

Antibodies to *Klebsiella*, *Proteus*, and HLA-B27 Peptides in Japanese Patients with Ankylosing Spondylitis and Rheumatoid Arthritis

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ABSTRACT. *Objective.* To determine whether patients with ankylosing spondylitis (AS) and patients with rheumatoid arthritis (RA) from Japan have antibodies to *Klebsiella pneumoniae* and *Proteus mirabilis* and to assess whether such antibodies are activated against peptides sharing sequences with HLA-B27.

Methods. Serum samples from 152 Japanese patients, 52 with AS, 50 with RA, and 50 healthy controls, were tested against 3 bacteria (*K. pneumoniae*, *P. mirabilis*, and *Escherichia coli*) and 3 synthetic peptides (HLA-B27, pullulanase-D, and scrambled pullulanase-D control peptide) by ELISA under coded conditions. Samples were tested for elevations in IgG, IgA, and IgM antibody classes in patients with active AS or RA, in patients with RA with probable disease, and in patients with inactive AS. Disease activity was determined by an elevated serum C-reactive protein (> 10 mg/l) level and elevated erythrocyte sedimentation rate (> 20 mm/h).

Results. Patients with active AS showed specific elevations in serum IgA antibody levels against *K. pneumoniae* compared to patients with RA and controls ($p < 0.001$). No such elevation was seen in the IgG and IgM antibody classes. Patients with inactive AS showed no elevation in any class of antibody against *K. pneumoniae* compared to controls or patients with RA. Patients with active or probably active RA showed significant elevations in IgG antibody levels against *P. mirabilis* compared to AS and controls ($p < 0.001$). Patients with AS (active or inactive), RA (active or probably active), and controls showed no elevations in any antibody class to *E. coli*. Both active and inactive AS patients had specific autoantibodies against HLA-B27 peptide compared to patients with RA and controls (active AS: IgG, IgA, IgM, $p < 0.001$; inactive AS: IgG and IgA, $p < 0.001$). Patients with active AS had IgG and IgA antibodies against pullulanase-D peptide, which contains a sequence that cross reacts with HLA-B27 compared to controls ($p < 0.001$).

Conclusion. These results provide the first evidence of AS and RA patients in Japan having specific elevations of antibody to *K. pneumoniae* and *P. mirabilis*, respectively. This suggests that *K. pneumoniae* in AS and *P. mirabilis* in RA may play a role in triggering and/or exacerbating these diseases. (*J Rheumatol* 1997;24:109-14)

Key Indexing Terms:

ANKYLOSING SPONDYLITIS
KLEBSIELLA

RHEUMATOID ARTHRITIS
PROTEUS

ANTIBODY
HLA-B27

Ankylosing spondylitis (AS) and rheumatoid arthritis (RA) are HLA linked inflammatory disorders. Over 90% of patients with AS possess HLA-B27 and a similar percentage of patients with RA possess HLA-DR1 or DR4¹⁻³. In Japan, the frequency of HLA-B27 is only 0.41% in the general population⁴, and the prevalence of AS is reported to be 0.03-0.04%^{5,6}. In contrast, the frequency of HLA-DR4 (mainly DW15 in Japanese) is about 40%, but the prevalence of RA is 0.3-0.5% in Japanese^{6,7}, lower than the

Caucasian rate^{8,9}. These data suggest that the pathogenesis of AS and RA are mediated by factors, such as the immunogenetic status of the patient and the involvement of environmental agents, similar to those observed in other countries.

An increase in anti-*Klebsiella* antibodies in patients with AS during the active phase has been reported in different geographical locations^{10,11}. Moreover, crossreactivity between *Klebsiella* and HLA-B27 has been reported by several groups¹²⁻¹⁴. In addition to the relationship between AS and *Klebsiella*, elevated levels of antibodies to *Proteus* have been identified in patients with active RA in Europe and America¹⁵⁻¹⁸. An amino acid homology between an outer membrane hemolysin protein of *P. mirabilis* and the susceptibility sequence in HLA-DR1 and DR4 subtypes (DW14, DW15) has been reported¹⁹.

