Cross-Reactivity between the Rheumatoid Arthritis-Associated Motif EQKRAA and Structurally Related Sequences Found in Proteus mirabilis

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Cross-reactivity or molecular mimicry may be one of the underlying mechanisms involved in the etiopathogenesis of rheumatoid arthritis (RA). Antiserum against the RA susceptibility sequence EQKRAA was shown to bind to a similar peptide ESRRAL present in the hemolysin of the gram-negative bacterium Proteus mirabilis, and an anti-ESRRAL serum reacted with EQKRAA. There was specificity with the EQKRAA antibody in that it did not cross-react with anti-ESRRAL to a peptide containing the EDERA sequence which is present in HLA-DRB1*0402, an allele not associated with RA. Furthermore, the EQKRAA and ESRRAL antisera bound to a mouse fibroblast transfectant cell line (Dap3) expressing HLA-DRB1*0401 but not to DRB1*0402. However, peptide sequences structurally related to the RA susceptibility motif LEIEKDFTTYGEE (P. mirabilis urease), VEIRAEGNRTFY (collagen type II) and DELSPETSPYVKE (collagen type XI) did not bind significantly to cell lines expressing HLA-DRB1*0401 or HLA-DRB1*0402 compared to the control peptide YASGASGASGAS. It is suggested here that molecular mimicry between HLA alleles associated with RA and P. mirabilis may be relevant in the etiopathogenesis of the disease.

The link of a set of related HLA-DR alleles, DRB1*0101 (DR1), DRB1*0401 (DR4/Dw4), DRB1*0404 (DR4/Dw14), DRB1*0405 (DR4/Dw15), and DRB1*1402 (DR6/Dw16), which share an amino acid sequence EQ(K/R)RRAA and also have an increased susceptibility to develop rheumatoid arthritis (RA) has been well established (11, 16). The mechanism by which the susceptibility motif predisposes to RA is at present unknown. However, it has been suggested that an environmental factor interacting with a genetic predisposition may be one mechanism involved in the pathogenesis of this debilitating disease (8). Recent studies have demonstrated cross-reactivity between a sequence QKRAA, which is present in the DnaJ heat shock protein of Escherichia coli and the RA susceptibility sequence (1). We have identified an amino acid sequence similarity between the RA susceptibility motif and the sequence ESRRAL found in the hemolysin of Proteus mirabilis (17). Furthermore, elevated levels of antibodies to EQKRAA (13) and ESRRAL (17) have been found in RA patients. Apart from reactivity with antibody, the RA associated motif has also been shown to play a role in peptide binding (7), and we have identified a structurally related peptide found in P. mirabilis urease LEIEKDFTTYGEE and collagen type XI, DELSPETSPYVKE, which satisfy the criteria for selectively binding the RA associated motif (19). Our studies were undertaken to determine the extent of cross-reactivity between the RA associated motif KDILEQRAAVDTCY (residues 65 to 79); HLA-DRB1*0402 peptide, CKDILEDERAADVTY (residues 65 to 79); and P. mirabilis hemolysin peptide, CL GSISERSRALQDSQR (residues 65 to 79). A cysteine residue was attached to all three peptides at the N terminus for coupling of the synthetic peptides to the carrier protein. The following peptides were labelled with the fluorescence marker dimethoxy-coumaryl-alanine: LEIEKDFTTYGEE (P. mirabilis urease, residues 27 to 42); DELSPETSPYVKE (type XI collagen, residues 543 to 555); VEIRAEGNRTFY (collagen type II, residues 1350 to 1361); and a control peptide, YASGASGASGAS.

