Induction of anti-Ro60/anti-La by immunisation with spectrin and induction of anti-spectrin by immunisation with Ro60 and 4-hydroxy-2-nonenal-modified Ro60 immunisation

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Abstract

Objective

The Ro ribonucleoprotein particle, targeted in systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), includes Ro60 (SSA) and La (SSA) autoantigens. Anti-Ro60 occurs in SLE and SS. The importance of α-fodrin and spectrin as well as anti-Ro and anti-fodrin/spectrin antibodies in SS and SLE, led us to hypothesise that rabbit immunisation with Ro60 or 4-hydroxy-2-nonenal-modified Ro60 would induce anti-spectrin. In addition, we hypothesised that antibodies to Ro60 and La will develop in animals immunised with spectrin.

Methods

Two NZW rabbits each were immunised with 4-hydroxy-2-nonenal-modified Ro60 or unmodified Ro60. Methods used included ELISA, including an inside-out RBC membrane ELISA, and Crithidia lucilae *assays.*

Results

Commercial anti-spectrin sera bound significantly to Ro60 (OD 2.6 ± 0.1), Ro60 multiple antigenic peptides (MAPs) (3 out of 21 Ro60 MAPs), La (OD 4.4±0.5), and La fragments as well as to double stranded DNA but not to BSA (OD 0.6±0.1). Anti-spectrin binding to purified spectrin could be inhibited by spectrin (>95%), and Ro60 or La (70%). When the binding of anti-spectrin was tested against a nested set of La fragments we found that a N4 fragment representing the C-terminal 250 aa (aa 159 to 408) bound the strongest (OD=4.12) followed by a N9 fragment (the C-terminal 36aa; aa373 to 408 (OD=1.36). Also, significant anti-spectrin antibody levels were induced by Ro60 and HNE-modified Ro60 immunisation.

Conclusion

We found intermolecular epitope spreading from Ro60/La to spectrin and vice versa, and this may have pathological significance in these animal models of autoimmunity.

Key words

spectrin, fodrin, Ro60 (SS-A), La (SS-B), SLE, SS

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Introduction

Systemic lupus erythematosus, a chronic autoimmune disorder, affects the skin, joints and several organ systems. Autoantibodies directed against self-antigens are commonly seen in this condition, including against Ro ribonucleoprotein. Anti-Ro60 occurs in up to 50% of patients with SLE and anti-La in substantially fewer patients (1, 2). The presence of anti-Ro60 is associated with photosensitive skin rash, subacute cutaneous lupus, deficiency of early complement components, renal disease, neonatal lupus, lymphopenia and neutropenia (3-5).

Anti-Ro60 occurs in up to 90% of patients with Sjögren's syndrome (SS) (6, 7). SS is characterised by lacrimal and salivary gland inflammation leading to keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). SS patients can have other systemic manifestations, like kidney, lung, skin, muscle, bone marrow, joints and vascular involvement. SS is considered primary when it occurs alone and secondary when SS occurs together with another inflammatory autoimmune disease such as rheumatoid arthritis, primary biliary cirrhosis, polymyositis, scleroderma or systemic lupus erythematosus (6). Severe fatigue is a common complaint in primary SS (8, 9).

α–fodrin, the non-erythroid homolog of spectrin, is an autoantigen in SS. Antibodies to α-fodrin occur in SS. Immunisation with α-fodrin induces SS in an animal model (10). Mucosal administration of α-fodrin has been demonstrated to inhibit experimentally induced SS in mice (11).

Spectrin consists of 2 non-identical subunits, α (MW 240,000) and β (MW 220,000 and constitutes a major component of the red blood cell (RBC) membrane skeleton. Spectrin localises on the cytoplasmic side of the membrane and interacts with a number of proteins, forming an intracellular network. The RBC shape as well as elasticity of the lipid bilayer are controlled by such interactions (10, 11).

Spectrin forms a tetramer by head-to-head association of αβ dimer pairs. An actin binding domain is located at either end of the tetramer in the N-terminal region of β spectrin. Protein 4.1 promotes the interaction of actin with spectrin. Actin filaments bring about the clustering of spectrin-4.1 complexes (spectrin/4.1/actin junctions). Tropomyosin, tropomodulin, adducin and dematin (4.9) are other proteins found in these junctions. Numerous membrane proteins are bound by protein 4.1, thus making these junctions to act as scaffolds for the assembly of protein complexes (10, 12).

