Autoantibodies to the HLA-B27 sequence cross-react with the hypothetical peptide from the arthritis-associated Shigella plasmid.

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Autoantibodies to the HLA-B27 Sequence Cross-react with the Hypothetical Peptide from the Arthritis-associated Shigella Plasmid

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Abstract

We previously reported elevated serum antibody levels to a peptide representing the HLA-B27 polymorphic region (B27 peptide) in HLA-B27(+) ankylosing spondylitis (AS) patients. A plasmid (pHS-2) isolated from arthritogenic Shigella flexneri strains had been shown to encode an amino acid sequence homologous to HLA-B27. Rabbit antibody to this peptide (pHS-2 peptide) strongly cross-reacted with B27 peptide, and, to a much lesser extent, with Klebsiella nitrogenase peptide. Serum antibody levels to pHS-2 peptide were studied in 160 spondylarthropathy patients. 12 of 115 (10.4%) AS patients, 2 of 45 (4.4%) patients with Reiter’s syndrome or reactive arthritis, as well as 6 of 147 (4.1%) normal controls were shown to have elevated anti-pHS-2 peptide antibodies. Antibody levels to B27 and pHS-2 peptides were significantly correlated in 134 HLA-B27(+) patients (r = 0.333, P < 0.001). 13 of 15 affinity-purified anti-B27 peptide antibodies from patients strongly cross-reacted with pHS-2 peptide, whereas only 3 weakly cross-reacted to nitrogenase peptide. Leucine appeared to be a critical residue for this cross-reaction. AS patients’ anti-B27 peptide antibodies reacted with HLA-B27 transfected L cells. These results may suggest that pHS-2 peptide more efficiently “mimics” B27 peptide than does nitrogenase peptide. Involvement of pHS-2 in pathogenesis of spondylarthropathy through molecular mimicry mechanisms requires further study. (J. Clin. Invest. 1990. 86:1193–1203.) Key words: HLA-B27 antigen • molecular mimicry • Shigella plasmid

Introduction

Although several hypotheses have been proposed, the actual mechanism for development of the seronegative spondylarthropathies and their relationship to HLA-B27 antigen still remain unclear (1). Recent advances in molecular biology and peptide synthesis technology have provided considerable new information concerning the theory of molecular mimicry (2–8). Thus, Schwimmbeck and associates (9) found a homologous six-amino acid sequence between the polymorphic region of B*2705 antigen1 and Klebsiella pneumoniae nitrogenase2 (Table I). They also detected elevated levels of serum antibodies against synthetic peptides encompassing the homologous sequence in a significant proportion of patients with ankylosing spondylitis (AS)3 or Reiter’s syndrome (RS). We previously demonstrated that this shared epitope is strongly expressed in the synovial tissue of patients with AS and HLA-B27 (14). We also confirmed the elevated level of serum antibodies to the peptide representing the HLA-B27 polymorphic region (B27 peptide) in some patients with AS, however, these human antibodies did not seem to cross-react with the peptide representing Klebsiella pneumoniae nitrogenase (nitrogenase peptide) (15).

Recently, a 2-MD plasmid, pHS-2, was identified in four arthritogenic Shigella flexneri strains (13). DNA sequence analysis revealed that one of the possible open reading frames encoded a 22-amino acid peptide which has a sequence homologous to the same polymorphic region of HLA-B27 (Table I). Initial Western blotting studies indicated that this putative gene product was expressed in the arthritogenic strains (16), however, subsequent attempts to define bacterial expression have not as yet yielded positive results. It is clear that the plasmid pHS-2 sequence codes for a homologous peptide but whether this product is transiently or uniformly expressed in infected patients is not clear. We have examined the presence of antibodies against a peptide representing the homologous region of pHS-2 (pHS-2 peptide), as well as the potential cross-reactivity of anti-B27 peptide antibodies with pHS-2 peptide, in a large number of patients with AS, RS, or reactive arthritis. Attempts have also been made to examine whether the anti-B27 peptide antibody would bind to the native B27 molecule. In addition, a series of synthetic peptides were used to attempt to establish critical residues for the cross-reactivity.

Methods

Patients and controls. 115 patients with AS, 45 patients with RS or reactive arthritis, as well as 147 normal controls were studied. Patient

1. A new WHO designation has recently been adopted (10), which has changed the nomenclature for HLA-B27 subtypes. The amino acid sequence of the old designation B27.1 which was used in previous reports, should now be referred to as B*2705.
2. The nitrogenase enzyme complex consists of two components. The homologous sequence studied in this paper occurs in component 2, or nitrogenase reductase (11). For simplicity, the peptide representing this sequence is referred to as “nitrogenase peptide” in this study.
3. Abbreviations used in this paper: AS, ankylosing spondylitis; CELISA, cell ELISA; RS, Reiter’s syndrome.

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Table I. Amino acid sequences of HLA-B27 antigens of Klebsiella pneumoniae nitrogenase, and pHS-2

<table>
<thead>
<tr>
<th>A. Original sequences</th>
<th>Residues</th>
<th>Amino acid sequences</th>
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<tbody>
<tr>
<td>pH5-2</td>
<td>183-200</td>
<td>GTVCAQTDRHSLSCIAMQ</td>
</tr>
<tr>
<td>K. pneumoniae nitrogenase</td>
<td>67-84</td>
<td>ICNSRTQLDELIIALA</td>
</tr>
<tr>
<td>HLA-B*2705</td>
<td>67-84</td>
<td>CKAQTDRDLRTLLRY</td>
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<tr>
<td>B*2704</td>
<td>67-84</td>
<td>CKAQTDRNLSLRTLLRY</td>
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B. Synthetic peptides used in this study

