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Autoantigen-Specific Memory B Cells in Primary Sjögren's Syndrome

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Abstract

Sjögren's syndrome (SS) is a systemic rheumatic autoimmune disease affecting the exocrine glandular function and is characterized by the presence of autoantibodies against the ribonucleoprotein particles, SS-A/Ro and SS-B/La, and mononuclear cell infiltration of exocrine tissues. Our aim is to characterize memory B cell pattern and function in relation to the progression of the disease, by analysing samples from a well-defined cohort of patients with primary SS. We have measured the number of Ro/La-specific plasma cells in peripheral blood mononuclear cells (PBMC) from 23 patients and 20 healthy controls by direct enzyme-linked immunospot (ELISPOT) assay. Furthermore, we quantified the Ro- and La-specific memory B cells in these individuals by a 6-day *in vitro* polyclonal stimulation of PBMC followed by an antigen-specific ELISPOT assay for the detection of memory B cells. In addition to this, ELISA profiling of autoantibodies was carried out using patients' plasma and supernatant, collected post-mitogen stimulation of PBMC. The average Ro60-, Ro52- and La48-specific plasma cells in PB was 9, 17 and 13 cells in 10^5 PBMC, respectively. After *in vitro* stimulation, these numbers increased to 43, 50 and 26 for Ro60, Ro52 and La48, correspondingly. However, the fraction of memory B cells activated into antibody-secreting cells was lower than the overall IgG B cell population. We conclude that these lower Ro/La-specific memory B cell levels may indicate that a greater portion of the Ro- and La-specific B cells are in an activated stage. This is in tune with previous reports.

Introduction

Primary Sjögren's syndrome (pSS) is a rheumatic autoimmune disease with systemic manifestations [1, 2]. It is characterized by chronic inflammation manifested by mononuclear cell infiltration of exocrine glands, lachrymal and salivary glands in particular. This results in an impaired tear and saliva production, which in turn contribute to dry eyes (keratoconjuctivitis sicca) and dry mouth (xerostomia). A common distinguishing feature of pSS is the presence of autoantibodies against the ribonucleoprotein particles Ro/SS-A and La/SS-B antigens, which are intracellular proteins involved in transcription and regulation [3, 4]. These are observed in 50–80% of the patients [5, 6].

B cells are important in the pathogenesis of SS, since B cell activation contributes to the high production levels of autoantibodies i.e. hypergammaglobulinaemia [7]. This is why patients with pSS generally show an increase in naïve B cells and consequently an elevated level of antibody-secreting $CD27^{high}$ plasma cells [7]. Still, it is not known whether B cell activation is a primary cause or a secondary effect in SS [8]. The autoantibodies in pSS possess activity for IgG, Ro/SSA and La/SSB. Nonetheless, IgG is the predominant isotype expressed by the infiltrating B cells, which have been found to make up 20% of the infiltrating cell population in exocrine glands [9].

Memory B cells and long-lived plasma cells are responsible for long-term humoral immunity [10, 11]. In humans, memory B cells constitute 40–60% of the total B cell population in peripheral blood (PB) [12]. Inactivated B cells are generally recognized by their expression of surface marker CD20, whereas memory B cells are specifically $CD20^+$ $CD27^+$ [12–14]. Patients with pSS show a general reduction of their $CD27^+$ memory B cell levels in peripheral blood, while an accumulation/retention of these cells is observed in their salivary glands [15]. This build up of $CD27^+$ memory B cells in the exocrine glands seems to contribute to the diminished peripheral

$CD27^+$ memory B cell population in SS [16]. Thereby, the demonstration of $CD20^+$ and $CD27^+$ B cells in salivary glands may help in the diagnosis of the disease [17]. In addition, abnormal B cell differentiation also results in a depressed percentage of circulating memory B cells and elevated levels of soluble CD27 that also correlate with serum IgG concentrations [18].

