Cytotoxicity Responses to Peptide Antigens in Rheumatoid Arthritis and Ankylosing Spondylitis

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ABSTRACT. Objective. To measure levels of IgG antibodies against structurally related synthetic peptides of HLA-DRB1*0404, type XI collagen, and Proteus mirabilis in patients with rheumatoid arthritis (RA) and HLA-B*2705 in Klebsiella pneumoniae in patients with ankylosing spondylitis (AS), and to determine whether sera from RA and AS patients are cytotoxic for sheep red blood cells (SRBC) coated with HLA-DRB1*0404, type XI collagen, or HLA-B*2705.

Methods. Sera from 51 patients with RA, 34 with AS, and 38 healthy controls were tested against synthetic EQRRAA, ESRRAL, LRREI, and IRRET peptides by ELISA. Sera from patients and controls were also tested for reactivity in complement mediated cytotoxicity with SRBC coated with EQRRAA and HLA-B*2705, LRREI peptides.

Results. Antibodies to synthetic peptides containing EQRRAA, ESRRAL, LRREI, and IRRET were significantly increased in RA patients compared with AS patients (p < 0.001) and controls (p < 0.001). The percentage lysis data for SRBC coated with EQRRAA and LRREI peptides were significantly higher for RA sera (p < 0.001) compared to control sera. Percentage lysis for SRBC coated with HLA-B*2705 peptide was significantly higher for AS sera (p < 0.001) compared to control sera.

Conclusion. Our results suggest that antibodies against antigenic determinants of P. mirabilis in RA and K. pneumoniae in AS have cytotoxic properties on structurally related host proteins. These cytotoxic antibodies together with T cell interactions could be relevant in the etiopathogenesis of RA and AS.

Key Indexing Terms:
CYTOTOXICITY
RHEUMATOID ARTHRITIS
PEPTIDE ANTIGENS

The HLA-DR alleles, DRB1*0401, *0404, *0405, *0101, and *1402, which share an amino acid sequence EQR(K)RAA in the DRB1 chain, have been linked to susceptibility to develop rheumatoid arthritis (RA)1,2. It has been suggested that an environmental factor interacting with a genetic predisposition contributes to the pathogenesis of the disease. We have reported that RA patients with active disease have elevated levels of antibodies against Proteus mirabilis3,4, and this observation has been confirmed by several independent groups5-7. Further, the level of anti-Proteus antibodies correlates with urinary isolation rates of Proteus in RA8. Amino acid sequence homologies9 and immunological cross-reactivity10 between the susceptibility motif EQR(K)RAA and P. mirabilis hemolysin ESRRAL as well as type XI collagen LRREI and P. mirabilis urease IRRET have been reported9. Patients with RA have been shown to have antibodies against both the hemolysin protein and a 16-mer synthetic peptide containing the ESRRAL sequence by 2 groups9,11.

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatological disorder of which 95% of patients are HLA-B*2705 positive. Increased levels of antibodies to the bacterium Klebsiella pneumoniae have been reported, suggesting a role for this microbe as an etiological agent in the disease. Amino acid sequence homologies have been identified between HLA-B*2705 (QTDRED) and 2 enzymes present in K. pneumoniae, nitrogenase reductase (QTDRED)12 and pullulanase secretion protein pul D (DRDE)13. Patients with AS have been shown to possess elevated levels of antibodies to all 3 peptide sequences compared to control groups. We investigated whether humoral immune responses against antigenic determinants of P. mirabilis in the sera of patients with RA and K. pneumoniae in the sera of patients with AS are cytotoxic for cells expressing structurally related peptides from DRB1*0404, type XI collagen, and B*2705 and whether antibodies against these molecules correlate with percentage lysis.

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MATERIALS AND METHODS

Patients. Sera were collected from patients with active RA [erythrocyte sedimentation rate (ESR) 15 mm/h] attending the Rheumatology Department at the Lister Hospital, Stevenage, England, and patients with active AS attending the AS Research Clinic at the Middlesex Hospital, London. The diagnosis of RA was made according to the American Rheumatism Association criteria and that of AS by the New York criteria.

