Association of anti-Ku autoantibodies and DNA repair proteins in connective tissue diseases


Objective. To analyse the autoimmune response to DNA damage response factors in systemic autoimmune rheumatic disease (SARD) patients and to determine their association to Ku antigen.

Methods. We have screened the serum of 238 patients suffering from SARD, including systemic lupus erythematosus, systemic sclerosis and rheumatoid arthritis to detect the occurrence of anti-Ku autoantibodies and four other DNA damage response proteins that form macromolecular complexes with Ku using an immunoprecipitation assay.

Results. We identified samples positive for anti-Ku autoantibodies (20.5%), DNA-dependent protein kinase catalytic subunit (DNA-PKcs, 8.4%) and poly(ADP-ribose) polymerase (PARP-1, 5.6%), and reported the first time the occurrence of anti-Ku autoantibodies directed against Ku and four other DNA repair proteins, Werner (6.3%) and Mre11 (9.6%). Remarkably, we found a striking correlation between the production of antibodies to Ku and the other four Ku-binding factors. Sixty-five percent of anti-Ku-positive sera were found to contain at least one of the four anti-DNA repair antibodies vs only 10% of the anti-Ku-negative sera.

Conclusion. Our results suggest that the autoimmune response to Ku is elicited by macromolecular protein complexes containing Ku and the associated DNA damage proteins. The presence of anti-Ku autoantibodies directed against macromolecular complexes known to play roles in the DNA damage response provides evidence that B-cell responses to latent or persistent DNA damage may be present at the onset or during the development of autoimmune disease.

KEY WORDS: Autoantigen, Autoimmunity, Autoantibody association, Ku, DNA damage.

Introduction

A common feature of systemic autoimmune rheumatic diseases (SARDs) is the presence of autoantibodies directed against nuclear proteins [1]. Although the role of these autoantibodies in the initiation or progression of the diseases is still unclear, they are important diagnostic markers [2, 3]. Some nuclear protein antibodies are highly specific for particular disease manifestations, whereas in other cases, the spectrum of autoantibodies helps define the pathology. The presence and titre of these autoantibodies sometimes can be used to monitor the progression or even predict the outcome of the disease [2, 4].

The processes through which nuclear proteins trigger an immune response are still largely unknown. Autoantibodies often target specific conserved epitopes, suggesting that their production may be epitope-driven [5, 6]. They also have been shown to be directed to linked components of multi-protein complexes, a sign of a concerted autoantigen-driven response to specific molecular processes or pathways. Several studies have also revealed that some autoantibodies recognize modified or protease-cleaved forms of nuclear proteins that could be generated by a cellular stress response [10, 11]. Thus, the production of autoantibodies appears to reflect the physiological status of metabolically active target proteins at the onset of the autoimmune reaction.

Autoantibodies directed against the Ku antigen were originally identified in patients suffering from scleroderma–polymyositis (PM) overlap syndrome [12], but have been subsequently found in a broad spectrum of SARD [13, 14]. Depending on the study and method of detection, anti-Ku autoantibodies have been reported in up to 55% of overlap PM/systemic sclerosis (SSc) and SLE patients, with titres that can exceed $10^7$ in ELISA [13, 15, 16]. Thus, the Ku complex can provoke very strong autoimmune responses and anti-Ku antibodies may have significant impact on the disease.

Ku (Ku70/Ku80) is an abundant DNA-binding protein that plays a fundamental role in DNA repair, being required for the repair of double-stranded DNA breaks through the non-homologous end-joining (NHEJ) pathway [17, 18]. Ku is also essential for V(D)J recombination, telomere protection and has been implicated in having roles in DNA replication and the regulation of gene transcription [18]. In many of these processes, Ku is associated with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a Ser/Thr kinase of the large phosphatidylinositol-3-kinase (PI3-K)-like kinase family [19]. Interaction with a number of other nuclear proteins including several factors involved in DNA repair or the DNA damage response pathway also appears to be fundamental to Ku activity [18]. Physical and functional interactions of Ku with the DNA ligase IV/XRCC4 complex [20], Werner syndrome protein (WRN) [21–23] and poly(ADP-ribose) polymerase-1 (PARP-1), also referred to as PARP [24] have been reported, as has evidence for the interaction of Ku with Mre11 [25] and DNA polymerase-μ [26]. Autoantibodies against DNA-PKcs and other components of the NHEJ repair pathway have been found in the serum of SARD patients [5, 15, 27, 28]. In some cases, conformational epitopes recognizing the Ku–DNA-PKcs complex have been characterized, suggesting a specific response to the DNA-bound Ku/DNA-PKcs complex [27, 28].

