

Anti-elongation factor-1 α autoantibody in adult atopic dermatitis patients

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Abstract

Adult atopic dermatitis (AD) patients develop severe facial lesions, which sometimes distribute in sun-exposed areas similar to the rash of systemic lupus erythematosus. To declare autoimmunity in the pathogenesis of AD, we investigated serum antinuclear antibody (ANA) in 256 adult AD patients and identified its ligands. A high titer of ANA was found in 31.3% of AD patients and 75% of the ANA showed a homogenous pattern. Sixty-five percent of ANA⁺ sera reacted to a 52 kDa protein (p52) in HeLa cell immunoblots. By screening the HeLa cell cDNA expression library with anti-p52 sera, a clearly positive clone was isolated. The sequence of this cDNA was identical to human elongation factor (hEF)-1 α . The eluate of IgG bound to hEF-1 α -glutathione S-transferase (GST) fusion protein recognized a band at 52 kDa in a HeLa cell immunoblot, and stained Hep-2 cell nuclei and cytoplasm as reported in hEF-1 α distribution. The anti-p52 AD sera recognized the hEF-1 α -GST fusion protein. The anti-hEF-1 α antibody-positive AD patients were characterized by higher facial involvement and lower white blood cell counts compared with antibody-negative patients. The present results suggest the possible involvement of autoimmunity in the pathogenesis of adult AD.

Introduction

Hypersensitivity to environmental antigens has been widely accepted as the pathomechanism of atopic dermatitis (AD). Elimination of the causative exogenous antigens, including house dust and mite antigens, is sometimes remarkably effective. However, in some cases dermatitis persists for a long time even after the elimination of such antigens from the patient's environment. The majority of infants suffering from AD show symptom improvement by the time they reach adolescence (1,2). However, in some patients AD persists until adulthood. Recently, adult cases of AD have been increasing and the treatment of these cases has become an important issue (3,4). In adult cases of AD, the face is commonly involved (5,6) and sometimes the symptoms are exacerbated after sun exposure (7). In addition, facial manifestations occasionally distribute in sun-exposed areas similar to the malar rash of systemic lupus erythematosus (SLE). Our preliminary study suggested a high anti-nuclear antibody (ANA) level in adult AD patients (6). Scratching of the skin lesions, sunburn, herpes simplex and other infections frequently exacerbate AD. In such cases, the destroyed or altered self-antigens are exposed to the immune system.

This implies the possibility of hyper-reactivity to endogenous antigens, as well as to exogenous environmental antigens, in AD. Thus, to clarify the pathomechanism of autoimmunity in AD, we evaluated the clinical and laboratory manifestations of ANA⁺ AD patients and characterized the ligands of the autoantibody by molecular cloning. It was revealed that the antigen for ANA is human elongation factor (hEF)-1 α .

Methods

Patients

Two-hundred and fifty-six patients (124 male and 132 female) with AD (mean age 23 years) were diagnosed according to Hanifin–Rajka's diagnostic criteria (8). Sixty age- and sex-matched healthy volunteers with no history of atopy served as normal controls. All experiments were performed after obtaining informed consent.

Clinical and laboratory investigations

Patients were divided into two clinical groups with or without marked facial involvement.

Complete blood cell counts, measurement of serum liver enzymes and renal function tests were performed. Serum IgE levels were measured by radioimmunoassay and specific RAST scores for antigens were measured using a cap-RAST kit (Pharmacia-Japan, Kusatsu, Japan). Anti-DNA antibodies were detected by radioimmunoassay. Anti-SS-A(Ro), anti-SS-B(La), anti-RNP and anti-Sm antibodies were measured using the double-immunodiffusion method (9).

ANA

The ANA of serum samples from 256 AD patients was screened with an indirect immunofluorescent ANA detection system (HEPANA test; MBL, Nagoya, Japan) using the human epithelioid carcinoma cell line (Hep-2 cell) substrate and fluorescein-conjugated goat anti-human IgG. Sera from 60 healthy volunteers were used as normal controls. Antibody titers of the samples were determined with serially diluted serum. A sample that recognized ANA at a >40-fold dilution was considered to be positive.

