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The interaction between anti-Ro/SSA and anti-La/SSB autoantibodies and anti-infectious antibodies in a wide spectrum of auto-immune diseases: another angle of the autoimmune mosaic

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Abstract

Objective

The presence of anti-Ro/SSA and anti-La/SSB antibodies has been linked with autoimmunity in general and with several autoimmune diseases (AID) in particular. In the current study we evaluated these antibodies in a wide spectrum of AID as well as the links between them and anti-infectious antibodies.

Methods

We examined 2082 sera from patients with 16 different AID compared to 524 sera from geographically-matched healthy controls, for the presence and titres of anti-Ro/SSA and anti-La/SSB. All samples were also tested for a variety of anti-infectious agents’ antibodies using the BioPlex 2200-immunoassay (Bio-Rad, USA).

Results

Anti-Ro/SSA was more prevalent, with significantly higher titre in 5 autoimmune diseases namely Sjögren’s syndrome (SS), systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS) both primary and APS linked to SLE, systemic sclerosis (SSc) and primary biliary cirrhosis (PBC). Anti-La/SSB was more prevalent with higher titers in SS, SLE, APS linked to SLE and PBC. Prevalence, but not titers, of both antibodies were higher also in polymyositis (PM). Additionally, we found a correlation between anti-Ro/SSA antibodies and antibodies of the IgM and IgG subtypes directed at cytomegalovirus as well as IgG-antibodies directed at Epstein-Barr virus (EBV) and toxoplasma (p<0.001). Anti-La/SSB antibodies correlated with the presence of IgG antibodies against EBV early antigen (p<0.001).

Conclusion

In a large cohort of patients with autoimmune diseases we found an association between anti-Ro/SSA and anti-La/SSB antibodies and 6 autoimmune diseases, amongst which primary APS and PM. Additionally, we observed linkages between these autoantibodies and anti-infectious antibodies directed at Epstein-Barr virus, toxoplasma and cytomegalovirus. Our findings support the concept of interplay between infectious agents and autoimmunity, such as the plausibility of an infectious agent that trigger the immune system to produce specific antibodies which will later result in a unique group of AID.

Key words

anti-Ro/SSA antibody, anti-La/SSB antibody, autoimmunity, Sjögren’s syndrome, antiphospholipid syndrome, systemic lupus erythematosus
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In this cross-sectional study we analysed 2606 serum samples from 2082 patients with autoimmune conditions and 524 healthy controls. Patients were followed and classified according to the ACR classifications and by their local physicians either in Europe or in South-Amercia. 812 patients were from South American including 199 patients with SLE, 82 patients with SS, 152 patients with rheumatoid arthritis (RA), 180 patients with type 1 diabetes mellitus (DM), 199 patients with multiple sclerosis (MS). While 1270 European patients consisted of 101 with polymyositis (PM), 98 with primary antiphospholipid syndrome (APS), 63 with antiphospholipid syndrome associated with SLE (APS+SLE), 91 with SLE, 78 Hepatitis C virus associated cryoglobulinaemic vasculitis (HCV+Cryo), 80 with SSC, 35 with RA, 69 with primary biliary cirrhosis (PBC), 119 with inflammatory bowel disease (IBD), 197 with autoimmune thyroid disease (Graves’ disease and Hashimoto’s thyroiditis), 173 with ANCA associated vasculitis (AAV), 35 with giant cell arteritis (GCA), 41 with AID associated mixed cryoglobulinaemic vasculitis (MIXC) (27) and 90 with coeliac diseases. Of the 524 matched controls, 277 were from South American and 247 were European. Controls were healthy individuals with no known inflamma-

Methods

Sera collection

In this cross-sectional study we analysed 2606 serum samples from 2082 patients with autoimmune conditions and 524 healthy controls. Patients were followed and classified according to the ACR classifications and by their local physicians either in Europe or in South America. 812 patients were from South American including 199 patients with SLE, 82 patients with SS, 152 patients with rheumatoid arthritis (RA), 180 patients with type 1 diabetes mellitus (DM), 199 patients with multiple sclerosis (MS). While 1270 European patients consisted of 101 with polymyositis (PM), 98 with primary antiphospholipid syndrome (APS), 63 with antiphospholipid syndrome associated with SLE (APS+SLE), 91 with SLE, 78 Hepatitis C virus associated cryoglobulinaemic vasculitis (HCV+Cryo), 80 with SSC, 35 with RA, 69 with primary biliary cirrhosis (PBC), 119 with inflammatory bowel disease (IBD), 197 with autoimmune thyroid disease (Graves’ disease and Hashimoto’s thyroiditis), 173 with ANCA associated vasculitis (AAV), 35 with giant cell arteritis (GCA), 41 with AID associated mixed cryoglobulinaemic vasculitis (MIXC) (27) and 90 with coeliac diseases. Of the 524 matched controls, 277 were from South American and 247 were European. Controls were healthy individuals with no known inflamma-

