Case Report

Autoantibodies to NOR 90/hUBF: Longterm Clinical and Serological Followup in a Patient with Limited Systemic Sclerosis Suggests an Antigen Driven Immune Response

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ABSTRACT. We describe the clinical and serological followup of a 9-year-old girl with anti-nucleolar organizing region 90/human upstream-binding factor (anti-NOR 90/hUBF) who had features of systemic sclerosis over a period of 17 years, from childhood into adulthood. We review the associations of anti-UBF autoantibodies, and provide evidence that anti-NOR 90/UBF immune response is antigen driven. (J Rheumatol 2002;29:1543–7)

Key Indexing Terms:
AUTOANTIBODIES AUTOANTIGENS SCLERODERMA

Nucleolar organizing regions (NOR) are the initiation sites for nucleologenesis. Ribosomal RNA synthesis is integrally linked to active NOR[1,2]. In 1987, using autoantibodies from patients with systemic sclerosis (scleroderma, SSc), Rodriguez-Sanchez, *et al* described a novel 90 kDa nucleolar protein, NOR 90[3]. Subsequently, Chan, *et al* showed that the autoantigen recognized by human anti-NOR 90 autoantibodies is identical to human upstream-binding factor (hUBF)[4]. UBF is an RNA polymerase I-specific transcription factor that binds directly to the promotor region of the ribosomal RNA genes and, by interacting with other factors, plays a central role in transcriptional regulation of these genes[5-9]. In mammalian cells, UBF occurs in 2 isoforms of 97 and 94 kDa (referred to as UBF1 and UBF2), which are derived from the same gene by alternative splicing events of the pre-mRNA[10].

To date, anti-UBF autoantibodies have been reported predominantly in adult patients with systemic autoimmune rheumatic diseases or malignancies[11-14]. Only 2 pediatric patients with anti-NOR 90/hUBF have been described[15]. In most instances, longterm followup of these autoantibodies and associated clinical features is unavailable. We report the clinical and serological followup of a patient with anti-NOR 90/hUBF and SSc features over an exceptionally long period of 17 years from childhood into adulthood. We review the associations of anti-UBF autoantibodies. We also provide evidence that anti-NOR 90/UBF immune response is antigen driven.

CASE REPORT

At 9 years of age, a French Canadian girl developed triphasic Raynaud's phenomenon involving the 4 extremities. Her family history was positive for hyperthyroidism in her mother. At age 13.8 years, when first seen by us, dilated nailfold capillaries visible with the naked eye were noted, as well as digital tip pitting scars, without sclerodactyly or telangiectasias. Nailfold capillary microscopy revealed changes characteristic of SSc, i.e., extremely dilated capillaries, moderate capillary loss, and capillary telangiectasias[16]. By indirect immunofluorescence, antinuclear antibodies (ANA) were strongly positive on HEp-2 cells, with discrete speckles in interphase nucleoli and in mitotic cells consistent with NOR staining in a titer of 1:5120. This staining was observed with anti-human IgG (γ chain-specific), and not with μ or α chain-specific reagents. Detailed laboratory tests were negative, including erythrocyte sedimentation rate, complete blood cell count, urinalysis, serum CK and creatinine, liver enzymes, C3, C4, CH50, rheumatoid factor, VDRL, Coombs, serum immunoglobulin levels, and autoantibodies to centromeres, topoisomerase I, U1RNP, Sm, Ro, La and DNA. Radiographs of the chest and hands and an electrocardiogram (ECG) were normal. Esophageal manometry revealed a slightly hypotonic lower

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sphincter. A diagnosis of early limited SSc was made. She was followed regularly. At age 16, clinically visible capillary telangiectasias were noted on her hands.

At age 26, after 17 years of evolution, extensive reevaluation revealed persistence of Raynaud's phenomenon. Sicca symptoms and signs were absent. Visible dilated nailfold capillaries remained. Telangiectasias of the hands and lips were noted. Skin sclerosis remained absent. Nailfold capillary microscopy was unchanged. ANA measured yearly revealed persistence of a speckled nucleolar pattern suggestive of NOR in a titer of 1:2560 to 1:20,480. Again, extensive biochemical and autoimmunity tests remained normal, including absence of autoantibodies to cardiolipin, Jo-1, mitochondria, and smooth muscle. Esophageal scintigraphy and ECG were normal. Pulmonary function tests disclosed a decreased carbon monoxyde diffusing capacity (DLCO) without restrictive syndrome (DLCO 66%, forced vital capacity 100%, forced expiratory volume-1 113% of predicted normal value). Bidimensional mode echocardiogram was normal with an estimated pulmonary artery pressure of 26/8 mm Hg. HLA typing revealed that her HLA haplotype was HLA-A*01,32; B*27,57; DQA1*0201; DQB1*0303, 0402; DRB1*0701, 0801; DRB4*0101/3/5.

