Autoantibodies to the Nucleolar Organizer Antigen NOR-90 in Children with Systemic Rheumatic Diseases

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ABSTRACT. ***Objective.*** To determine the frequency of autoantibodies directed against the nucleolar organizer antigen NOR-90 [human upstream binding factor (hUBF)] in pediatric patients with systemic rheumatic diseases. Two children with antibodies to NOR-90 are reported.

Methods. Two hundred thirty-eight sera from children with systemic rheumatic diseases were screened for autoantibodies directed against hUBF by indirect immunofluorescence and immunoblotting using MOLT-4 cell extracts. Reactivity with hUBF was confirmed by immunoprecipitation of the recombinant hUBF protein.

Results. Two sera out of 238 (<1.0%) with reactivity against hUBF were identified. One patient had recurrent abdominal pain, headache, and Raynaud's phenomenon. There has been no evidence of an active systemic rheumatic disease in more than 8 years of followup. The 2nd patient had Raynaud's phenomenon and systemic lupus erythematosus.

Conclusion. Antibodies to hUBF are rare in children with systemic rheumatic diseases. Raynaud's phenomenon was a clinical feature common to both patients. (*J Rheumatol 1995;***22:521–4)**

Key Indexing Terms:
AUTOANTIBODIES NUCLEOLAR ORGANIZER NUCLEOLUS
HUMAN UPSTREAM BINDING FACTOR

Autoantibodies directed against nucleolar components are commonly seen in the sera of patients with systemic sclerosis (SSc) and less commonly in the sera of patients with other systemic rheumatic diseases. The autoantigenic targets in nucleoli include a 35 kDa protein of the U3 RNP complex known as fibrillarin, the 80 and 110 kDa proteins of the PM/Scl antigen, the 210 kDa protein of the RNA polymerase I complex, the 110 kDa phosphoprotein nucleolin, a 40 kDa component of the Th ribonucleoprotein (RNP) particle, and B23, a 37 kDa component of the nucleolus[1-3].

In 1987, Rodriguez-Sanchez, *et al* described another autoantibody that bound to a 90 kDa protein that was identified as discrete foci in the nucleolus and metaphase chromosomes[4]. This antigen, which colocalized to silver staining regions of the nucleolar organizer, was referred to as NOR-90[5,6]. Autoantibodies to NOR-90 were originally described in 6 patients, 4 of whom were diagnosed with scleroderma[4]. Subsequently, this autoantibody was reported to be uncommon[7], and it was noted in a more recent study that 4 of 8 patients had Raynaud's phenomenon (RP)[5].

NOR-90 has been identified as the human upstream binding factor (hUBF)[7], which binds to the ribosomal RNA gene promoter and enhances the transcriptional activity of RNA polymerase I[8]. NOR-90/hUBF exists as distinct molecular forms having calculated molecular weights of 97 and 94 kDa[8], although in sodium dodecyl sulfate polyacrylamide gels they migrate at 93 and 89 kDa[9]. cDNA encoding hUBF and NOR-90 have been cloned, and it has been shown that both cDNA sequences are identical except for a 37 amino acid (111 nucleotide) deletion in the low M_r form [7]. In an immunoprecipitation assay, anti-NOR-90 sera were shown to recognize both forms of the *in vitro* transcription and translation products derived from cDNA[7].

To date, all reported patients with anti-NOR-90 were adults. We studied the frequency of anti-NOR-90 in pediatric patients with systemic rheumatic diseases and report the clinical features of 2 patients identified with this autoantibody.

MATERIALS AND METHODS.

Patients and sera. We studied sera from 238 patients at the Hospital for Sick Children in Toronto, the University of Calgary, and in Porto Alegre, Brazil. The age range was 10 months to 16 years. Clinical diagnoses included

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juvenile arthritis (28%), myositis and dermatomyositis (14%), systemic lupus erythematosus (SLE) (12%), overlap syndromes and mixed connective tissue disease (6%), scleroderma (5%), Sjögren's syndrome (4%), and other diseases (vasculitis, HLA-B27 related diseases, etc.) (31%). Serum from a patient with RP previously shown to react with recombinant NOR-90[5] was used as a positive control. Sera were stored at −20°C until required for assays.