Immunoblot analysis

Hep-2 and HeLa cells were purchased from ATCC (Rockville, MD). They were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Cansera, Ontario, Canada), penicillin (50 μ g/ml) and streptomycin (50 μ g/ml). Hep-2 cells were suspended in a SDS-PAGE sample buffer and electrophoresed in a 12.5% polyacrylamide gel under reduced conditions with 2-mercaptoethanol. The electrophoresed protein was transferred to a nitrocellulose membrane (Schleicher & Schnell, Kassel, Germany). After blocking with 3% skimmed milk for 15 min, the membrane was incubated with the AD sera serially diluted from 80 times. After rinsing with 10 mM Tris buffer, the bound human IgG was detected with a system comprising alkaline phosphatase-conjugated anti-human IgG antibody (Promega, Madison, WI) and a Western Blue substrate (Promega).

cDNA cloning by antibody screening

Escherichia coli strain Y1090 was used for the propagation of a HeLa cell λ gt11 cDNA library (HL5013b; Clontech, Palo Alto, CA) on a magnesium-free LB agar plate with 50 mM ampicillin and incubated overnight at 37°C. The phage plaques were plotted onto a nitrocellulose membrane after incubation for 3.5 h at 37°C. After blocking with 3% skimmed milk for 15 min, the membranes were incubated for 1 h at 37°C with the 20-fold diluted sera of AD patients, which were previously absorbed by Sephadex columns coated with the lysate of wild-type *E. coli* Y1090. The bound IgG was detected with an alkaline phosphatase-conjugated anti-human IgG antibody and a Western Blue substrate (Promega). Positively stained plaques were picked up and a strongly positive clone that reacted to three ANA⁺ (homogenous and peripheral pattern) sera, but not to normal sera, was subjected to further analysis.

PCR and sequencing

The cDNA insert of the phage DNA was amplified by PCR using Ex-Taq polymerase and λ gt11 primers (Takara, Kusatsu, Japan) complementary to the β -galactosidase-encoding regions which flank the *Eco*RI site of the phage vector. Twenty-

five cycles of PCR were performed at 95°C for 1 min, at 65°C for 2 min and at 72°C for 3 min, and the amplified DNA was sequenced using the dideoxy-chain termination method. A homology search was performed using the GenBank and the National Center for Biotechnology Information.

Expression of *hEF-1 α* protein

RNA samples prepared from 1×10^9 cells of HeLa and Hep-2 cell lines were transcribed into cDNA using superscript II reverse transcriptase (Gibco/BRL, Grand Island, NY), and an obtained *hEF-1 α* antisense primer. Then, the coding region of the *hEF-1 α* cDNA was amplified by PCR with the antisense primer and a *hEF-1 α* sense primer, both of which were designed to allow the cDNA to adjust to *Bam*HI–*Sal*I sites in the glutathione S-transferase (GST) gene fusion vector (pGEX4T-3; Pharmacia-Japan). The sequences of the primers were as follows: sense: 5'-GGG ATC CGG AAA GGA AAA GAC TCA TAT CAA CAT TGT C-3'; antisense: 5'-GGT CGA CTC ATT TAG CCT TCT GAG CTT TCT GGG CAG A-3'. PCR was performed as described above. The PCR product (1696 bp) was ligated into a pGEX 4T-3 vector and introduced into *E. coli* BL21(DE21)pLysS competent cells (Stratagene, La Jolla, CA). The transformant was cultured in LB medium containing 1 mM isopropylthio- β -D-galactoside (IPTG) for 2 h at 37°C. Cell pellets were collected by centrifugation and dissolved in a SDS-PAGE sample buffer. The samples were electrophoresed and immunoblotted with anti-p52⁺ sera and the anti-GST antibody (Pharmacia-Japan). Bound human and goat IgG were detected with alkaline phosphatase-conjugated anti-human IgG antibody, anti-goat antibody (Zymed, San Francisco, CA) and a Western Blue developing system.

Purification of anti-*hEF-1 α* autoantibody (AEFA) and intercellular distribution of *hEF-1 α* protein

The electrophoresed *rhEF-1 α* -GST fusion protein was transferred to a PVDF membrane (Immobilon-P; Millipore-Japan, Tokyo, Japan). A slip of the PVDF membrane was cut out at 80 kDa and immersed in the IgG fraction of anti-p52 high-titer AD sera. The bound IgG was eluted with a 0.2 M glycine-HCl buffer (pH 3.5) and neutralized immediately with 1 M Tris buffer (pH 8.0). The eluate was dialyzed against distilled water and was vacuum condensed. The purified anti-*hEF-1 α* IgG was applied to the indirect immunofluorescent ANA detection system and immunoblotting.