<table>
<thead>
<tr>
<th></th>
<th>Amino acid sequences</th>
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<tbody>
<tr>
<td>B27 peptide</td>
<td>AKAQTDRDLRTLLRY</td>
</tr>
<tr>
<td>Nitrogenase peptide</td>
<td>CNSRTQDRDELI</td>
</tr>
<tr>
<td>pH5-2 peptide</td>
<td>CAQTRHLSY</td>
</tr>
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<tr>
<td>R2L9</td>
<td>-R------------E-</td>
</tr>
<tr>
<td>A2E9</td>
<td>GMEVRWCATSDGEG</td>
</tr>
<tr>
<td>R2E9</td>
<td></td>
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Table II. Characteristics of the Patients and Normal Controls Studied

<table>
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<tr>
<th>Patients</th>
<th>Total</th>
<th>HLA</th>
<th>Sex</th>
<th>Norway</th>
<th>USA</th>
<th>Canada</th>
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<tbody>
<tr>
<td>Ankylosing spondylitis</td>
<td>115</td>
<td>B27+</td>
<td>M</td>
<td>50</td>
<td>26</td>
<td>9</td>
</tr>
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<td>13</td>
<td>2</td>
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<td></td>
<td></td>
<td>B27-</td>
<td>M</td>
<td>10</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Reiter’s syndrome or reactive arthritis</td>
<td>45</td>
<td>B27+</td>
<td>M</td>
<td>6</td>
<td>7</td>
<td>12</td>
</tr>
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<td></td>
<td>F</td>
<td></td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B27-</td>
<td>M</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Normal controls</td>
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<td>B27+</td>
<td>M</td>
<td>11</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td></td>
<td>4</td>
<td>9</td>
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<td></td>
<td></td>
<td>B27-</td>
<td>M</td>
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<td>32</td>
<td>20</td>
</tr>
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<td></td>
<td>F</td>
<td></td>
<td>30</td>
<td>17</td>
<td>1</td>
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</tbody>
</table>

Amino acid sequences are shown in single-letter code. The sequences of pH5-2 and K. pneumoniae homologous at B*2705 are underlined. HLA-B27 subtypes are designated according to the new WHO nomenclature (10), and the sequences are based on references 11-13.

and control sera were collected at the Rheumatology Department, University of Tromso (Norway), University of New Mexico (USA), and Toronto Western Hospital (Canada) (Table II). An additional serum from the original reactive arthritis patient, from whom pH5-2 positive S. flexneri was isolated, was collected at Southwestern Medical Center in Dallas, TX. All patients and controls had been HLA-typed. The controls consisted of healthy medical staff, hospital, and laboratory personnel. The age distribution of controls and patients from Norway was 39.8±11.1 (21-62 yr) and 38.3±11.4 (20-69 yr), respectively, and that from the USA was 40.2±12 (25-66 yr) and 42.4±13.6 (16-76 yr). The age distribution of Canadian patients was 28.9±8.2 (16-52 yr), which was matched with that of Canadian controls.

Initial findings in the patients from Norway or USA have been summarized in a previous report (15). All patients with AS fulfilled the New York criteria (17) for a definite diagnosis. RS was defined as a seronegative peripheral arthritis in combination with at least one of the following characteristics: mucocutaneous lesions, inflammatory eye disease, or urethritis. Reactive arthritis was a seronegative peripheral arthritis in the absence of extra-articular features, which followed an antecedent gastrointestinal or genitourinary tract infection (18).

The 45 patients with RS or reactive arthritis, 5 were post-Chlamydia, 6 were post-Salmonella, 4 were post-Campylobacter, and 4 were post-Shigella reactive arthritis. In the other 26 patients, causative microorganisms were not identified. Canadian samples from RS or reactive arthritis patients were collected when the articular symptoms were active. Also, about half of AS or RS patients from Norway or the USA were clinically active, as determined by concurrent clinical assessment and serum C-reactive protein determination (15).

Synthetic peptides. All peptides used in this study were prepared as originally described by Merrifield (19) (Table I), and were analyzed for purity by high-performance liquid chromatography. The peptides used were AKAQTDRDLRTLLRY residues 69-84 of HLA-B*2705 (B27 peptide), CNSRTQDRDELI residues 184-196 of Klebsiella pneumoniae nitrogenase (nitrogenase peptide), and CAQTRHLSY of pH5-2 (pH5-2 peptide). The carboxyl-terminal tyrosine (Y) of pH5-2 peptide (introduced to allow radioiodination) was not found in the original sequence. Three peptides with single or double amino acid substitutions from the original pH5-2 peptide, namely, CRQTRHLSRY (R2L9), CAQTRHSESY (A2E9), and CRQTRHSESY (R2E9) were also synthesized. These specific substitutions were employed to attempt to define amino acid residues most likely to be involved in cross-reacting determinants. A 14-mer peptide derived from human melanoma antigen P-97, GMEVRWCATSDGEG (P-97 peptide) kindly provided by Dr. A. Latif Kazim, Department of Cell Biology, University of New Mexico, was used as a control.

An antiserum was raised in the rabbit against pH5-2 peptide cross-linked to keyhole limpet hemocyanin, and subsequently antibodies were affinity-purified using the same peptide directly coupled to agarose.

