Chapter 16
Molecular Similarity Between the Rheumatoid Arthritis Associated Motif EQKRAA and Structurally Related Sequences of *Proteus*

Contents

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Introduction: The Association of the 'Shared Epitope' with Sequences of *Proteus* and Transfectant Cell Lines

The HLA-DR alleles, DRB1*0401, *0402, *0405, *0101 and *1402, which share an amino acid sequence EQR(K)RAA in the DRB1 chain, have been linked with a susceptibility to develop rheumatoid arthritis.

It has been suggested that an environmental factor interacting with a genetic predisposition contributes to the pathogenesis of rheumatoid arthritis and studies from our group have suggested that antigens present in the urinary microbe *Proteus mirabilis* are somehow involved in the pathology of this disease.

The mechanism by which the susceptibility motif predisposes to the disease is to some extent unknown, although the two main theories have been that the cavity of the 'shared epitope' presents as yet some unknown antigen to lymphocytes (Hammer et al. 1995) or the sequences making up the cavity share some 'molecular mimicry' with environmental agents such as the urinary microbe *Proteus mirabilis* (Wilson et al. 2000).

Our studies were undertaken to determine the extent of cross-reactivity between the rheumatoid arthritis associated motifs **EQKRAA** and **EQRRAA** with the non-rheumatoid arthritis associated motif **EDERAA** and compare them to the *Proteus mirabilis* bacterial haemolysin sequence **ESRRAL**.

Peptide Synthesis

The peptides were assembled by using an automated Milligen Biosearch model 9050 Pepsynthesizer on NovaSyn TG flow resin with a loading value of 0.41 mmol·g/l, functionalized with a Rink amide linker. Acylation cycles with (four equivalents) Fmoc (9-fluorenylmethoxycarbonyl) amino acid, preactivated with 2(1-H-benzo-triazo-1-yl)-1,1,9-tetramethyl uranium

tetrafluoroborate (TBTU) and di-iso-propyl-ethylamine (DIPEA) in a molar ratio (1:1:1.5 by volume) were carried out in DMF for 30–40 min. Fmoc deprotection was achieved with 20% piperidine in DMF. N-terminal acetylation was carried out by using pentafluorophenyl acetate (4 equivalents)for 30 min. Reactions were monitored by UV detection of the column effluent at 365 nm and colour tests for free amino groups (Kaiser et al. 1980). Complete peptidyl resins were washed successively with DMF, methanol and diethyl ether before being dried in vacuo. The peptidyl resin was treated with a solution (10 ml) of trifluoroacetic acid (TFA)-H_2O-tri-iso-propyl-silane (94:5:1 by volume) for 90 min at room temperature. The resin was then filtered through a sintered funnel and washed with TFA, and the combined filtrate was dried by rotary evaporation. Residual TFA was azeotroped with diethyl ether. The products were lyophilized from water and purified by preparative high-performance liquid chromatography with a Vidac 218TP54 column on a Waters 900 photodiode array system. Solvent A was 0.1% TFA and solvent B was 10% solvent A plus 90% acetonitrile. All peptides used had a purity in excess of 90% and their masses were determined by using a Bioanalysis MALDI-TOF instrument with a-cyano-4-hydroxycinnamic acid as the matrix.

The molecular mimicry sequences are shown in bold. The sequences of the peptides were as follows:

1. The HLA-DRB1*0401 peptide, CKDLL**EQRRAA**VDTYC (residues 65–79) which is associated with rheumatoid arthritis.
2. The HLA-DRB1*0402 peptide, CKDIL**EDERAA**VDTYC (residues 65–79) which is NOT associated with rheumatoid arthritis.
3. The *Proteus mirabilis* haemolysin peptide, CLGSIS-**ESRRAL**QDSQR (residues 27–42).

A cysteine residue was attached to all three peptides at the N terminus for coupling of the synthetic peptide to the carrier protein.

Peptide Antisera

Synthetic peptides of HLA-DRB1*0401, HLA-DRB1*0402 and *Proteus mirabilis* haemolysin sequence were conjugated to the carrier protein keyhole limpet haemocyanin (KLH) (ICN Biomedical Ltd) by using m-maleiimido-benzoyl-N-hydroxysuccinimide ester (Green et al. 1982).

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 of a 1 mg/ml solution (synthetic peptide conjugated to KLH) was added to 250 μl of Specol adjuvant (Central Veterinary Institute). Peptide antiserum reactivity was continuously monitored by measuring the immune response to various concentrations of both the target peptide and KLH alone. The rabbits were bled 14 days after the last immunisation, and the resultant sera were stored at –20°C.