Indirect immunofluorescence (IIF). The specificity of the autoantibodies for NOR-90 antigens was first identified on the basis of IIF microscopy on a commercial HEp-2 cell substrate (Immuno Concepts, Sacramento, CA) using a fluorescein conjugated polyvalent goat antihuman immunoglobulin (light and heavy chain) as described[10]. Slides were viewed on a Zeiss Universal fluorescent microscope and images were recorded on Ilford HP-5 film.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Proteins or cellular preparations from MOLT-4 cells were dissolved in SDS sample buffer and separated by discontinuous SDS-PAGE, and the separated proteins were transferred to nitrocellulose as described[5,9,11]. After transfer, the nitrocellulose sheets were blocked with 3% nonfat milk in PBS containing 0.05% Tween-20 (PBST), and again with PBST to remove any unbound antibody. Bound antibody was traced with ^{125}I-protein A ($2–4 \times 10^5$ cpm/ml; ICN Radiochemicals, Irvine, CA) or with the ECL blotting kit (Amersham) using a polyvalent, peroxidase conjugated goat antihuman immunoglobulin (Calbiochem-Behring Corp.).

NOR-90 clone. The NOR-90 clone (NOR-5) is a full length 2.3 kb cDNA, isolated and characterized by Dr. E.K.L. Chan[6].

In vitro transcription and translation. The NOR-90 clone was used for *in vitro* transcription and translation using a rabbit reticulocyte based kit (TnT; Promega, Madison, WI) and protocols as described[5,12]. One μg of the purified plasmid DNA was used as template for *in vitro* transcription with T3 RNA polymerase, added in a 50 μl translation reaction containing rabbit reticulocyte lysate, ^{35}S-methionine (trans-^{35}S-label, 70% methionine, 15% cysteine; ICN Biochemicals), and RNase block II (Stratagene Inc.), as suggested by the manufacturer. The reaction was carried out at 30°C for 1 h, followed by SDS-PAGE of a 2–5 μl aliquot to confirm the presence of translation products. Samples were stored at −70°C until required.

Immunoprecipitation. Immunoprecipitation of [^{35}S]-labelled *in vitro* translation products was performed using Protein A-sepharose beads[12]. Briefly, 5 μl of human serum and 50 μl of *in vitro* translation products were incubated with Protein A-sepharose beads for 1 h at 4°C. After incubation, the sepharose beads were washed 5 times with buffer and resuspended in SDS sample buffer. Samples were then analyzed by SDS-PAGE and autoradiography as descibed.

Patients. Patient 1. The patient (SP) was a 16-year-old Caucasian girl who presented with chronic abdominal pain of 1 year duration, recurrent headache, and RP. Other than RP involving the fingers and toes, examination was unremarkable. Photoelectric plethysmography revealed cold induced vasoconstriction compatible with a history of RP. Antinuclear antibodies (ANA) were positive with a punctate nucleolar pattern to a titer of > 1/1280 (Figure 1). Other laboratory investigations including serum complements, hemolytic complement, Coomb's test, cryoglobulins, and hemogram were normal. Over an 8 year followup, there were no new signs or symptoms of systemic rheumatic diseases. The RP persisted but was not complicated by digital ulceration, gangrene, or acroosteolysis. The speckled nucleolar autoantibody titer modestly decreased to a titer of 1/640.

Patient 2. The patient (CS) was a 7-year-old Caucasian girl who developed RP, photosensitivity, a butterfly rash, purpura, and arthritis. Laboratory investigation showed anemia and decreased levels of complement C3 and C4. Urinalysis revealed hematuria and cylinduria, but renal function was normal. The latex agglutination test for rheumatoid factor, LE cell test, and VDRL test were all negative or within normal limits. Tests for antibodies to histone and other chromatin components [dsDNA, (H2A/H2B)-DNA complex] were negative. The ANA on HEp-2 cells demonstrated a speckled nucleolar pattern and punctate staining of chromatin to a dilution of > 1/640. The patient met American Rheumatology Association criteria for the classification of SLE; unfortunately, she died in a motor vehicle accident.

RESULTS

Both sera produced a speckled nucleolar pattern of immunofluorescence on HEp-2 cells (Figure 1) characteristic of anti-NOR-90 antibodies. When the sera of both patients were tested by immunoblotting, they reacted with an 89/93 kDa doublet (Figure 2A, lanes CS, SP) having the same M_r as the prototype anti-hUBF serum (Figure 2A, lane JO). By comparison, these antibodies reacted with proteins of different molecular weight than prototype sera with reactivity to other nucleolar autoantibodies such as anti-fibrillarin (Figure 2A, lane Fb) or anti-PM/Scl (Figure 2A, lane PM/Scl).