Here we investigated the linkage between the production of autoantibodies against Ku and other Ku-interacting proteins implicated in the DNA damage response. Using an immunoprecipitation (IP) assay with a panel of sera from patients diagnosed with several rheumatic diseases, we have investigated the profile of autoimmune responses to Ku and four DNA damage proteins that interact with Ku, DNA-PKcs, Mre11, PARP-1 and WRN. Our results add anti-Mre11 and anti-WRN to...
the list of autoantibodies directed against DNA damage response proteins in autoimmune rheumatic disease. They also provide strong evidence for a concerted autoimmune response to DNA damage response proteins linked to Ku. This suggests that DNA damage may be a factor in the development or the progression of autoimmune rheumatic diseases.

Materials and methods

Patients and sera

Sera were obtained from 239 consenting patients with a range of rheumatic diseases in Ottawa and Calgary. Patients were diagnosed according to established classification criteria: SLE and RA: ACR revised classification criteria [29, 30]; SSc: [31]; PM/DM: [32]; Sjögren’s syndrome (SS): Revised European Criteria [33]; MCTD: Japanese Criteria [34]; UCTD: [35]. It should be noted that all PM/DM samples in this study were selected on the basis of anti-Jo1 positivity. Serum from 57 healthy control individuals was also obtained after informed consent. The sera collection and the study were reviewed and approved by the Ottawa Health Research Ethics Board and Health Research Ethics Board at the University of Calgary. Patients and controls gave informed consent.

Characterization of sera by IP analysis

Anti-Ku and associated protein autoantibodies were identified by IP of HeLa cell extracts with human sera followed by western blot analysis. For each IP reaction, 100–150 µg of HeLa cell whole cell extract (WCE), prepared with 1% NP-40 as previously described [36], was first pre-cleared for 30 min with 15 µl of protein A Sepharose, and then incubated with 10–15 µl of patient serum for 2–3 h in WCE buffer [150 mM NaCl, 50 mM Hapes (pH 7.4), 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.5 mM dithiothreitol and 1 mM PMSF]. In some experiments, the NaCl concentration was adjusted to 500 mM. After addition of 25 µl protein A Sepharose beads and a 30-min incubation, complexes were washed four times with the same buffer. Beads with bound IPs were resuspended in SDS loading buffer and resolved by 8% SDS PAGE, followed by transfer to Immun-Blot

Expression and purification of recombinant proteins

Plasmids were transformed into *Escherichia coli* BL21. Single clones were grown in 30–50 ml cultures and processed according to standard protocols (Amersham Biosciences, Piscataway, NJ, USA). Proteins were eluted from the glutathione-S-transferase (GST) beads with 10 mM glutathione and protein amounts quantified on Coomassie blue-stained gels.

IPs with purified proteins

IPs were carried out with purified DNA-PK (Promega, Madison, WI, USA) to confirm the results obtained by IP of whole cell extracts by selected sera. Reactions were performed as described above with 1 ml of purified DNA-PK (about 20 ng) in WCE buffer containing 1% NP-40 and 5% non-fat dry milk. Epitope mapping analysis with purified GST-Mre11 and WRN peptides was performed by IP of each of the fusion proteins separately with 5–10 µl of patient sera in WCE buffer containing 1% NP-40 and 5% non-fat dry milk as described above. IP proteins were analysed by western blot with a GST antibody (B-14, Santa Cruz Biotechnology).

