

## INVESTIGATIVE REPORT

# Serum Antibody Reactivity to Human Intracisternal A-Type Particle Retrovirus Proteins in Systemic Sclerosis Patients

Michelangelo La PLACA<sup>1</sup>, Tommaso BIANCHI<sup>1</sup>, Francesca VITONE<sup>2</sup>, Luigi MURATORI<sup>3</sup>, Claudio VAROTTI<sup>1</sup>, Davide GIBELLINI<sup>2</sup> and Maria Carla RE<sup>2</sup>

*Department of Clinical and Experimental Medicine, <sup>1</sup>Section of Dermatology and <sup>2</sup>Section of Microbiology and <sup>3</sup>Department of Internal Medicine, Cardiangiology and Hepatology, University of Bologna, Bologna, Italy*

**Serum antibodies against human intracisternal A-type particle (HIAP) endogenous retrovirus have been found to be associated with various autoimmune pathologies. To evaluate the presence of serum antibody reactivity to HIAP proteins in systemic sclerosis, a Western blot analysis was performed on sera from 42 patients with systemic sclerosis, in comparison with 18 sera from patients with primary biliary cirrhosis and 52 healthy subjects. A positive Western blot was found in 55.5% of serum samples from patients with primary biliary cirrhosis and in 66.0% of patients with systemic sclerosis. None of the 52 healthy subjects showed positive results. Although this difference may be attributable either to an autoimmune response to antigenically related cellular proteins or to a specific antibody response to HIAP proteins expressed as an incidental consequence of attendant pathological processes, the high prevalence of antibodies against HIAP proteins demonstrated in patients with systemic sclerosis may be considered a hallmark of this disease.**

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Dr Michelangelo La Placa, Department of Clinical and Experimental Medicine, Section of Dermatology, Via Massarenti, 1, 40138 Bologna, Italy. E-mail: dermolap@med.unibo.it

Systemic sclerosis (SSc) is a connective tissue disease characterized by excessive deposition of collagen in the skin and various internal organs, and by vascular abnormalities (1). SSc is considered to be an autoimmune disease. Although both cellular microchimerism (2) and molecular mimicry of some common infectious agents, such as cytomegalovirus and parvovirus B19 (3), have been implicated in its pathogenesis, the aetiology of SSc remains unknown.

Several publications have described the presence of retroviral sequences associated with virions, produced by cells of patients with autoimmune diseases. In recent reviews (4, 5), these viruses, identified as human endogenous retroviruses (HERVs), have been associated with Sjögren's syndrome, type 1 or insulin-dependent

diabetes mellitus, multiple sclerosis, rheumatoid arthritis, congenital heart block, systemic lupus erythematosus and SSc. Serum antibodies specific for human intracisternal A-type particles (HIAP), a HERV recognized by monoclonal antibody against HIV-1 p24 capsid protein (6), have been found in primary biliary cirrhosis (PBC) (7), familial erosive arthritis (8) and some patients with SSc, systemic lupus erythematosus, Still's disease and idiopathic T-lymphocytopenia (9, 10).

To further investigate serum antibody reactivity to HIAP proteins in SSc, we performed a Western blot analysis of a substantial number of sera from SSc patients, in comparison with sera from PBC patients and healthy subjects.

## MATERIALS AND METHODS

### *Serum samples*

A total of 42 serum samples from patients with SSc (diffuse and limited subsets), 18 serum samples from patients with PBC and 52 serum samples from blood donors, were examined. All patients' sera were collected between January 1989 and July 2001, after informed consent was obtained, and stored at  $-80^{\circ}\text{C}$  without preservatives until used.

SSc patients enrolled in the study included 8 men and 34 women (median age 54.5 years), while PBC patients were 2 men and 16 women (median age 56.9 years). All SSc and PBC patients fulfilled the diagnostic criteria as described in the literature (1, 11).

### *Western blot (immunoblot) and other serological analyses*

HIAP-infected and uninfected RH9/MC lymphoblastoid cell lines were obtained from the Cell Culture Collection Centre of the Istituto Zooprofilattico Sperimentale di Brescia (Brescia, Italy) and maintained in RPMI (GIBCO, Grand Islands, NY, USA) plus 10% fetal calf serum. Cells were harvested by low speed centrifugation and disrupted in hypotonic buffer. The HIAP-containing microsomal fraction was isolated by density gradient ultracentrifugation over a 36–68% sucrose gradient. Proteins were resolved by sodium dodecyl sulphate/12% polyacrylamide gel electrophoresis, run for 40 min at 200 V, using a Biorad mini Protean II apparatus, and blotted onto nitrocellulose. Nitrocellulose strips were used for immunoblotting analysis by overnight incubation at room temperature in the presence of 2 ml of blotting buffer (Genelabs Diagnostics Pte, Ltd, Singapore) containing 20  $\mu\text{l}$  of serum sample. Nitrocellulose strips were then washed and further