Antibodies against synthetic 15/16-mer peptides containing ESRRAL, EQQRRA, LRREI, IRREI, EDERAA, QTDRED, and DRED and control sequences were measured in sera from 51 patients with RA [18 men, 33 women, mean age 49 yrs (range 28–70), mean ESR 45.4 (SE 3.6) mm/h]; 34 patients with AS [26 men, 8 women, mean age 46 yrs (range 23–69), mean ESR 48.2 (SE 3.7) mm/h]; and 38 healthy controls [18 men, 20 women, mean age 40 yrs (range 24–57)].

ELISA. Peptides were prepared by solid phase synthesis and analyzed for purity by high performance liquid chromatography. All peptides had a purity of at least 90%. The test peptides were SQKDLLEEQRRAA VDTY of HLA-DRB1*0404; LGSSERRALQDSQR of P. mirabilis hemolysin; GSLDSLRRREIEQMR of type XI collagen; FAESRIRREITIAED of P. mirabilis urease, control peptide SQKDLLEERAAVDTY of HLA-DRB1*0402; CKAAXQDRELDRTL of HLA-B*2705; RPTVR-DREYRQAQS of K. pneumoniae pullulanase; CNSRQDTREDELII of K. pneumoniae nitrogenase; ASLHEEGIKAQLE of human myosin; KKLHEKEAELQAKLE of Streptococcus; LGSSRSEITQDQR of scrambled hemolysin; and RPTVRSIDYRQAEQR of scrambled pullulanase. The ELISA was carried out as follows: 96 well flat bottom rigid polystyrene microtiter plates (Dynatech, Billingham, UK) were coated with the synthetic peptides dissolved in carbonate buffer (5.0 µg/well) overnight at 4°C. After adsorption and washing with phosphate buffered saline (PBS)-TWEEN 20, the plates were saturated with 1% bovine serum albumin (BSA) in PBS-Tween and incubated 1 h at 37°C, followed by further washing with PBS-Tween. Serum samples (200 µl) at 1:50 dilution in PBS-Tween were added and plates incubated for 90 min at 37°C, followed by washing with PBS-Tween and peroxidase conjugated rabbit anti-human class-specific IgG (Dako Ltd., Bucks, UK) diluted 1:500 in PBS-Tween added (200 µl) and the plates incubated for 90 min at 37°C. After washing, the substrate (2, 2′-azinobis(3)-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical, Dorset, UK) was added to each well (200 µl) and the plates developed at room temperature for 20 min; the reaction was stopped with sodium fluoride (100 µl) (2 mg/ml) and the optical density (OD) measured at wavelength 630 nm. All assays were carried out in duplicate and under code, so that the status of each serum sample under investigation was not known to the tester.

Preparation of sheep red blood cells (SRBC) and cytotoxicity assay. A 20 µl aliquot of sheep blood (Unipath Ltd., Hampshire, UK) was washed twice with 0.85% sodium chloride (saline) in a centrifuge (Heraeus, minifuge) at 750 g (3000 rpm) for 15 min. Supernatant was discarded and 600 µl of the packed cells were removed and placed into each of 2 universal containers. The cells in each container were resuspended in 10 ml saline, and 10 ml of a 1:20,000 tannic acid (Aldrich Chemical Co., Dover, UK) in saline added to each container, followed by mixing and incubation for 15 min at 37°C in a water bath. After centrifugation for 5 min, the supernatant was discarded and cells resuspended in 20 ml saline and washed. One container of cells was later used as a negative control. The cells in the other container were resuspended in 10 ml saline and 10 ml antigen (2 mg/ml) added, followed by mixing and incubation for 30 min at 37°C in a water bath. After washing by centrifugation for 5 min, the supernatant was discarded; cells were washed 3 times with saline and both coated and uncoated (control) cells resuspended in 50 ml of saline.