These reports suggest the possibility of bacteria being the causative agents of AS and RA. To explore these possibili-

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ties, we measured IgG, IgA, and IgM class specific antibodies to *K. pneumoniae*, *P. mirabilis*, and synthetic peptides containing HLA-B27 sequences by ELISA in Japanese patients with AS and RA.

MATERIALS AND METHODS

Patients and controls. Serum samples from 152 Japanese patients were studied: 29 from patients with active AS (New York criteria) (27 men/2 women) [mean age 42 years, range 20–69; mean erythrocyte sedimentation rate (ESR) 44.9 mm/h (standard error: SE \pm 3.7), mean C-reactive protein (CRP) 24.8 mg/l (\pm 3.1)]; 23 patients with inactive AS (21 men/2 women) [mean age 42 years, range 25–68; mean ESR 12.2 mm/h (\pm 2.1), mean CRP 3.9 mg/l (\pm 1.1)]; 30 patients with active RA (American Rheumatism Association criteria) (5 men/25 women) [mean age 51 years, range 23–70; mean ESR 77.7 mm/h (\pm 5.9), mean CRP 40.2 mg/l (\pm 5.2)]; 20 patients with probably active RA (5 men/15 women) [mean age 55 years, range 30–77; mean ESR 38.3 mm/h (\pm 3.4), mean CRP 5.9 mg/l (\pm 1.3)], and 50 healthy controls (25 men/25 women) (mean age 47 years, range 30–60), control samples were supplied by the Red Cross Blood Center, Otsu, Japan. Active patients were deemed to be those who had both ESR > 20 mm/h and serum CRP > 10 mg/l. Probable active patients were considered those with at least one of these variables elevated and inactive patients were those with both ESR < 15 mm/h and serum CRP < 10 mg/l¹⁰.

All patients with AS except one inactive patient were HLA-B27 positive. The percentage positive of active RA associated tissue types, DR1 or DR4, was 93.3%, and 40% had both DR1 and DR4 alleles. Control subjects were not tissue typed.

Bacterial cultures. *K. pneumoniae* (K54), *P. mirabilis*, and *Escherichia coli* were urinary tract isolates obtained from the Department of Microbiology, King's College, London. Bacterial cultures were grown aerobically in 250 ml conical flasks on an orbital shaker for 16–18 h at 37°C in nutrient broth (Oxoid: 25 g/l) to obtain a stationary phase culture. Cells were harvested by centrifugation (MSE 18, 6 \times 250 ml rotor) and washed 3 times in 0.15 M phosphate buffered saline (PBS), pH 7.4. The cells were resuspended in 20 ml of PBS and then diluted in carbonate buffer, 0.05 M, pH 9.6, to give an optical density (OD) reading of 0.25 on the spectrophotometer (Corning, Model 258), which is equivalent to 6 \times 10⁸ cells/ml.

Peptide synthesis. Three 16 mer peptides were constructed: the HLA-B27 sequence (residues 67–83) CKAKAQTdredLRtLL, the pullulanase-D (pulD) secretion protein sequence (residues *590–605) RPTVIRdrdeYRQASS, and a control peptide sequence made from a scrambled sequence of the pulD peptide, RPTVRSDDIDYRQAESR. All synthetic peptides were prepared by solid phase synthesis; purity assays were performed by