ELISA

Antibody responses were measured by peptide enzyme-linked-immunosorbent assay (ELISA) as previously described.

For inhibition studies, the inhibitors, KDLL**EQKRAA**-VDTYC, LGSIS**ESRRAL**QDSQR and KDIL**EDERAA** (100 μg/ml), were incubated overnight at 37°C with the three peptide antisera, before ELISAs were carried out. All ELISAs were carried out in triplicate, and the mean OD value (± standard error) was calculated for each sample.

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 Prof. R. Lechler of the Department of Immunology, Hammersmith Hospital, London, England.

Both sets of transfected and untransfected cells were maintained as previously described (Barber et al. 1991). However, transfected cells were also grown in the presence of G418 (200 μl/ml) (Gibco). The formation of an adherent monolayer indicates healthy growth.

Dilution Studies with Peptide Antisera

The results obtained in this study demonstrate cross-reactivity between KDLL**EQKRAA**VDTYC and LGSIS**ESRRAL** but not with the Dw 10 motif KDIL**EDERAA**VDTYC when tested by ELISA.

Increased binding activity by the HLA-DRB1*0401 peptide antiserum and the *Proteus mirabilis* haemolysin peptide antiserum was present compared to the HLA-DRB1*0402 peptide antiserum.

The mean optical density (OD) (± standard error) at a dilution of 1/1,600 of the DRB1*0401 peptide antiserum tested against the *Proteus mirabilis* haemolysin peptide was 0.87 ± 0.02 OD units which was significantly higher than the value of 0.11 ± 0.03 OD units obtained for binding with the DRB1*0402 peptide antiserum or the value of 0.02 ± 0.02 OD units obtained with the pre-immune serum (Fig. 16.1).

Furthermore the DRB1*0401 antiserum bound to the *Proteus mirabilis* haemolysin peptide up to a dilution of 1/51,200, while DRB1*0402 antiserum stopped reacting at 1/6,400 (Fig. 16.1).

Binding activity of the *Proteus mirabilis* haemolysin peptide antiserum and the DRB1*0401 peptide antiserum was increased compared to the DRB1*0402 peptide antiserum.

The mean (± standard error) OD units binding at 1/1,600 dilution of the *Proteus mirabilis* peptide antiserum was 0.77 ± 0.04 OD units which was significantly higher than the value of 0.05 ± 0.02 OD units obtained for the DRB1*0402 peptide antiserum or the value of 0.04 ± 0.01 OD units obtained with the pre-immune serum (Fig. 16.2).

The *Proteus mirabilis* haemolysin peptide antiserum bound to DRB1*0401 peptide at 1/25,600, whereas the DRB1*0402 peptide antiserum reacted at dilutions of up to 1/6,400 (Fig. 16.2).

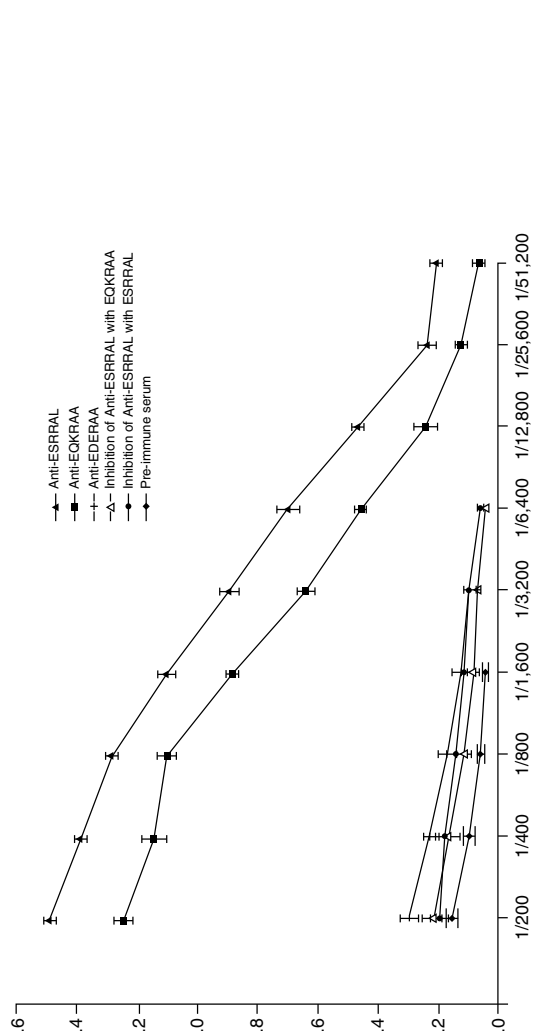

FIGURE 16.1 Anti-peptide antiserum dilutions response curves with ELISA. The antisera were raised against the following KLH conjugates of peptide CKDLLE**QKRAA**VDTYC, CLGSIS**ESRRAL**QDSQR and CKDIL**EDERAA**VDTYC. The binding of the antisera and pre-immune serum was determined by using the uncoupled peptide LGSIS**ESRRAL**QDSQR adsorbed onto the ELISA plate. Also shown is the inhibition by the indicated peptide (With permission from Tiwana et al. (1999))