Statistical analysis

Chi-square tests of independence were used to evaluate whether (i) the presence of antibodies against one, two or three of the DNA-repair proteins (e.g. DNA-PKcs, Mre11, WRN and PARP) is related to Ku, (ii) antibodies against individual DNA-repair proteins occur more frequently in patients with Ku-positive sera compared with Ku-negative sera and finally (iii) whether the co-occurrence of antibodies against DNA-repair and Ku was associated with certain pathologies. Where significant chi-square tests were evident, Cramer’s Phi was calculated to examine the relative strength of the relationship between variables assessed. A P-value of <0.05 was used to indicate statistical significance.

Results

Screening for autoantibodies to Ku and DNA repair proteins

The sera of 239 patients diagnosed with SLE (n = 99), SSc (n = 57), RA (n = 38), PM/DM (n = 23), SS (n = 10) and MCTD/UCTD (n = 12) (Table 1) were screened using an IP assay. In this assay, we examined the ability of the sera to IP Ku or Ku-interacting factors present in HeLa whole cell extracts, using conditions that precluded co-immunoprecipitation of Ku and the Ku-binding proteins tested (Fig. 1A, see Materials and methods section). Detection of the antigens immunoprecipitated was by western blot, with successive re-probing of the membrane performed with antibodies to Ku70, DNA-PKcs, Mre11, WRN and PARP.

This methodology was employed because it allowed the screening of several antigens in the same experiment and was determined to be more specific and sensitive for these antigens than ELISA (data not shown). Further, this technique allowed the identification of autoantibodies that recognized antigens in their native conformation, an important feature of autoantibodies that bind to Ku [38]. A representative example of the antigens immunoprecipitated by SARD patient sera is shown in Fig. 1A. Analyses were repeated independently on all serum samples at least twice with all positive signals being reproduced.

The immunoprecipitation of the Ku dimer by the sera was detected using a Ku70 antibody. Control experiments employing both Ku70 and Ku80 antibodies showed that antibodies to either subunit were effective in detecting the Ku dimer in the immunoprecipitates (data not shown), as described previously [16]. The levels of anti-Ku antibodies varied greatly (e.g. Fig. 1A, lanes 3 and 6), consistent with previous reports of anti-Ku antibodies [16]. A total of 49 anti-Ku positive sera were identified,
TABLE 1. Frequency of autoantibodies to DNA repair proteins in SARD sera

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Total samples</th>
<th>Ku</th>
<th>DNA-PKcs</th>
<th>hMre11</th>
<th>WRN</th>
<th>PARP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE (%)</td>
<td>99</td>
<td>27</td>
<td>10 (10.1)</td>
<td>8 (8.1)</td>
<td>8 (8.1)</td>
<td>5 (5.1)</td>
</tr>
<tr>
<td>SSc (%)</td>
<td>57</td>
<td>8</td>
<td>14 (3.9)</td>
<td>8 (14)</td>
<td>3 (5.3)</td>
<td>4 (7.0)</td>
</tr>
<tr>
<td>RA (%)</td>
<td>38</td>
<td>6</td>
<td>15 (6.8)</td>
<td>2 (5.3)</td>
<td>4 (10.5)</td>
<td>1 (2.6)</td>
</tr>
<tr>
<td>PM/DM (%)</td>
<td>23</td>
<td>6</td>
<td>26 (1.1)</td>
<td>21 (7.7)</td>
<td>2 (8.7)</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>SJögren’s syndrome (%)</td>
<td>10</td>
<td>1</td>
<td>10 (10.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MCTD/UCTD (%)</td>
<td>12</td>
<td>1</td>
<td>8 (8.3)</td>
<td>0</td>
<td>1 (8.3)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>239</td>
<td>49</td>
<td>20 (8.4)</td>
<td>23 (9.6)</td>
<td>15 (6.3)</td>
<td>14 (5.9)</td>
</tr>
<tr>
<td>Healthy (%)</td>
<td>57</td>
<td>1</td>
<td>1 (1.8)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. Analysis of human sera by IP assay. (A) Example of IP of HeLa whole cell extract with six different sera. Whole cell extract from HeLa cells (100 μg/reaction) was IP with 10 μl of serum from six individual patients (lanes 3–8) or with a Ku70 antibody (lane 1) as described in Materials and methods. Lane 5 contains 50 μg of HeLa whole cell extract as input (I). The samples were analysed by western blot and the membrane was probed successively with antibodies to Ku70, DNA-PKcs, Mre11, WRN, and PARP, as indicated. (B) IP of purified DNA-PK with six individual SARD sera (lanes 3–8) and one healthy individual (H, lane 9). Samples were processed as described in Materials and methods and the blot was probed with an anti-DNA-PKcs antibody. Lane 1 (I), 10% input; lane 2 (PK) is an IP performed with anti-DNA-PKcs antibody.

