Fluctuation of Anti-Endothelial Cell Antibody Titers in Mixed Connective Tissue Disease

Laís V. Lage MD PhD[1], Jozélio F. de Carvalho MD PhD[1], Maria T.C. Caleiro MD PhD[1], Natalino H. Yoshinari MD PhD[1], Licia M.H. da Mota MD PhD[2], Munther A. Khamashta MD, PhD, FRCP[3] and Wilson Cossermelli MD PhD[1]

[1]Rheumatology Division of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil
[2]Rheumatology Service, Hospital Universitário de Brasília, Universidade de Brasília, Brasília-DF, Brazil.
[3]Lupus Unit, St. Thomas' Hospital, London. UK

ABSTRACT: **Background:** Antibodies directed against endothelial cell surface antigens have been described in many disorders and have been associated with disease activity. Since the most prominent histopathologic feature in mixed connective tissue disease (MCTD) is the widespread and unique proliferative vascular lesion, our aim was to evaluate the frequency of anti-endothelial cell antibodies (AECA) in this condition.

Objectives: To evaluate the frequency of AECA in this disease and assess its clinical and laboratory associations.

Methods: Seventy-three sera from 35 patients with MCTD (Kasukawa's criteria), collected during a 7 year period, were tested for immunoglobulins G and M (IgG and IgM) AECA by cellular ELISA, using HUVEC (human umbilical vein endothelial cells). Sera from 37 patients with systemic lupus erythematosus (SLE), 22 with systemic sclerosis (SSc) and 36 sera from normal healthy individuals were used as controls. A cellular ELISA using HeLa cells was also performed as a laboratory control method.

Results: IgG-AECA was detected in 77% of MCTD patients, 54% of SLE patients, 36% of SSc patients and 6% of normal controls. In MCTD, IgG-AECA was associated with vasculitic manifestations, disease activity and lymphopenia, and was also a predictor of constant disease activity. Immunosuppressive drugs were shown to reduce IgG-AECA titers. Since antibodies directed to HeLa cell surface were negative, AECA was apparently unrelated to common epitopes present on epithelial cell lines.

Conclusions: AECA are present in a large proportion of patients with MCTD and these antibodies decrease after immunosuppressive treatment.

IMAJ 2012; 14: 84–87

KEY WORDS: mixed connective tissue disease (MCTD), Sharp's disease, anti-endothelial cell antibodies, endothelial cells, antibody fluctuation

For Editorial see page 119

Mixed connective tissue disease, first described by Sharp et al. in 1972 [1], is an inflammatory disease clinically characterized by features of systemic lupus erythematosus, systemic sclerosis and polymyositis, and associated with high titers of circulating antinuclear antibody with specificity for nuclear ribonucleoprotein.

Anti-endothelial cell antibodies have been described in several connective tissue diseases such as SLE, SSc, rheumatoid arthritis, microscopic polyangiitis, Kawasaki disease, Wegener's granulomatosis, and antiphospholipid syndrome [2,3]. Specifically in MCTD, some studies have shown that AECA might be present in the serum of patients with this disease [4-9]. In one study, high levels of AECA were associated with the presence of pulmonary fibrosis [4] and with disease activity [7]. However, little information is available regarding disease activity, and longitudinal evaluation of these antibodies has not yet been performed.

The objectives of the present study were to ascertain the frequency of AECA in patients with MCTD and to evaluate the association of these antibodies and their titers with clinical and laboratory manifestations of MCTD cross-sectionally and longitudinally.

PATIENTS AND METHODS

The study group comprised 33 consecutive patients with MCTD who were prospectively followed in our outpatient clinic at the Rheumatology Division of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. All patients fulfilled the classification criteria for MCTD according to Kasukawa [10]. Exclusion criteria included positivity for anti-Sm and anti-dsDNA antibodies (to exclude SLE). The control group comprised 37 patients with SLE, 22 with systemic sclerosis, and 36 healthy individuals. Clinical and laboratory data of the patients were collected through an extensive review of their medical charts and by clinical examination. All participants provided written informed consent and the study was approved by the local ethics committee. Blood samples were collected from all study participants.

SLE = systemic lupus erythematosus
SSc = systemic sclerosis
AECA = anti-endothelial cell antibodies
MCTD = mixed connective tissue disease

DISEASE ACTIVITY

The disease was considered active if the patient presented two major manifestations (cutaneous vasculitis, increase of pulmonary fibrosis, central nervous system manifestations, severe myositis, or urinary casts/proteinuria), or one major plus two minor manifestations (arthritis, new rash, diarrhea, mild myositis, or weight loss), or three minor manifestations plus abnormal laboratory test (lymphopenia, thrombocytopenia, increased erythrocyte sedimentation rate or C-reactive protein, or increased gammaglobulin levels) [11].