high performance liquid chromatography. They had a purity of at least 90%. ELISA. All serum samples were tested against 3 bacterial antigens and 3 synthetic peptides by ELISA. All assays were carried out under code, so that the status of each serum sample under investigation was not known to the tester. Briefly, bacteria (100 μ l or 100 μ l of 5 μ g/ml peptide solution) were fixed in polystyrene microtiter ELISA plates (Dynatech, McLean, VA, USA) overnight at 4°C. After absorption and washing with PBS containing 0.1% (v/v) Tween 20 (Sigma, St. Louis, MO, USA), the plates were saturated with 0.5% (w/v) bovine serum albumin (Sigma) PBS-Tween 20 and incubated for 1 h at 37°C to block nonspecific binding. Serum samples (1/200 dilution in PBS-Tween on bacterial studies and 1/50 dilution in PBS-Tween on peptide studies) were added to the plates, incubated for 2 h at 37°C, followed by washing with PBS-Tween and peroxidase conjugated rabbit antihuman class specific IgG, IgA, or IgM (DAKO Ltd.) diluted 1/500 in PBS-Tween was added and the plates incubated for 2 h at 37°C. After washing, the substrate solution, 0.5 ml 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid) ABTS (Sigma) in citrate/phosphate buffer, pH 4.1, containing 0.98 mM H₂O₂ (Sigma), was added to each well. Development of the plates took place at room temperature in the dark for 20 min. The reaction was stopped with 2 mg/ml sodium fluoride solution (Sigma) and the OD measured at wavelength 630 nm with a micro-ELISA plate reader (Dynatech, MR 600).

Statistical analysis. Data were analyzed using Student's t test and coefficient of correlation (r).

RESULTS

ELISA studies on bacteria. ELISA results with whole *K. pneumoniae*, *P. mirabilis*, and *E. coli* are shown in Table 1. The patients with AS with active disease had elevated IgA antibodies to *K. pneumoniae* compared to controls ($t = 5.72$, $p < 0.001$) (Figure 1A). IgA anti-*Klebsiella* antibody levels correlated with both ESR ($r = 0.606$, $p < 0.001$) and serum CRP ($r = 0.540$, $p < 0.001$). Antibodies of the IgG and IgM class showed no significant elevation to *K. pneumoniae*. Patients with inactive AS showed no significant elevation in IgG, IgA, or IgM antibody class compared to controls. No antibody elevation of any class was seen against *P. mirabilis* in active or inactive AS patients compared to controls. Similarly, no elevation in any antibody class against *E. coli* was seen in active AS or inactive AS compared to controls.

Patients with active RA showed elevated IgG ($t = 14.10$,

Table 1. IgG, IgA, and IgM ELISA antibody (OD units) (mean \pm SE) results against whole bacteria (*K. pneumoniae*, *P. mirabilis*, and *E. coli*) in patients and controls. OD₆₃₀ \pm SE.

	Active AS (n = 29)	Inactive AS (n = 23)	Active RA (n = 30)	Probably Active RA (n = 20)	Controls (n = 50)
<i>Klebsiella pneumoniae</i>					
IgG	0.898 \pm 0.051	0.769 \pm 0.043	0.790 \pm 0.040	0.556 \pm 0.074	0.785 \pm 0.038
IgA	0.661 \pm 0.060*	0.154 \pm 0.022	0.272 \pm 0.041	0.205 \pm 0.041	0.222 \pm 0.019
IgM	0.458 \pm 0.037	0.357 \pm 0.023	0.336 \pm 0.025	0.344 \pm 0.029	0.334 \pm 0.019
<i>Proteus mirabilis</i>					
IgG	0.220 \pm 0.020	0.199 \pm 0.024	0.752 \pm 0.045*	0.456 \pm 0.035*	0.219 \pm 0.012
IgA	0.051 \pm 0.011	0.046 \pm 0.008	0.034 \pm 0.006	0.035 \pm 0.005	0.031 \pm 0.003
IgM	0.105 \pm 0.011	0.102 \pm 0.012	0.144 \pm 0.014*	0.104 \pm 0.008	0.093 \pm 0.005
<i>Escherichia coli</i>					
IgG	0.730 \pm 0.043	0.619 \pm 0.051	0.691 \pm 0.028	0.721 \pm 0.046	0.820 \pm 0.025
IgA	0.134 \pm 0.016	0.111 \pm 0.016	0.129 \pm 0.015	0.069 \pm 0.008	0.116 \pm 0.009
IgM	0.348 \pm 0.038	0.329 \pm 0.034	0.408 \pm 0.038	0.361 \pm 0.026	0.385 \pm 0.026

* $p < 0.001$ compared to controls.