Free radical-mediated damage has been shown to be actively involved in the pathogenesis of SLE and other diseases (13-15). Reactive lipid peroxidation products can form adducts with lysine, histidine and cysteine targets (16). One of the most common and reactive lipid oxidation products is 4-hydroxy-2-nonenal (HNE) (16). Higher levels of HNE-modified proteins have been found in autoimmune diseases (13). HNE-protein adducts are potential neoantigens, and so could be involved in the pathogenesis of autoimmune diseases (17, 18).

We have reported oxidatively modified proteins in the red cell membrane of SLE patients. Specifically, we found that catalase bound to red cell membrane is a possible protein target for 4-hydoxy-2-nonenal (HNE) (a by-product of oxidative damage to lipids) modification. We have also previously shown that immunisation with HNE modified Ro60 induced accelerated autoimmunity by bringing about rapid intra and intermolecular epitope spreading.

We were interested to see whether animals immunised with Ro60 or HNE Ro60 would break tolerance to the spectrin autoantigen. We also hypothesised that the reverse would be true as well. That is, immunisation of animals with human spectrin will bring about anti-spectrin antibodies and autoimmunity.

Materials and methods

Materials

4-hydroxy-2-nonenal was purchased from Cayman Scientific, Ann Arbor, MI. *Crithidia lucillae* immunofluorescent anti-nDNA test kits were from Inova Diagnostics, San Diego, CA. Poly-lysine coated ELISA plates were from Fisher Scientific, Dallas, TX. Purified

bovine Ro60 was from Immunovision (Springdale, AK). Purified human spectrin, and anti-human spectrin were from Sigma Chemical Co., St. Louis, MO. Anti-rabbit IgG fluoroisothiocyanate was from Jackson Laboratories, Bar Harbor, ME. All other chemicals were of reagent grade.

Animals
The rabbits were fed standard rabbit feed and water *ad libitum*. The animal protocol was approved by the Institutional Animal Care and Use Committee according to established guidelines.

Ro60 multiple antigenic peptides (MAPs)
Twenty-one MAPs were synthesised from the sequence of the Ro60 autoantigen (19-21) at the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center, Oklahoma City by a manual stepwise solid phase procedure. An unrelated MAP with the sequence PPPGMRPP (22) from the Sm autoantigen was also synthesised.

Preparation of Ro60 and HNE Ro60 for immunisation
Purified Ro60 was modified by the addition of 10 mM 4-hydroxy-2-nonenal (HNE) at room temperature for 24 hour in the presence of 10 mM sodium cyanoborohydride and then dialysed against 0.1 N NaCl using a 10K molecular weight cut-off membrane. Ro60 treated with sodium cyanoborohydride and dialysed parallel to HNE-Ro, served as the control (17).

Ro 60 rabbit immunisation
New Zealand White rabbits were immunised with either unmodified Ro60 or HNE-modified Ro60. On day 1, 500 µg of either Ro60 or HNE-modified Ro60 was emulsified in 500 µl of complete Freund's adjuvant and injected intraperitoneally and subcutaneously into the rabbit. Subsequent boosts, with 500 µg antigen in incomplete Freund's adjuvant each time, were administered on day 26, 53, 99 with a final intravenous boost on day 152. The animals were bled weekly and sera obtained were stored at -20° C. Pre-immunisation serum was collected from all animals.

Anti-spectrin antibody
Anti-spectrin antibody (Sigma Chemical Co.) was made by the manufacturer by immunising rabbits with spectrin purified from freshly isolated red cell membranes. This antibody interacts specifically with the α and β chains of human erythrocyte spectrin.

Enzyme linked immunosorbant assay (ELISA)
Solid phase ELISA assays for anti-spectrin and anti-spectrin binding to Ro60 MAPs, Ro60 or La were performed as previously described (23, 24). For inhibition studies, serum samples were incubated one hour at RT with inhibitor, Ro60, spectrin or La, at 10 µg/ml.

Detection of antibodies to double stranded DNA by CLIF
Anti-dsDNA assays using *Crithidia Lucillae* Immunofluorescence (CLIF) dsDNA kit was carried out according to the manufacturer's instructions.

Anti-nuclear antibody (ANA) testing
ANA testing using HEp-2 cells was carried out following the instructions of the manufacturer.