Serum samples were inactivated in a water bath at 56°C for 30 min. The cytotoxicity assay was carried out as follows: 100 µl of serum diluted 1:8 in saline was added to 96 well microtiter plates (Dynatech) together with 100 µl of peptide coated (SRBC) and the plates incubated at 37°C for 30 min. A 100 µl aliquot of guinea-pig complement (Calbiochem Ltd., Nottingham, UK) diluted 1:10 in saline was added followed by mixing, then incubation at 37°C for 30 min and later at 4°C overnight to allow the unlysed cells to settle. A 100 µl aliquot of test supernatant was removed and placed into wells of a microtiter plate and absorbance measured at 570 nm. Minimum lysis was calculated from peptide coated cells treated with saline plus complement, and absorbance value obtained from the minimum lysis was subtracted from each test value. One hundred percent lysis was calculated from uncoated cells (100 µl) treated with 200 µl of distilled water (maximum lysis). Percentage lysis was determined using the following formula:

\[
\% \text{ Lysis} = \frac{\text{(Test lysis – Minimum lysis)}}{\text{(Maximum lysis – Minimum lysis)}} \times 100
\]

All assays were carried out in duplicate and under code, i.e., the tester did not know which sera came from patients or controls.

Antibody absorption assay. Serum samples from 5 RA patients with high antibody levels to the individual peptides were absorbed with packed SRBC (250 µl) coated with the ESRRAL, EQQRRA, IRREI, and LRREI peptide sequences in a plastic tube overnight at 4°C, with gentle rotation. The absorption process was repeated until the antibody level for each sample was below the mean value of the control subjects, measured by ELISA. The absorbed sera were then tested for cytotoxic activity against SRBC coated with EQQRRA and LRREI peptides as described above.

Statistical analysis. The mean OD units of class-specific IgG antibodies against the various synthetic peptides in the different groups were compared with Student’s t test. The association between anti-Proteus or anti-Klebsiella peptide antibodies and percentage lysis of SRBC was analyzed using the correlation coefficient (r).

RESULTS

Antibodies to peptide antigens in RA patients. Table 1 shows the results of the IgG antibody studies in the 3 groups of patients. Concentrations of antibodies to HLA-DRB1*0404 peptide SQKDLLEEQRRAA VDTY of the IgG class were significantly higher in RA patients (0.537 ± 0.013 OD units; mean ± SE) compared with AS patients (0.134 ± 0.006) (t = 23.65, p < 0.0001) and controls (0.152 ± 0.010) (t = 21.62, p < 0.0001). The difference between the levels in AS patients and controls tested against the DRB1*0404 peptide was not significant. Concentrations of antibodies to P. mirabilis hemolysin peptide LGSSERRALQDSQR of the IgG class were significantly higher in RA patients (0.590 ± 0.015) compared with AS patients (0.155 ± 0.008) (t = 22.17, p < 0.0001) and controls (0.170 ± 0.010) (t = 21.38, p < 0.0001). The difference between the levels in AS patients and controls tested against the DRB1*0404 peptide was not significant.

Concentrations of antibodies to type XI collagen peptide GSLDSLRRREIEQMR of the IgG class were also significantly elevated in RA patients (0.490 ± 0.009) compared with AS patients (0.133 ± 0.007) (t = 30.08, p < 0.0001) and controls (0.165 ± 0.010) (t = 26.17, p < 0.0001). The difference between the levels in AS patients and controls tested against the type XI collagen peptide was not significant. Concentrations of antibodies to type XI collagen peptide GSLDSLEQMR of the IgG class were also significantly higher in RA patients (0.514 ± 0.010) compared with AS patients (0.146 ± 0.007) (t = 26.13, p < 0.0001) and controls.
The difference between the levels in AS patients and controls tested against the *P. mirabilis* urease peptide was not significant. There was no significant reactivity by RA and AS patients or controls against the DRB1*0402 peptide SQKDLEDERAAVDTY (Table 1).