Enzyme-linked immunosorbent assay (ELISA). Serum antibody levels against synthetic peptides were assayed by ELISA as previously described (15). Briefly, duplicate wells of microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 0.1 ml of peptide solution (10 μg/ml) in carbonate buffer (0.05 M Na2CO3, 0.05 M
Duplicate control wells received carbonate buffer alone. After overnight incubation, wells were washed three times with phosphate-buffered saline (PBS), containing 1% heat-inactivated horse serum (Sigma Chemical Co., St. Louis, MO) and 0.1% Tween 20, and the remaining protein binding sites were saturated with dilution buffer (PBS containing 5% heat-inactivated horse serum and 0.1% Tween 20). After washing, 0.1 ml of serum, diluted 1:50 in dilution buffer, was added to peptide-coated or uncoated wells. After incubation for 1.5 h, wells were washed five times with washing buffer, and 0.1 ml of peroxidase-conjugated goat anti-human IgG + IgA + IgM (American Qualex, Inc., La Mirada, CA), diluted 10,000-fold in dilution buffer, was added. After incubation for 1.5 h, wells were washed six times with washing buffer. 0.1 ml of enzyme substrate solution, consisting of phosphate-citrate buffer with 0.4 mg/ml 0-phenylenediamine (Sigma Chemical Co.) and 0.01% H2O2, were added for 30 min. The reaction was stopped by 2.5 M H2SO4 and the absorbance was read at 490 nm. Absorbance for each sample in the peptide-coated wells was corrected for nonspecific binding by subtracting the absorbance in the corresponding peptide-uncoated wells. To ensure consistency between assays, five sera including those with negative, moderate, and strong reactivity were used as reference controls in each microtiter plate, and the arbitrary ELISA unit was calculated for each sample using an intrassay standardization of each series of tests. Interassay variability was examined by using five reference sera in 14 assays. Standard deviation for each assay was 1.2, 1.4, 1.8, and 2.0, respectively.

The same procedure was used for the assay of class-specific serum antibodies using peroxidase-conjugated affinity-purified goat anti-human IgG, anti-human IgA, or anti-human IgM (Tago, Inc., Burlingame, CA).

The reactivity of affinity-purified rabbit anti-pHS-2 peptide and control rabbit IgG (Jackson Immuno Research, West Grove, PA) was examined similarly, using 1% bovine serum albumin (BSA, Sigma Chemical Co.) instead of horse serum and peroxidase-conjugated goat anti-rabbit IgG (Tago, Inc.) as a developing antibody. Affinity-purification of human anti–B27 peptide antibodies. Human anti–B27 peptide antibodies were affinity-purified from 17 sera which were found to contain elevated levels of antibodies. B27 peptide, representing HLA-B*2705 sequence, was directly coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia, Uppsala, Sweden) (coupling efficiency > 95%). 0.3–1.5 ml of serum was diluted 1:3 in PBS and applied to the B27 peptide-Sepharose 4B column. After the column was washed, bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.5) and neutralized immediately with 1 M Tris-HCl (pH 8.5). After dialysis against PBS, eluted antibody solutions were concentrated to 1.5 ml using YM-10 membrane (Diaflow, Amicon Corp., Danvers, MA), and their immunoglobulin concentrations measured by ELISA. These human anti–B27 peptide antibodies were assayed for their reactivity with a panel of peptides by ELISA as described above, using PBS containing 1% BSA and 0.1% Tween 20 as washing and dilution buffer. In order to detect all possible cross-reactivity, the maximum concentration available for each antibody solution was used. Peroxidase-labeled goat anti–human IgG + IgA + IgM was used as the developing antibody. In some experiments, the peptide coating solution was completely air-dried in the wells to make sure that the same quantity of each peptide remained coated on the microtiter plate.

The results were expressed as the percentage of specific binding to B27 peptide, i.e.: 100 × (OD of Ag peptide–coated wells – OD of uncoated wells)/(OD of B27 peptide–coated wells – OD of uncoated wells) × %.

Transfected mouse L cell lines. Transfected mouse L cell lines were generously provided by Dr. Joel D. Taurog, University of Texas Southwestern Medical Center at Dallas (20). One cell line was doubly transfected with the HLA-B*2705 gene and the human β2-microglobulin gene, the other was transfected only with the β2-microglobulin gene. Cell ELISA (CELSIA). CELISA was performed according to Baumgarten (21) with some modifications. Transfected L cells were washed and resuspended in PBS at 1 × 10⁶ cells/ml. 50 μl of the cell suspension was delivered to each well of flat-bottomed microtiter plates (Dynatech Laboratories, Inc.) and the plates were centrifuged at 1,200 rpm for 5 min. 30 μl of the supernatant was carefully removed by micropette, and the plates were air-dried. After 200 μl of methanol (100%) was added to each well, the plates were centrifuged for 10 min at 1,500 rpm. After washing with PBS, nonspecific binding sites were blocked by adding 200 μl PBS containing 1% BSA and 0.02% sodium azide. After incubation overnight at 4°C, the plates were washed, and 70 μl of antibody solution in PBS containing 1% BSA was added to each well. After incubation for 2 h, plates were washed four times, 75 μl of peroxidase-conjugated F(ab′)2 fragment of goat anti–human IgG (Tago, Inc.), anti-human IgM (Jackson Immuno Research) or goat anti–mouse IgG (Fc fragment specific, Accurate Chemical & Scientific Corp., Westbury, NY), 1:10,000 dilution, was added to each well. After incubation for 1.5 h the plates were washed five times, and the color was developed as described in the ELISA protocol. Murine MAb ME1, an IgG1 that reacts with HLA–B27, B7, Bw22, and Bw42 (20), was kindly provided by Dr. J. D. Taurog and used as a positive control. MOPC 31C (Sigma Chemical Co.), an IgG1 MAb without known specificity, was used as a negative control.

As a control for human IgG and IgM, IgG from Cohn fraction II (Sigma Chemical Co.) isolated using DEAE Sephadel (Pharmacia) or polyclonal human IgM (Calbiochem-Behring Corp., La Jolla, CA) were used respectively.