A well-established method for the assessment of the presence of anti-Ro/SSA and anti-La/SSB in peripheral blood of patients with SS is the enzyme-linked immunospot (ELISPOT) assay [19]. This has been demonstrated successfully in previous studies [9, 20, 21]. Tracking of human antigen-specific *memory* B cells in particular has also been made feasible over the years through a generalized and sensitive ELISPOT assay system that allows the quantization of memory B cells in peripheral blood [22]. This assay utilizes a 6-day polyclonal stimulation of peripheral blood mononuclear cells (PBMC) with a mitogen-mix, consisting of pokeweed mitogen [23], a CpG oligonucleotide [24, 25] and pansorbin [26, 27]. This is followed by an antigen-specific ELISPOT assay for the detection of memory B cells that have differentiated into antibody-secreting cells (ASCs) *in vitro*. Memory B cell ELISPOT assay has been used previously to quantify the number of memory B cells present in peripheral blood in relation to different vaccines [28–32]. In this study, we have adapted this memory B cell ELISPOT assay to examine the Ro/SSA- and La/SSB-*specific* memory B cell pattern in the peripheral blood of patients with pSS. Then, by assessing the total amounts of Ro/SSA and La/SSB ASC in the peripheral blood of these same patients, as performed previously [21], we tried to establish a more complete picture of the B cell repertoire specific for Ro/SSA and La/SSB in patients with pSS.

Materials and methods

Study population. Heparinized whole (Table 1) blood (~20 ml) was collected from 23 patients with pSS and 20 healthy controls (Table 2). These consecutive patients, fulfilling the criteria for the American-European Criteria Group for pSS (AECC) [33], were enrolled outpatients at the Department of Rheumatology, Haukeland University Hospital in Bergen, Norway. The healthy controls were recruited from the same geographical area as the patients with pSS. Age and gender of the healthy individuals were matched as much as made feasible to that of the patients. Informed consent was obtained from all participants, and the Committee of Ethics at the University of Bergen approved the study.

Recombinant proteins. cDNA encoding the Ro 60 kD, Ro 52 kD and La 48 kD was obtained as described for-

Table 1 Clinical and laboratory characteristics of the patients with pSS included in the study.

Patient no.	Age (years)	Gender	Leucocytes (per 10^9/l)	Lymphocytes (per 10^9/l)	ANA[a]	SSA	SSB	RF titre	IgG (g/l)	IgA (g/l)	IgM (g/l)	Focus[b] score	Sicca sum (mm)	Schirmer's test	Saliva[c]	Dry mouth
pSS-137	53	F	5.2	1.9	+	(+)	−	−	15.4	1.25	0.64	0	NT	NT	NT	+
pSS-138	58	F	6.8	5.3	−	−	−	+	7.62	3.78	0.47	4	NT	NT	NT	NT
pSS-139	48	F	4.4	3.2	+	+	−	−	12.5	2.89	0.37	NT	NT	+	6.7	+
pSS-141	64	F	4.2	1.5	+	+	+	+	21.4	4.38	3.1	3	3	+	1.0	−
pSS-144	69	F	11.4	2.2	+	+	−	−	6.58	1.08	0.85	2	1	−	12.0	−
pSS-146	79	F	3.9	1.1	+	+	+	+	11.6	2.37	0.73	−	4	−	NT	NT
pSS-147	77	F	7.4	1.8	+	−	−	−	14.4	6.59	0.28	1	5	+	−	−
pSS-148	65	F	6.3	3.9	+	+	+	+	13.3	2.28	1.07	0	NT	NT	NT	+
pSS-149	71	F	4.8	3.0	+	+	−	+	9.08	1.85	1.18	4	NT	NT	0.2	+
pSS-150	60	F	6.6	1.4	+	−	−	+	9.76	3.13	1.08	4	2	−	0.0	+
pSS-151	62	F	7.6	1.8	+	+	−	−	11.5	4.04	0.86	2	NT	−	2.0	+
pSS-152	60	F	7.5	1.5	+	+	−	−	7.89	2.21	1.00	0	NT	+	1.4	+
pSS-153	50	F	4.4	1.2	+	+	+	−	14.7	2.32	0.61	2	6	−	1.0	+
pSS-155	68	F	6.2	1.6	+	+	+	+	17.6	2.76	2.37	NT	NT	−	0.7	+
pSS-156	27	F	5.6	1.8	+	+	−	+	14.4	2.17	0.85	NT	NT	−	NT	NT
pSS-158	30	F	5	1.1	+	+	+	−	39.4	2.39	1.16	0	5	+	NT	NT
pSS-159	67	F	4.9	1.1	+	+	+	−	27.2	3.25	1.05	−	4	+	1.0	+
pSS-160	42	F	10.3	8.4	+	−	−	+	11.1	1.82	1.00	2	2	−	0.5	+
pSS-161	68	M	5.2	3.2	+	+	+	−	14.3	3.29	1.41	NT	4	+	1.1	+
pSS-162	69	F	4.9	2.2	+	+	+	+	14.0	2.72	1.06	−	4	+	NT	NT
pSS-163	65	F	3.2	1.5	+	+	−	−	15.7	2.17	0.63	1	2	−	0.0	+
pSS-165	51	F	4.0	1.7	−	−	−	−	8.49	1.94	0.59	1	4	+	0.6	+
pSS-166	67	F	5.8	1.4	+	+	−	−	8.11	2.15	1.47	NT	3	+	2.4	+