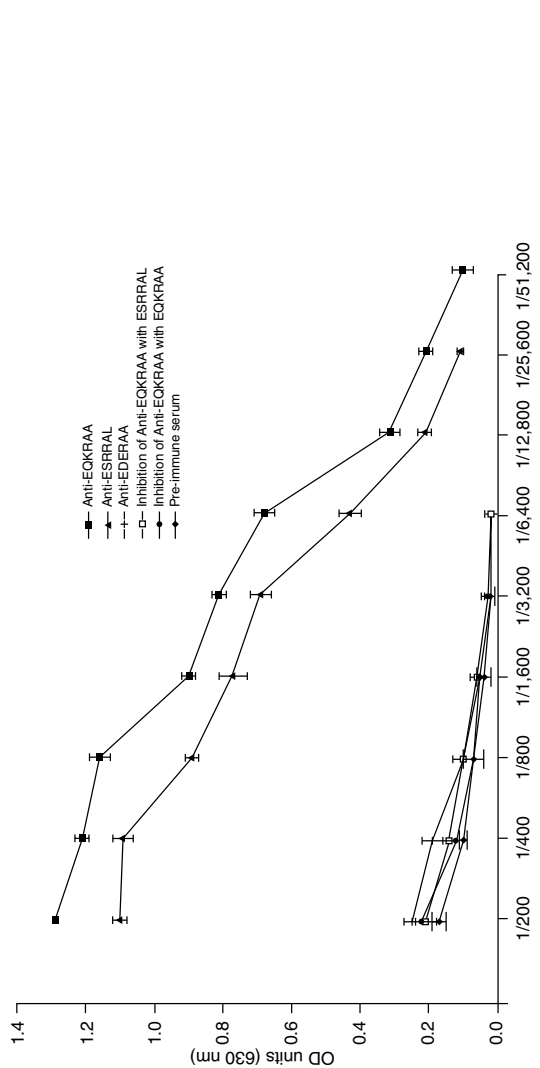

FIGURE 16.2 Anti-peptide antiserum dilutions response curves with ELISA. The antisera were raised against the following KLH conjugates of peptide CKDLL**EQKRAA**VDTYC, CLGSI**SERRAL**QDSQR and CKDIL**EDERAA**VDTYC. The binding of the antisera and pre-immune serum was determined by using the uncoupled peptide KDLLE**EQKRAA**VDTYC adsorbed onto the ELISA plate. Also shown is the inhibition by the indicated peptide (With permission from Tiwana et al. (1999))

However, there was no increased binding activity between DRB1*0401 and *Proteus mirabilis* haemolysin peptide antisera, respectively to DRB1*0402 (Fig. 16.3).

Furthermore, there was no reactivity to keyhole limpet haemocyanin (KLH) when all three peptide antiserum samples were tested.

Inhibition Studies

Peptide antiserum raised against DRB1*0401 was inhibited by pre-incubation with 100 μg of LGSIS**ESRRAL**QDSQR peptide per ml, as well as with KDLL**EQKRRA**VDTYC peptide.

In a similar way, antisera raised against the *Proteus mirabilis* haemolysin sequence was also inhibited by preincubation with 100 μg/ml of KDLL**EQKRAA**VDTYC (Fig. 16.1) or preincubation with 100 μg/ml of LGSIS**ESRRAL**QDSQR (Fig. 16.2).

The anti-CLGSIS**ESRRAL**QDSQR antiserum prior to incubation with DRB1*0401 peptide had a mean (± standard error) antibody-binding activity of 1.49 ± 0.02 OD units 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 dilution of 1/200 and reacted at dilutions up to 1/6,400.

Similar results were obtained with LGSIS**ESRRAL**QDSQR peptide. The DRB1*0401 antiserum had a mean (± standard error) to the DRB1*0401 peptide of 1.29 ± 0.01 OD units at 1/200 dilution and bound up to a dilution of 1/51,200.