The protein quantity of the wells after multiple washing procedures was estimated by dye-binding assay (21). Plates were coated and treated identically as described above without adding BSA or antibodies in the buffer. The plates were then washed four times with methanol and air-dried, and 30 μl of protein assay solution (Bio-Rad Laboratories, Richmond, CA) was added. The plates were shaken for 5 min, water (120 μl) was added, and the plates were shaken again. The optical density was read at 630 nm with reference at 490 nm.

Reactivity of anti–pHS-2 antibody with Shigella strains. In order to ascertain whether the pH5-2 peptide encompassing the HLA–B27 cross-reactive sequence was actually expressed in Shigella bacterial strains which possessed the original plasmid and which had actually been isolated from B27-positive patients with documented post-Shigella reactive arthritis, a series of studies were undertaken. Direct reactivity between rabbit affinity-purified anti–pHS-2 peptide antibody and various Shigella strains was examined both by ELISA and immunoprecipitation techniques. A series of four pH5-2 plasmid-positive Shigella strains which had been isolated from reactive arthritis patients (6115, ELDL, CDH, and 6335) in parallel with several pH5-2 plasmid-negative strains not known to be associated with arthritis (7061 and C1250) were grown up in quantity, heat killed, and used either as whole bacterial suspensions or to prepare membrane and cytosol enriched fractions for ELISA testing. When whole bacteria were directly coated on ELISA plates, bacterial suspensions were adjusted to equal optical density on the spectrophotometer, applied in pH 9.6 carbonate buffer and allowed to fix to ELISA polystyrene microtiter plates (Dynatech Laboratories, Inc.) overnight at room temperature. Isolation of outer bacterial membranes employed lysis by sonications, followed by removal of unbroken cells by centrifugation for 1 min at 15,600 g in a microfuge (22). The supernatant fluid was sedimented for 30 min in a microfuge at 4°C to concentrate membranes and the pellet was resuspended in 300 μl of 10 mM Tris, pH 8.0, 5 mM MgCl2–2% Triton X-100. The supernatant was used as presumed residual cytosol components.

Rabbit antibody produced against pH5-2 peptide CAQTDHSLSY was isolated by adsorption to and elution from an epoxy-activated Sepharose 4B affinity column linked to the pH5-2 peptide. The affinity-isolated antibody was studied in parallel with normal rabbit IgG in ELISA assays. In several experiments, affinity-purified rabbit anti–pHS-2 antibody and normal rabbit IgG were pepsin digested to produce F(ab′)2 fragments used in the comparative ELISA assays. This was done to rule out nonspecific binding of rabbit Ig fractions to bacteria through any Fc receptor mechanism.

After blocking with 1% BSA, incremental concentration of affin-
ity-purified anti–pHS-2 peptide antibody was applied to duplicate wells and incubated for 3 h. After washing, bound antibodies were developed by addition of peroxidase-labeled anti–rabbit Fc of IgG at 1:5,000 dilution.

Immunoprecipitation assays utilized [S^35]methionine and [S^35]-cysteine labeling of *Shigella* strains followed by addition of affinity-purified anti–pHS-2 peptide antibody, precipitation with protein A, and subsequent SDS-PAGE separation followed by autoradiography. The possibility that molecular mimicry between the *Shigella* strains and HLA-B27 could be more directly related to other cross-reactive epitopes aside from those ascribed to the pHS-2 plasmid was also studied. Affinity-purified human antibodies in concentration ranging from 10 ng to 5 µg/ml isolated from HLA-B27 peptide affinity columns (see Table IV) were applied to microtiter plates coated with *Shigella* organisms including pHS-2 plasmid-positive strains isolated from patients with reactive arthritis as well as other plasmid-negative strains not known to be arthritis related. After blocking with 1% BSA, ELISA reactivity was developed by addition of peroxidase conjugated goat anti–human IgG, IgA, and IgM. Similar experiments were also done and developed with peroxidase-labeled anti–IgM alone since most human cross-reactivity occurred in IgM.

**Statistical analysis.** The significance of correlation was determined by linear regression analysis. Student’s t test was used to compare the mean results among different groups.

**Results**

Cross-reactivity of rabbit anti–pHS-2 peptide antibody to B27 peptide. Reactions of the affinity-purified rabbit anti–pHS-2 peptide antibody with the four peptides are shown in Fig. 1. Strong reactivity with pHS-2 peptide was present. In addition, clear cross-reactivity with B27 peptide, 1/16 as strong as with pHS-2 peptide, was observed. Weak cross-reactivity was also observed with nitorgenase peptide (1/1024 as strong as with pHS-2 peptide). Control P-97 peptide showed no significant binding; control rabbit IgG tested at the same range of concentrations was negative for all peptides tested (data not shown).

Detection of serum anti–peptide antibodies. The patients and controls from Norway and USA, previously studied for antibody to nitrogenase and B27 peptide (15), were examined for the presence of serum antibodies to pHS-2 peptide. Fig. 2 demonstrates IgG + IgA + IgM antibody levels of Norwegian and American subjects. The cutoff value was set at normal mean plus 2 SD of the sex-matched controls of the respective Norwegian or American group. Unlike anti–B27 peptide antibody levels (15), differences related to previous pregnancy were not observed in female individuals. In Norwegian subjects, 7 of 60 (11.7%) male AS patients, 3 of 16 (18.8%) female AS patients, as well as 2 of 25 (8%) normal male controls and 1 of 34 (2.9%) normal female controls were positive. Among American subjects, 2 of 26 (7.7%) male AS patients as well as 1 of 38 (2.6%) normal male controls and 1 of 26 (3.8%) normal female controls were positive. Although statistically not significant, the proportion of antibody-positive individuals and the antibody levels of the positive sera were higher in AS patients than normal controls.

