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Anti-alpha-actinin antibodies are part of the anti-cell membrane antibody spectrum that characterize patients with lupus nephritis

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ABSTRACT

Anti-membrane autoantibodies (MbA) have been reported in sera from patients with lupus nephritis (LN) but the targets of the MbA remain to be explored, which is the aim of the current study. Sera were collected from 40 patients with LN determined by renal biopsy, and from 30 systemic lupus erythematosus (SLE) patients without clinical evidence of LN. Thirty autoimmune disease control patients (rheumatoid arthritis, Sjögren's syndrome and systemic sclerosis), and 30 healthy controls were also included. Using flow cytometry, the presence of anti-MbA was explored revealing that IgG anti-MbA positivity was associated with LN (62.5% vs 13.3%) when compared to non-LN SLE patients, autoimmune disease patients (6.7%) and healthy controls (0%). Next, using purified plasma membrane fractions from human embryonic kidney (HEK) cells, the more prominent targets and their occurrence rates were located at 50 kDa, 60/65 kDa, 90 kDa, 110 kDa, 180 kDa and 220 kDa. Alpha-actinin (110 kDa) autoAb was characterized as a major target in LN patients positive for anti-MbA, and anti-MbA binding activity was reduced (36.9 ± 13.7%) in the presence of α-actinin. Laminin (200 kDa) was also characterized as a minor target, which was not the case for annexin A2 (36 kDa). Finally, anti-MbA IgG subclass analysis indicated a predominance of IgG2. In conclusion, IgG anti-MbA were detected at high levels in LN patients supporting a primary pathogenic role for anti-MbA and anti-MbA/α-actinin+ in LN that needs further research.

1. Introduction

Systemic lupus erythematosus (SLE) is the prototype of autoimmune diseases characterized by a loss of self-tolerance and production of a large panel of antibodies (Ab) directed against nuclear, cytoplasmic and cell-membrane targets [1]. The latter, anti-cell-membrane Ab (MbA), have revealed a correlation with lupus nephritis (LN), the most common and severe complication in SLE [2]. Although, most of the studies have been conducted with cells originating from kidneys, the same correlation with LN was also noted when using non-kidney cells such as endothelial cells [3] and peripheral blood lymphocytes [4].

Several techniques, such as glomerular proteome array, antigen array, or 2-dimensional gel electrophoresis coupled with or without mass spectrometry, have been used to elucidate the molecular targets associated with LN [5–8]. Accordingly, these

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techniques have revealed a large panel of nephrotoxic Ab, such as laminin, proteoglycan, heparin, myosin, collagen, annexin A2, α-actinin, and α-enolase [9]. However, most of these technologies focus on gene expression levels rather than cellular localization, which can be critical when considering cell-membrane antigens. Therefore, this study was designed to explore whether or not the kidney derived cell line HEK-293 (HEK) can be used to detect and characterize IgG anti-MbA present in serum from LN patients, using both a flow cytometry approach for screening, and a plasma-membrane purified approach for proteomic characterization. Our results confirmed the presence of IgG anti-MbA in the sera of LN patients. Furthermore, results from plasma-membrane purified Western blotting revealed several targets and α-actinin was the predominant one.

2. Material and methods

2.1. Patients and controls

Samples were collected from 70 patients with SLE fulfilling at least four of the 1982 American College of Rheumatology (ACR) revised criteria [10]. There were 34 patients at Brest University Medical School Hospital, France, and 36 at the Center for Autoimmune Diseases Research (CREA) in Bogota, Colombia. Forty patients had LN as determined by kidney biopsy [11], blood samples were collected on the same day before the biopsy procedure, while the remaining 30 SLE patients had no clinical or laboratory signs of nephritis. SLE activity was assessed by the SLE disease activity index (SLEDAI) [12]. SLE patient characteristics are reported in Table 1. The control group consisted of 30 healthy controls (HC), and 30 non-SLE rheumatic disease controls (DC) including 10 with rheumatoid arthritis (RA), 10 with systemic scleroderma (SSc), and 10 with Sjögren's syndrome (SS). All sera were stored at −80 °C until used.

2.2. Autoantibody assays

As described previously [13,14], anti-dsDNA Ab were determined using indirect immunofluorescence on *Crithidia luciliae* (CLIFT) and sera were considered as positive at serum dilutions above 1/10. IgG specific ELISA for anti-α-actinin, anti-laminin, anti-annexin A2, and anti-C1q Abs were tested following published protocols [15–19]. Briefly, microplates were coated with 10 μg/ml of target antigen (Sigma–Aldrich, St Louis, MO) in bicarbonate buffer, pH 9.6. Sera were diluted 1:200 in PBS–BSA 1% (plus NaCl 1M for anti-C1q Ab), and incubated for 90 min at 37 °C. Bound Abs were visualized using horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fragment of goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA). To avoid false positive results, the absorbance value of antigen-free wells was subtracted from each sample. Eighty serum aliquots from healthy volunteers obtained from members of the staff and blood bank donors were used to optimize the in-house ELISA, and the positive cut-off level was fixed at 3SD above the mean value of these sera.