Preparation of inside-out red cell membrane
Inside-out cell RBC membranes have been prepared using polylysine coated glass beads (25, 26). We basically adapted this procedure to obtain inside-out RBC membranes on ELISA plates. Peripheral blood collected in a heparinised tube was spun to remove plasma. Lymphocytes were removed using Lymphoprep according to instructions of the manufacturer.
200 µl of packed RBC's were washed twice with PBST, containing 220 mM sucrose. From the washed packed cells, 12.5 µl was removed and diluted to 20 ml. A poly-D-lysine coated ELISA plate (96-well) was washed with PBST. 200 µl of diluted cells were added to the buffer-washed polylysine plate and incubated overnight at 4ºC. The following day, the plate was washed once with PBS and blocked for 2 h with 0.03% milk. One half of the plate was sonicated (10 sec/well; setting n. 4, Branson Sonicator, Danbury, CT). The wells were washed twice with PBST and then blocked with 3% milk for 2 h at room temperature. Samples were added to the wells (1:100 dilution in 0.03% milk) and incubated at room temperature for two hours. The plate was then washed with PBST, followed by the addition of secondary antibody conjugated with alkaline phosphatase (1:5000) and incubated further for 1 hour at room temperature. The plate was washed with PBST 4-5 times and color developed with p-nitrophenyl phosphate substrate.

Results
Earlier data from our laboratory show that autoimmunity is induced in experimental animals upon immunisation with either the whole Ro60 autoantigen or peptides derived from Ro60 antigen (17, 18, 22, 24, 27-29). Immunisation with Ro60 or HNE-modified Ro60 led to epitope spreading to La, Sm B/B' and 70K autoantigens. Here, we investigated epitope spreading to human spectrin in Ro60 and HNE-Ro60 immunised animals as well as epitope spreading to Ro60 and La in animals immunised with spectrin.
Figure 1 shows the binding of anti-Ro60 and anti-HNE Ro60 antibodies to purified spectrin by ELISA. Commercial anti-spectrin binds to solid phase spectrin with an OD of about 2.9. Anti-Ro60 as well as anti-HNE Ro60 antibodies bound to spectrin. Anti-spectrin antibodies were induced strongly by the 9th bleed in the Ro60 immunised rabbits, while it was induced by the 6th bleed in HNE Ro60 immunised rabbits.
We prepared inside-out red cell membrane, to see whether anti-Ro60 and anti-HNE Ro60 rabbit sera would bind to spectrin lining the inner side of the cells. Commercial anti-spectrin antibody bound to the spectrin in the inside-out cell membrane preparation (Fig. 2). However, the binding was not robust as the binding to purified spectrin observed in Figure 1. Anti-Ro60 and anti-HNE Ro60 antibodies also bound to the inside-out cell membrane preparation.
Figure 3 shows the binding of commercial anti-spectrin sera to La, Ro60 and BSA. As hypothesised, anti-spectrin

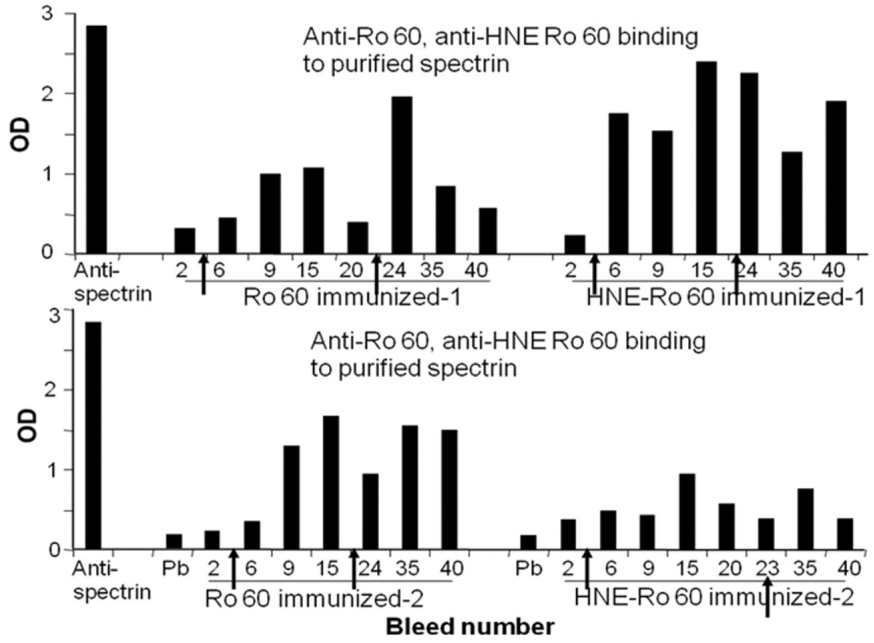

Fig. 1. Serial bleeds of rabbits immunised with Ro60 or 4-hydroxy nonenal (HNE)-modified Ro60 analysed for binding to purified spectrin. Spectrin was coated on ELISA plates and binding of the rabbit anti-sera to spectrin was determined by ELISA. Anti-spectrin was used as the positive control. Arrow indicates time of boost given to the animals.