Cytotoxicity studies in RA patients. Figure 1 and Figure 2 show the results of cytotoxicity studies in the 3 groups of patients. Sera from RA and AS patients and controls were tested for reactivity in complement mediated cytotoxicity with SRBC coated with HLA-DRB1*0404 and type XI collagen peptides. The percentage lysis for SRBC coated with peptide SQKDLEQRRAAVDTY was significantly higher with RA sera (83.3 ± 2.2) compared with AS sera (13.7 ± 0.8) (t = 25.63, p < 0.0001) and controls (15.5 ± 1.2) (t = 25.06, p < 0.0001) (Figure 1). The difference between the levels in AS patients and controls tested against the DRB1*0404 peptide was not significant. The percentage lysis for SRBC coated with peptide GSLDSLREIEQMRR was significantly higher with RA sera (65.7 ± 2.2) compared with AS sera (11.5 ± 0.9) (t = 19.16, p < 0.0001) and controls (13.7 ± 1.3) (t = 18.43, p < 0.0001).

There was a significant correlation between anti-ESRRAL and anti-IRRET antibodies and percentage lysis of SRBC coated with EQRRAA (Figure 2A) (r = 0.970, p < 0.0001) or LRREI (Figure 2B) (r = 0.940, p < 0.0001) peptides for the RA patients and controls, respectively. There was also a significant correlation between anti-EQRRAA and anti-LRREI antibodies and percentage lysis of SRBC coated with EQRRAA (r = 0.954, p < 0.0001) or LRREI (r = 0.948, p < 0.0001) peptides for the RA patients and controls, respectively (not shown).

Absorbed sera from 5 RA patients were tested for reactivity in complement mediated cytotoxicity with SRBC coated with EQRRAA and LRREI peptides. The mean percentage lysis for SRBC coated with EQRRAA and LRREI peptides for the 5 RA sera was 100% and 82%, respectively. However, after absorption with SRBC coated with EQRRAA, ESRRAL, LRREI, and IRRET, the mean percentage lysis was 13.7% and 12.2%, respectively (Table 2). Absorption analyses with EQRRAA, ESRRAL, LRREI, and IRRET thus resulted in the reduction of percentage lysis.

Antibodies to peptide antigens in AS patients. Table 3 shows the results of the IgG antibody studies in the 3 groups of patients. Concentrations of IgG antibodies to HLA-B27 peptide CKAKAQTDRDELRTLL were significantly elevated in AS patients (0.491 ± 0.011) compared to RA patients (0.181 ± 0.012) (t = 18.52, p < 0.0001) and controls (0.223 ± 0.015) (t = 14.15, p < 0.0001). No significant difference was seen in the levels of antibodies to the *Klebsiella* nitrogenase peptide CNSRQT-DREDELIIA of the IgG class were significantly higher in AS patients (0.529 ± 0.011) compared to RA patients (0.197 ± 0.012) (t = 18.89, p < 0.0001) and controls (0.233 ± 0.014) (t = 15.96, p < 0.0001). The difference between the levels of antibodies to the *Klebsiella* nitrogenase peptide was not significantly different between RA patients and controls. Levels of IgG antibodies to *K. pneumoniae* pullulanase peptide RPTVIRDREYRQASS were also significantly elevated in AS patients (0.530 ± 0.010) compared to RA and controls (0.290 ± 0.012) (t = 17.89, p < 0.0001).

Table 1. IgG antibodies to synthetic peptides in controls and patients with RA and AS. Mean ± SE of the absorbance values are given. Molecular mimicry sequences are shown in bold.