For the data analysis, these patients were divided into two subgroups: group A – patients who had taken immunosuppressive drugs (chlorambucil 2–6 mg/day, intravenous cyclophosphamide or azathioprine 50–100 mg/day) associated or not with glucocorticoid (11/19); group B – patients who had received exclusively prednisone without other immunosuppressive drugs (8/19).

ANTI-ENDOTHELIAL CELL ANTIBODY ASSAY

ECV-304 endothelial cells were used to identify the anti-endothelial antibodies as previously described [12,13]. Briefly, these cells were expanded in plastic flasks appropriate for cell culture and seeded onto sterile polystyrene 96-well (6 mm in diameter) microplates at a concentration of 2 x 104 cells/well in 100 µl medium 199 containing 10% fetal bovine serum. After culture for 24–48 hours at 37ºC in an incubator in a 5% CO_2 atmosphere, the plates were washed twice with magnesium-containing Hank's solution, fixed with 0.1% glutaraldehyde for 10 minutes at 4ºC, and then washed with distilled water. Next, 100 µl 2% bovine serum albumin in phosphate-buffered saline was added to each well to block non-specific immunoglobulin receptors, and the plates were incubated for 2 hours at 37º C. After three washes with PBS, test serum diluted 1/200 in PBS-2%BSA were plated in duplicate and incubated for 2 hours at 37ºC. After a new cycle of three washes, 100 µl PBS-2%BSA containing alkaline phosphatase-conjugated human anti-immunoglobulins G or M was added to each well and incubated for 1 hour at 37ºC. After a new cycle of three washes with PBS, 100 µl p-nitrophenyl phosphate at a concentration of 1 mg/ml in 10% (v/v) di-ethanol-amine buffer, pH 9.8, was added to each well, and absorbance was read every 15 min with an enzyme-linked immunosorbent assay spectrophotometer. The reaction was stopped when the standard serum reached an optical density of 1. Each plate contained two positive sera as standards, and two aliquots of 60 pooled normal sera with known OD were used as negative controls. A serum was considered positive when the OD was superior to the mean OD of negative control sera plus three standard deviations.

PBS = phosphate-buffered saline
BSA = bovine serum albumin
OD = optical density

STATISTICAL ANALYSIS

Results are presented as the mean and standard deviation for continuous variables and as the number (%) for categorical variables. Data were compared by *t*-tests or by the Mann-Whitney test for continuous variables to evaluate differences between groups. For categorical variables, differences were assessed by chi-square test or Fisher's exact test. *P* values < 0.05 were considered significant.

RESULTS

CLINICAL MANIFESTATIONS

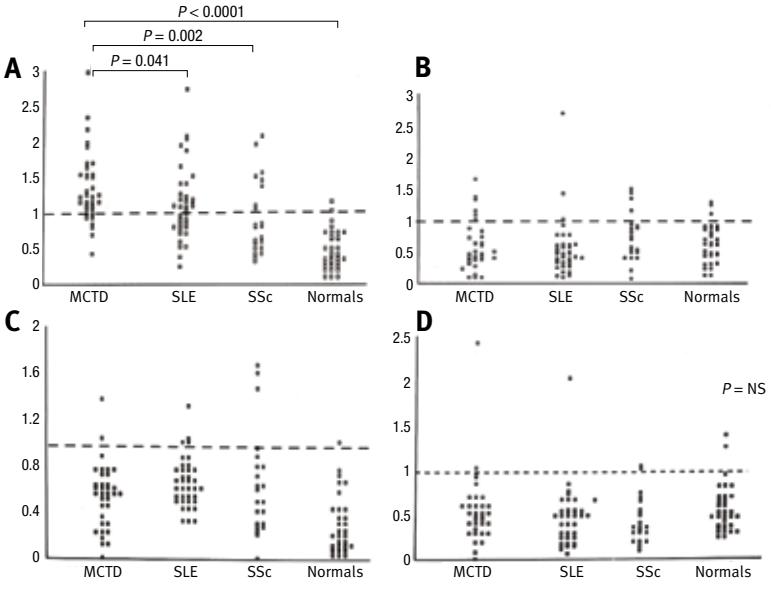
The mean age of the MCTD patients was 43 ± 11 years and they had a mean disease duration of 7.0 ± 4.0 years; 95% were female and 55% were Caucasian.