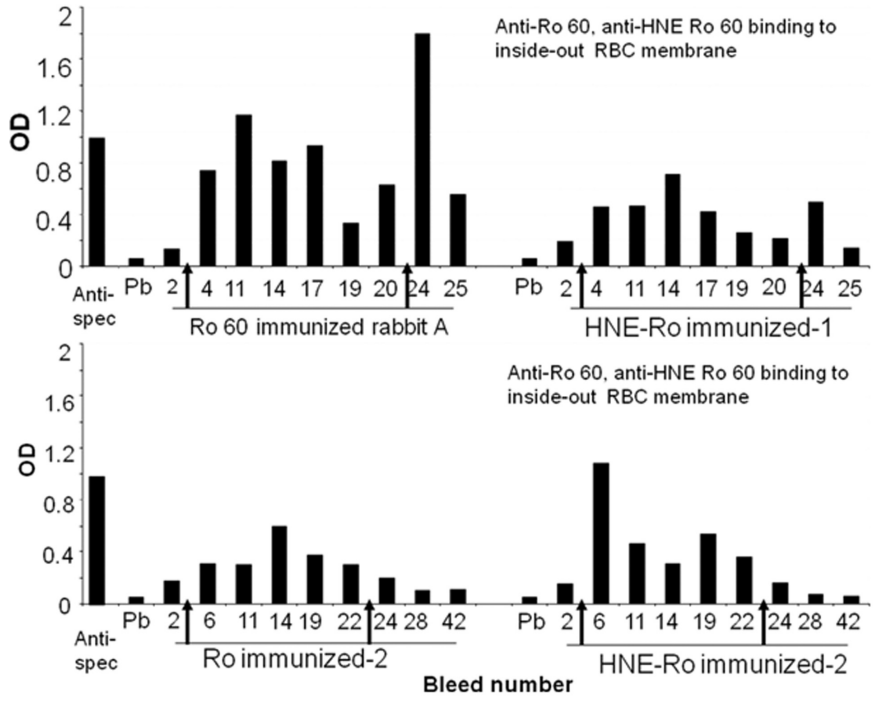

Fig. 2. Serial bleeds of rabbits immunised with Ro60 or HNE-modified Ro60 analysed for binding to inside-out cell membrane immobilised to polylysine coated ELISA plates. Inside-out cell membrane was prepared as mentioned in Materials and methods. The plates were blocked and anti-Ro60 or anti-HNE Ro60 rabbit sera were added (1: 100 dilution). After addition of appropriate alkaline phosphatase conjugate, paranitrophenol phosphate substrate was added and the color developed was read.

bound very significantly to La and Ro60 (p<0.0001), but not to BSA (Fig. 3).

To test the specificity of binding, anti-spectrin was incubated with La, Ro60 or spectrin and tested for binding to spectrin used as solid phase antigen. As shown in Figure 4, spectrin completely inhibited the binding of anti-spectrin to spectrin. However, Ro60 or La inhibited the binding of anti-spectrin to spectrin by only 70%.

Since anti-spectrin bound Ro60 and La, we were interested to identify the epitopes recognised by anti-spectrin on Ro60 and La. We used 21 Ro60 multiple antigenic peptides (Table I) as the solid phase antigen and investigated the binding of anti-spectrin to these peptides. We found three specific epitopes recognised by the anti-spectrin sera (Fig. 5), namely TFIQFKKDLKES, LAVTKYKQRNGWSHK and LPMIWAQKTNTP (MAP n. 4, 7 and 20 – Table I). However, anti-spectrin did not bind to a peptide PPPGMRPP derived from Sm B/B'. Figure 6 shows anti-spectrin binding to human La recombinant fragments, coated on an ELISA plate. N4 fragment, spanning C-terminal 256 aa (aa 159 to 408) amino acids bound to anti-spectrin the strongest.

Finally we determined whether immunisation with spectrin would bring about a lupus-like phenotype, in the form of induction of antibodies to double stranded DNA. When we tested anti-dsDNA levels in the anti-spectrin anti-sera we found that anti-dsDNA antibodies were induced upon immunisation with spectrin (Fig. 7).