representing just over 20% of our patient group (Table 1). In contrast, only one positive signal for Ku was detected in control sera from 57 healthy individuals.

Twenty SARD patient sera had anti-DNA-PKcs antibodies while 14 contained anti-PARP autoantibodies (8.4 and 5.9% of all patients, respectively, Fig. 1A and Table 1). We also identified 23 samples displaying anti-Mre11 antibodies and 15 with anti-WRN antibodies (Fig. 1A, Table 1). These data expand the autoantigen profile of Ku-associated DNA repair factors. Moreover, the frequency of both these newly identified autoantibodies (9.6 and 6.3%, respectively) was similar to that of anti-DNA-PKcs and PARP. The association with SARD was highly specific as none of the control sera from the 57 healthy individuals was positive for any of these four autoantibodies (Table 1).

In each experiment, a control IP with a Ku70 antibody was performed to ensure that the experimental conditions prevented the co-immunoprecipitation of any of the four factors with Ku (Fig. 1A, lane 1). Additionally, anti-DNA-PKcs-positive and -negative sera were retested for the ability to IP purified DNA-PK (Fig. 1B). All the samples identified as positives for anti-DNA-PKcs antibodies using the whole cell extract IP assay were confirmed positives in this second assay. Similarly, sera positive for anti-Mre11 and anti-WRN autoantibodies also recognized recombinant peptides from these proteins purified from E. coli (Fig. 3 and data not shown). Again, all the sera found positive for antibodies against either one of these proteins in our initial assay using whole cell extracts were able to immunoprecipitate the recombinant purified protein. Attempts to identify previously characterized anti-XRCC4/DNA ligase IV antibodies [5] failed due to the presence of non-specific cross-reactive products at the molecular mass where the XRCC4/DNA ligase IV bands were expected (data not shown).

Correlation between anti-Ku and DNA repair factor autoantibodies

To assess the possible linkage between the anti-Ku response and responses to DNA-PKcs, MRE11, WRN and PARP, we compared the frequency of autoantibodies with the four DNA repair proteins in anti-Ku-positive and anti-Ku-negative sera (Fig. 2). A total of 52 samples were found positive for one or more autoantibodies to DNA-PKcs, Mre11, WRN and PARP, while 49 sera were positive for anti-Ku antibodies. Of the 49 anti-Ku positive sera, 32 (65%) were also positive for at least one of the other four antigens. In contrast, only 20 of 190 (10.5%) anti-Ku-negative patients were positive for antibodies to DNA-PKcs, Mre11, WRN or PARP (Fig. 2A and B).

Interestingly, of the 65% of anti-Ku sera positive for other DNA damage proteins, 35% had one additional antibody, 25% two additional antibodies and 6% three additional antibodies (Fig. 2C). This was in sharp contrast with the small percentage of anti-Ku-negative sera containing autoantibodies against one (9.5%) or two proteins (1%), with none of the Ku-negative samples being positive for antibodies to three of the other factors. Chi-square analyses verified the significance of these differences (P < 0.001). These results strongly imply a link between the presence of autoantibodies to Ku and to these DNA repair proteins in the SARD sera.

We next compared the distribution of individual markers in anti-Ku-positive and anti-Ku-negative patient sera (Table 2). Chi-square analyses verified that antibodies against individual DNA repair proteins were significantly more frequent in patients with anti-Ku-positive sera compared with anti-Ku-negative sera (P < 0.001 in all cases). A 5-fold (Mre11) to 20-fold (PARP) higher frequency was observed in anti-Ku-positive vs -negative sera.