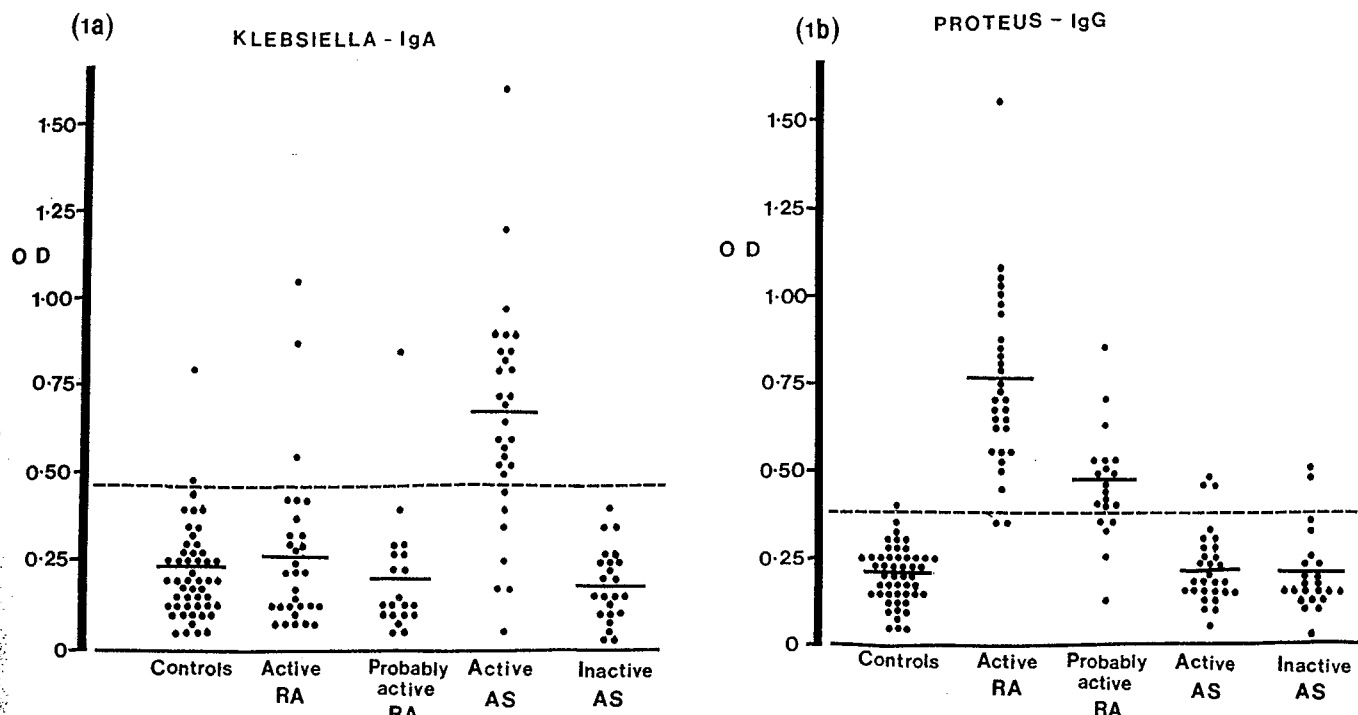


Figure 1. IgA *Klebsiella* antibodies (1A) and IgG *Proteus* antibodies (1B) in patients with RA (active and probably active), patients with AS (active and inactive) compared to control subjects. The broken line represents 95% confidence limit of the distribution of the controls (bars = means).

$p < 0.001$) and IgM ($t = 4.01$, $p < 0.001$) antibodies against *P. mirabilis* compared to controls (Figure 1B). However, IgA antibody was not significantly elevated. Similar results were seen in the probably active RA patients, with IgG antibody levels elevated compared to controls ($t = 8.30$, $p < 0.001$), whereas IgA and IgM levels were not. There was no significant correlation between IgG anti-*Proteus* antibody levels and the acute phase reactants ESR and serum CRP. Antibodies against *K. pneumoniae* were not elevated in active or probably active patients. No elevation in any class of antibody was seen against *E. coli* in active or probably active patients.