Discussion

Spectrin plays a vital role in the structural organisation of plasma membranes of erythrocytes, and also in other cells in the form of its structural homologue fodrin. Spectrin has been shown to be associated with the membrane of organelles like exocytotic vesicles (30, 31) or with electron-dense cytoplasmic

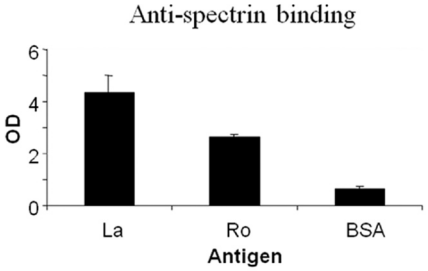

Fig. 3. Anti-spectrin binding to purified recombinant human La and to bovine serum albumin by ELISA. Ro 60, La or BSA were coated on ELISA plates and binding by anti-spectrin to these antigens was determined.

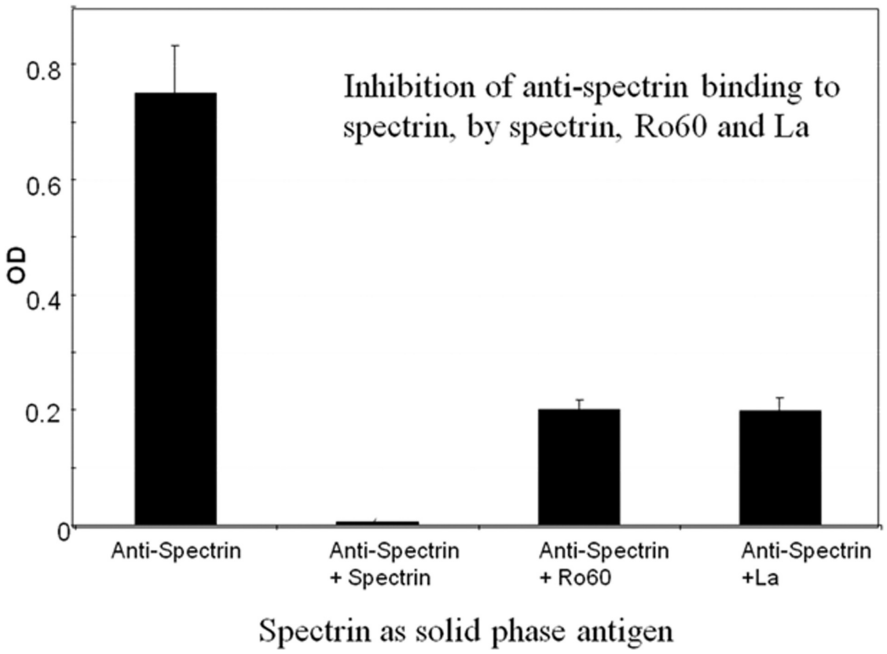

Fig. 4. Inhibition of anti-spectrin binding to spectrin in the absence and presence of spectrin, Ro or La. The antigens were incubated at room temperature for one hour with anti-spectrin (1:500 dilution) and binding of anti-spectrin or anti-spectrin + antigen to spectrin was investigated.

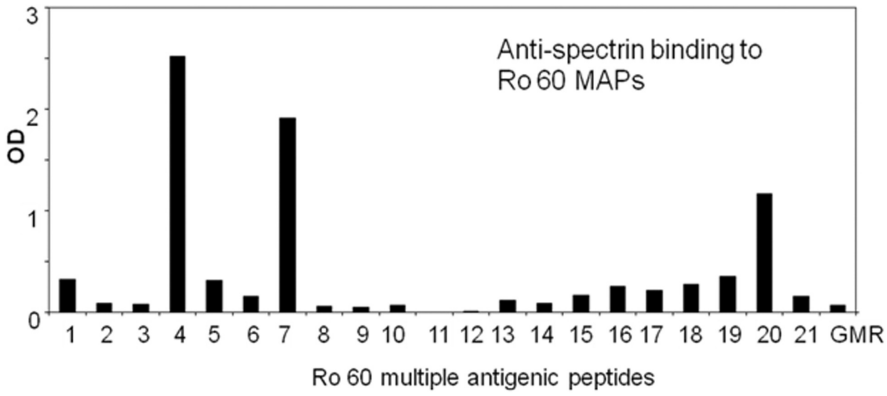

Fig. 5. Intermolecular epitope spreading of anti-spectrin to Ro MAPs. MAPs constructed from the 60 kD Ro sequence were coated on ELISA plates, blocked and incubated with anti-spectrin rabbit antisera. A MAP from the Sm autoantigen, PPPGMRPP was used as control.

islets (32). In addition, a β-spectrin homologue (bIS*) is associated with the Golgi apparatus (33).