Linked sets of anti-Ku/DNA repair proteins were predominantly found in SLE, SSc and PM/DM patients (Table 3). While categorizing these antibody sets according to disease gave rise to smaller numbers that precluded formal statistical analyses, we observed that over 60% of the DNA repair autoantibodies were detected in anti-Ku-containing sera from patients diagnosed with these diseases (with the exception of anti-Mre11, 50% in SSc patients). This frequency was 50% and lower in RA sera.

Mapping of the antigenic regions of WRN and Mre11

To begin to investigate a potential link between the autoantibody association and epitope specificity, we set out to map the regions in WRN and Mre11 recognized by the autoantibodies by IP analysis of bacterially expressed N-, central and C-terminal regions of WRN with 12 of the 15 anti-WRN-positive sera (Fig. 3). GST-WRN 3 peptide corresponding to the C-terminus of the protein (amino acids 949–1432) was immunoprecipitated by all anti-WRN-positive sera, 32 (65%) were also positive for at least one of these four autoantibodies (Table 3). These data expand the autoantigen profile of Ku-associated DNA repair factors. Moreover, the frequency of both these newly identified autoantibodies (9.6 and 6.3%, respectively) was similar to that of anti-DNA-PKcs and PARP. The association with SARD was highly specific as none of the control sera from the 57 healthy individuals was positive for any of these four autoantibodies (Table 1).

In each experiment, a control IP with a Ku70 antibody was performed to ensure that the experimental conditions prevented the co-immunoprecipitation of any of the four factors with Ku (Fig. 1A, lane 1). Additionally, anti-DNA-PKcs-positive and -negative sera were retested for the ability to IP purified DNA-PK (Fig. 1B). All the samples identified as positives for anti-DNA-PKcs antibodies using the whole cell extract IP assay were confirmed positives in this second assay. Similarly, sera positive for anti-Mre11 and anti-WRN autoantibodies also recognized recombinant peptides from these proteins purified from E. coli (Fig. 3 and data not shown). Again, all the sera found positive for antibodies against either one of these proteins in our initial assay using whole cell extracts were able to immunoprecipitate the recombinant purified protein. Attempts to identify previously characterized anti-XRCC4/DNA ligase IV antibodies [5] failed due to the presence of non-specific cross-reactive products at the molecular mass where the XRCC4/DNA ligase IV bands were expected (data not shown).
samples also contained antibodies directed against the C-terminus of the protein. Thus, clearly, the C-terminal region of WRN is the most highly reactive, but there was no correlation between this epitope reactivity and the presence of anti-Ku antibodies.

Mre11 autoantigenic determinants were tested with the N-terminal (amino acids 1-319), C-terminal (amino acids 409-708) and central (amino acids 217-498) regions of the protein expressed as GST proteins by the IP assay. All previously identified anti-Mre11-positive sera, but not control sera were able to efficiently immunoprecipitate the C-terminal portion of Mre11. However, none of them recognized the peptides corresponding to the Mre11 central and N-terminal regions (data not shown).

**Discussion**

We report the occurrence of linked sets of autoantibodies directed against Ku and DNA repair proteins in the serum of patients suffering from various SARD. All the factors tested in this study, DNA-PKcs, Mre11, WRN and PARP, have been reported to interact with Ku, indicating that the production of linked sets of anti-Ku and anti-DNA damage response protein antibodies may be the result of the association of these proteins in vivo. The occurrence of autoantibodies to DNA-PKcs, Mre11, WRN and PARP was 5- to 20-fold higher in anti-Ku-containing sera, suggesting that DNA damage may be one of the underlying events in the development of the autoimmune response.