ANA, antinuclear antibodies; RF, rheumatoid factor; NT, not tested; pSS, primary Sjögren's syndrome.
[a]Values are attained by ELISA ranging at 0–9.99.
[b]Values are the number of inflammatory infiltrates/4 mm^2 area containing >50 mononuclear cells
[c]Values are in ml/15 mins (unstimulated flow); normal flow >1.5 ml/15 min.

Table 2 Clinical characteristics of the healthy controls included in the study.

Control no.	Age	Gender
1	57	F
2	45	F
3	66	F
4	54	F
5	49	F
6	55	F
7	57	F
8	64	F
9	55	F
10	54	F
11	50	F
12	45	F
13	49	F
14	53	F
15	58	F
16	41	F
17	30	F
18	23	F
19	29	F
20	59	M

merly [20, 34, 35]. Recombinant proteins were cloned into the pMAL vector (New England Biolabs, Beverly, MA, USA) as performed earlier [36] to express soluble recombinant fusion proteins, where maltose-binding protein (MaBP) was the vector-encoded fusion partner. The pMAL constructs were transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). The cultures were grown at 37 °C. Expression of the recombinant protein was induced by the addition of 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG; Pharmacia, Uppsala, Sweden). Cell extracts were prepared using the manufacturer's protocol of BugBuster Protein Extraction Reagents (Novagen). The recombinant proteins were then purified using an amylose column according to the production company's guidelines of pMAL protein fusion and purification system (New England Biolabs). Protein-containing fractions were determined by the BioRad Protein assay (BioRad Laboratories, Richmond, CA, USA). This was followed by SDS-PAGE and Western blotting against MaBP and Ro- or La proteins, respectively, to estimate the purity of these constructs. In addition to this, mass spectrometry analyses indicated that the Ro/La-MaBP fusion proteins are at least 95% pure, although the antigens consisted of full-length fusion protein and partially synthesized peptides.

Cell and serum isolation. Peripheral blood mononuclear cells were isolated from fresh blood using 10-ml Vacutainer cell preparation tubes containing 2 ml of Ficoll (Becton Dickinson, Plymouth, UK) by centrifugation for 20 min at 1650 *g* at room temperature. The plasma was then collected and stored at −20 °C until tested. The layer of mononuclear cells was carefully harvested and washed, by resuspending the pellet in sterile phosphate buffer saline (PBS), then centrifuged at 300 *g* for 10 min at 4 °C. This was followed by diluting the pellet in a solution of 90% fetal bovine serum gold (FBS) (PAA laboratories GmbH, Pasching, Austria) and 10% dimethylsulphoxide (DMSO) (Merck, Darmstadt, Germany). The sample was stored at −70 °C till use.

Thawing and counting of peripheral blood mononuclear cells. The isolated PBMC were thawed and rapidly resuspended in B cell media (BCM): RPMI-1640 containing 10% FBS gold, 1% streptomycin (Sigma Aldrich, St. Louis, MO, USA) and supplemented with ultraglutamine 1 (Lonza, Verviers, Belgium). The cells were then washed once in BCM and centrifuged at 300 *g* for 15 min at room temperature.

To count the mononuclear cells, Casy Cell Counter system was used (Schärfe System GmbH, Reutlingen, Germany).