Immunoblotting. Nuclei were isolated from cultured human HEp-2 cells as described[17] and the proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis[18] using 12% acrylamide. For immunoblots, proteins were electrophoretically transferred to nitrocellulose, the membrane was blocked by overnight incubation at 4˚C in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat dry milk followed by incubation with autoimmune serum diluted 1:1000 in TBS for 2 h at ambient temperature. After several washes in TBS, peroxidase coupled secondary antibodies [goat anti-human IgG (Dianova, Hamburg, Germany) diluted 1:10,000 in TBS containing 5% dry milk] were added for 1 h. Blots were washed as above and developed using the enhanced chemiluminescence detection system (ECL, Amersham Buchler, Braunschweig, Germany).

Preparation of recombinant human UBF. UBF1 was expressed as an amino terminally FLAG-tagged protein in baculovirus infected Sf9 cells. Briefly, following infection of Sf9 cells for 40 h, the insect cells were lysed in buffer AM (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 10% glycerol, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) supplemented with 600 mM KCl (AM-600), protease inhibitors (leupeptin, aprotinin, and pepstatin, 2 μg/ml each), and 0.5% NP-40. The lysate was cleared by ultracentrifugation and incubated overnight at 4˚C with anti-FLAG antibodies (M2; Kodak) bound to agarose beads. After washing the beads with AM-1000/0.5% NP-40 and AM-400/0.5% NP-40, bead-bound UBF was eluted with buffer AM-300/0.1% NP-40 containing 400 μg of FLAG peptide (Kodak) per ml of extract.

Results. Patient sera were associated with a strong fluorescent ANA pattern on HEp-2 cells reminiscent of NOR distribution (data not shown). Nucleoli of interphase cells were strongly labeled in a distinctly punctate fluorescence pattern and, during mitosis, several chromosomes displayed a fluorescent spot, most likely reflecting the persistence of UBF at the mitotic NOR (UBF distribution in interphase and mitotic cells as described[19,20]). Because proteins associated with NOR are present in nuclei, immunoblotting was performed on total proteins from HEp-2 nuclei. As shown in Figure 1, patient serum 2002 obtained at first evaluation and all subsequent samples obtained over 12 years reacted with a doublet of roughly 90 kDa molecular weight (Figure 1, lanes 1–9). The same 2 polypeptide bands were revealed with rabbit antibodies raised against recombinant mouse UBF (data not shown). Further, the patient's serum reacted with recombinant hUBF1, which comigrated with the upper band of the doublet (Figure 1, lane 11), confirming the anti-hUBF specificity of the patient's serum. As judged from the immunoblots, patient serum (at the dilution tested) was monospecific for UBF since there was no reaction with the numerous other nuclear protein bands present (visualized by Coomassie blue staining of the gel, lane 10). Discrete changes in band intensity were noted in the sequential immunoblotting results shown in Figure 1, in keeping with the slight changes in HEp-2 fluorescent ANA titers noted over time.

To characterize patient serum further, samples obtained 12 years apart and diluted 1:10,000 were incubated with transblotted hUBF. As shown in Figure 2, both sera strongly bound to hUBF (lanes 1–8). Even at 1:20,000 serum dilution, as little as 5 ng of UBF protein were readily detected (Figure 2, lanes 9 and 10).

DISCUSSION

This is the first report on the clinical and serological followup of a patient with anti-NOR 90/hUBF and features of SSc over an exceptionally long period, 17 years, that spanned childhood to adulthood. Disease manifestations in this patient were suggestive of limited SSc, i.e., Raynaud's phenomenon, digital pitting scars, telangiectasias, visible nailfold capillaries, characteristic nailfold capillary microscopy changes, hypotonic lower esophageal sphincter, and decreased DLCO.