Competing interests: none declared.
tory or AID, or a history of a chronic infectious disease, including tuberculosis and human immunodeficiency virus. Patients in the HCV+Cry cryoglobulinaemia tested positive for Hepatitis C virus (HCV) infection while patients in the MIXC tested negative for HCV. The study protocol was approved by the Ethical Review Board of our institution and procedures were in accordance with the ethical standards laid down in Helsinki Declaration, as revised in 2000.

Autoantibody testing
The Bio-Rad BioPlex 2200 (Bio-Rad Laboratories, Hercules CA, USA) system is an automated analyser that uses multiplex bead technology to simultaneously detect antibodies to several antigens in a single tube. This device uses 25 different 8-μm magnetic beads, which are dyed with two fluorophores for classification purposes. Each bead is coated with specific proteins, according to the different assay being tested, thus representing a different target antigen. The amount of antibody bound to the bead was determined by fluorescence analysis; raw data were subsequently converted to the fluorescence ratio using a pre-dyed internal normaliser the detector signal. Elevated titres were determined as above the cut-off of 2 standard deviations from the normal control. The technology applied in this work had already been published and evaluated prior to this study in previous publications by our group as well as in by others (28-30). The BioPlex 2200 ANA Screen is intended for the qualitative screening of ANA, the quantitative detection of antibody to dsDNA, and the anti-human-IgG peroxidase and appropriate substrate. Positive results were calculated according to the manufacturer’s equations for cut-off value determination. Antibodies to hepatitis B virus core protein (recombinant HBc antigen) and Helicobacter pylori (HP) were tested using MONOLISA anti-HBc plus commercial kit and MONOLISA pylori - IgG commercial kit (Bio-Rad, Hercules, CA, USA), respectively, according to the manufacturer’s instructions. Of note, in the analysis of anti-infectious agents’ antibodies we considered differences between groups, only according to titles above the manufacturer protocol, similarly to clinical practice; In other words different titles below positivity cut-off may not be of clinical significance.

### Table 1. The prevalence of Anti-Ro/SSA and anti-La/SSB antibodies in different autoimmune diseases.

<table>
<thead>
<tr>
<th>Autoimmune disease</th>
<th>Anti-Ro/SSA</th>
<th>Anti-La/SSB</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>European subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls n=247</td>
<td>0.8%</td>
<td>0.8%</td>
<td></td>
</tr>
<tr>
<td>APS n=98</td>
<td>12.2%</td>
<td>1%</td>
<td>&lt;0.000001 NS</td>
</tr>
<tr>
<td>APS associated with SLE n=63</td>
<td>28.5%</td>
<td>9.5%</td>
<td>&lt;0.000001 0.001&gt;</td>
</tr>
<tr>
<td>SLE n=91</td>
<td>49.4%</td>
<td>18.7%</td>
<td>&lt;0.000001 &lt;0.000001&gt;</td>
</tr>
<tr>
<td>SSC n=80</td>
<td>11.2%</td>
<td>3.8%</td>
<td>0.0001&gt; NS</td>
</tr>
<tr>
<td>RA n=35</td>
<td>2.9%</td>
<td>0%</td>
<td>NS NS</td>
</tr>
<tr>
<td>HCV+ Cryoglobulinaemia n=78</td>
<td>12.8%</td>
<td>6.4%</td>
<td>0.00001&gt; 0.01&gt;</td>
</tr>
<tr>
<td>MIX. Cryoglobulinaemia n=41</td>
<td>2.4%</td>
<td>2.4%</td>
<td>NS NS</td>
</tr>
<tr>
<td>Polymyositis n=101</td>
<td>35.6%</td>
<td>14.8%</td>
<td>&lt;0.000001 &lt;0.000001&gt;</td>
</tr>
</tbody>
</table>

| South-American subjects              |            |            |               |
| Controls n=277                       | 3.30%      | 2.20%      |               |
| SLE n=199                            | 32.2%      | 9.5%       | <0.000001 0.001> |
| SS n=82                              | 62.2%      | 37.8%      | <0.000001 <0.000001> |
| RA n=152                             | 9.9%       | 3.9%       | 0.01> NS      |
| DM1 n=180                            | 1.1%       | 0.6%       | NS NS         |
| MS n=199                             | 2%         | 2%         | NS NS         |