Direct ELISPOT assay. The ELISPOT assay was performed to detect single ASCs against the recombinant Ro 60-kD, Ro 52-kD and La 48-kD proteins in the peripheral blood (PB) of 23 patients with pSS [19]. The assay was performed using 96-well filter plates (MSHA N45 50; Millipore, Billerica, MA, USA). The plates were coated with recombinant Ro 60-kD, Ro 52-kD and La 48-kD proteins diluted with sterile PBS to a final concentration of 10 μg/ml, and 100 μl was added to each well. The plates were incubated overnight at 4 °C. For the enumeration of total IgG-producing cells in PB, wells were coated in parallel with high-affinity purified goat anti-human heavy-chain-specific IgG (Sigma, Schnelldorf, Germany), at a concentration of 4 μg/ml in sterile PBS. Control wells were coated with PBS. For blocking of non-specific binding sites, the wells were filled with 250 μl of BCM, and the plates were incubated for at least 2 h at 37 °C in a humid atmosphere containing 5% CO_2. Individual wells were filled with 100 μl aliquots containing 10^5–20^5 PBMC in BCM. All cultures were performed in triplicates. The cells were incubated overnight under the same conditions as the blocking (37 °C, 5% CO_2). After incubation, the plates were washed with PBS once, followed by six consecutive washes with PBS containing 0.05% Tween-20 (PBST). Subsequently, for spot detection, 100 μl of peroxidase-conjugated goat anti-human IgG (Sigma) was added to each well, with a dilution of 1:1000 made in PBST. The plates were incubated for 2 h followed by another washing step with PBS and thereafter PBST. Then, the plates were developed by the addition of 100 μl of tetramethylbenzidine (TMBH) (Moss Inc., Pasadena, MD, USA) to each well. The reaction was stopped by washing the plates thoroughly with tap water. Enzyme activity was visualized on the nitrocellulose membrane as blue spots, where each spot represents one ASC. The membranes were punched out on sealing tape and scanned. Spots

were counted using the ELISPOT assay plate reader (ImmunoSpot software; Cellular Technology Ltd., Bonn, Germany).

Memory B cell assay. The memory B cell assay was essentially carried out as previously described [22]. PBMC from 20 patients obtainable for testing were plated in 24-well dishes (Nunc A/S, Roskilde, Denmark) at 5×10^5 cells/well in BCM supplemented with an optimized mix of polyclonal mitogens: 1:100 000 pokeweed mitogen extract (PWM) (Sigma-Aldrich, Oslo, Norway), 3 μg/ml fully phospho*rothioated* CpG ODN-2006 [25] (Integrated DNA Technologies, Coralville, IA, USA), and 1:10 000 Pansorbin (Calbiochem/Merck, Darmstadt, Germany). Eight wells were cultured per individual, four of which were stimulated with the optimized mix of polyclonal mitogens, while the other four were non-stimulated negative control wells cultured in BCM alone. Cells were cultured for 6 days at 37 °C, 5% CO_2. Lymphocyte blasting and clustering were observed in the stimulated culture wells from days 3 to 6.

Similar to the direct ELISPOT assay, 96-well filter plates were coated with recombinant Ro 60-kD, Ro 52-kD and La 48-kD proteins (10 μg/ml) and goat anti-human IgG (4 μg/ml) in sterile PBS, while control wells were coated with PBS alone. 100 μl of antigen was added to each well, and the plates were incubated overnight at 4 °C. For blocking of non-specific binding sites, the wells were blocked with 250 μl of BCM, and the plates were incubated for at least 2 h at 37 °C, 5% CO_2. The cultured PBMC were harvested on the sixth day, and the supernatant (SN) was collected for both stimulated and non-stimulated cells and stored at −70 °C for further testing. The cells were then washed thoroughly in PBS containing 5% FBS Gold. A series of 1:2 dilutions of 50 000–400 cells in BCM were prepared for each individual for both stimulated and non-stimulated cells; 100 μl of each dilution was plated onto the ELISPOT assay plates in duplicates and incubated overnight at 37 °C, 5% CO_2. Plates were then washed with PBS followed by PBST and incubated with peroxidase-conjugated goat anti-human IgG for 2 h for detection. This was followed by a second washing step and developed using TMBH, where each blue spot represents a memory B cell that has differentiated and proliferated into an ASC. The plates were then scanned and analysed as before (see Direct ELISPOT assay for details).