AECA FREQUENCY

IgG-AECA was detected in 77% of MCTD patients, 54% of SLE patients, 36% of SSc patients and 6% of healthy controls. Analysis of results showed that IgG-AECA was more frequently found in the studied diseases than in healthy controls (odds ratio = 10.53, $P < 0.0001$). Moreover, IgG-AECA was more common in MCTD than in SLE and SSc ($P < 0.001$) [Figure 1A]. There was no sig-

Ig = immunoglobulin

Figure 1. Dot plots representing the ELISA binding. Titers were arbitrarily defined by the ratio between the sample optical density (OD) and the normal reference value (the OD obtained for 36 normal individuals plus three standard deviations) Values ≥ 1 were considered positive. **[A and B]** Binding of IgG and IgM class antibodies, respectively, to endothelial cells in sera from 35 patients with MCTD, 37 with SLE, 22 with SSc and 36 healthy controls. **[C and D]** Binding of IgG and IgM class antibodies, respectively, to HeLa cells in sera from 35 patients with MCTD, 37 with SLE, 22 with SSc patients and 36 healthy controls.



nificant difference between the presence of IgM-AECA [Figure 1B] and any of the studied groups (OR = 1.77, $P > 0.05$).

ANTI-HELA ANTIBODIES

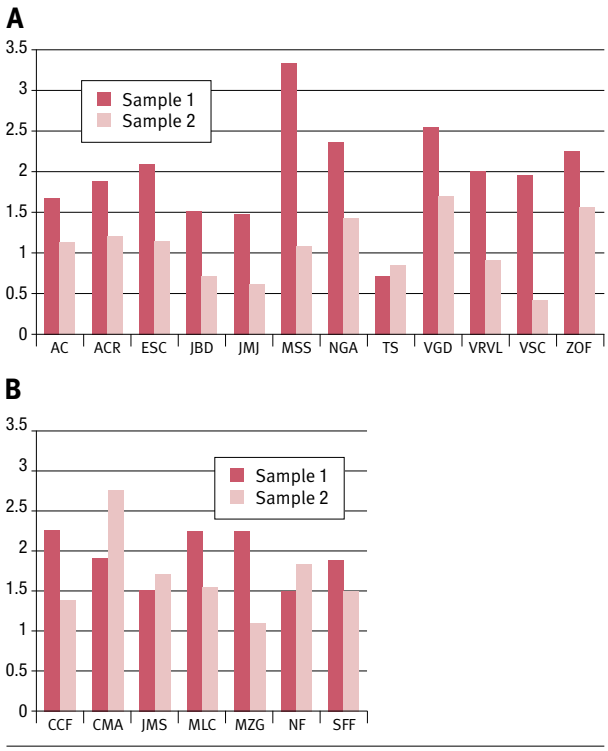
IgG and IgM anti-HeLa cells using ELISA assay showed no statistical differences between the studied connective tissue diseases (MCTD, SLE and SSc) and healthy controls [Figures 1C and D].

AECA AND CLINICAL MANIFESTATIONS

A positive association of circulating IgG-AECA was found between IgG-AECA and fever, weakness, weight loss, cutaneous vasculitis, palmar erythema, current disease activity, persistent disease activity, lymphopenia and gamma-globulin (> 2 g/dl) ($P < 0.001$). Clinical manifestations of myositis, interstitial lung disease (vital capacity < 70%), arthritis and laboratory parameters such as elevated erythrocyte sedimentation rate, raised C-reactive protein levels, anemia and leukopenia were not associated with the presence of IgG-AECA.

OR = odds ratio
ELISA = enzyme-linked immunosorbent assay

Figure 2. Comparison between IgG-AECA values along time. Letters on the x axis are the patients' initials. **[A]** IgG-AECA values obtained for patients from group A, i.e., those who used immunossupressive drug during the follow-up of this study. **[B]** IgG-AECA for patients from group B, i.e., those who did not use immunossupressive drugs during the follow-up. There was no difference between groups A and B for the values of IgG-AECA in sample 1; however, IgG-AECA measured in sample 2 was significantly lower in group A than in group B. Moreover, there was a significant difference between IgG-AECA values between sample 1 and sample 2 for group A, but there was no difference for group B.



AECA FLUCTUATION AND IMMUNOSUPPRESSIVE THERAPY

During a 5 year follow-up period, sera were collected from 19 of the 28 studied patients, allowing the evaluation of AECA oscillations and their relation with clinical and therapeutic manifestations. We observed that there was a tendency for IgG-AECA titers to decline in parallel to clinical activity reduction. In this context, patients from group A presented a significant decrease in IgG-AECA titers, when comparing levels at the start of the study with those measured during the follow-up period ($P < 0.001$) [Figure 2A]. On the other hand, in patients from group B, IgG-AECA titers also diminished during the study period, but this difference did not reach significance [Figure 2B]. As a whole, the interval between the collections of the two different samples of serum was 44 ± 17 months, which was not different between the two groups. It is noteworthy that the initial IgG-AECA levels were not different between groups A and B.