**Materials and Methods**

**Peptide synthesis.** The peptides were assembled by using an automated Milligen Biosearch model 9050 Pepsynthesiser on NovaSyn TG flow resin with a loading value of 0.41 mmol/g liter, functionalized with a Rink amide linker. Acylation cycles with (four equivalents) Fmoc (9-fluorenylemethoxycarbonyl) amino acid, preactivated with 2(1-H-benzoтриazole-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate (TBTU) and di-isopropyl ethylamine (DIPEA) in a molar ratio (1:1:1:5 by volume) were carried out in DMF for 30 to 40 min. Fmoc deprotection was achieved with 20% piperidine in DMF. N-terminal acetylation and the combined filtrate was dried by rotary evaporation. Residual TFA was continuously monitored by measuring the immune response to various concentrations with PD10 columns (Sigma Chemical Ltd.). New Zealand White rabbits were immunized with 1-mg/ml solution (synthetic peptide conjugated to KLH) by using m-maleimidobenzoyl-N-hydroxysuccinimide ester (6). The conjugate was purified by gel filtration with PD10 columns (Sigma Chemical Ltd.). New Zealand White rabbits received three subcutaneous injections at 2-week intervals. A 250-μl aliquot of a 1-mg/ml solution (synthetic peptide conjugated to KLH) was added to 250 μl of Specel adjuvant (Central Veterinary Institute). Peptide antiserum reactivity was continuously monitored by measuring the immune response to various concen-
tations of both the target peptide and KLH alone. The rabbits were bled 14 days after the last immunization, and the resultant sera were stored at −20°C.

ELISA. Antibody responses were measured by peptide enzyme-linked immunosorbent assay (ELISA). Briefly, the assay was carried out as follows: flat-bottomed, rigid polystyrene microtiter plates (Dynatech) were coated with 100 μl of synthetic peptide (5.0 μg peptide per ml) and KLH (10 μg/ml) overnight at 4°C. After adsorption and being washed with phosphate-buffered saline (PBS; pH 7.4) containing 0.1% (vol/vol) Tween 20 (Sigma), the plates were saturated with 1% (wt/vol) bovine serum albumin (Sigma)-PBS-Tween 20 and incubated for 1 h at 37°C, followed by further washing with PBS-Tween 20. Different dilutions of peptide rabbit antisera in PBS-Tween were then added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween. Peroxidase-conjugated goat anti-rabbit class-specific immunoglobulin G (Dako, Ltd.) diluted 1/500 in PBS-Tween 20 was added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween 20. Different dilutions of peptide rabbit antisera in PBS-Tween were then added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween. Peroxidase-conjugated goat anti-rabbit class-specific immunoglobulin G (Dako, Ltd.) diluted 1/500 in PBS-Tween 20 was added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween 20. Different dilutions of peptide rabbit antisera in PBS-Tween were then added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween. Peroxidase-conjugated goat anti-rabbit class-specific immunoglobulin G (Dako, Ltd.) diluted 1/500 in PBS-Tween 20 was added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween 20. Different dilutions of peptide rabbit antisera in PBS-Tween were then added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween. Peroxidase-conjugated goat anti-rabbit class-specific immunoglobulin G (Dako, Ltd.) diluted 1/500 in PBS-Tween 20 was added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween 20. Different dilutions of peptide rabbit antisera in PBS-Tween were then added, and the plates were incubated for 90 min at 37°C, followed by washing with PBS-Tween.

Mouse fibroblast (Dap.3) cells. Mouse fibroblast Dap.3 cells transfected with HLA-DRB1*0401, HLA-DRB1*0402, and untransfected cells, together with L243 (anti-DRα) in supernatant form, were kindly provided by R. Lechler of the Department of Immunology, Hammersmith Hospital, London, England. Both sets of transfected and untransfected cells were maintained as described previously (2). However, transfected cells were also grown in the presence of G418 (transfected or untransfected) were incubated on ice for 1 h with L243, with a second antibody only, as well as with different dilutions of rabbit peptide antisera. After a washing in PBA, the mean cell fluorescence values, defined by the use of the negative control peptide, were detected with a FACScan.

RESULTS

Dilution studies with peptide antisera. The results obtained in this study demonstrate cross-reactivity between KDILLEQRRAAVIDTYC and LGSISSESRALDQSR (Fig. 1A and B) but not with the Dw10 motif KDILLEDERAAVIDTYC when tested by ELISA (Fig. 1C).

Increased binding activity by the HLA-DRB1*0401 peptide antisera and the P. mirabilis hemolysin peptide antisera was present compared to the HLA-DRB1*0402 peptide antisera (Fig. 1A). The mean OD (± the standard error [SE]) was calculated for each sample.