<table>
<thead>
<tr>
<th>Test Peptide</th>
<th>Source</th>
<th>Controls</th>
<th>RA</th>
<th>AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQKDLEQRRAAVDTY</td>
<td>HLA-DRB1*0404</td>
<td>0.152 ± 0.010</td>
<td>0.537 ± 0.013†</td>
<td>0.134 ± 0.006</td>
</tr>
<tr>
<td>LGSSESRRLQDSQR</td>
<td><em>P. mirabilis</em> hemolysin</td>
<td>0.170 ± 0.010</td>
<td>0.590 ± 0.015†</td>
<td>0.155 ± 0.008</td>
</tr>
<tr>
<td>GSLDSLREIEQMRR</td>
<td>Type XI collagen</td>
<td>0.136 ± 0.011</td>
<td>0.490 ± 0.009†</td>
<td>0.133 ± 0.007</td>
</tr>
<tr>
<td>FAESRIRRETIAAED</td>
<td><em>P. mirabilis</em> urease</td>
<td>0.171 ± 0.012</td>
<td>0.514 ± 0.010†</td>
<td>0.146 ± 0.007</td>
</tr>
<tr>
<td>SQKDLEDERAAVDTY</td>
<td>HLA-DRB1*0402</td>
<td>0.131 ± 0.009</td>
<td>0.132 ± 0.007</td>
<td>0.113 ± 0.008</td>
</tr>
</tbody>
</table>

† p < 0.0001.

Figure 1. Percentage lysis of sheep red blood cells coated with HLA-DRB1*0404 (EQRRAA), 0402 (EDERAA), and type XI collagen (LRREI) peptides, via complement mediated cytotoxicity, by control sera and patients with RA and AS.
Figure 2. Correlation of anti-

\(P.\) mirabilis hemolysin (A) and anti-

\(P.\) mirabilis urease (B) IgG antibody levels for AS and RA patients and controls and percentage lysis of sheep red blood cells coated with HLA-DRB1*0404 and type XI collagen peptides, respectively.

Table 2. Antibody absorption assay on RA sera (n = 5). The mean ± SE of the absorbance values (630 nm) and the percentage lysis are given.

<table>
<thead>
<tr>
<th>Absorbing Test Peptides</th>
<th>ELISA (OD) Units</th>
<th>Cytotoxicity (% lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preabsorption</td>
<td>Postabsorption</td>
</tr>
<tr>
<td>HLA-DRB1*0404</td>
<td>0.63 ± 0.005</td>
<td>0.13 ± 0.009</td>
</tr>
<tr>
<td>(P.) mirabilis hemolysin</td>
<td>0.72 ± 0.004</td>
<td>0.11 ± 0.010</td>
</tr>
<tr>
<td>Type XI collagen</td>
<td>0.56 ± 0.009</td>
<td>0.12 ± 0.007</td>
</tr>
<tr>
<td>(P.) mirabilis urease</td>
<td>0.59 ± 0.010</td>
<td>0.13 ± 0.010</td>
</tr>
</tbody>
</table>

ND: not done.
with RA patients (0.183 ± 0.012) (t = 20.15, p < 0.0001) and controls (0.218 ± 0.016) (t = 16.32, p < 0.0001). The difference between levels in RA patients and controls tested against *Klebsiella* pullulanase was not significant. No significant reactivity by AS or RA patients or controls was observed when tested against human myosin, *Streptococcus*, or hemolysin scramble or pullulanase scramble peptides used in this study (Table 3).

**Cytotoxicity studies in AS patients.** Sera from AS and RA patients and controls were tested for complement mediated cytotoxicity against SRBC coated with HLA-B*2705 synthetic peptide. The percentage lysis for SRBC coated with CKAKAQTDREDLRTL was significantly higher with AS sera (69.2 ± 3.1) compared with RA sera (14.8 ± 1.1) (t = 19.28, p < 0.0001) and controls (16.6 ± 1.4) (t = 16.13, p < 0.0001) (Figure 3). No significant difference was observed between the RA patients and healthy controls. No significant differences in cytotoxic activity were found between the AS and RA patients and controls when testing SRBC coated with either human myosin or hemolysin scrambled or pullulanase scrambled peptides.