Table 1 summarizes the characteristics of 39 patients with anti-NOR 90/UBF autoantibodies[11-15]. It can be seen that these autoantibodies are most commonly associated with various autoimmune diseases, particularly the connective tissue diseases systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis, and systemic lupus erythematosus (SLE). These autoantibodies have also been observed rarely in patients with malignancies, and in a single patient with osteoarthritis and coronary artery disease (Table 1). We have shown previously that anticentromere and anti-topoisomerase I autoantibodies in patients with isolated Raynaud's phenomenon are strong predictors of the future onset of SSc, usually over the course of several years[21]. Whether patients without apparent autoimmune disease at the time that anti-NOR 90/UBF autoantibodies are found might later develop an autoimmune disease with prolonged followup remains unknown.

Table 1 also shows that anti-NOR 90/UBF have been reported to date in only 2 children[15]. In their study, Fritzler, *et al* reported a 7-year-old girl with Raynaud's phenomenon and SLE who died of an unrelated cause. The second child was a 16-year-old girl with Raynaud's phenomenon who did not develop signs and symptoms of systemic rheumatic diseases over an 8 year followup[15]. Although Raynaud's phenomenon was the presenting feature in our patient and definitive signs of limited SSc progressively became apparent, she did not develop evidence of severe visceral involvement over the course of 17 years. Since prospective studies of anti-NOR 90/UBF have not been done, their predictive value remains to be determined. However, the data to date are consistent with anti-NOR 90/UBF as markers for a protracted disease course in children.

Several features of our patient's anti-NOR 90/UBF autoantibody are strongly suggestive of an antigen driven autoimmune response. First, the strikingly persistent high titer, IgG isotype restricted reactivity with human UBF is characteristic of autoantigen driven responses, as observed in other autoantibody systems, such as anti-topoisomerase I and anti-CENP-B autoantibodies[22]. Similarly, anti-NOR

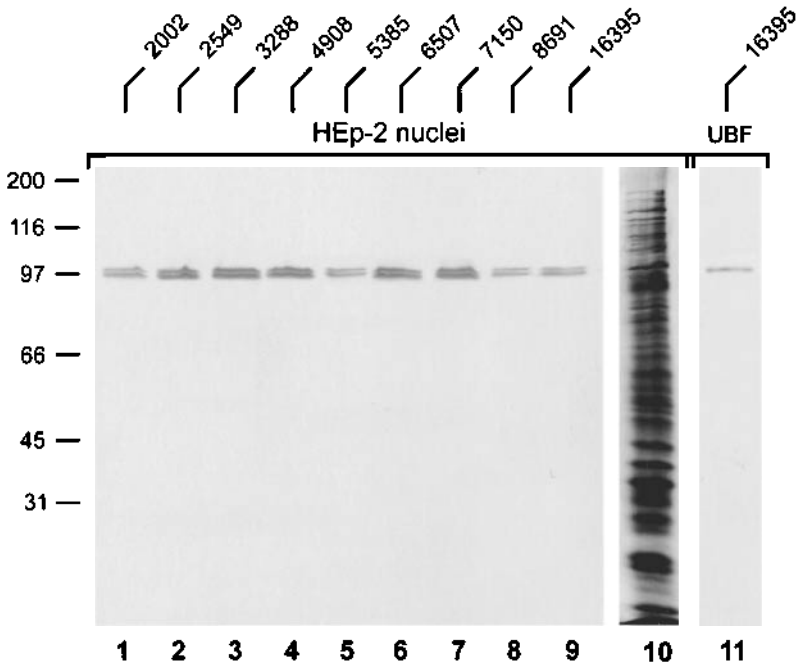

Figure 1. Over a period of 12 years, the patient's serum shows persistent reactivity with a 90 kDa doublet by immunoblotting on total proteins from HEp-2 nuclei. Serum 2002 obtained at first evaluation and subsequent sera obtained over 12 years were frozen at –80°C until used, thawed, and diluted 1:1000. Only the 90 kDa doublet was recognized, suggesting that her serum contained a single autoantibody specificity (lanes 1–9). Normal control serum and control sera with antibodies to La and nuclear lamin B1 were unreactive with the 90 kDa doublet (not shown). To illustrate the monospecificity of the serum, one lane of the gel was stained with Coomassie blue prior to the transfer of the proteins (lane 10). Recombinant hUBF1 comigrated with the upper band of the doublet and was recognized by the patient serum (lane 11). Ages corresponding to each serum sample were: 2002, 13.8 yrs; 2549, 14.4 yrs; 3288, 15.3 yrs; 4908, 16.6 yrs; 5385, 16.9 yrs ; 6507, 17.8 yrs; 7150, 18.3 yrs; 8691, 19.5 yrs; 16395, 26 yrs.

