibodies that bind to HLA B27 peptides in B27− AS patients or B27− controls theoretically has no pathogenic role in HLA B27− related arthritis, within the hypothesis of molecular mimicry and crossreacting antigenic determinants. Other HLA class I molecules may play a role in B27− AS patients. It has been shown that HLA B27− AS patients often carry HLA antigens
from the B7 CREG group (21), and B7 and B40 are crossreactive with B27 (17). Our own studies have shown that AS sera contain antibodies that bind to peptides synthesized from the hypervariable regions of B7 and B40 (unpublished data).

The epitopes we have identified on *Klebsiella* and HLA B27.1 are antibody binding sites and are therefore B cell epitopes. Moreover, the methodology used in this study can only locate sequential epitopes. We cannot infer from the data the nature of the *Klebsiella* peptides that may be presented to T cells in the initiation of the immune response. However, the size of the epitopes identified on *Klebsiella* are consistent with the projected size of linear amino acid sequences that could form T cell epitopes, i.e., five to seven amino acids (22, 23). Further investigations using systematically shortened peptides, and T cell clones are required to determine which *Klebsiella* peptides can activate a proliferative T cell response and thereby initiate a crossreactive immune response. Crossreaction of antibacterial antibodies with an HLA molecule, i.e., a self protein, may be sufficient to constitute a pathogenetic mechanism, resulting in inflammatory processes and tissue damage. The presence of antibodies bound to an HLA molecule may also interfere with HLA function.

Summary

74 overlapping peptides of varying lengths from *Klebsiella pneumoniae* nitrogenase reductase (residues 181-199) and from the HLA B27.1 molecule (residues 65-85) were synthesized and tested by ELISA against sera from HLA B27* ankylosing spondylitis (AS) patients, and sera from HLA B27* and HLA B27- healthy first-degree relatives. Antibody activity in AS sera to *Klebsiella* peptides of four to eight amino acids was maximal with the peptide NSRQTDR. Activity to HLA B27 peptides was maximal with the peptide KAKAQTDR (named epitope I). These peptides overlap with, but are proximal to the NH$_2$ terminus from QTDRED, which is homologous in HLA B27.1 and *K. pneumoniae* nitrogenase reductase. A second weaker reactive site was noted in the HLA B27.1 peptides, proximal to the COOH terminus from the homologous sequence, namely peptide REDLRTL (named epitope II). Little activity was seen against peptides that included the entire homologous sequence. Sera from 50 AS patients showed higher total Ig activity against peptides KAKAQTDR ($p < 0.001$) and NSRQTDR ($p < 0.02$) than did sera from 22 B27* and 22 B27- healthy controls. These data indicate that AS patient sera contain antibodies that bind to *K. pneumoniae* nitrogenase peptides and HLA B27.1 peptides, and that there are at least two epitopes on HLA B27.1 in the αβ domain, at the MHC groove region, that are autoantigenic in AS patients. Epitope I may be a site for crossreactivity between HLA B27 and *Klebsiella*.

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*Addendum:* A new World Health Organization (WHO) designation has changed the nomenclature for HLA B27 subtypes (24). The old designation B27.1, used in this paper, refers to B27.5 in the new nomenclature.
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