There was a significant correlation between anti-nitrogenase (QTDRED) (r = 0.826, p < 0.0001) (Figure 4A) and anti-pullulanase (DRDE) (r = 0.823, p < 0.0001) (Figure 4B) antibodies and percentage lysis of SRBC coated with QTDRED (HLA-B*2705) peptides for the AS and RA patients and controls. There was also a significant correlation between anti-HLA-B*2705 antibodies and percentage lysis of SRBC coated with QTDRED (HLA-B*2705) peptide (r = 0.817, p < 0.0001) for the AS and RA patients and controls (not shown).

**DISCUSSION**

Patients with RA have been shown to have increased levels of antibodies against synthetic peptides derived from *P. mirabilis* hemolysin ESRRAL, DRBI*0404 EQRRAA, type XI collagen LRREI, and *P. mirabilis* urease IRRET. However, we were unable to find any significant increase in concentrations of antibodies to the DRB1*0402 peptide. These findings confirm our previous reports and those of others, that RA patients have increased levels of antibodies against synthetic peptides containing ESRRAL and EQRRAA sequences. Further, RA sera are cytotoxic for sheep red blood cells coated with DRB1*0404 and type XI collagen peptides. There was significant correlation between elevated levels of anti-ESRRAL and anti-IRRET antibodies and percentage lysis of SRBC coated with EQRRAA or LRREI peptides. Similarly, AS patients have been shown to have increased levels of antibodies against synthetic peptides derived from *K. pneumoniae* and HLA-B*2705*. Furthermore, AS sera are cytotoxic for SRBC coated with HLA-B*2705* and there was a significant correlation between anti-HLA-B*2705* antibodies and percentage lysis of SRBC coated with HLA-B*2705* peptide. A similar situation is known to occur in rheumatic fever, where anti-streptococcal antibodies were reported to be cytotoxic for heart and fibroblast cell lines, because of molecular similarity between the streptococcal M protein and human cardiac myosin. The RA and AS patients used in the study were deemed active in that their ESR values were elevated. The
high levels of *P. mirabilis* hemolysin, urease, DRB1*0404*, and type XI collagen peptide antibodies observed in the RA patients were not due to nonspecific effects of inflammation, because although the AS patients were active, their levels of antibodies were similar to those in healthy control subjects. Antibodies against type XI collagen have been described in RA, and type XI collagen was found to be arthritogenic in DBA/1 mice. The alpha 2 subunit of type XI collagen is a component of hyaline cartilage and is also present in noncartilaginous tissue such as vitreous humor, which could be of relevance in episcleritis and scleromalacia perforans, conditions occurring in severe RA.

*P. mirabilis* hemolysin and urease proteins are both virulence factors contributing to the pathogenesis of the microorganism, and hemolysin proteins can cause the release of inflammatory mediators such as histamine and leukotrienes from neutrophils and mast cells. The hemolysin from *P. mirabilis* has been shown to be a potent cytotoxic agent against human renal proximal tubular epithelial cells. Some of the systemic manifestations such as vasculitis and renal disease seen in RA could occur as a result of the biological properties of this molecule.

Our results suggest that patients with active RA and AS have significantly increased levels of antibodies against...
antigenic determinants of *P. mirabilis* and *K. pneumoniae*, respectively, which are structurally related to host proteins. Further, RA and AS sera have cytotoxic antibodies that contribute to the development of autoimmunity by activating either the complement cascade or natural killer cells, leading to tissue damage. It is known that IgG subclasses can differ in their affinity to the complement components. Hence there may be variation in the IgG subclasses between patients and controls against the peptides used in this study. Studies are required to determine whether a decrease in antibodies against cross-reactive determinants of *P. mirabilis* present in RA and *K. pneumoniae* in AS will slow down the progression of these diseases and in what way do helper and cytotoxic T cells contribute to the development of these cytotoxic antibodies and pathological lesions.

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**REFERENCES**