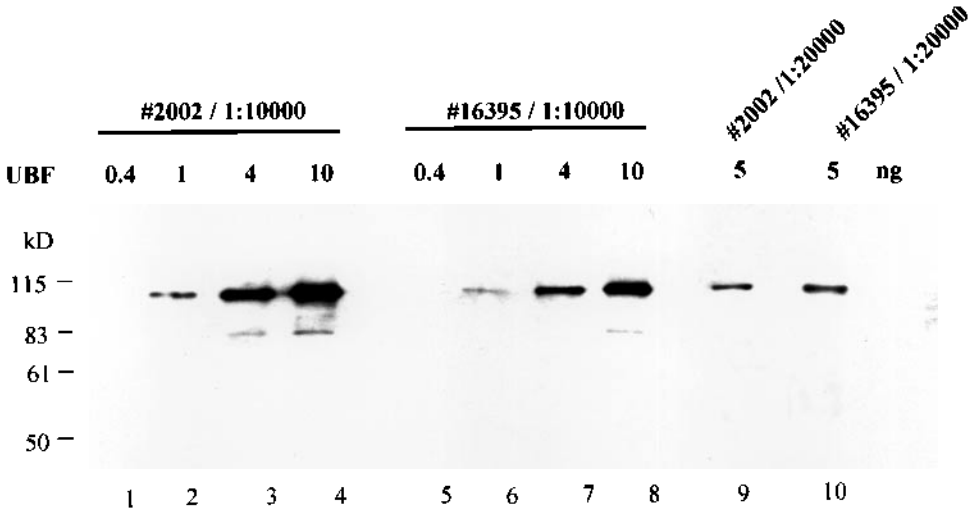

Figure 2. Strong binding of patient's serum to recombinant human UBF (hUBF1). Serum samples 2002 and 16395, obtained 12 years apart, were diluted 1:10,000 and used for immunoblot reactivity with increasing UBF concentrations, ranging from 0.4 ng to 10 ng (lanes 1–8). Note that 5 ng of UBF protein are readily detected by 1:20,000 serum dilution (lanes 9 and 10).

Table 1. Disease associations of anti-NOR 90/UBF autoantibodies in 39 patients.

Study	Ethnicity	No. of Patients	Fluorescent ANA Titers	Sex, Age, yrs	Diagnoses
Rodriguez-Sanchez [3]	Spanish	10	1:320 to 1:10,000		SSc (n = 5) SS Arthritis Angioedema Unknown (n = 2)
Imai[11]	Japanese	1	> 1:100		Hepatocellular carcinoma, positive HBsAg
Imai[12]	Japanese	8	1:100 1:1600 to 1:12,800	7 F: 1 M 23 to 65	RP, early SSc RP, melanoma RP, RA RP, SLE SLE UCTD Arthritis, IgA nephropathy, hypothyroidism Same patient as reference 11*
Fritzler[15]	Caucasian Brazillian	2 of 238 children with systemic rheumatic diseases	> 1:640	2 F 7 and 16	RP, abdominal pain RP, SLE
Dick[13]	German	9 of 26,631 patients screened for rheumatic diseases	1:160 to 1:2560	9 F 24 to 78	Limited SSc RA (n = 2) SLE (n = 2) UCTD SS Alveolar proteinosis Osteoarthritis, coronary artery disease
Fujii[14]	Japanese	9 of 91 patients with rheumatic diseases and antinucleolar antibodies		7 F: 2 M 48 to 78	SSc (n = 2) SSc, RA, SS RA, (n = 3) SS (n = 3)
This report	French Canadian	1	1:2560 to 1:20,480	F 9	RP, limited SSc

RP: Raynaud's phenomenon, SSc: systemic sclerosis, UCTD: undifferentiated connective tissue disease, SS: Sjögren's syndrome. * Counted once.

90/UBF in other reports were usually high titer IgG autoantibodies (Table 1)[3,4]. Second, the persistent expression in this patient of anti-NOR 90/UBF over 12 years as single autoantibody activity is also consistent with this concept. Third, the striking binding characteristics of the patient's sera (Figure 2) suggest a high affinity for hUBF, another feature of antigen driven immune responses[23]. Finally, although epitope mapping was not done with this patient's serum, Fujii, *et al* have used recombinant fusion proteins expressed from several cDNA encoding the NOR 90/hUBF autoantigen to show that multiple epitopes on the hUBF molecule are bound by anti-UBF autoantibodies[14]. Taken altogether, these data strongly suggest that hUBF itself drives the anti-UBF autoimmune response.

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