incubated for 1 h at room temperature with peroxidase-conjugated anti-human IgG rabbit serum (Sigma, Milan, Italy) in PBS containing 1% bovine serum albumin. Specific results were revealed with the enhanced chemiluminescent (ECL) Western blotting detection reagent (Amersham, Arlington Heights, IL, USA). All the sera were tested with immunoblots from both HIAP-infected and uninfected cells, to assess specific reactivity to HIAP proteins, and all the tests were performed in duplicate to check reproducibility. HIAP immunoblots were considered positive when serum samples reacted with two or more proteins with electrophoretic mobility corresponding to that of known HIAP proteins (6, 7).

All the sera were also subjected to Western blot analysis for the presence of antibodies against HIV-1/2 and HTLV-I and -II specific proteins, employing commercially available reagents (HIV Blot 2.2, Genelabs Diagnostics; HTLV-I/II Immunoblot, Cambridge Biotech, Worcester, MA, USA).

All the serum samples from the 42 SSc patients were also screened by Western blot analysis for IgG autoantibody reactivity to Scl-70 (DNA topoisomerase I) and other (U1-nRNP, Sm, SS-A, SS-B, Jo-1) extractable nuclear antigens (ENAs) employing a commercially available reagent kit (ProfilePlus, Euroimmun GmbH, Lübeck, Germany). Anti-nuclear antibodies (ANA), including anti-centromere IgG antibodies, were evaluated by microscopy with an image analyser, after indirect immunofluorescence on HEP-2 cell slides (Kallestad/Sanofi Diagnostics Pasteur, Chaska, MN, USA).

#### Statistical analysis

Statistical analysis was performed using Student's two-tailed *t*-test.

## RESULTS

Duplicate immunoblots revealed reproducible results, and none of the sera examined had antibody reactivity

to immunoblots from uninfected cells. No serum sample showed reactivity to HIV-1/2 or HTLV-I and -II proteins; only a faint reactivity restricted to HIV-1 p24 was observed in 8 of 42 (19.0%) SSc and 3 of 18 (16.6%) PBC serum samples. Representative HIAP immunoblots are illustrated in Fig. 1.

No serum sample out of 52 obtained from control healthy blood donors showed positive results against two HIAP proteins. Only five samples showed a (usually weak) reactivity restricted to p46 (four samples) and p66 (one sample).

As expected, 10 of 18 (55.5%) serum samples from PBC patients, reacted against two or more HIAP proteins with an antibody pattern including p46, p60, p66 and p80 (eight samples) or p46 and p60 (two samples) (Table I).

A similar ( $p=0.48$ ) percentage (66.0%) of serum samples from SSc patients showed an identical antibody pattern. In fact, 28 of 42 serum samples clearly reacted against p46, p60, p66 and p80 (19 samples) or p46 and p60 (9 samples).

Table I. Western blot antibody reactivity to two or more human intracisternal A-type particle (HIAP) proteins in serum samples from patients with systemic sclerosis (SSc) and primary biliary cirrhosis (PBC) and healthy subjects

Serum samples	Number of sera examined	Number (%) of sera reacting with two or more HIAP proteins
SSc patients	42	28 (66.0)
PBC patients	18	10 (55.5)
Healthy subjects	52	0