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Peptide antisemur assay. An aliquot of 10^6/ml per fluorescence-activated cell sorter tube (Falcon) of cells was suspended in PBS (PBS plus 0.1% [wt/vol] plus 1% [wt/vol] azide), the tubes centrifuged at 4°C at 1,500 rpm (Omnifuge 20RS; Heraeus Sepatech) for 5 min, and the supernatant was discarded. Mouse cells (transfected or untransfected) were incubated on ice for 1 h with L243, with a second antibody only, as well as with different dilutions of rabbit peptide antisera. After a washing in PBA, aliquots of swine anti-rabbit fluorescein isothiocyanate-labelled total immunoglobulin (Dako) diluted in PBA (1/24) were added to the assay tubes and incubated for 1 h on ice. The cells were washed and resuspended in PBA. The percentage of cells which fluoresced above an arbitrarily set level by the use of the negative control (second antibody only) were detected with a FACScan by using the software provided (Becton Dickinson) with the permission of N. Staines of the Infection and Immunology Section, Division of Life Sciences, King’s College, London, England.

Peptide binding assay. Mouse transfectants of DRB1*0401 and DRB1*0402 and untransfected cells were incubated on ice for 90 min, with concentrations of test or control peptide as described above that ranged between 0.25 and 1 mg/ml. After a washing in PBA, the mean cell fluorescence values, defined by the use of the negative control peptide, were detected with a FACScan.

FIG. 1. Anti-peptide antiserum dilution response curves with ELISA. The antisera were raised against the following KLH conjugates of peptide: CKDLLLEQRRAAVIDTYC, LGSISSESRALDQSR, and KDILLEDERAAVIDTYC. The binding of antisera and preimmune serum was determined by using the uncoupled peptides LGSISSESRALDQSR (A), KDILLEQRRAAVIDTYC (B), and KDILLEDERAAVIDTYC (C) adsorbed onto the ELISA plate. Also shown is the inhibition of binding by the indicated peptide.
between DRB1*0401 and *P. mirabilis* hemolysin peptide antisera, respectively, to DRB1*0402 (Fig. 1C). Furthermore, there was no reactivity to KLH when all three peptide antiserum samples were tested (data not shown).

**Inhibition studies.** Peptide antiserum raised against DRB1*0401* was inhibited by preincubation with 100 μg of LGSIES RRALQDSQR peptide per ml, as well as with KDLLEQKRAA VDTYC peptide. In a similar way, antisera raised against the *P. mirabilis* sequence was also inhibited by preincubation with 100 μg of KDLLEQKRAA VDTYC and LGSIESRRALQDSQR peptides per ml (Fig. 1A and B). The anti-CLGSIESRRALQDSQR antiserum prior to incubation with DRB1*0401* peptide had a mean antibody binding activity of 1.49 (±0.02) at a dilution of 1:200 and reacted at dilutions of up to 1:51,200. After incubation, the binding activity was reduced to 0.20 (±0.04) at a 1:200 dilution and reacted at dilutions up to 1:6,400. Similar results were obtained with the LGSIESRRALQDSQR peptide (Fig. 1A). The DRB1*0401* antiserum had a mean antibody binding activity to the DRB1*0401* peptide of 1.29 (±0.01) at 1:200 and bound at a dilution of up to 1:51,200. However, after incubation with the LGSIESRRALQDSQR peptide, the activity was reduced to 0.21 (±0.02) at 1:200, and the serum reacted at a dilution of up to 1:6400. Furthermore, similar results were obtained with the KDLLEQKRAA VDTYC peptide (Fig. 1B).