To ensure the reliability of this adapted method for the stimulation of memory B cells, the technique was verified by the use of a memory B cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Memory B cells were isolated from PBMC of patients with pSS through the depletion of unwanted non-B cells and subsequent positive selection with CD27Microbeads. The purity of the different eluates was evaluated by flow cytometry. The different cell populations isolated were then cultured and stimulated with PWM as previously described. As anticipated, lymphocyte blasting and clustering were only observed in the stimulated culture when memory B cells were present.

ELISA. Autoantibody levels were measured in the plasma and supernatant of both stimulated and non-stimulated cells for all individuals. The recombinant Ro 60-kD, Ro 52-kD and La 48-kD proteins and polyvalent goat anti-human IgG (Southern Biotech, Birmingham, AL, USA) were used to coat the ELISA plates (Greiner Bio-One GmbH, Frickenhausen, Germany). These were dissolved in PBS to a final concentration of 10 and 2 μg/ml, respectively, and 100 μl was added to each well and incubated overnight at 4 °C. This was found to be optimal in primary experiments [20]. Non-specific binding sites were blocked with 10% FBS Gold in PBS, and 200 μl were added to each well for 1 h, with gentle shaking. Blood plasma was diluted 1:100 and 1:50, while the supernatant was not diluted. Human IgG (Sigma) was used as a standard with twofold dilutions from 200 to 1.5 ng/ml. All dilutions were made in PBS containing 10% FBS Gold; 100 μl was added per well, and the plates were incubated for 2 h at room temperature with gentle shaking. The plates were then washed five times in PBST. This was followed by the addition of 100 μl of peroxidase-conjugated goat anti-human IgG (Sigma), diluted 1:2000 in PBS containing 10% FBS Gold, to each well for 1 h with gentle shaking at room temperature. After a second wash with PBST, the plates were developed by the addition of 100 μl of substrate solution to the wells. This consisted of four tablets of 2 mg ortho-phenylenediamine diluted in 12 ml of ddH_2O and 5 μl of 30% H_2O_2. The reaction was stopped after 15 min at room temperature by adding 100 μl of 0.5 M H_2SO_4 to each well. The absorbance was then measured at 490 nm, and absorbance values 1 SD above mean values obtained from 20 healthy individuals (negative controls) was considered positive.

Statistical analysis. Statistical significance was evaluated by the Student's *t*-test and presented as mean. Differences were considered significant when $P \leq 0.05$. The Pearson correlation test was used to examine the association between the different parameters.

Results

Ro- and La-specific circulating B cells in direct and memory B cell ELISPOT assay for patients with pSS and healthy controls

The direct ELISPOT assay was utilized to estimate the frequencies of PB lymphocytes producing antibodies against Ro/SS-A and La/SS-B antigens. In addition, the memory B cell ELISPOT assay was applied to the same individuals to enumerate the Ro- and La-specific memory

B cells. We observed Ro- and La-specific ASC in both the direct and the memory B cell ELISPOT assays. In the case of memory B cell ELISPOT assay, no spots were seen in the non-stimulated wells, as expected. IgG-positive ASC were present in both assays.

In the direct ELISPOT assay, the average Ro60-specific plasma cells in PB were lower than that of Ro52 and La48 (9 per 1×10^5 PBMC). The mean Ro52-specific ASC, on the other hand, was the highest (17 per 1×10^5 PBMC), while the La48-specific ASCs had an average of 13 cells in 1×10^5 PBMC. After *in vitro* stimulation with pokeweed mitogen and utilizing the memory B cell ELISPOT assay, we noticed that these values increased to an average of 43, 50 and 26 ASCs per 1×10^5 PBMC, for Ro60, Ro52 and La48, respectively. To investigate whether this rise was of statistical relevance, we applied the Student's *t*-test. This confirmed that the elevation in ASC levels post-stimulation was statistically significant for both Ro60 and Ro52, with *P*-values of ≤0.0005 and ≤0.0117, respectively. As anticipated, the healthy controls only expressed IgG-positive ASC (Fig. 1A).