Because the number of RS or reactive arthritis patients was rather small in both groups, 20 additional patients with RS or reactive arthritis and 11 patients with AS as well as 24 normal controls collected in Toronto were also studied in parallel for the presence of antibodies to B27 peptide, nitrogenase peptide and pHS-2 peptide. Since the numbers of female patients and controls were small (six and two, respectively), statistical analysis focussed principally on male subjects. The results of IgG + IgA + IgM antibodies are presented in Fig. 3. 2 out of 11 (18.2%) Canadian AS patients and 2 out of 14 (14.3%) male patients with RS or reactive arthritis were positive for anti–B27 peptide antibodies, whereas none of the 22 male controls was positive. Two values, one in AS and one post-*Chlamydia* reactive arthritis, were extremely elevated. All four patients with elevated anti–B27 antibody levels were HLA-B27(+), while two normal male HLA–B27(+) controls from this Canadian population showed negative reactions. When antibody levels to pHS-2 peptide were compared, 2 of 14 (14.3%) patients with RS or reactive arthritis as well as 1 of 22 (4.5%) normal male controls were positive. Both of the anti–pHS-2 positive patients were also positive for the anti–B27 peptide antibodies. On the other hand, 3 of 22 (13.6%) normal Canadian male controls as well as only 1 of 11 (9.1%) AS patients and 2 of 14 (14.3%) Canadian patients with RS or reactive arthritis were positive for the anti–nitrogenase peptide antibodies. None of these positive individuals had concurrent elevated antibody levels to B27 peptide or pHS-2 peptide. Thus, some Canadian AS patients and RS or reactive arthritis patients showed clear elevations of anti–B27 peptide antibody, whereas differences between controls and patients were not apparent with respect to anti–nitrogenase peptide reactions, confirming our previous observations in two population groups (Norway and USA) (14).

When Canadian patients with reactive arthritis were analyzed with respect to types of antecedent infections, both patients positive for anti–B27 peptide and anti–pHS-2 peptide antibodies were post-*Chlamydia* arthritis; thus, two out of three HLA–B27(+) male patients with post-*Chlamydia* arthritis had elevated antibody levels to both peptides. Surprisingly, none of the three Canadian patients with post-*Shigella* arthri-
Elevated ELISA were not peptide 1 (Unit) = 15, expressed normal controls observed in HLA-B27(+). The three isolated peptide from the reactivity was activated tissues [two male and one female, all HLA-B27(+)] showed elevated reactivity to either the B27 or the pHS-2 peptides. Serum from the original Dallas HLA-B27(+) female reactive arthritis patient (from whom the pHS-2 containing S. flexneri strain was isolated) also showed no significant reactivity with any of the three peptides.

Correlation between antibody levels to B27 peptide and pHS-2 peptide. As shown in Fig. 4, the antibody levels against B27 peptide and pHS-2 peptide were significantly correlated in HLA-B27(+) patients with AS, RS, or reactive arthritis (n = 134, r = 0.333, P < 0.001). No significant correlation was observed in HLA-B27(−) patients (n = 26, r = 0.169; NS) or normal controls (n = 147, r = 0.015; NS). As previously reported (15), the antibody levels to B27 peptide and nitrogenase peptide were not significantly correlated in any of the groups.

Among all 307 normal control and patient sera, 35 showed elevated ELISA reactions with B27 peptide. Sera showing two or three specificities were examined. Among 10 such individuals with elevated anti–B27 peptide antibody, 7 were also positive for anti–pHS-2 peptide and 5 were positive for anti–nitrogenase peptide, including 2 positive for both antipeptide antibodies. The class distributions of the antibodies, as expressed by the relative level compared with normal controls, are shown in Table III. Most of the individuals positive for both anti–B27 peptide and anti–pHS-2 peptide antibodies had IgM-dominant distribution of the antibodies to both peptides. On the other hand, among individuals positive for antibodies to B27 peptide and nitrogenase peptide, the class distribution pattern of the antibodies to both peptides was not similar except for one AS patient N334.

Cross-reactivity of affinity-purified human anti–B27 peptide antibodies. Direct cross-reactivity of affinity-purified human anti–B27 peptide antibodies with pHS-2 peptide and nitrogenase peptide was studied. 15 sera from HLA-B27(+) patients showed significant reactivity with both peptides. The distribution of the antibodies was similar to that for the B27 peptide. N1 showed no cross-reactivity with the B27 peptide. N2 showed weak reactivity with the B27 peptide.

![Figure 2](image-url) 
Figure 2. Serum antibody levels of Norwegian and American individuals against synthetic peptide representing amino acid sequence of pHS-2 homologous to HLA-B*2705 antigen. The box shows the mean value (m) plus 2 SD of the respective sex-matched control group (either Norway or USA). (○) HLA-B27(+) individuals; (●) those negative for HLA-B27. M, male; F, female; AS, ankylosing spondylitis; RS, Reiter’s syndrome; ReA, reactive arthritis.