<table>
<thead>
<tr>
<th></th>
<th>Anti Ro/SSA vs. Control*</th>
<th>Anti La/SSB vs. Control**</th>
</tr>
</thead>
</table>
| SLE: systemic lupus erythematosus; SS: Sjögren’s syndrome; RA: rheumatoid arthritis, DM1: diabetes mellitus type 1, MS: multiple sclerosis; APS: antiphospholipid syndrome; SSC: systemic sclerosis; HCV+ cryoglobulinaemia: hepatitis C virus associated cryoglobulinaemic vasculitis; PBC: primary biliary cirrhosis; IBID: inflammatory bowel disease; AITD: autoimmune thyroid disorders, GCA: giant cell arteritis; MIXC: mixed cryoglobulinaemia; NS: not significant; AAV: ANCA-associated vasculitis includes the following granulomatosis with polyangiitis (Wegener’s) (GPA); microscopic polyangiitis (MPAN) and eosinophilic granulomatosis with polyangiitis (Churg-Strauss syndrome).

*\( p \)-value for the comparison between the prevalence of anti Ro/SSA antibodies in autoimmune disease patients compare with healthy control.

**\( p \)-value for the comparison between the prevalence of anti La/SSB antibodies in autoimmune disease patients compare with healthy control.

Anti-infectious autoantibodies in autoimmune diseases / N. Agmon-Levin et al.
Statistical analysis
Data was analysed using SPSS version 22.0 software. Mean and standard deviation (SD) were calculated for quantitative variables. The percentage and titres of each autoantibody was compared between groups. Statistical tests used were student’s t-test, Pearson chi-squared, Fisher’s exact test, and spearman correlation test as appropriate. Tests were considered significant when p-value was <0.05.

Results
In the current study we analysed sera from 2082 patients with 16 different AID compared to 524 geographically matched healthy controls, for the presence and titres of anti-Ro/SSA and anti-La/SSB and their correlation to a profile of anti-infections antibodies. 

Anti-Ro/SSA and anti-La/SSB prevalence in different autoimmune diseases
Out of 16 AID, prevalence and titers of anti-Ro/SSA were significantly higher among 5 autoimmune diseases (p<0.001) namely Sjögren’s syndrome, SLE, APS both primary disease and APS linked to SLE, systemic sclerosis (SSc), and primary biliary cirrhosis (PBC). Prevalence and titers of anti-La/SSB were significantly higher (p<0.001) in Sjögren’s syndrome, SLE, APS linked to SLE and PBC as described in Table I. It is of note, that in polymyositis (PM) as well as in HCV+Cryo the prevalence but not titres of both autoantibodies were found to be higher among European patients.

In contrast, both anti-Ro/SSA and anti-La/SSB were not found to be more prevalent among patients with IBD,AITD,AAV, coeliac, GCA, and MS and type 1 DM.
Notably, the prevalence of anti-Ro/SSA and anti-La/SSB differ significantly amid the two control groups with higher prevalence among controls from South America compare to Europeans (Table I). This observation was previously reported by our group (31, 32).

In addition, we analysed the association of anti-Ro/SSA and anti-La/SSB among the different patient populations and compared them to controls. Notably as specified in Table I, other than the conditions reported earlier in the results section, we tested all sera for distinctive positivity to anti-Ro52, anti-Ro60 and total anti-Ro/SSA. The data is not specified as specific serology to Ro52/Ro60 autoantibodies was in agreement with the positivity found to the total Anti-Ro/SSA. Not surprisingly, we noticed as others before us (8, 33), that some patients were seropositive to a single epitope (-Ro52 or -Ro60) while others were seropositive to both.

Titre of anti Ro/SSA and anti La/SSB in various autoimmune diseases
Significant differences between titres of anti-Ro/SSA and anti-La/SSB in several conditions are described in Figure 1. Titres for both antibodies reached statistical significance in patients with SLE, SS, APS+SLE, PBC and SSc. Additionally we found high titres of anti-Ro/SSA only in primary APS. Generally high antibody titres were found in the same conditions that exhibited high antibody prevalence. Similarly, in accordance with their prevalence, also the titres of the autoantibodies were greater in South American controls compared to European controls.