Confirmation that the patient's sera reacted with NOR-90 was demonstrated when both immunoprecipitated the 90 kDa *in vitro* translated product of the NOR-90 cDNA (Figure 2B).

DISCUSSION

Sera from 2 pediatric patients were shown to produce a speckled nucleolar pattern of immunofluorescence on HEp-2 cells typical of antibodies to NOR-90. Reactivity with NOR-90 was confirmed by immunoblotting and immunoprecipitation of an *in vitro* transcribed and translated protein derived from a NOR-90 cDNA clone[6]. This is the first report of this autoantibody in children with features of a systemic rheumatic disease. This autoantibody appears to be rare, since it was found in only 2 of 238 sera from children with systemic rheumatic diseases. This may represent a higher frequency than expected in an unbiased sample because the sera from the 3 centers were selected from existing serum banks. The patient from Brazil (CS) was 1 of 18 with a diagnosis of juvenile SLE followed in that center. In another study, only 9 of 26,631 sera tested were found to have the typical anti-NOR-90 speckled pattern of immunofluorescence and immunoblotted a 90 kDa protein doublet[13]. In that report, 8 of 9 anti-NOR-90 positive

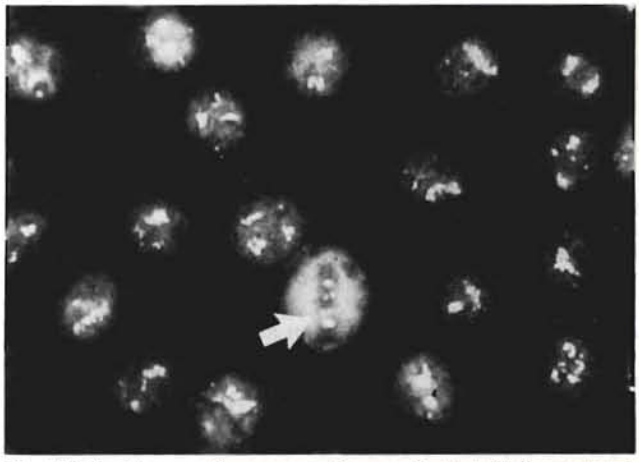

Fig. 1. Indirect immunofluorescence of serum of patient SP demonstrated a punctate nucleolar pattern of immunofluorescence on HEp-2 cells. The staining is seen in both interphase cells and on some metaphase chromosomes (arrow). Original magnification × 600.

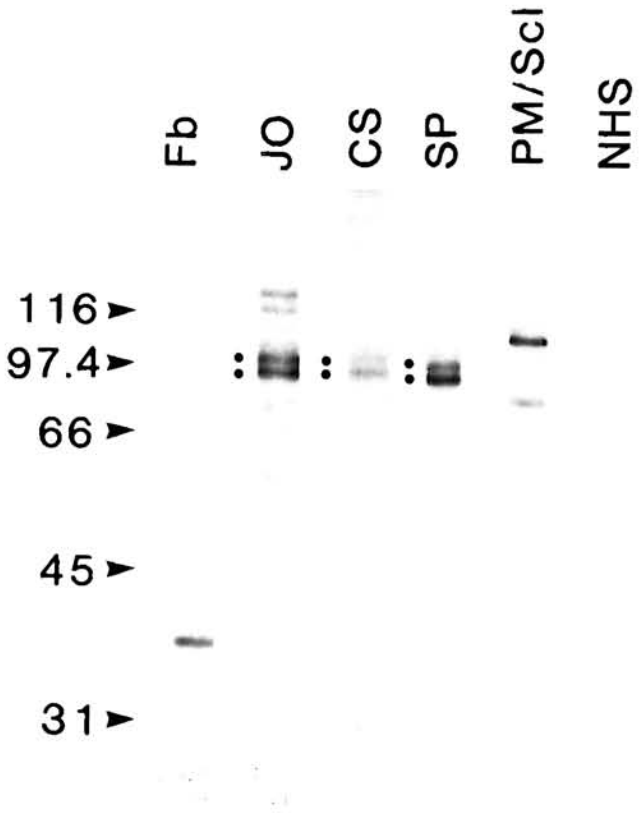

Fig. 2A. Immunoblotting profile of 2 pediatric patients. MOLT-4 whole cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and probed with sera diluted 1:100. Lane 1, prototype anti-fibrillarin (Fb) serum; lane 2, prototype anti-NOR-90 (JO) serum, lanes 3 and 4, sera from Patients CS and SP showing reactivity with a 90 kDa protein doublet (dots). Lane 5, reactivity of a prototype serum with anti-PM/Scl antibodies (PM/Scl). Lane 6, normal human serum (NHS). Molecular mass markers are indicated on the left.