![Figure 3](image-url) 
Figure 3. Serum antibody levels of Canadian subjects against synthetic peptides representing residues 69–84 of HLA-B*2705, residues 184–196 of K. pneumoniae nitrogenase or pHS-2 sequence. The box shows the mean value (m) plus 2 SD of normal male control group. (○) HLA-B27(+) individuals; (●) those negative for HLA-B27. M, male; F, female; AS, ankylosing spondylitis; RS, Reiter’s syndrome; ReA, reactive arthritis.
patients as well as 2 from normal, previously pregnant female controls were examined; all sera were positive for anti-B27 peptide antibody in the whole-serum assay. Cross-reactivity, expressed as percentage of binding to B27 peptide, was considered to be significant when values were greater than mean plus 3 SD of the percentage of binding to the control P-97 peptide in each individual (Table IV). Significant cross-reaction with the pHS-2 peptide was observed in 13 of 15 affinity-purified samples from patients, including those which were determined to possess only borderline or no reactivity to pHS-2 peptide in the whole-serum assay. On the other hand, only 3 of 15 patients’ samples showed weak cross-reactivity with nitrogenase peptide. Although four samples were positive or borderline for nitrogenase peptide in the whole serum assay, only one of them weakly cross-reacted when the purified anti–B27 peptide antibodies were examined (N136), suggesting that the anti–nitrogenase peptide antibodies detected in the whole serum actually belonged to different antibody clones from those producing anti–B27 peptide antibodies. Among the two positive samples from normal female controls, one showed slight but significant cross-reactivity with pHS-2 peptide and nitrogenase peptide (A64). Overall, the average cross-reactivity of purified anti-B27 peptide antibodies with pHS-2 peptide (26.2±21.3%) was highly significant when compared with cross-reaction with control P-97 peptide (4.1±5.6%) (P < 0.001). In contrast, cross-reactivity with the nitrogenase peptide (3.1±3.0%) was not different from that with P-97 peptide.

There are two amino acids which are shared by HLA-B*2705 and pHS-2 sequences but not by nitrogenase peptide, i.e., alanine (Ala) and leucine (Leu) at positions 71 and 78 of B*2705 sequence (Table I). To test the possibility that either one or both of these residues was critical for the cross-reactivity, we synthesized three peptides homologous to pHS-2 peptide except either one or both the Ala or Leu had

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**Table III. Class Distribution of Serum Antipeptide Antibodies from Individuals with Elevated Antibodies to More Than One Peptide**

<table>
<thead>
<tr>
<th>Sample* (diagnosis)</th>
<th>Anti-B27 peptide</th>
<th>Anti-pHS-2 peptide</th>
<th>Anti-nitrogenase peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
<td>IgM</td>
</tr>
<tr>
<td>N334 (AS)</td>
<td>0</td>
<td>±</td>
<td>1+</td>
</tr>
<tr>
<td>N1064 (N1)</td>
<td>0</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>N287 (AS)</td>
<td>0</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>N518 (AS)</td>
<td>0</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>N797 (AS)</td>
<td>1+</td>
<td>±</td>
<td>3+</td>
</tr>
<tr>
<td>C774 (ReA)</td>
<td>0</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>C11 (ReA)</td>
<td>1+</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>N212 (AS)</td>
<td>0</td>
<td>0</td>
<td>1+</td>
</tr>
<tr>
<td>A44 (AS)</td>
<td>0</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>A21 (N1)</td>
<td>2+</td>
<td>0</td>
<td>2+</td>
</tr>
</tbody>
</table>

* Abbreviations: N, Norway; A, America; C, Canada; AS, ankylosing spondylitis; ReA, reactive arthritis; NL, normal female with previous pregnancy.

† Data on class-specific antipeptide antibody levels in whole serum. 0, < normal mean (m) + 1 SD; ±, <m + 2 SD; 1+, <m + 3 SD; 2+, <m + 4 SD; 3+, ≥m + 4 SD. § NT, not tested. The IgG + IgA + IgM antibody level was within normal range.
been replaced by arginine (Arg) or glutamic acid (Glu), which appeared at the corresponding position in nitrogenase peptide (Table I). In all 13 samples tested, significant cross-reactivity was observed against the peptide with the single substitution Ala → Arg (designated R2L9), however, cross-reactivity was abrogated in the two peptides which included a substitution Leu → Glu, regardless of the presence of Ala → Arg substitution (A2E9, R2E9) (Table IV). Of great interest, in all samples tested, R2L9 peptide had even higher cross-reactivity than the original pHS-2 peptide showed with the anti–B27 peptide antibodies. Thus, the average cross-reactivity with R2L9 peptide (56.2±34.8%) was significantly higher than that with the original pHS-2 peptide (26.3±21.3%) (P < 0.01).

Some experiments were repeated with the peptides air-dried in the microtiter plate wells so that the identical quantity of the various peptides were uniformly coated on the plates. The results were essentially identical.

**Binding of anti-B27 peptide antibodies to HLA-B27-transfected mouse L cells.** Whether the purified human anti-B27 peptide antibodies bind to the whole HLA-B27 molecule was examined by CELISA. In order to avoid nonspecific binding due to surface human immunoglobulins and Fc receptors, mouse L cells doubly transfected with HLA-B*2705 and human β2-microglobulin genes were used. L cells transfected only with β2-microglobulin gene were used as controls. Results are shown in Table V. IgG dominant anti-B27 peptide antibody from patient N34 bound significantly stronger to B27-transfected cells than to the cells transfected only with β2-microglobulin at all concentrations tested. Control IgG showed slightly enhanced binding to B27-transfected cells at the highest concentration, but no significant difference was observed at lower concentrations. Among four IgM dominant anti–B27 peptide antibodies examined, one (A44) showed significantly stronger binding to B27-transfected cells, while other patients' antibodies and control polyclonal IgM did not show significant differences. Mouse MAb ME1 reacted strongly to B27-transfected cells, while MOPC 31C (control IgG 1) did not.

To exclude the possibility that a different number of the cells were adhering in the wells after the washing procedures essential for CELISA, the dye-binding assay was performed to estimate the protein quantity within the wells after vigorous washing. No difference was observed between two transfected cell lines (Table V).

**Anti-pHS-2 peptide antibody binding to Shigella strains.** Whole Shigella bacteria as well as bacterial membrane and cytosol fractions were assayed by ELISA using affinity-purified rabbit anti–pHS-2 peptide antibody to determine whether plasmid-positive strains presumably responsible for enhanced
expression of HLA-B27 cross-reactive epitopes showed enhanced reactivity with antibody to pHS-2 peptide. Six experiments were performed using intact IgG from affinity-purified rabbit anti-pHS-2 peptide and control normal rabbit IgG and two experiments with pepsin digested F(ab')2 fragments of the same preparations.