Results

ANA

Ninety-three of 256 (31.3%) serum samples of AD patients were positive for ANA at a 40-fold dilution, which was significantly higher than that of the normal control (seven of 60, 11.7%, $P < 0.001$, Fisher's exact test). Samples taken from 33.8% of females and 30.8% of the samples from males tested positive. The ANA titer was from 40 $\times$ to 1250 $\times$. Nuclei of Hep-2 cells were stained in a homogenous pattern (HO, 55.8%), homogenous and speckled pattern (HO/SP, 19.2%), speckled pattern (SP, 15.4%), nucleolar pattern (NU, 5.8%) or a proliferating cell nuclear antigen pattern (PCNA, 3.8%). Staining along nuclear membranes (peripheral pattern) and

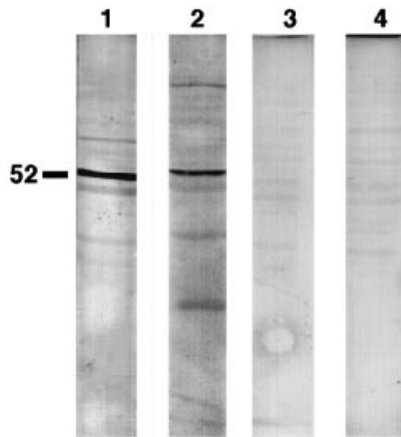


Fig. 1. Immunoblot analysis of serum from an AD patient (ANA titer 1:640, homogeneous and peripheral pattern) to Hep-2 (lane 1) and HeLa cell lysates (lane 2). Clear immunoreactive bands were detected at 52 kDa. No specific immunoreactive bands for Hep-2 cells were obtained by incubation with a serum sample from an AD patient (ANA 1:640, speckled pattern) (lane 3) or an ANA⁻ healthy control serum (lane 4).

cytoplasm was observed in the homogenous pattern ANA. Subsequently, the homogenous ANA pattern was found in 75% of ANA⁺ patients. Antibody titers varied in the ANA patterns (HO, 268.6 ± 222.0 , mean times dilution $\pm$ SD; HO/SP, 685.0 ± 539.0 ; SP, 65.0 ± 42.2 ; NU, 93.3 ± 61.1 ; PCNA 80). A significant difference was observed between mean ANA titers of the HO and SP pattern groups. No significant differences in ANA patterns and titers were identified between the male and female groups.

Immunoblotting of AD sera

From a single to several immunoreactive bands to the Hep-2 cell lysate were detected in ANA⁺ patients' sera. A band at 52 kDa was detected (Fig. 1) in 52 of 80 (65.0%) serum samples from ANA⁺ patients at a dilution of 1/80. Other immunoreactive bands were not so clearly or reproducibly detected.

Using sera positive for the 52 kDa bands, other human cell lines were screened. The carcinoma cell line (HeLa), hepatoma cell line (HepG-2), squamous carcinoma cell line (SCC) and the SV-40 transformed keratinocytes cell line (RHEK) expressed the same 52 kDa immunoreactive protein (data not shown). This implied the 52 kDa protein (p52) is commonly expressed as a cellular protein.

Forty-four of 56 (78.6%) homogenous pattern samples were p52⁺, but p52 reactivity was detected in only eight of 24 (33.3%) other ANA pattern samples. Thus, anti-p52 antibody was related to the homogenous pattern ANA ($P < 0.01$, Fisher's exact test).

cDNA cloning for the ANA antigen using antibody screening

In order to identify the 52 kDa protein reactive with ANA sera, a HeLa cell cDNA expression library was screened with the patients' sera. After three cycles of antibody screening, one clearly positive clone was isolated, and the nucleotide sequences of the 5' and 3' ends of the cDNA insert were

5'- 1	CCTAAAAGCAAAATGGGAAAGGAAAAGACTC	31
32	ATATCAACATTGTCGTCATTGGACACGTAGAT	63
	GAT	64
64	TCGGGCAAGTCCACCACTACTGGCCATCTGAT	95
65	TCGGGCAAGTCCACCACTACTGGCCATCTGAT	96
96	CTATAATGCGGTGGCATCGGACAAAAGAACCA	127
97	CTATAATGCGGTGGCATCGGACAAAAGAACCA	128
128	TTGAAAATTTGAGAAGGAGGCTGCTGAGATCG	159
129	TTGAAAATTTGAGAAGGAGGCTGCTGAGATCG	160
160	GGAAAGGGCTCCTTCAAGTATGCCTGGG	189
161	GGAAAGGGCTCCTTCAAGTATGCCTGGG	190

Fig. 2. The obtained 1.6 kb flanking sequence selected by anti-p52 antibody-positive sera (upper lane) is identical to the reported sequence of the 5' region in human EF-1 α (10) (lower lane).

determined. A homology search for these sequences revealed that the cDNA encoded a part of the sequence of hEF-1 α (Fig. 2) (10). Using obtained sequence primers, full-length hEF-1 α cDNA was obtained. The mol. wt of hEF-1 α is known to be 51 kDa, which is close to that of the ANA sera-reactive protein in HeLa cells, suggesting that the ANA sera-reactive 52 kDa protein in HeLa cells is hEF-1 α .