Table 1
Baseline characteristics in systemic lupus erythematosus (SLE) patients with and without lupus nephritis (LN).

	LN ($n = 40$)	Non-LN (n = 30)	*P*	OR
Age (mean years [min–max])	32.7 [13–73]	35.0 [18–74]	NS	–
Sex ratio [F/M]	3 [30/10]	16 [29/1]	0.01	0.10
SLEDAI (mean [min–max])	10.1 [0–29]	5.6 [0–10]	NS	–
Autoantibodies (Ab)				
Anti-dsDNA Ab (CLIFT)	37.5%	10.0%	0.01	5.4
Anti-C1q Ab	57.5%	10.0%	0.0001	12.2
Anti-MbA Ab (HEK)	62.5%	13.3%	0.002	10.8
Therapeutics				
Antimalarial	40.0%	90.0%	0.0002	0.07
Steroids	70.0%	56.7%	NS	–
Immunosuppressors	37.5%	16.7%	NS	–

Abbreviations: F: female; M: male; SLEDAI: SLE disease activity index; CLIFT: *Crithidia luciliae* immunofluoresence; OR: odd-ratios; NS: non significant.

2.3. Tissue culture

Mesangial cells were prepared from healthy kidney tissues obtained from donors undergoing tumor nephrectomy as described [20]. Fibroblast contamination was further excluded by using medium in which L-valine was substituted by D-valine, a condition in which human mesangial cells (hMC) but not fibroblasts survive. The hMC were used at passage three to exclude macrophages and endothelial cells contamination.

The human embryonic kidney HEK cell line, the larynx epithelioma cell line HEp-2, and the human cervix epithelial cell line HeLa were purchased from the American Type Culture Collection (Manassas, VA). HEp-2 cells were those routinely used in our laboratory for the detection of antinuclear antibodies (ANA). Cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 2 μM L-glutamine (Promega, Mannheim, Germany), 10% fetal calf sera (Eurobio, Les Ulis, France), and antibiotics. At confluence, the cells were harvested using 0.25% trypsin–EDTA (Eurobio), washed, and cultured overnight in suspension before staining as described [21].

2.4. Flow cytometry analysis

The ability of sera to bind cell membrane was evaluated by flow cytometry. Briefly, tubes containing 5×10^5 cells were incubated 15 min at 4 °C with a buffer containing 5% human serum (HS, Invitrogen) and 1% bovine serum albumin (BSA; Sigma–Aldrich) in phosphate-buffered saline (PBS), washed twice with the same buffer, supernatant was removed, and cells were incubated for 1 h at 4 °C with 5 μL of serum. After washing twice again with 5% HS and 1% BSA in PBS, supernatant was removed and cells were incubated for 30 min at 4 °C in the dark with 0.5 μL of fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ anti-human IgG (Dako, Carpinteria, CA) and the four-color Epics XL flow cytometer (Beckman–Coulter, Hialeah, FL) was used to evaluate their staining after washing. Living cells were identified and selected through the forward and side scatters. In selected experiments FITC-conjugated anti-human IgG1, IgG2a + IgG2b, IgG3 and IgG4 (Sigma–Aldrich) were used. DNA dependence was tested following pretreatment of the cells 30 min at 37 °C with *DNase*I (Thermo Scientific, Rockford, IL), or in the presence of 50 μg/mL dsDNA. Inhibition assays were performed using 50 μg/mL of BSA, α-actinin, and laminin (Sigma–Aldrich). Results of subsequent staining were expressed as mean fluorescence intensities (MFI) and the cutoff point for positivity was defined using healthy controls (mean MFI ± 3SD).

2.5. Cell surface biotinylation and extraction

HEK cell surface proteins were biotinylated using the impermeant EZ-link Sulfo-NHS-LC-biotin cell surface protein isolation kit (Ref 89881; Pierce, Rockford, IL). Briefly, HEK cells were grown to 90% confluence in 75 cm^2 flasks, washed twice with ice-cold PBS solution, and then living cells were incubated with the ice-cold biotinylation solution for 30 min at 4 °C in a rocking platform. The biotinylation reaction was stopped using 500 μL of the provided quenching solution (Pierce) and cells were gently scraped, transferred in a 50 mL conical tube, and washed with PBS. Next, cells were sonicated twice in 500 μL of the provided lysis buffer