As shown in Fig. 5, anti-pHS-2 peptide antibody showed strong reactivity with plasmid-positive strain 6115, while weak reactivity with normal rabbit IgG was present. Another plasmid-positive strain EDL showed moderate reactivity with anti-pHS-2 peptide and no binding with normal rabbit IgG. Surprisingly, two plasmid-negative strains (C1250, 7561) also showed moderate reactivity with anti-pHS-2 peptide. They also showed higher reactivity with normal rabbit IgG than in the case with plasmid-positive strains.

Similar results were obtained with whole bacteria, membrane or cytosol preparations, and likewise similar findings were noted using intact antibody or F(ab')2 fragments. Thus, the strong binding between anti-pHS-2 peptide antibody and plasmid-positive strain 6115 suggested the presence of pHS-2 peptide sequence on the bacterial membrane, however, data obtained with plasmid-negative strains also suggested the presence of cross-reacting epitopes on membrane regardless of whether the strains contained the pHS-2 plasmid.

To address this question more directly, a series of comparative immunoprecipitation experiments using plasmid-positive or negative Shigella strains were performed. Both pHS-2 plasmid-positive and negative strains showed several components reacting with affinity-purified anti-pHS-2 peptide antibody. No consistent difference in autoradiograph patterns was noted when plasmid-positive and plasmid-negative Shigella strains were compared.

**Studies with human affinity-purified anti-B27 antibodies.** Similar comparisons were next made using six different affinity purified human anti–HLA B27 antibodies (Tables III and IV) added to ELISA plates coated with plasmid-positive arthritogenic Shigella and plasmid-negative neutral strains. Only one of six human affinity-purified anti–HLA B27 antibodies showed positive reactions with Shigella. The antibody was derived from a patient with classic AS. No significant difference in reactivity between plasmid-positive and plasmid-negative Shigella strains was noted. Thus, the human antibody reacting with HLA-B27 epitopes failed to distinguish between arthritogenic Shigella strains and those not associated with reactive arthritis.
Discussion

In the present study, we have demonstrated the presence of serum antibodies in some patients with AS or reactive arthritis, which reacted with synthetic peptides representing the polymorphic region of HLA-B27 antigen or a homologous putative sequence derived from the DNA nucleotide sequence of a plasmid isolated from S. flexneri strains associated with reactive arthritis. It is still not yet clear whether the pH5-2 peptide is actually expressed during reactive arthritis in these cases. 13 of 15 affinity-purified anti–B27 peptide antibodies from patients showed strong cross-reactivity with pH5-2 peptide, whereas 3 of the 15 only weakly cross-reacted with Klebsiella nitrogenase peptide. One of the two anti–B27 peptide antibodies from normal female controls with previous pregnancy showed only weak cross-reaction with the pH5-2 and the nitrogenase peptides. These results may suggest that, although both the Klebsiella nitrogenase and pH5-2 share a similar degree of primary sequence homology (Table I), pH5-2 peptide “mimics” the B27 sequence epitope in a more efficiently recognized by human B cells than is the case with the nitrogenase peptide. Although the finding of definite anti–HLA-B27 peptide reactivity in the 15 patient sera studied in Table IV provides clear evidence for the degree of cross-reactivity of such affinity-isolated human antibodies for other similar sequential epitopes, it does not provide direct insight into how such patients actually are capable of making antibodies against antigenic epitopes theoretically contained within their own MHC phenotypes. Actually the data presented in Figs. 2 and 3 show that with the putative pH5-2 peptide, a relatively low level of reactivity can be demonstrated even when large numbers of AS and RS/reactive arthritis patients are studied. Moreover, the data presented here combined with our previous work (15) indicate that only a minority of patients can be shown to have humoral antibody to B27 peptide or similar cross-reacting sequences.

However, the finding that a proportion of patients have antibodies to HLA-B27 sequence which cross-react with the putative sequence encoded by the plasmid represents a finding of considerable interest, because plasmids may be exchanged among various Enterobacteriaceae. It is well known that various microorganisms including Shigella, Salmonella, Campylobacter, Yersinia, and Chlamydia may be involved in the pathogenesis of reactive arthritis (23). Unexpectedly, antibodies to B27 peptide and pH5-2 peptide were elevated in two out of three HLA-B27(+) male post–Chlamydia arthritis patients, while none of the four post–Shigella arthritis patients, including one patient from whom the S. flexneri strain with the pH5-2 plasmid was isolated, were positive for the antibodies. These data could suggest that a DNA sequence encoding a pH5-2-like peptide is expressed by arthriogenic Chlamydia strains. Also, further study with a larger number of patients will be necessary in order to assess whether pH5-2 may be involved in the pathogenesis of post–Shigella arthritis.

It has already been recognized that, in reactions between peptide antigens and antipeptide antibodies, the degree of amino acid sequence homology does not always correlate with the degree of antigenic cross-reactivity (24). Extensive studies by Getzoff et al. (25), during which one amino acid residue within a hexamer peptide was systematically replaced with one of the other 19 residues, revealed that some residues appeared to be critical for binding to antibodies raised against the original protein, whereas replacement of other residues did not result in significant reduction of the reactivity. Our data obtained using pH5-2 analogue peptides clearly indicated that leucine, in the context of QTDR sequence, was critical for the cross-reactivity. Unexpectedly, the peptide with Ala → Arg substitution (R2L9) more strongly cross-reacted with anti–B27 peptide antibodies than did the original pH5-2 peptide. This phenomenon might be related to the biochemical similarity between arginine and lysine (which latter is found at position 70 of the B27 sequence), because both have positively charged polar side chains. This point must also be considered in terms of the critical leucine residue, since the Klebsiella nitrogenase peptide also has arginine at the same position but failed to cross-react with human sera.