DISCUSSION

To the best of our knowledge, the present study demonstrates for the first time that IgG-AECA are more frequent in MCTD patients than in SLE or SSc patients, and these antibodies fluctuate following MCTD treatment. In addition, the presence of AECA is associated with a number of clinical and laboratory findings in MCTD.

The higher frequency of AECA in MCTD than in SLE or SSc suggests that the participation of the vascular system may play an important role in the striking features of this particular disease. The presence of AECA was less common in SSc than in MCTD. In SSc, denuded and deleted vessels are often found, a finding not described for MCTD [14]. Therefore, it is conceivable that the vascular changes encountered in SSc might result from circulating active molecules that lead to irreversible vascular damage [15], while in MCTD immunoglobulins with affinity for endothelial cells could somehow be involved in the vascular structural reorganization. Furthermore, our data showed that in MCTD the presence of IgG-AECA was associated with cutaneous vasculitis, active disease and persistent clinical activity, findings similar to those previously published by others and ourselves in SLE patients [16-18]. This point is particularly meaningful in MCTD, where few laboratory parameters are available to determine disease activity. Unlike SLE, complement levels are rarely altered in this disease. Anti-RNP titers, the serological marker of MCTD, do not oscillate much along time [1]. Perhaps, IgG-AECA may prove useful in this respect.

Data from the experiments with HeLa cells illustrate that the binding of immunoglobulins to endothelial cells was not related to epitopes commonly present on epithelial cell lines. This confirms specificity of the AECA assay in detecting altered circulating antibodies to vascular surface.

Previous studies showed that some sera with anti-endothelial cell activity may also recognize antigens present on fibroblasts, erythrocytes, monocytes, lymphocytes, platelets and subendothelial matrix [19]. We found an association between the presence of IgG-AECA and lymphopenia, but no association of IgG-AECA with anemia or thrombocytopenia. Therefore, it is possible that the former association simply reflects active disease and may not be directly implicated in the pathogenic process. Several articles that review AECA in various tissue connective diseases are available [20-25].

We found that significant decreases of IgG-AECA titers occurred with the use of immunossupressive drugs. These observations, although preliminary, deserve further analysis in controlled trials.

In conclusion, this study showed that IgG-AECA were more frequent in MCTD than in other connective diseases, and these antibodies were associated with various clinical and laboratory findings in MCTD; titers of AECA can decrease with use of immunosuppressive drugs.

Correspondence author:
Dr. J.F. de Carvalho
Rheumatology Division, Faculdade de Medicina da Universidade de São Paulo, Av. Dr. Arnaldo 455, 3º andar, São Paulo – SP, 01246-903, Brazil.
Tel/Fax: 5511-30617490
email: jotafc@gmail.com

Acknowledgments
Supported by grants from FAPESP (92/4508-0) and Federico Foundation and CNPq (300665/2009-1) to J.F. Carvalho.