Alpha-fodrin genes are strictly conserved across species, while the mammalian spectrin genes have diverged rapidly. The alpha-chains of spectrin and fodrin are mainly composed of homologous 106-amino-acid repeat units. Spectrin α chain lacks a 37 amino-acid sequence bearing the calmodulin-binding site of α-fodrin. The prominent degree of homology between the alpha-chains of spectrin and fodrin resides in a central atypical segment that is not related to the canonical repeat sequence. The important central portion of β-spectrin is comprised of repeat units of 106 amino-acids, just like α-spectrin (34).

In addition to its structural role, fodrin has been reported to play an important regulatory role in secretion and exocytosis (35). Perrin *et al*., using permeabilised chromaffin cells, found that anti-fodrin inhibited secretion (36), showing that fodrin and the cytoskeleton take part in the exocytosis mechanism.

Owing to the position of spectrin or fodrin on the internal side of the membrane should, in theory, exclude any interaction with antibodies. However, data in literature and our experience both indicate the interaction of anti-spectrin antibodies with the RBC under certain conditions. Membrane complexes containing IgG, globin, band 3 and other polypeptides including spectrin, have been found in the denser RBC population (37), in sickle RBCs (38) and also in RBCs from β-thalassemic subjects (39). The ascertained anti-spectrin specificity of some RBC-bound IgG (40-42), as well as the appearance of antibodies against spectrin in some experimental haematological conditions (43) show that antibodies can interact with spectrin.

Haneji *et al*. first identified the 120 kDa cleavage product of alpha-fodrin as a candidate autoantigen in experimental Sjögren's syndrome (44) and cleaved alpha-fodrin has since been found as apopoptic marker in salivary glands of SS patients (45) (salivary glands specifically expresses alpha-fodrin). Antibodies targeting α-fodrin have been found in SS and SLE patients (44,46-48). SS has been induced by immunisation with α-fodrin in an animal model (10). Mucosal administration of α-fodrin inhibits experimentally induced SS in mice (11). Savuz *et al*. observed IgA and IgG anti-spectrin with a frequency similar to anti-Ro/SSA and anti-La/SSB in the serum of patients with SS (47). Haneji *et al*. first showed (44) that anti-alpha-fodrin has a higher specificity and sensitivity than anti-Ro60 and anti-La antibodies in adult SS patients. Ro60 is a common target in SLE and SS. Immunisation of experimental animals with Ro60 or HNE-Ro60 induces intermolecular epitope spreading to La and other autoantigens (17, 18, 24, 28, 29). Similarly immunisation with La brings about intermolecular epitope spreading to the Ro60 autoantigen (49).

In this work we demonstrate that immunisation with Ro60 or HNE Ro60 induces intermolecular epitope spreading to the spectrin autoantigen. We also show that the reverse is possible. That is, immunisation with spectrin leads to cross-reactive intermolecular epitope spreading to the Ro60 and La autoantigens. The interaction of anti-spectrin with Ro60 is mediated via binding through three specific sequences on

Table I. Ro 60 multiple antigenic peptides (MAPs), their sequence, amino acid position and molecular weight (measured by mass spectrometry).

Map no.	Amino acid sequence	Location on protein	M. Wt. by mass spec. (in kD)
1	TYYIKEQKLGL	45-55	11.69
2	SQEGRTTKQ	81-89	9.12
3	STKQAAFKAV	106-115	9.25
4	TFIQFKKDLKES	126-137	12.7
5	MKCGMWGRA	139-147	9.2
6	MWGRALRKAIA	143-153	11.02
7	LAVTKYKQRNGWSHK	166-180	15.37
8	LRLSHLKPS	183-191	9.25
9	VTKYITKGWKEVH	198-210	13.55
10	LYKEKALS	212-219	8.45
11	TEKLLKYL	222-229	8.9
12	EAVEKVKRTKDELE	230-243	14.23
13	HLLTNHLKSKEVWKAL	257-272	16.18
14	ALLRNLGKMTA	280-290	10.34
15	NEKLLKKARIHPFH	310-323	14.69
16	YKTGHGLRGKLKWRP	331-345	15.22
17	AAFYKTFKTV	355-364	10.25
18	VEPTGKRFL	364-372	9.21
19	MVVTRTEKDSY	401-411	11.4
20	LPMIWAQKTNTP	449-460	12.04
21	ALREYRKKMDIPAK	482-495	14.59
22	PPPGRRPP	Sm	7.63