Interactions between anti Ro/SSA and anti La/SSB autoantibodies and anti-infectious agents
We analysed the associations between a wide profile of anti-infectious antibodies and anti-Ro/SSA and anti-La/SSB autoantibodies (Table II). We found positive correlation between anti-Ro/SSA and antibodies of the IgM-subtype directed at cytomegalovirus as well as of the IgG-subtype directed towards toxoplasma, cytomegalovirus and Epstein-Barr virus. Anti-La/SSB antibodies correlated only with the presence of IgG antibodies against EBV early antigen. Interestingly, an inverse correlation was found between anti-Ro/SSA and antibodies of the IgM-subtype directed at cytomegalovirus as well as of the IgG-subtype directed towards toxoplasma, cytomegalovirus and Epstein-Barr virus. Anti-La/SSB antibodies correlated only with the presence of IgG antibodies against EBV early antigen. Similar observation was noted for anti -La/SSB, although for these autoantibodies the effect did not reach statistical significance.

Discussion
The links between autoimmunity (e.g. the presence of autoantibodies), auto-
immune diseases and infectious agents have been extensively studied (34-37). Herein we report associations of the same nature between anti-Ro/SSA and anti-La/SSB antibodies anti-infectious antibodies in a large cohort of patients with various autoimmune diseases.

We found an association between these antibodies and 6 autoimmune diseases namely SS, SLE, SSc, PBC, APS and PM. These associations are well established for 4 diseases (namely SS (1, 38-40), SLE (41, 42), PBC (43-45), SSc (5, 46)) that were further consolidated in this large cohort. In addition, new association between anti-Ro/SSA and primary APS as well as between anti-La/SSA and PM were described in this study for the first time to the best of our knowledge.

It is of interest that the 6 AID related to anti-Ro/SSA (SS, SLE, PBC, SSc, APS, PM) are interlinked and may overlap both clinically and serologically. One plausible explanation for having high anti-Ro/SSA in patients with no overt clinical manifestations of SS or SLE is that those patients are already suffering from various autoimmune diseases and CMV (23, 54). In line with this concept Poole et al. (55) showed that immunisation of rabbits with epitopes derived from EBV (EBNA-1 58-72 peptide) develop anti-ENBA as well as specific anti-RO autoantibodies linked between these autoimmune diseases and CMV.

The data about Anti-La/SSB prevalence in different inflammatory diseases is less abundant. This antibody which is thought to be more specific to SS interact with a 47-kD protein, which shuttles between the nucleus and cytoplasm but which is predominantly found in the nucleus (47). Our finding of increased prevalence of anti-La/SSB not only in SS and PM, but also in PBC, is important and further supports the notion that PM is in overlap with other connective tissue diseases. An additional interesting finding was the increased prevalence of anti-La/SSB in HCV+Cryo. This finding might be explained by secondary Sjögren’s syndrome found in up to 20% of the populations with cryoglobulinaemia.

In this study we found an association between anti-infectious agents’ antibodies and the existence of anti-Ro/SSA. This was especially noticeable among patients exposed to CMV and EBV. The link between SLE and SS to previous infection with EBV was reported both by our group and by others (50-53), and so was the link between these autoimmune diseases and CMV (23, 54) in line with this concept Poole et al. (55) showed that immunisation of rabbits with epitopes derived from EBV (EBNA-1 58-72 peptide) do develop anti-ENBA as well as specific anti-RO autoantibodies followed by clinical manifestation of SLE-SS-like disease. Hence, one may suggest that a past exposure to those infectious agents, can lead to the presence of anti-Ro/SS, which serves as marker for early autoimmunity (13, 15)
and as a preliminary stage in the later development of an AID. Why would one patient develop a certain AID out of the 6 AID related to anti-Ro/SSA described earlier and not another? This might be related to exposure to other environmental factors and/or exposure to other infectious agents as well as different genetic background. Unlike Anti-Ro/SSA, anti-La/SSB, correlated only with EBV-early antigen (EBVEA). This direct correlation between anti-La/SSB and EBVEA is reported herein for the first time to the best of our knowledge. However a related finding of high levels of EBVEA in SS patients was recently published (53), Further supporting the theory that in SS patients a subclinical EBV reactivation may trigger or perpetuate articular involvement.

The current study evaluated specific autoantibodies in a large cohort including patients with different AID and controls in order to compare between these AID. However it is limited by its retrospective design, the absence of exact matching between patients’ groups as well as association with clinical data. Notably, a more particular analysis regarding each group of patients matched with controls was analysed in former studies from our group which dealt more thoroughly with different sub-groups and specific clinical manifestations relevant to each entity (27, 56, 57).

To summarise, in this study done on a large group of patients with different AID and controls in order to compare between these AID. However it is limited by its retrospective design, the absence of exact matching between patients’ groups as well as association with clinical data. Notably, a more particular analysis regarding each group of patients matched with controls was analysed in former studies from our group which dealt more thoroughly with different sub-groups and specific clinical manifestations relevant to each entity (27, 56, 57).

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