However, after incubation with the LGSIS**ESRRAL**-QDSQR peptide, the peptide activity was reduced to 0.21 ± 0.02 at 1/200 dilution and the serum reacted at a dilution of 1/6,400 (Fig. 16.2).

Furthermore, similar results were obtained with the KDLL**EQRRAA**VDTYC peptide (Fig. 16.2).

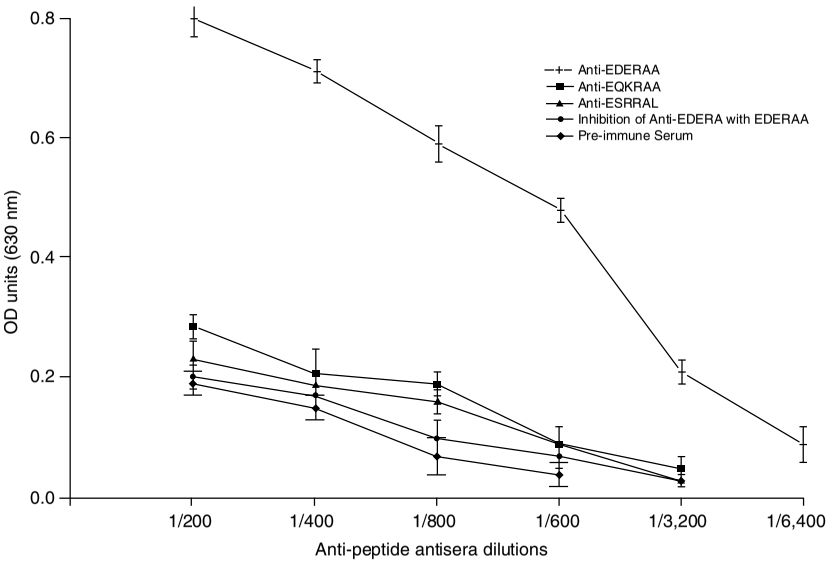

FIGURE 16.3 Anti-peptide antiserum dilutions response curves with ELISA. The antisera were raised against the following KLH conjugates of peptide CKDLL**EQKRAA**VDTYC, CLGSIS**ESRRAL**QDSQR and CKDIL**EDERAA**VDTYC. The binding of the antisera and pre-immune serum was determined by using the uncoupled peptide KDIL**EDERAA**VDTYC adsorbed onto the ELISA plate. Also shown is the inhibition by the indicated peptide (With permission from Tiwana et al. (1999))

Binding by Mouse Fibroblast Cell Lines

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.

At an antibody dilution 1/160,88% of DRB1*0401 transfected cells were positively bound by the CKDLL**EQKRAA**VDTYC and 79% were bound by the CLGSIS**ESRRAL**QDSQR peptide antisera compared to 21% bound by the anti-CKDIL-**EDERAA**VDTYC and 5% bound by the pooled pre-immune rabbit sera (Fig. 16.4).

Both anti-CKDLL**EQRRAA**VDTYC and anti-CLGSIS**ESRRAL**QDSQR antiserum samples bound to the transfected cell lines at dilutions of up 1/10,240, whereas anti-CDIL**EDERAA**VDTYC stopped reacting at 1/ 2,560 and the pooled pre-immune serum stopped reacting at 1/ 640 (Fig. 16.4).

Furthermore, increased binding to transfectants expressing DR4/Dw10 was demonstrated by using antiserum raised to DRB1*0402 peptide at dilutions 1/40, 1/80 and 1/160 compared to anti-CKDLL**EQKRAA**VDTYC, anti-CLGSIS**ESRRAL**QDSQR and pooled pre-immune rabbit sera.

At an antibody dilution of 1/80, 22% of the DRB1*0402 transfected Dap.3 cells were positively bound by the anti-CKDIL**EDERAA**VDTYC, 7% by the anti-CKDLL**EQKRAA**VDTYC and 10% by the anti-CLGSIS**ESRRAL**QDSQR and 9% were bound by the pooled pre-immune rabbit sera.

All three peptide antisera and the pre-immune serum stopped binding to the DR4/Dw10 cells at a dilution of 1/ 2,560 (Fig. 16.5).

However, there was no difference in binding between the individual peptide antiserum and the pooled pre-immune rabbit serum to the untransfected cells.

Discussion and Conclusions

In this study, peptide antisera raised in rabbits against the rheumatoid arthritis susceptibility sequence EQKRAA reacted with the *Proteus mirabilis* haemolysin ESRRAL peptide.