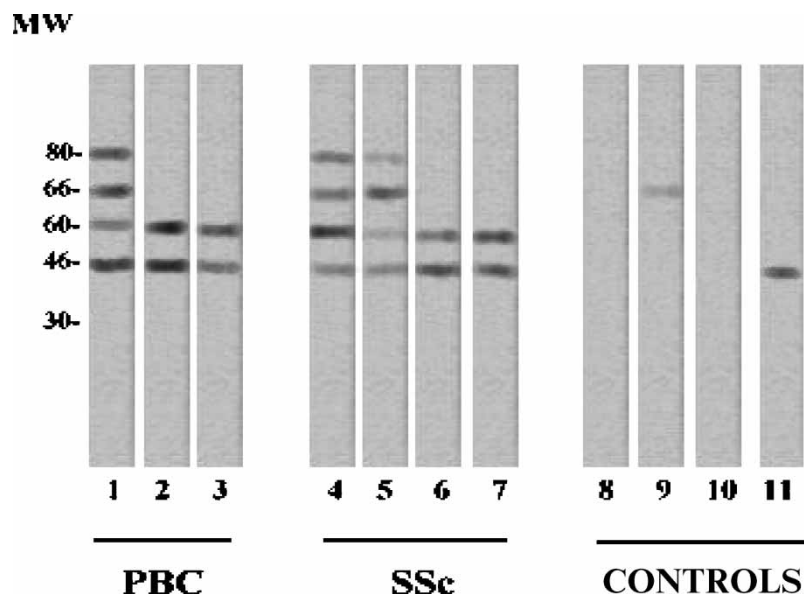


Fig. 1. Representative human intracisternal A-type particle (HIAP) Western blot (immunoblot) results in serum samples from primary biliary cirrhosis (PBC) and patients with systemic sclerosis (SSc), and healthy subjects (controls). Showing PBC serum samples positive against four (lane 1) or two (lanes 2 and 3) HIAP proteins, and SSc serum samples positive against four (lanes 4 and 5) or two (lanes 6 and 7) HIAP proteins.

All serum samples from SSc patients were also tested for autoantibody reactivity. Results obtained were in accordance with previous reports (1). In particular, 18 (42.8%) patients showed a variable positivity to ANA test by immunofluorescence. Within this subgroup, 14 patients (33.3%) were positive to anti-centromere antibody and 11 (26.1%) patients were positive to Scl-70. Furthermore, seven patients reacted against other extractable nuclear antigens (U1-RNP in four cases, Jo-1 in two cases and SS-A in one case). Autoantibody reactivity was more common in the group of HIAP-positive patients than in negative patients, but without statistical significance.

## DISCUSSION

The results of our study confirmed the presence of serum antibodies against HIAP retrovirus proteins in a high percentage (55.5%) of PBC patients (7), and demonstrated an identical antibody pattern in an even greater percentage (66.0%) of SSc patients. In addition, none of the HIAP-positive samples reacted against microsomal fraction proteins from the uninfected cell line, thus demonstrating the specificity of immunoblot serum reactivity against HIAP-infected cell microsomal proteins.

These results, compared with findings in healthy blood donors, clearly indicate a very significant ( $p \leq 0.0001$ ) association between the presence of serum antibodies against HIAP proteins and the autoimmune pathologies investigated. The significance of these findings in relation to the aetiology and pathogenesis of autoimmune pathologies, and of SSc in particular, is not clear.

The concept of severe immune dysregulation caused by persistent infections, as exemplified by HIV infection, or permanent untoward autoimmune phenomena caused by exogenous viruses (12–15), is now a familiar one. In recent years, the potential capacity of HERVs to move about the genome and to modify host gene expression (16) expressing much of their own genomic potential, thereby modulating immune responses (17), imply that these persistent viruses might also have a role in autoimmune diseases (4, 5, 18, 19).

HERVs or increased amounts of HERV gene expressions (4, 5, 20), and/or high prevalence of specific serum antibodies (21–24), in particular against HIAP retrovirus proteins (7–10), have been demonstrated in various autoimmune disorders. This evidence is however, still circumstantial, and the role of HERVs in autoimmune diseases remains elusive (25). Moreover, the human origin of a putative HERV (HRV-5) detected in patients with arthritis and systemic lupus erythematosus has recently been questioned (26).

In particular it is still unclear which antigens are actually inducing the antibody response against HERV proteins and how the possible link between this immune

response and the autoimmune pathology is accomplished. One possibility is that antibodies reacting with HERV proteins are merely the results of molecular mimicry of these proteins with auto-antigens (27). In addition, particularly in the immune dysregulation associated with autoimmune disease, they could also be the consequence of the production of cross-reacting antibodies. In fact, more recent studies, using combinatorial peptide libraries have revealed, in various instances, surprisingly high levels of cross-reactivity of both antibodies (28) and T cell receptors (29), often not predicted by database searches for sequence homology. Alternatively, the expression of HERV proteins, and the consequent induction of a specific immune response, might be the outcome of HERV activation induced by ongoing inflammatory responses (30), providing another model which could explain the association between the presence of antibodies against HERV-specific proteins and autoimmune diseases. Therefore, while on one hand, the existence of serum antibodies reactive with endogenous retroviral proteins does not necessarily indicate that these antigens are actually driving the immune response (not necessarily implying that the related viral proteins are expressed), HERV protein expression and the cognate antibody response could on the other hand, only represent incidental consequences of attendant pathological processes.

In conclusion, our study has shown that the presence of serum antibodies reacting with HIAP retrovirus proteins is significantly associated with systemic sclerosis. Whether these results indicate the possible direct involvement of an A-type retrovirus, in the origin and development of systemic sclerosis, is still an open question that requires further research efforts. There is, however, a growing body of evidence, including the present findings, suggesting that serum antibodies to HERV proteins, and HIAP proteins in particular, might be considered a hallmark of autoimmune disease.

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