References

1. Sharp GC, Irwin WS, Tan EM, Gould RG, Holman HR. Mixed connective tissue disease – an apparently distinct rheumatic disease associated with a specific antibody to an extractable nuclear antigen (ENA). *Am J Med* 1972; 52: 148-59.
2. Praprotnik S, Blank M, Meroni PL, Rozman B, Eldor A, Shoenfeld Y. Classification of anti-endothelial cell antibodies into antibodies against microvascular and macrovascular endothelial cells: the pathogenic and diagnostic implications. *Arthritis Rheum* 2001; 44: 1484-94.
3. Ronda N, Leonardi S, Orlandini G, Gatti R, Bellosta S, Bernini F, Borghetti A. Natural anti-endothelial cell antibodies (AECA). *J Autoimmun* 1999; 13: 121-7.
4. Watanabe H, Kaise S, Takeda I, et al. Anti-endothelial cell antibodies in the sera of patients with mixed connective tissue disease – the clinical significance. *Fukushima J Med Sci* 1997; 43: 13-28.
5. Bodolay E, Bojan F, Szegedi G, et al. Cytotoxic endothelial cell antibodies in mixed connective tissue disease. *Immunol Lett* 1989; 20: 163-7.
6. Naniwa T, Sugiura Y, Banno S, Yoshinouchi T, Matsumoto Y, Ueda R. Ribosomal P protein P0 as a candidate for the target antigen of anti-endothelial cell antibodies in mixed connective tissue disease. *Clin Exp Rheumatol* 2007; 25: 593-8.
7. Vegh J, Szodoray P, Kappelmayer J, et al. Clinical and immunoserological characteristics of mixed connective tissue disease associated with pulmonary arterial hypertension. *Scand J Immunol* 2006; 64: 69-76.
8. Bodolay E, Csipo I, Gál I, et al. Anti-endothelial cell antibodies in mixed connective tissue disease: frequency and association with clinical symptoms. *Clin Exp Rheumatol* 2004; 22: 409-15.
9. Sasaki N, Kurose A, Inoue H, Sawai T. A possible role of anti-endothelial cell antibody in the sera of MCTD patients on pulmonary vascular damage relating to pulmonary hypertension. *Ryumachi* 2002; 42: 885-94.
10. Kasukawa R. Preliminary diagnostic criteria for classification of mixed connective. In: Kasukawa R, Gordon GC, eds. Mixed Connective Tissue Disease and Anti-Nuclear Antibodies. Amsterdam: Excerpta Medica, 1987: 41-7.
11. Lage LV, Caleiro MC, Carvalho JF. Proposed disease activity criteria for mixed connective tissue disease. *Lupus (Basingstoke)* 2010; 19: 223-4.
12. Rosenbaum J, Pottinger BE, Woo P, et al. Measurement and characterisation of circulationg anti-endothelial cell IgG in connective tissue diseases. *Clin Exp Immunol* 1988; 72: 450-6.
13. Souza RC, Lage L, Goldesntein-Schainberg C, Macedo AR, Carrasco S, Gonçalves CR. Anti-endothelial cell antibodies and central nervous system involvement in Behçet's disease. *Clinics* 2007; 62: 685-90.
14. Abraham DJ, Krieg T, Distler J, Distler O. Overview of pathogenesis of systemic sclerosis. *Rheumatology (Oxford)* 2009; 48: iii 3-7.
15. Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, Wick G. Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis Rheum* 2000; 43: 2550-62.
16. Azeka E, Mendonça LLF, Cossermelli-Messina W, Bonfá E, Khamashta MA, Hughes GRV. Anti-endothelial cell antibodies: marker of ongoing disease acivity in systemic lupus erythematosus. *Arthritis Rheum* 1999; 42: s149.
17. D'Cruz DP, Houssiau FA, Ramirez G, et al. Antibodies to endothelial cells in systemic lupus erythematosus: a potential marker for nephritis and vasculitis. *Clin Exp Immunol* 1991; 85: 254-61.
18. Yazici ZA, Raschi E, Patel A, et al. Human monoclonal anti-endothelial cell IgG-derived from a systemic lupus erythematosus patient binds and activates human endothelium in vitro. *Int Immunol* 2001; 13: 349-57.
19. Hasselaar P, Derden RHWM, Blokzijl L, De Groot PG. Crossreactivity of antibodies directed against cardiolipin, DNA, endothelial cells and blood platelets. *Thromb Haemost* 1990; 63: 169-73.
20. Meroni PL, Khamashta MA, Youinou P, Shoenfeld Y. Mosaic of anti-endothelial antibodies: review of the first international workshop on AEA. Clinical and pathological significance. *Lupus* 1995; 4: 95-9.
21. Damianovich M, Gilburd B, George J, et al. Pathogenic role of anti-endothelial cell antibodies in vasculitis. *J Immunol* 1996; 156: 4946-51.
22. Kollmeier A, Kreditor M, George J, Levy Y, Shoenfeld Y. Functional properties of antiendothelial cell antibodies. *Clin Rev Allergy Immunol* 1998; 16: 297-311.
23. Soltész P, Kerekes G, Dér H, et al. Comparative assessment of vascular function in autoimmune rheumatic diseases: considerations of prevention and treatment. *Autoimmun Rev* 2011; 10: 416-25.
24. Tomasoni L, Sitia S, Borghi C, et al. Effects of treatment strategy on endothelial function. *Autoimmun Rev* 2010; 9: 840-4.
25. Domiciano DS, Carvalho JF, Shoenfeld Y. Pathogenic role of anti-endothelial cell antibodies in autoimmune rheumatic diseases. *Lupus* 2009; 18: 1233-8.

"No great discovery was ever made without a bold guess"

Isaac Newton (1642-1727), English physicist, mathematician, astronomer, natural philosopher, alchemist, and theologian, considered by many to be the greatest and most influential scientist who ever lived. In his monograph *Philosophiæ Naturalis Principia Mathematica*, Newton described universal gravitation and the three laws of motion, which dominated the scientific view of the physical universe for the next three centuries