Antibodies against Ku predominated among the 239 SARD patients, with highest incidence in SLE and PM/DM. The frequency of anti-Ku-positive patients in our cohort matches the frequency of anti-DNA-PKcs was higher than previously reported [27, 28]. This could be explained by differences in IP conditions, as previous studies included 0.5 M NaCl in the IP buffer to prevent the co-purification of DNA-PKcs via its interaction with Ku. Upon inclusion of 0.5 M NaCl in our buffer, we noticed that some sera lost their ability to immunoprecipitate the DNA-PKcs, Mre11, WRN, and PARP in anti-Ku-positive and anti-Ku-negative sera. (A) The anti-Ku-positive (n=49) or -negative (n=190) sera from the panel of 239 sera analysed in this study were analysed for their reactivity to DNA-PKcs, Mre11, WRN and PARP. Fifty-two sera were found positive for autoantibodies to these proteins and classified in the following groups: total positives indicate samples positive for at least one of the four autoantibodies (n=52); 1: positive for one DNA repair factor autoantibody (n=35); 2: positive for two (n=14); 3: positive for three (n=3). They were further sub-classified into the anti-Ku-positive and anti-Ku-negative category, as indicated. (B) Graphic presentation of the proportion of anti-DNA repair protein antibody-containing sera in the anti-Ku-positive (grey bar) and the anti-Ku-negative (black bar) samples. (C) Graphic representation of percentage of samples positive for 1, 2 or 3 antibodies to DNA-PKcs, Mre11, WRN and PARP in anti-Ku-positive (grey bar) and anti-Ku-negative sera (black bars). The values displayed in (A) were expressed as a percentage and represented as a bar graph with the percentage value indicated at the top of each bar. *For one autoantibody: χ² (1, n=239) = 68.67, Cramer’s Phi = 0.54, P < 0.001. **For two autoantibodies: χ² (1, n=239) = 11.78, Cramer’s Phi = 0.46, P < 0.001. ***For three autoantibodies: χ² (1, n=239) = 11.78, Cramer’s Phi = 0.40, P < 0.001.

**Table 2. Proportion of anti-DNA-PKcs, Mre11, WRN and PARP antibodies in anti-Ku-positive and anti-Ku-negative patients**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Total positives</th>
<th>In anti-Ku+ sera</th>
<th>Percentage in anti-Ku+</th>
<th>Percentage in anti-Ku-</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-PKcs (%)</td>
<td>20</td>
<td>14 (70)</td>
<td>14/49 (28.6)</td>
<td>6/190 (3.2)</td>
</tr>
<tr>
<td>Mre11 (%)</td>
<td>23</td>
<td>13 (57)</td>
<td>13/49 (26.5)</td>
<td>10/190 (5.3)</td>
</tr>
<tr>
<td>WRN (%)</td>
<td>15</td>
<td>11 (73)</td>
<td>15/49 (22.4)</td>
<td>4/190 (2.1)</td>
</tr>
<tr>
<td>PARP (%)</td>
<td>14</td>
<td>12 (86)</td>
<td>14/49 (24.5)</td>
<td>2/190 (1.1)</td>
</tr>
</tbody>
</table>

Chi-square analysis: DNA-PKcs: χ² (1, n=239) = 82.81, Cramer’s Phi = 0.37, P < 0.001; Mre11: χ² (1, n=239) = 20.26, Cramer’s Phi = 0.29, P < 0.001; WRN: χ² (1, n=239) = 27.41, Cramer’s Phi = 0.34, P < 0.001; PARP: χ² (1, n=239) = 88.80, Cramer’s Phi = 0.40, P < 0.001.