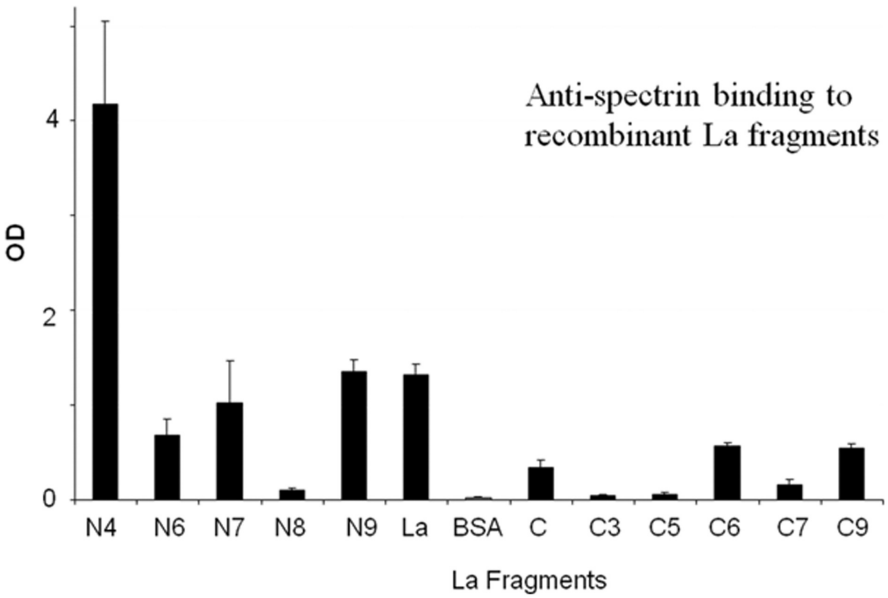

Fig. 6. Anti-spectrin binding to fragments of La showing intermolecular epitope spreading. BSA, La and fragments of La were coated on ELISA plates and the binding of anti-spectrin to these antigens was determined by ELISA. N4 corresponds to the C-terminal 256 amino acids (aa 159 to 408). N9 corresponds to the C-terminal 46 amino acids (aa 373 to 408).

Ro60, namely amino acids 126-137, 166-180 and 449-460. Interestingly, anti-spectrin binds to two epitopes of Ro60 (Ro 166-180 and Ro 126-137) that are among the targets bound by some human SLE patients that are positive for anti-Ro60 (50). Ro 169-180 sequence is the epitope targeted first by these SLE patients, as they develop the disease. Following this, the antibody binding spreads to other regions of the Ro60 molecule in these patients, including Ro 126-137 and Ro 449-460 (50). The interaction with La is mediated by binding to amino acids 159 to 408 on the La autoantigen. It is interesting that Ro60 or La do not share any significant sequence similarity with spectrin or fodrin, and yet these antigens are able to inhibit anti-spectrin binding to spectrin by 70%. The observed reactivity is likely a result of cross-reactive conformational epitopes brought about by epitope spreading. The Ro60, especially the HNE Ro60, immunised animals developed anti-Ro60, anti-La, anti-Sm, anti-RNP A and anti-double stranded DNA, thus developing a lupus-like condition. The fact that spectrin immunisation induced anti-Ro60, anti-La and antibodies to double stranded DNA also suggests the development of a lupus phenotype.

We have not monitored salivary flow or lymphocytic infiltration in the salivary glands of the Ro60, HNE-Ro60 or the spectrin immunised animals. However, it is our hypothesis that these animals will have a normal salivary flow and would not have lymphocytic infiltration of the salivary glands owing to the observed development of anti-dsDNA antibodies in these animals. Anti-dsDNA is normally not seen in primary SS patients. Therefore, anti-spectrin development in the Ro60/HNE-Ro60 immunised animals and anti-Ro60/anti-La development in the spectrin immunised mice points more to these animals as being a model of SLE rather than primary SS.