As proliferation occurs during the 6-day culture, the number of spots observed in this assay is not a direct reflection of the number of antigen-specific memory B cells seeded in cell culture. For this reason, we assayed all PBMC samples for both total IgG memory B cells (spots observed in anti-IgG-coated ELISPOT assay wells) and antigen-specific memory B cells (spots observed in antigen-coated ELISPOT assay wells) to quantify the antigen-specific memory B cells as a *percentage* of total IgG+ memory B cells. When comparing the percentage of Ro- and La-specific IgG+ B cells per 1×10^5 PBMC in direct ELISPOT assay, we found that Ro60 had the lowest percentage (21%), while both Ro52- and La48-specific IgG+ B cells were present in almost similar levels (43% and 42%, respectively). In post-mitogen stimulation, the percentage of IgG+ Ro60-, Ro52- and La48-specific ASC levels decreased greatly. La48 showed the greatest decrease (1%), whereas Ro60 and Ro52 decreased to 2% and 3%, correspondingly. The Student's *t*-test illustrated that this decrease is statistically significant for all Ro60, Ro52 and La48, with a *P*-value of ≤0.0001 in all three instances (Fig. 1B).

Concentration of autoantibodies against the recombinant Ro 60-kD, Ro 52-kD and La 48-kD in plasma and supernatant of patients with pSS and healthy controls

For the comparison of the number of Ro- and La-specific ASC in PB with the levels of autoantibodies secreted in the same individuals, the ELISA was applied to measure the concentration of antibodies against each of recombinant Ro 60-kD, Ro 52-kD and La 48-kD proteins in both plasma and supernatant post-mitogen stimulation (for both stimulated and non-stimulated cells). Levels of IgG+ antibodies were also measured in all instances as a positive control to confirm the presence of antibodies in these individuals. Absorbance values 1 SD and 1 SEM

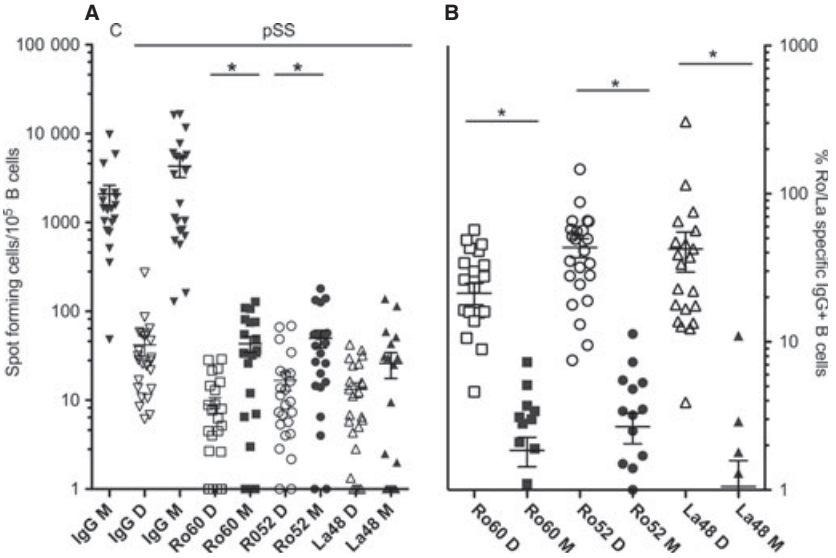

Figure 1 Ro- and La-specific circulating B cells in direct and memory B cell enzyme-linked immunospot (ELISPOT) assay for patients with primary Sjögren's syndrome and healthy controls. (A) The sample material is indicated on top. The antigens tested for are shown on the *x*-axis, where the autoantigen-specific spot-forming cells are measured by either direct (D, open data points) or memory B cell ELISPOT assay (M, closed data points) and presented as a concentration of positive spot-forming cells per 10^5 B cells on the *y*-axis. (B) The *x*-axis shows the autoantigens studied for both the direct (D, open data points) and memory (M, closed data points) B cell ELISPOT assay. The *y*-axis represents the percentage of IgG+ Ro- and La-specific B cell levels out of the total IgG+ B cell population. (*P-value ≤ 0.05).

above mean values obtained from 20 healthy individuals (negative controls) were considered positive.

After measuring the levels of autoantibodies in plasma, we deduced that five patients had high levels of anti-Ro60 (>10 μg/ml), whereas seven patients had lower concentrations of 1–5 μg/ml. This resulted in a mean of 3.3 μg/ml anti-Ro60 in plasma. Ten patients had somewhat similar levels of plasma anti-Ro52 at ~6 μg/ml, while six patients showed lower concentrations of 0.1–1 μg/ml. This lead to a mean value of 2.5 μg/ml plasma anti-Ro52. Eight patients expressed very high levels (>17 μg/ml) of plasma anti-La48; meanwhile, six patients exhibited much lower levels of 0.2–4 μg/ml anti-La48. This gave a mean of plasma anti-La48 at 6.8 μg/ml (Fig. 2). The healthy controls were only positive for plasma anti-IgG, with the exception of one individual expressing anti-Ro60 and anti-La48 autoantibodies at 7.4 and 2.0 μg/ml, respectively.