**Table 3. Frequency of anti-Ku antibodies in SARD sera positive for anti-DNA-PKcs, -Mre11, -WRN and -PARP antibodies**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>DNA-PKcs (%)</th>
<th>hMre11</th>
<th>WRN</th>
<th>PARP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE (%)</td>
<td>7/10 (70)</td>
<td>5/8 (62)</td>
<td>6/8 (75)</td>
<td>5/5 (100)</td>
<td>22/30 (73)</td>
</tr>
<tr>
<td>SSc (%)</td>
<td>2/3 (66)</td>
<td>4/8 (50)</td>
<td>3/3 (100)</td>
<td>4/4 (100)</td>
<td>13/18 (72)</td>
</tr>
<tr>
<td>RA (%)</td>
<td>1/2 (50)</td>
<td>2/4 (50)</td>
<td>0/1</td>
<td>1/3 (33)</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>PM/DM (%)</td>
<td>4/5 (80)</td>
<td>2/2 (100)</td>
<td>2/2 (100)</td>
<td>1/1 (100)</td>
<td>9/10 (90)</td>
</tr>
<tr>
<td>Sjögren’s syndrome</td>
<td>0/0</td>
<td>0/0</td>
<td>1/1 (100)</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>MCTD/UCTD (%)</td>
<td>0/0</td>
<td>0/0</td>
<td>1/1 (100)</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>
and the results were scored as positive (autoantibody epitope mapping. Twelve different sera identified as positive for WRN C were analysed by western blot with a GST antibody and immunoprecipitates immunoprecipitated by human sera. Equivalent amount of each GST-WRN peptide as GST fusion proteins. (WRN protein, with the bars below depicting the three regions of WRN expressed is primarily directed against Ku and may extend to associated postulate that in a subset of patients, the autoimmune response presence of anti-Ku autoantibodies. Based on these results, we WRN and PARP autoantibodies was significantly linked to the proliferating cell nuclear antigens (PCNA, 2–7% in SLE) [3, 4, 39]. It was also comparable with the frequency of some previously identified autoantibodies recognizing antigens found in various SARD such as anti-U1 RNP antibodies (found in 5–8% of SSc), which are considered disease markers for MCTD, anti-RAP74 (the large subunit of transcription factor TFIIIF, 4% in SLE) or proliferating cell nuclear antigens (PCNA, 2–7% in SLE) [3, 4, 39]. Remarkably, the occurrence of anti-DNA-PKcs, Mre11, WRN and PARP autoantibodies was significantly linked to the presence of anti-Ku autoantibodies. Based on these results, we postulate that in a subset of patients, the autoimmune response is primarily directed against Ku and may extend to associated proteins, consistent with the intermolecular spreading of B-cell responses suggested by others [6, 10]. By all accounts, it appears that autoimmunity in a subset of SARD is primarily targeting Ku, with a spreading to its associated factors. One reason that could explain why Ku is targeted predominantly is that Ku is an abundant protein (~4 × 10^5 molecules/cell) [16]. Also, Ku is highly antigenic, with epitopes broadly distributed throughout both Ku70 and Ku80 subunits [16, 38]. The fact that autoantibodies against DNA damage proteins are found predominantly in association with anti-Ku antibodies suggests that they become more frequent targets of the autoimmune process by virtue of their association with Ku in the cell.

Linked sets of anti-Ku and anti-DNA-PKcs autoantibodies in autoimmune sera have been described previously [27, 28]. Both studies found strong evidence of a linkage between anti-Ku and anti-DNA-PKcs antibodies in SARD patients. These characteristics were even more prevalent amongst autoantibodies produced against the two Ku subunits (Ku70 and Ku80), suggesting a possible correlation between the incidence and/or stability of the interaction between two proteins and the rate of production of dual sets and conformational antibodies recognizing the complex [42]. Thus, our results seem to indicate that more stable/frequent complexes are formed between Ku and PARP or DNA-PKcs or WRN than between Ku and Mre11. While the significance of the interaction of DNA-PKcs with Ku is well documented [17–19], these results may indicate the potential physiological importance of the interactions of PARP and WRN with Ku.

Epitope analysis of the anti-Mre11 and anti-WRN autoantibodies indicated the existence of one major epitope for each factor. Thus, antigenicity was not influenced by the autoantibody linkage to Ku. Our results suggest the existence of a dominant epitope in the C-terminus of Mre11. This region comprises a putative DNA-binding domain at its extreme C-terminus, but does not contain the conserved phosphoesterase motif, nor the Ku-interacting region, both of them identified in the N-terminus of Mre11 [25]. Anti-WRN autoantibodies were also found to target predominantly the C-terminus of the protein, in a region devoid of any enzymatic function. Previous reports have shown that both the C- and the N-terminus of WRN interact with Ku [22, 23]. Taken together, these results support the notion that the generation of anti-WRN and anti-Mre11 autoantibodies is autoantigen-driven and occurs in a highly conserved manner between individuals. Further studies will be needed to map more precisely the antigenic determinants for these two factors.