**Flow cytometric analysis.** Mouse fibroblast transfected cell lines expressing intact DRB1*0401* and DRB1*0402* molecules, together with the untransfected cells, were also used for
cross-reactive studies. An arbitrarily set value of $10^4$ for cell fluorescence was used as the cutoff point, as only 2% of the DRB1*0401 and DRB1*0402 transfected cells fluoresced positively with the negative control (second antibody only) (Fig. 2A1), since a positive control L243 was used (Fig. 2A2). At an antibody dilution of 1:160, 88% of DRB1*0401 transfected cells were positively bound by the CKDLLKRAAQDVC peptide and pooled preimmune serum binding to the mouse fibroblast transfected cell line Dap.3 expressing HLA-DRB1*0401 (DR4/Dw4). The percentages of cells which fluoresce at levels greater than the arbitrarily set level of $10^3$ are shown. (C) Immunofluorescence profiles for $10^4$ transfected DR4/Dw4 Dap.3 cells with fluorescence greater than $10^1$. Fluorescence intensity (x axis) versus cell number (y axis). Quadrant plots show binding of negative control (secondary antibody only) (panel 1), L243 (anti-DRa) (panel 2), anti-EQKRAA (panel 3), anti-ESRRAL (panel 4), anti-EDERA (panel 5), and pooled preimmune serum (panel 6). The peptide and preimmune serum were used at a 1:160 dilution. The marker (M1) indicates the positive area of binding. (D) Dilution studies of antisera raised against CKDLLKRAAQDVCTC, CLGSIESRRALQDSQR, and CKDLEDERAAQDVCTC peptides and pooled preimmune rabbit serum binding to mouse fibroblast transfected cell line Dap.3 expressing HLA-DRB1*0402 (DR4/Dw10). The percentages of cells which fluoresce at levels greater than the arbitrarily set level of $10^3$ are shown.
SQR (Fig. 2A4) peptide antiserum compared to 21% bound by the anti-CKDILEDERAAVDTYC (Fig. 2A5) and 5% bound by the pooled preimmune rabbit (Fig. 2A6) sera. Both anti-CKDILEDERAAVDTYC and anti-CLGSISESRRALQDSQR antiserum samples bound to the transfected cells at dilutions of up to 1:10,240, whereas anti-CKDILEDERAAVDTYC stopped reacting at 1:2,560 and the pooled preimmune serum stopped reacting at 1:640 (Fig. 2B). Furthermore, increased binding to transfecants expressing DR4/Dw10 was demonstrated by using antiserum raised to DRB1*0402 peptide at dilutions of 1:40, 1:80, and 1:160 compared to anti-CKDILEEQKRAAVDTYC, anti-CLGSISESRRALQDSQR, and pooled preimmune rabbit sera. At an antibody dilution of 1:80, 22% of the DRB1*0402 transfected Dap.3 cells were positively bound by the anti-CKDILEEQKRAAVDTYC (Fig. 2C1), 7% by the anti-CKDILEEQKRAAVDTYC (Fig. 2C2), and 10% by the anti-CLGSISESRRALQDSQR (Fig. 2C3) peptide antiserum and 9% were bound by the pooled preimmune rabbit sera (Fig. 2C4). All three peptide antiserum and the preimmune serum stopped binding to the DR4/Dw10 cells at a dilution of 1:2,560 (Fig. 2D). However, there was no difference in binding between the individual peptide antiserum and the pooled preimmune rabbit serum to the untransfected mouse cells (Table 1).

**HLA-peptide binding.** Fluorescent labelled peptides from type II as well as type XI collagens and *P. mirabilis* bound without appreciable differences to mouse fibroblast transfec tant cell lines expressing HLA-DRB1*0401 and DRB1*0402 molecules compared to the control peptide. The mean cell fluorescence as determined by the FACSscan did not differ when the two transfecants and individual peptide concentration of the test and control peptides were used (Table 2).

**DISCUSSION**

In this study, peptide antiserum raised in rabbits against the RA susceptibility sequence EQKRAA reacted with *P. mirabilis* hemolysin ESRRAL peptide. In a reciprocal manner, antiserum against the hemolysin sequence demonstrated greater binding affinity towards the RA susceptibility motif than the EDERAA peptide sequence of HLA-DRB1*0402 found in the HLA-Dw10, an allele not associated with RA. These observations are compatible with the recent report of cross-reactivity between the RA susceptibility motif QKRAA and the DnaJ protein of *E. coli* QKRAA (1). In addition, the structurally related peptides LEIEKDFTTYGEE of *P. mirabilis* urease and DELS,PETSPYVK of type XI collagen showed low reactivity with HLA-DRB1*0401 molecules. Furthermore, recent studies have shown that the type II collagen sequence VEIRAEGNRFTY bound with high affinity to the RA-associated motif but not to the nonassociated DRB1*0402 (7). However, in this study, the