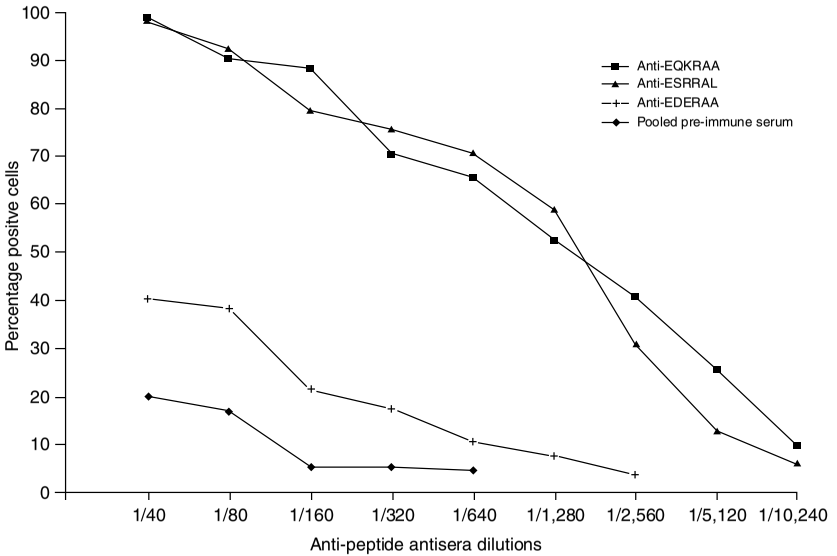

FIGURE 16.4 Dilution studies of antisera raised against CKDLL**EQKRAA**VDTYC, CLGSIS**ESRRAL**QDSQR and CKDIL**EDERAA**VDTYC peptides and pooled pre-immune rabbit serum binding to mouse fibroblast transfected cell line Dap.3 expressing HLA-DRB1*0401 (DR4/Dw4). The percentage of cells which fluoresce at levels greater than the arbitrarily set level of 10^1 are shown (With permission from Tiwana et al. (1999))

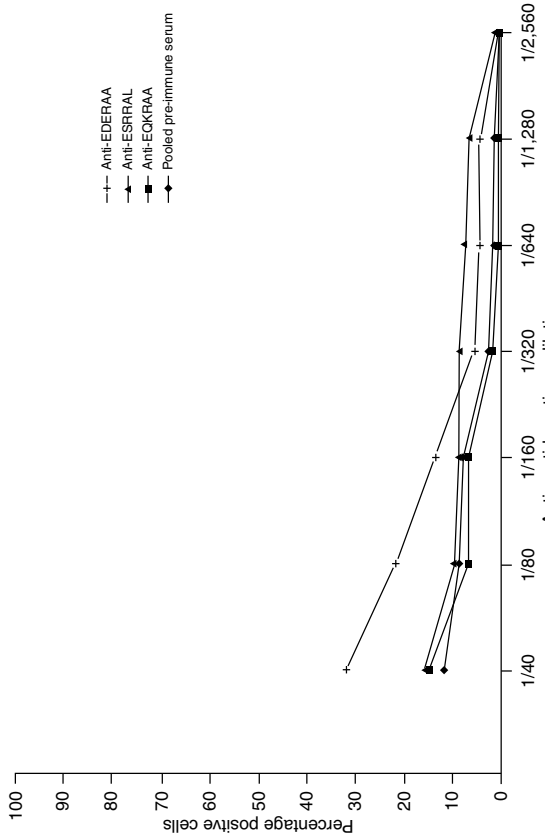

FIGURE 16.5 Dilution studies of antisera raised to CKDLL**EQKRAA**VDTYC, CLGSIS**ESRRAL**QDSQR and CKDIL**EDERAA**VDTYC peptides and pooled pre-immune serum binding to the mouse fibroblast transfected cell line Dap.3 expressing HLA-DRB1*0402 (DR4/Dw10). The percentage of cells which fluoresce at levels greater than the arbitrarily set level of 10^1 are shown (With permission from Tiwana et al. (1999))

In a reciprocal manner, antisera raised against the *Proteus mirabilis* haemolysin sequence demonstrated greater binding affinity towards the rheumatoid arthritis susceptibility motif than the EDERAA peptide sequence of HLA-DRB1*0402 found in the HLA-Dw10 complex, an allele not associated with rheumatoid arthritis.

The results presented in this study suggest that antibodies raised against *Proteus mirabilis* ESRRAL antigens during urinary tract infections could possible bind, albeit with lower affinity, to HLA-DR4-positive cells in tissues expressing the class II HLA antigens EQKRAA and EQRRAA, fix complement and so initiate local inflammation that could lead to destruction of self-tissues in the joints by antibody-dependent cell cytotoxicity (Tiwana et al. 1999).

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