ELISA studies on synthetic peptides. ELISA results with 3 synthetic peptides are shown in Table 2. Active AS patients showed significant elevation in all antibody classes against the HLA-B27 peptide compared to controls (IgG; $t = 11.94$, $p < 0.001$), (IgA; $t = 8.298$, $p < 0.001$), (IgM; $t = 7.503$, $p < 0.001$) (Figure 2A). Similar results were seen in IgG ($t = 5.908$, $p < 0.001$) and IgA ($t = 5.776$, $p < 0.001$) antibodies in the inactive AS patients. There was no significant correlation between antibody levels to HLA-B27 peptide and the acute phase reactants ESR and serum CRP, except between IgG anti-B27 peptide antibody levels and CRP ($r = 0.332$, p

Table 2. IgG, IgA, and IgM ELISA antibody (OD units) (mean \pm SE) results against peptides (HLA-B27, pullulanase-D, and control) in patients and control subjects. OD₆₃₀ \pm SE.

	Active AS (n = 29)	Inactive AS (n = 23)	Active RA (n = 30)	Probably Active RA (n = 20)	Controls (n = 50)
HLA-B27 peptide					
IgG	0.446 \pm 0.027*	0.281 \pm 0.024*	0.153 \pm 0.010	0.123 \pm 0.012	0.159 \pm 0.010
IgA	0.195 \pm 0.012*	0.161 \pm 0.015*	0.073 \pm 0.010	0.036 \pm 0.008	0.076 \pm 0.008
IgM	0.261 \pm 0.018*	0.191 \pm 0.021	0.128 \pm 0.009	0.130 \pm 0.012	0.136 \pm 0.007
Pullulanase-D peptide					
IgG	0.505 \pm 0.034*	0.196 \pm 0.021	0.186 \pm 0.015	0.200 \pm 0.014	0.215 \pm 0.009
IgA	0.224 \pm 0.035*	0.086 \pm 0.017	0.096 \pm 0.021	0.087 \pm 0.021	0.064 \pm 0.007
IgM	0.508 \pm 0.035	0.298 \pm 0.027	0.426 \pm 0.024	0.385 \pm 0.017	0.444 \pm 0.018
Control peptide					
IgG	0.677 \pm 0.041	0.589 \pm 0.040	0.602 \pm 0.032	0.668 \pm 0.038	0.696 \pm 0.028
IgA	0.238 \pm 0.026	0.207 \pm 0.027	0.208 \pm 0.027	0.142 \pm 0.015	0.211 \pm 0.014
IgM	0.469 \pm 0.027	0.441 \pm 0.034	0.474 \pm 0.031	0.454 \pm 0.034	0.487 \pm 0.022

* $p < 0.001$ compared to controls.