The explanations for the discrepancy between the present study and the study by Schwimmbeck et al. (9) with respect to anti–nitrogenase peptide antibody were discussed in our previous paper (15). Firstly, in the original study the cross-reactivity of human antipeptide antibodies had not been demonstrated by methods such as correlation analysis, inhibition, or affinity-purification. Secondly, procedures to correct for nonspecific binding which varies from serum to serum were not clearly mentioned in the original study, whereas we always used peptide-uncoated wells for every serum sample as negative controls (15). This kind of procedure seems to be essential in the ELISA for the antigen-specific antibodies since the presence of antibodies that bind to plastic assay plates has been clearly demonstrated (26). Methods similar to ours which provide an internal correction for nonspecific adsorption have already been used by other investigators (27).

Our CELISA results indicated that at least a proportion of patients’ anti–B27 peptide antibodies were capable of binding to the native HLA-B*2705 molecule. This agrees with our previous observations using rat antipeptide antibodies (13), and those by Rojo et al. (28), which demonstrated rabbit antipeptide to 63-84 peptide of HLA-B*2705 moleucle were cytotoxic to HLA-B*2705 lymphoblastoid cell lines. Whether the absence of significant reaction of the other anti–B27 peptide antibodies was due to the actual lack of binding capability of our assay conditions is not yet clear. Slightly enhanced binding of the pooled human IgG (Cohn fraction II) to the B27-transfected cells could be related to the presence of anti–class I antibodies among normal females with previous pregnancy (14, 29). One of the possibilities suggested by the present study is that bacterial plasmid components such as pH5-2 could play a role in initiating disease through a mechanism of molecular mimicry. Patients with elevated anti–B27 peptide antibodies without significant cross-reaction to pH5-2 peptide could have been triggered by other microorganisms such as Y. pseudotuberculosis with cross-reaction with the same region on HLA-B27 antigen (7).

Alternatively, it might also be possible that antibodies to HLA-B27 polymorphic region are produced by a genuine autoimmune mechanism, and that the cross-reactions with various bacterial components are merely an apparent phenomenon without pathogenic significance. Autoantibodies to HLA-class II antigen have been described in patients with systemic lupus erythematosus (30). Finally, anti–B27 peptide antibodies could originally be directed at B27 polymorphic regions.

Cross-reactions of Autoantibodies to HLA-B27 Antigen
modified by binding with some endogenous or exogenous ligands, as postulated by Seager et al. (31) and Geczy et al. (32, 33). Crystallographic studies have suggested that the amino acid sequence of the HLA-B27 antigen studied in this report corresponds to a part of the presumed antigen-peptide binding cleft (34, 35). Also, the possible role of the unpaired cysteine residue at position 67 in interacting with ligands has been suggested (20). All of these possibilities are not mutually exclusive.

As noted above it still is not clear whether the cross-reacting pHS-2 peptide encoded by the plasmid DNA sequence is expressed in the Shigella strains in question. Direct testing of plasmid-positive and negative strains with affinity-purified rabbit antipeptide-2 antibody showed strong reactions with several of the plasmid-positive strains, however, moderate reactions were also noted when plasmid-negative strains were used. These results may support the possibility of multiple cross-reacting epitopes recognized by the rabbit antibody regardless of the presence of pHS-2 plasmid. Similar observations were reported by Raybourne et al. (8) using anti-HLA MAbs.

Most relevant is the problem that expression of the pHS-2 peptide in vivo in bacteria within the infected patient may not be comparable to that with the same bacterial strains cultivated in vitro. An alternative answer to the problem of identifying links or clues to molecular mimicry in this situation was therefore to examine affinity-purified anti–B27 human antibody to determine whether such populations would show selective reactions with arthritic plasmid-positive Shigella strains. Only one of six affinity-isolated human anti–B27 antibodies reacted with Shigella strains; however, this antibody did not differentiate between arthritogenic and nonarthritogenic organisms. The question must remain open since the antibody was derived from a patient with AS. Ideally the same experiment eventually needs to be repeated using anti-B27-reacting antibody obtained from a patient with shigellosis and reactive arthritis.

How naturally occurring anti–B27 peptide antibodies are related to the pathological process still remains speculative. Our preliminary results of microcytotoxicity assay using HLA-B27 lymphocytes did not show significant cytotoxicity in the sera from patients with AS (data not shown). It is likely, therefore, that the antibodies bind to the class I molecule and could modify cellular functions, rather than directly damaging the tissues through complement mediated lysis. Class I MHC molecules have reportedly been implicated in T cell activation (36). The presence of antibodies to class I molecules thus could possibly result either in proliferative signals through cross-linking of class I molecules or inhibitory effects through blocking of activation signals. The latter mechanism might explain the impaired cellular immunity in patients with reactive arthritis after Salmonella typhimurium infection (37). Immune complexes formed by soluble HLA-class I antigens (38) and anti-class I antibodies might also be involved. The strong expression of this sequential epitope on synovial tissues might partially explain the tissue localization of the inflammation (13). Prolonged persistence of chlamydia or yersinia antigens within synovial tissue or synovial-fluid cells has been reported (39–42), suggesting the possibility of local molecular mimicry phenomena.

The present study provides evidence for cross-reactivity of hypothetical pHS-2 sequence with the HLA-B27 molecule. Additional studies using pHS-2 as well as other bacterial epitopes may eventually clarify the relationship between HLA-B27, antecedent infection and development of spondyloarthropathies.

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