Even though studies have shown elevated anti-fodrin activity in Sjögrens syndrome, other studies report the non-specificity of these antibodies in SS (51, 52). Anti-spectrin also has been shown to be part of the normal repertoire in humans and animals (51, 53-57). A more recent study (58) has shown that anti-alpha fodrin is present in almost twice as many non-SS (with sicca symptoms only) compared to SS patients with anti-Ro60, anti-La and sicca. This study also showed using sialoscintigraphy that almost thrice as many of the non-SS subjects had grade III impairment of the salivary gland compared to SS subjects. The authors suggest that sialoscintigraphy could be used along with serum anti-alpha-fodrin, anti-Ro and anti-La to distinguish between SS and non-SS sicca (58). Nordmark *et al* found anti-fodrin antibodies in 16/56 (29%) of subjects with primary SS and in 25/53 (47%) of subjects with SLE (without secondary SS). However, these authors suggest that alpha-fodrin autoantibod-

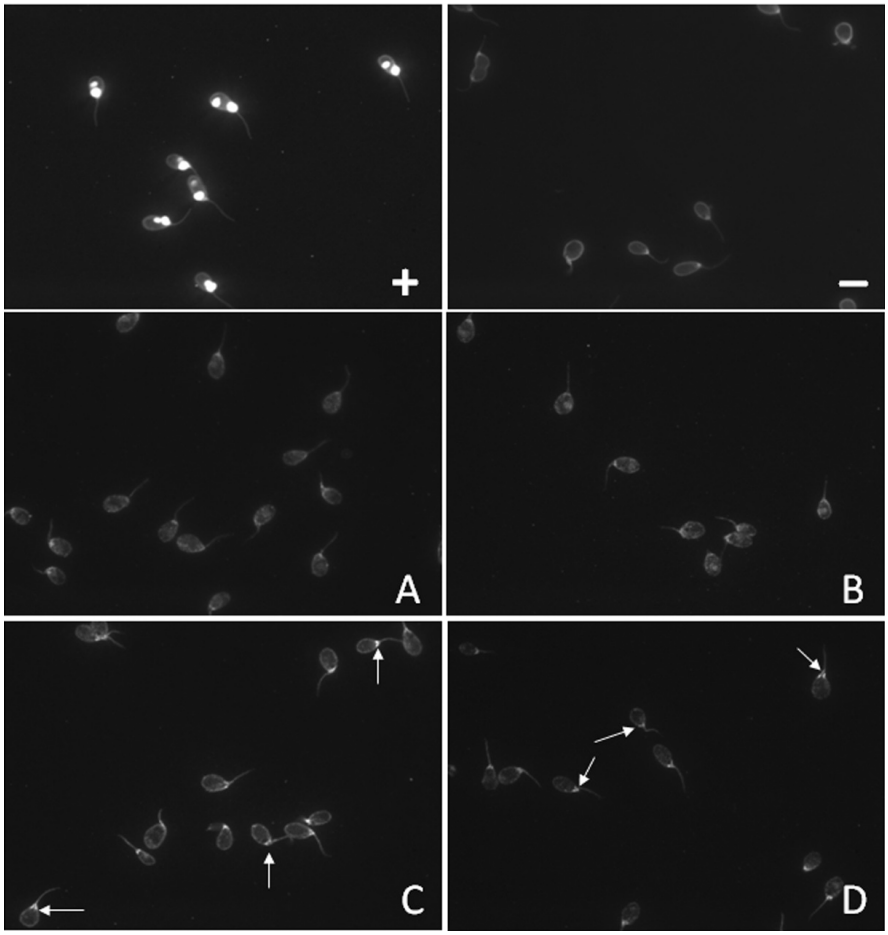

Fig. 7. Antibodies to dsDNA detected by CLIF assay in rabbits immunised with spectrin, haemoglobin, or Freund's control. **A**: Haemoglobin immunised; **B**: Freund's immunised. **C**: Spectrin immunised; **D**: Spectrin immunised.

ies are mainly related to non-organ-specific autoimmunity in primary SS and SLE and only have limited discriminating value (48). SLE serum, mediated by complement fragment deposition on RBC membrane, has been reported to induce dephosphorylation of β-spectrin bringing about reduced RBC membrane deformability and increase in RBC production of nitric oxide (59). Thus, complement activation in SLE patients is thought to lead to calcium-dependent changes in the RBC cytosketeleton making RBCs less deformable. This can impair the flow through capillaries and negatively affect oxygen delivery to the tissues (59).

Anti-spectrin has not been described in animal models of autoimmunity, as far as we know. The fact that we have observed anti-spectrin antibodies in our animal models of autoimmunity suggests that these antibodies might have a pathogenic role.

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