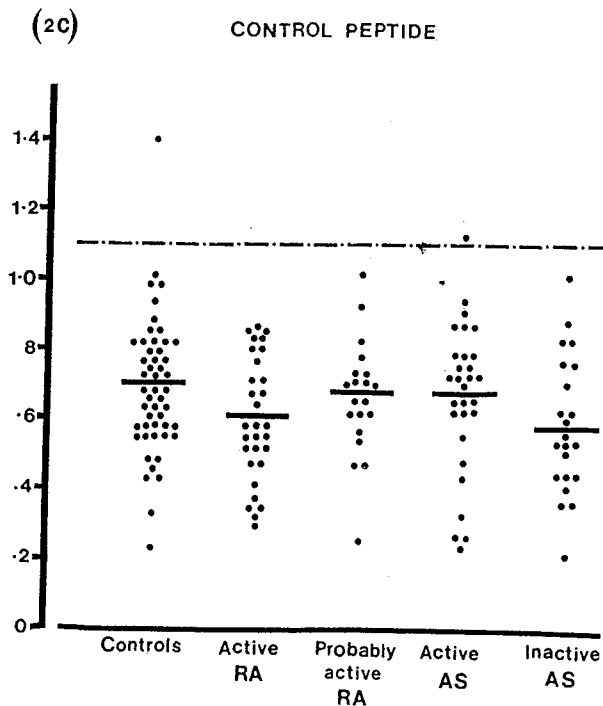
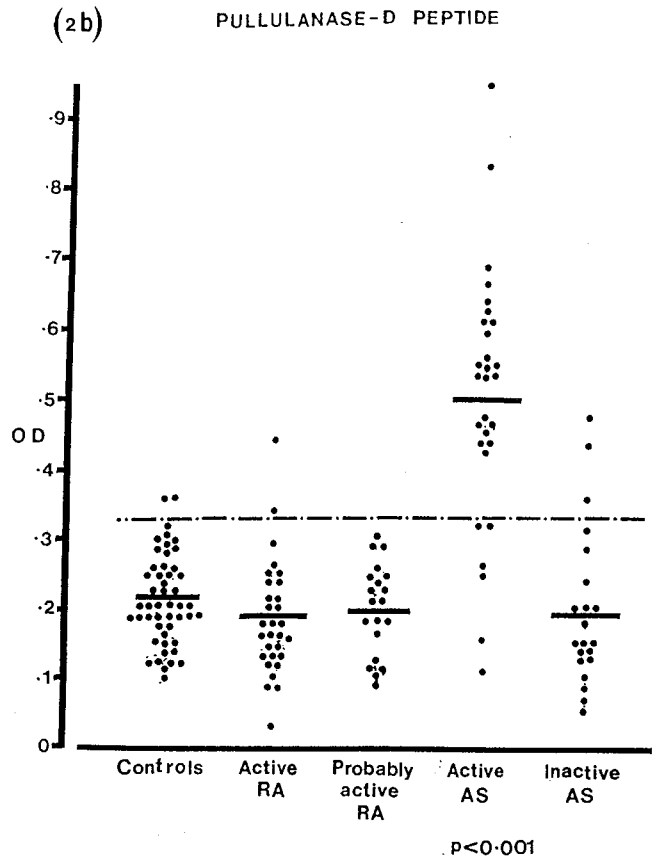
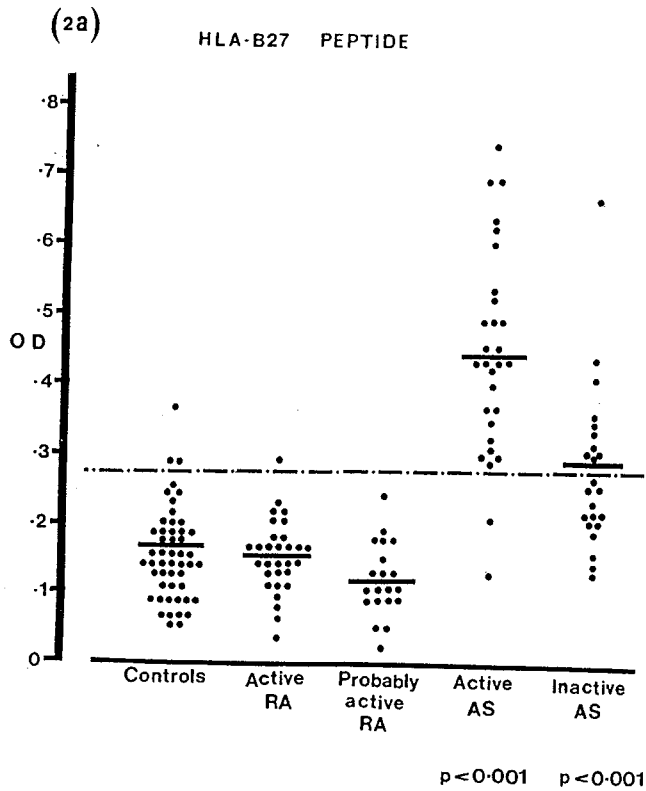


Figure 2. IgG antibodies (bars = means) in patients with RA (active and probably active), patients with AS (active and inactive) compared to control subjects, tested against HLA-B27 peptide (2A), pullulanase-D peptide (2B), and scrambled pullulanase-D control peptide (2C). The broken line represents 95% confidence limit of the distribution of the controls (significant p values compared to controls are indicated).