When determining the concentration of autoantibodies in the supernatants of stimulated cells, we found two patients to have similar levels of anti-Ro60 at 0.02 μg/ml, while five patients showed lower levels of anti-Ro60 (≤0.01 μg/ml) in the SN. This lead to a mean of 0.003 μg/ml of anti-Ro60. A somewhat similar pattern was observed in the case of anti-Ro52, where five patients expressed autoantibodies reactive against this recombinant antigen at levels ≤0.01 μg/ml, whereas one patient had 0.016 μg/ml of anti-Ro52 in the SN. This in turn resulted in a mean value of 0.002 μg/ml of SN anti-Ro52. In case of anti-La48, four patients had levels below 0.01 μg/ml in their SN, yet three patients displayed higher levels (≥0.015 μg/ml) of anti-La48. This gave a mean of 0.007 μg/ml of anti-La48 in the SN (Fig. 2). The supernatant of both stimulated and non-stimulated cells expressed high levels of anti-IgG for all 25 patients. Still, non-stimulated cells of these same patients did not express detectable levels of anti-Ro and anti-La autoantibodies. As for the healthy individuals, we did not find anti-Ro and anti-La autoantibodies being produced in the SN of neither stimulated nor non-stimulated, while anti-IgG was detected at high levels in both cases for all individuals.

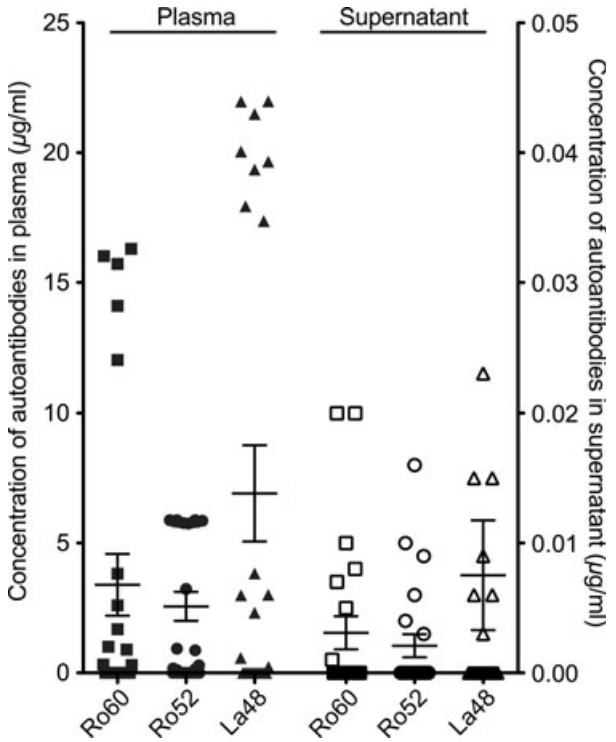

Figure 2 Concentration of Ro and La autoantibodies in plasma and supernatant of patients with primary Sjögren's syndrome was analysed by ELISA. The antigens tested for are indicated on the *x*-axis. The plasma concentrations (P, enclosed data points) are demonstrated on the left *y*-axis (μg/ml), while the concentrations of the supernatant (SN, open data points) are shown on the right *y*-axis (μg/ml). The mean concentration ± SEM for each of the antigens analysed is indicated in the figure.

Finally, by applying Pearson correlation test, we examined the covariation between the autoantibody concentrations detected in plasma and supernatant with the levels of Ro- and La-specific spot-forming cells in both the direct and memory B cell assays. We found an association between the levels of Ro60 and Ro52 autoantibodies in the supernatant in relation to their concentration as ASC in the memory B cell ELISPOT assay (Ro60: $r^2 = 0.2625$, $P = 0.0176$, Ro52: $r^2 = 0.2145$, $P = 0.0345$). Also, a correlation was seen between the level of Ro60 autoantibodies in the plasma and the concentration of Ro60 ASC in the direct ELISPOT assay ($r^2 = 0.4094$, $P = 0.0008$). Otherwise, we found no correlation between the various parameters (data not shown).