**TABLE 1. Binding of peptide antiserum raised against CKDILEEQKRAAVDTYC, CLGSISESRRALQDSQR, and CKDILEDERAAVDTYC and pooled preimmune serum to mouse untransfected fibroblast cell line**

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<th>Doubling dilution</th>
<th>Anti-CKDILEEQKRAAVDTYC</th>
<th>Anti-CLGSISESRRALQDSQR</th>
<th>Anti-CKDILEDERAAVDTYC</th>
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*Values indicate the percentage of cells which fluoresced at levels greater than the arbitrarily level of 10^1 when measured by FACSscan analysis.
VEIRAEGNRFTY peptide showed low reactivity with HLA-DRB1*0401 molecules. These findings could be due to a difference in methodology and further studies with affinity-purified HLA-DRB1*0401 molecules may increase the sensitivity of the peptide binding assay. The results obtained provide some evidence for a possible role of E. coli and P. mirabilis in the pathogenesis of RA. It has been reported that RA patients with active disease have specific antibodies against P. mirabilis (3, 5, 12), a finding which correlates with both levels of C-reactive protein (3) and isolation rates of P. mirabilis (18). A decrease in anti-Proteus antibody levels and a decrease in a modified Stoke disease activity index was observed in RA patients treated with a high fluid and vegetarian diet (10). Furthermore, RA patients were reported to have elevated antibodies to the 63-kDa hemolysin protein of P. mirabilis and to a 16-mer synthetic peptide containing the ESRRAL sequence (13). The P. mirabilis peptide binding by RA sera has recently been confirmed by an independent group (4). In a related study, it was reported that Japanese patients with RA have increased antibodies against a 16-mer synthetic peptide of DRB1*0409 which also contains the EQRRAA sequence (13).

The ESRRAL motif was found in 5 of 77,573 sequences according to the Protein Information Resource database: P. mirabilis, Serratia marcescens, Vibrio cholerae, Rickettsia tsutsugamushi, and Brucella ovis. Two other organisms, Pseudomonas aeruginosa DORRAA and E. coli EKQRAA have a sequence identity with EQRRAA, the RA susceptibility motif. However, we were unable to find any antibody levels against E. coli, Serratia sp., and Pseudomonas sp. in RA patients, although significant titers were present against Proteus sp. (15). Apart from such antibody responses against microorganisms in RA, T-cell reactions might also play a role in the development of the disease. The presentation of such microbial peptides as LEIEKDFTYYGEE of P. mirabilis by the RA-associated DR motif to CD4+ T cells could lead to the initiation of disease because of cross-reactivity with HLA-presented peptides of self-antigens of collagen type II and collagen type XI due to molecular mimicry. This CD4+ response to such a peptide needs to be measured in individuals with the appropriate HLA-DR alleles with or without RA.

The results presented in this study suggest that antibodies raised against P. mirabilis ESRRAL antigens during urinary tract infections could subsequently bind, albeit with lower affinity, to DR4-positive cells in tissues expressing the class II HLA antigens EOKRAA and EQRRAA, fix complement, and so initiate local inflammation that could lead to destruction of self-tissues by antibody-dependent cell cytotoxicity. It is important to note that E. coli is responsible for 80% of urinary tract infections in women, while P. mirabilis accounts for approximately 15 to 18% of cases. Interestingly, it is known that RA patients suffer an increased incidence of urinary tract infections (14). Furthermore, Proteus urease contains a sequence, IRRET, which cross-reacts with the sequence LRREI found in the α2 chain of type XI collagen (17), a component of hyaline cartilage, and this cross-reaction could play a role in the development of erosions found in this disease.

The mechanism of RA disease pathogenesis is as yet unknown; however, the possible involvement of microorganisms in RA would appear to be gaining greater acceptance. The most likely candidates for this disease pathogenesis are E. coli and P. mirabilis.

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