< 0.05). Furthermore, IgG ($t = 10.37$, $p < 0.001$) and IgA ($t = 5.646$, $p < 0.001$) antibodies against the pulD peptide were also found to be elevated in active patients compared to controls (Figure 2B). IgG antibodies to pulD peptide were significantly correlated with ESR ($r = 0.498$, $p < 0.001$) and CRP ($r = 0.426$, $p < 0.01$), while IgA antibodies to pulD

were significantly correlated only with ESR ($r = 0.339$, $p < 0.05$). No elevation in any class of antibody was seen against the HLA-B27 peptide or the pulD peptide in patients with RA. No antibody elevation of any class was seen against the control peptide in AS patients, RA patients, or controls (Figure 2C).

DISCUSSION

We report that Japanese patients with active AS have specific IgA antibodies to *K. pneumoniae* and Japanese patients with RA have specific IgG antibodies to *P. mirabilis*. Furthermore, IgA anti-*Klebsiella* antibody levels in patients with AS correlated with the degree of inflammation as measured by the acute phase reactants ESR and CRP. No such correlation was seen with anti-*Proteus* antibodies in RA patients; however, the group comprised only active and probably active patients and lacked patients with inactive disease, which is necessary for adequate correlation studies. This is the first report from Japan that specific antibacterial antibodies are present in patients with AS and RA. This finding confirms reports from other countries^{10-12,16-18}. The significant elevation of IgA antibody titers against *K. pneumoniae* in AS suggests the site of infection is the gut. The elevation in IgG antibodies but not IgA antibodies against *P. mirabilis* in patients with RA suggests the source of infection could be a nonmucosal site, probably the kidney. Clinically, the increased levels of occult chronic intestinal inflammation in patients with AS and urinary tract infection in patients with RA have been reported^{20,21}. These observations may suggest that local, subclinical bacterial infection could be related to the pathogenesis of these disorders. It has been reported that total serum IgA is elevated in AS²². However, in this study only patients with AS with active disease had significant elevations of antibodies against *Klebsiella*, while patients with RA had antibodies only against *Proteus*. These findings exclude the possibility that these results are due to a nonspecific effect of immunoglobulin. Furthermore, the observation of elevated anti-*Proteus* antibodies in patients with RA has been shown not to be due to rheumatoid factor activity²³.

These relationships between AS, RA, and bacterial infections could be explained by crossreactivity and molecular similarity in amino acid sequences between human leukocyte antigens (HLA) and bacterial proteins. In 1987, Schwimmbeck, *et al* reported homology and crossreactivity between HLA-B*2705 and *K. pneumoniae* nitrogenase (QTDRED)²⁴. Recently, a further sequence homology between HLA-B*2705 (DRED) and the terminal secretion protein of pullulanase enzyme system, pulD (DRDE), has been identified²⁵. In our study, elevation of antibody levels against the HLA-B27 peptide and the pulD peptide in patients with AS were observed, confirming the Schwimmbeck, *et al* report²⁴.

These results support the possibility that the molecular mimicry hypothesis may explain the high frequency of HLA-B27 in patients with AS. However, further studies are needed, in which each amino acid within the pulD and HLA-B27 synthetic peptides is substituted one by one to assess its importance in reactivity with test sera. In RA, molecular similarity has also been identified between protein sequences of *P. mirabilis*, hemolysin (ESRRAL), and HLA-

DR antigens (DRB1*0101, DRB1*0404, DRB1*0405 and 1402; EQRRRA)¹⁹. Additionally, a second homology has been shown between the urease enzyme (IRRET) of *P. mirabilis* and type XI collagen (LRREI)²⁶.

Longitudinal studies are required to assess whether antibiotic intervention or diet therapies will reduce the specific bacterial antibody levels, thereby affecting the acute phase reactants in patients with AS and RA and slowing down the progression of arthritic disease.

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