Discussion

In this study, we have examined the Ro/SSA- and La/SSB-*specific* memory B cell pattern in the peripheral blood of 20 patients with pSS by adapting the memory B cell ELISPOT assay developed by Crotty *et al.* in 2004 [22]. The total amounts of Ro/SSA and La/SSB ASC in the peripheral blood of these same patients were also inspected by applying the direct ELISPOT assay, as performed previously [21]. Both methods allowed quantification of the secreted products at the cellular level. Hence, by combining both the methods, a more complete picture of Ro/SSA and La/SSB ASC pattern in patients with pSS could be established.

When comparing the average numbers of Ro- and La-specific spot-forming cells in both the direct and memory B cell ELISPOT assays, there was an increase in Ro- and La-specific spot-forming cells post-mitogen stimulation. This difference was significant in case of Ro60 and Ro52 in particular (Fig. 1A). The general

increase in Ro and La ASC after stimulation could indicate a continuous immune response in these patients. Nonetheless, when quantifying these antigen-specific memory B cells as a percentage of total IgG+ memory B cells, a significant decrease in IgG+ Ro- and La-specific memory B cell levels in comparison with the total IgG+ antigen-specific B cell population was observed (Fig. 1B). It has been reported in previous studies that the abnormal differentiation of B cells in pSS leads to a decline in circulating memory B cells and a subsequent increase in levels of plasma cells and long-lived plasma cells [18, 37]. Correspondingly, the accumulation of memory B cells in the inflamed salivary glands could be a reason for their reduction in peripheral blood [15, 18, 38]. Our findings are in tune with these reports. However, whether or not these observations are due to Ro- and La-specific memory B cells has not been shown. In this study, we have managed to confirm that these memory B cells in question are indeed Ro and La specific in patients with pSS.

We found anti-Ro and anti-La autoantibodies in both the plasma and the supernatant of the patients with pSS examined. Nonetheless, autoantibody levels measured in the supernatant were detected at a much lower level than in the plasma. This is because the supernatant examined only accounted for a small fraction of the total number of PBMC in these individuals (2 million PBMC per individual). Autoantibody levels measured in plasma, on the other hand, represent the total number of PBMC in these subjects. This great difference in autoantibody levels also implies that most of the ASCs not originating from memory B cells have been eliminated during the 6-day incubation post-mitogen stimulation, rendering this as a reliable method to study memory B cells in particular.

Upon comparison, the SSA and SSB positivity of the subjects included in this study were generally similar to the data attained from the clinic, with a few exceptions. Specifically, patients 144, 149 and 163 were now found to be SSA negative, while patients 165 and 166 tested positive for SSB. Also, patient 147 was found positive for both SSA and SSB. These varying observations could be due to the difference in techniques employed in our study to that of the clinic. These differences could also be the results of varying progression of pSS between the different individuals over time. The clinical data was attained from tests carried out at point of clinical admission of these patients, which in some cases dated back to 2005. Hence, some subjects could have developed autoantibody production over time. Related to this, the advancement of pSS in patients 147, 165 and 166 was perhaps great, which in turn resulted in autoantibody production in these individuals. Meanwhile, patients 144, 149 and 163 might have been more susceptible to the immunosuppressive treatment administered, which could have lead to the abolishment of SSA or SSB autoantibodies in these subjects.

Some patients had detectable titres of anti-Ro and anti-La antibodies in plasma, yet no autoantibody-producing cells were found in their peripheral blood. This indicates that autoantibodies are also produced in other body compartments, including salivary glands and lymph nodes. Hence, antibody-secreting plasma cells have a tendency to migrate to the lymph nodes and other organs. It is therefore not always possible to identify a direct correlation between the number of Ro- and La-specific ASCs from both ELISPOT assays, with the anti-Ro and anti-La antibody levels in plasma and supernatant of these same individuals.

In conclusion, we have shown that the decrease in memory B cell levels in the peripheral blood of patients with pSS is specific to anti-Ro and anti-La memory B cells. Further studies need to be conducted to examine whether the increase in memory B cell infiltrates in salivary glands is also caused by anti-Ro- and anti-La-specific memory B cells.

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