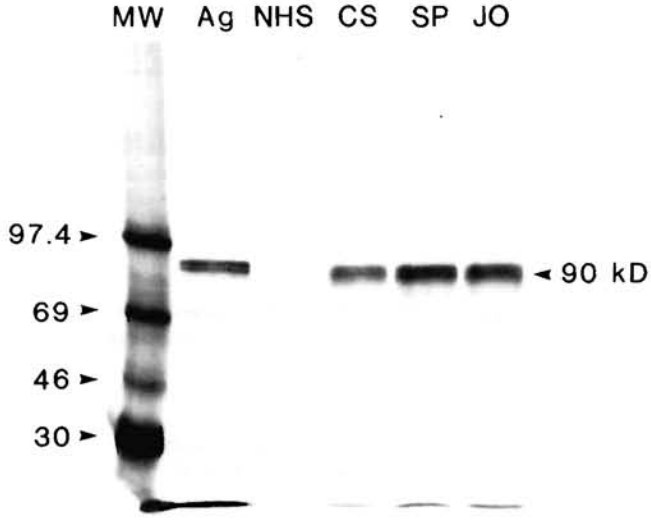

Fig. 2B. Immunoprecipitation on *in vitro* transcribed and translated NOR-90 cDNA. A NOR-90 cDNA was used as a template for *in vitro* transcription and translation to yield a 90 kDa protein product labelled with ^{35}S-methionine (Ag, lane 2). The radiolabelled protein was incubated with patient sera and the immune complexes were bound to Protein A-sepharose. The immune complexes were eluted, separated by SDS-PAGE, and the presence of antigen in the gels was detected by autoradiography. Lane 3, normal human serum (NHS). Lanes 4 and 5, patient sera from patients CS and SP immunoprecipitate the radiolabelled protein that is also precipitated by the prototype anti-NOR-90 serum (JO). ^{14}C-labelled molecular mass markers are indicated on the left.

patients expressed HLA-DR1, an observation identical to the HLA profile of a patient (SP) in this study (data not shown).

The initial report of clinical associations with anti-NOR-90 antibodies, detected by immunofluorescence and subsequently by immunoblotting, indicated that 5 of 10 patients had a diagnosis of SSc[4]. In a study by Dick, *et al*[13], only 1 of 108 patients with SSc had antibodies to NOR-90, and 1 of 9 patients with the typical speckled nucleolar pattern and immunoblotted 90 kDa protein had SSc. Other studies have found that antibodies to NOR-90 are uncommon in SSc[7], and that some patients had overlap connective tissue diseases and secondary Sjögren's syndrome[14]. In the report of 8 adult patients with anti-NOR-90 antibodies referred to earlier, a variety of clinical diagnoses were indicated, but a feature of 4 of the 8 patients was RP. Both the children in our report had RP. Patient 1 has been followed for 8 years and has not demonstrated new signs or symptoms of SSc or SLE. Unfortunately, the longterm observation of Patient 2, who had a diagnosis of SLE, was not possible because she died in a motor vehicle accident.

Many autoantibodies have been associated with clinical subsets of disease, and because of this it has been suggested that they might serve as prognostic indicators. For example, it has been reported that patients with SSc with antibodies directed against Scl-70 (topoisomerase I) have a higher frequency of pulmonary disease and acroosteolysis[2]. By comparison, patients with antibodies to centromere components CENP-A and CENP-B tend to have a limited form of SSc and a lower frequency of renal involvement[2]. Since prospective or systematic studies of patients who have NOR-90 antibodies have not been done, the clinical importance of this autoantibody is not clear. Followup of adults in the Calgary clinic has shown that the patients tend to have a protracted and relatively uncomplicated clinical course. For example, one patient with anti-NOR-90 antibodies and RP has been followed for 10 years, and only in the last 12 months has she developed signs of digital edema and acrosclerosis. This clinical course is similar to one of the patients (SP) reported here, who has continued to have RP but has not developed overt clinical features of SSc or other systemic rheumatic disease. The observation that the interval between the onset of symptoms and the presence of autoantibodies to the development of a disease such as scleroderma can often exceed 10 years emphasizes the importance of longterm fol-

lowup rather than cross sectional studies of patients before clinical correlations can be defined.

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