Our data support the concept that autoantibody production is driven by the existence/formation of macromolecular protein complexes in vivo. Indeed, previous studies have employed autoimmune sera to identify protein complexes in vivo [9, 43]. Reciprocally, analysis of the presence of autoantibodies directed against specific protein complexes and/or specific protein modifications have been suggested to reflect cellular events during which the autoimmune process developed [11]. Proteins involved in DNA damage responses and repair, such as the ones studied here, are expressed at steady levels and become activated by DNA damage either by interacting with the damaged DNA or by post-translational modifications and/or by forming complexes with other factors [17, 19]. For instance, DNA-PK activity is known to be activated following double-stranded DNA damage upon Ku binding to the DNA break, which induces the recruitment and activation of DNA-PKcs [19]. A synergistic interaction between WRN, a factor with helicase and exonuclease activities, and Ku on DNA has also been suggested to help processing broken DNA ends [21]. Moreover, this activity may be regulated by a PARP-Ku interaction [21]. Finally, it has been suggested that the two factors, together with Mre11-associated factors, Rad50 and NBS1, may cooperate in the double-stranded DNA break end-joining process [44, 45]. Thus, the associations of anti-Ku autoantibodies with those of factors known to interact with Ku in situations

---

**Fig. 3.** Analysis of WRN regions targeted by autoantibodies. (A) Schematic of WRN protein, with the bars below depicting the three regions of WRN expressed as GST fusion proteins. (B) Representative analysis of GST-WRN fusion proteins immunoprecipitated by human sera. Equivalent amount of each GST-WRN peptide was incubated separately with each individual sera (indicated by numbers above the gel) and immunoprecipitated as described in Materials and methods. Samples were analysed by western blot with a GST antibody and immunoprecipitates were compared with 10% input protein (Input). (C) Summary of the WRN autoantibody epitope mapping. Twelve different sera identified as positive for WRN autoantibodies by the HeLa cell extract IP assay (numbered 1–12, H, healthy patient control sera) were analysed by IP assay with GST-WRN peptides 1, 2 and 3 and the results were scored as positive (+) or negative (−) for the indicated WRN peptide. All samples were tested a minimum of two times with identical results. The column ‘Ku status’ indicates whether the serum tested positive (+) or negative (−) for anti-Ku autoantibodies.
where cells are facing a DNA injury suggest that DNA damage may be an underlying event occurring during the development of the autoimmune response.

The hypothesis that autoimmune disorders could result or evolve from an initial DNA damage event has previously been suggested by others [5]. Autoantibodies against DNA repair proteins involved in other DNA repair pathways, such as DNA mismatch repair proteins PMS1, PMS2, MLH1 and DNA excision repair protein hMYH, have also been found in the sera of autoimmune patients [46, 47]. In addition, the presence of autoantibodies directed against reactive oxygen species (ROS)-modified chromatin suggested underlying DNA damage at the onset of the autoimmune response [48]. Abnormal response to DNA damage has been observed in SS cells suggesting that it could be a component of the pathogenesis of the disease [49].

In another related study, an altered/delayed DNA repair has been noted in cells from SLE, juvenile RA and SSc patients [50]. While our report strengthens the link between an underlying DNA damage response and the pathogenesis of autoimmune connective tissue diseases, it remains to be elucidated whether increased DNA damage is a cause of the pathology or if autoantibodies directed against DNA repair proteins are causing an altered DNA damage response.

**Rheumatology key messages**

- Anti-Ku antibodies are associated with anti-DNA repair protein antibodies in the serum of SARD patients.
- DNA damage may be a factor in the development or the progression of SARD.

**Acknowledgements**

We thank P. Concannon and L. Comai for providing cDNA constructs for Mre11 and WRN, respectively. We also thank E. Atlas and S. Soubeyrand for critical reading of the manuscript. This work was supported by operating grants from the Canadian Institutes for Health Research to R.J.G.H., M.J.F. and R.G.

**Disclosure statement**

M.J.F. is a paid consultant to Immuno-Concepts Inc, Sacramento, USA. All other authors have declared no conflicts of interest.

**References**