Expression of recombinant hEF-1 α protein and immunoblot analysis

The hEF-1 α expression vector was constructed by the insertion of the obtained full coding region of hEF-1 α cDNA into the *E. coli* expression vector and the recombinant hEF-1 α protein was expressed in *E. coli* cells as an 80 kDa GST fusion protein, the mol. wt of which was expected to be 79 kDa (Fig. 3). As shown in Fig. 4(a), an 80 kDa protein was detected from the cell lysate of *E. coli* containing the EF-1 α expression vector by immunoblot analysis with ANA sera or anti-GST antibody (Pharmacia-Japan).

Furthermore, the immunoblot analysis was performed using the affinity-purified anti-hEF-1 α IgG as the first antibody. As shown in Fig. 4(b), immunoreactive bands at 52, 52 and 80 kDa were detected from the cell lysates of HeLa cells, Hep-2 cells and *E. coli* containing the hEF-1 α expression vector respectively. These results strongly suggest that the ANA sera-reactive 52 kDa protein in AD patients' sera is hEF-1 α .

Intracellular distribution of hEF-1 α protein

The IgG from anti-p52⁺ AD sera and purified anti-hEF-1 α IgG reacted with Hep-2 nuclei in a homogenous and peripheral pattern with scattered cytoplasmic staining (Fig. 5a and b). This distribution pattern was compatible with the reported intracellular distribution of hEF-1 α (11).

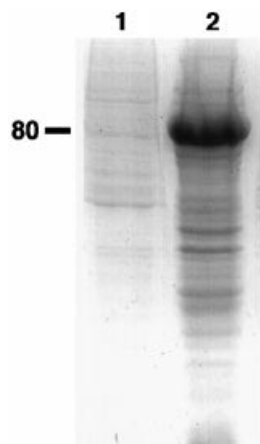


Fig. 3. Coomassie blue stained gel with protein extracts of *E. coli* BL21(DE3)pLysS transformed with pGEX4T-3-hEF-1 α cultured without IPTG (lane 1) and with 1 mM IPTG (lane 2) for 1 h. Human EF-1 α -GST fusion protein was expressed at 80 kDa.

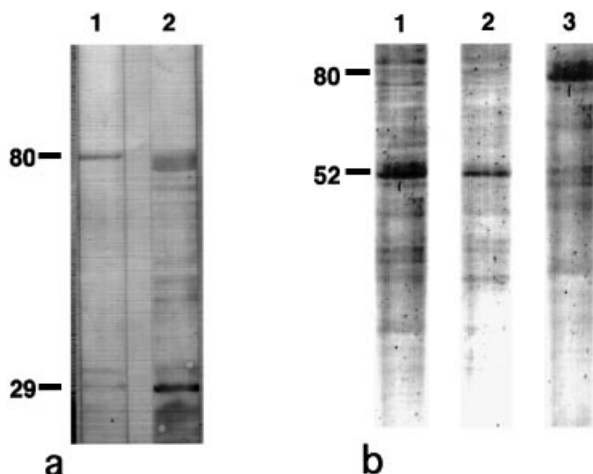


Fig. 4. (a) Immunoblot of the expressed pGEX4T-3-hEF-1 α with anti-p52⁺ sera (lane 1) and anti-GST antibodies (lane 2). The expected mol. wt of the hEF-1 α -GST fusion protein was ~80 kDa (29 kDa GST protein included) and the detected bands migrated at 80 kDa. (b) Immunoblot with the purified anti-hEF-1 α -GST IgG detected a band at 52 kDa in the Hep-2 lysate (lane 2) that was identical to a band detected in the HeLa lysate (lane 1). This anti-hEF-1 α -GST IgG reacted to the hEF-1 α -GST fusion protein at 80 kDa (lane 3).

Clinical and laboratory study

Marked facial involvement was observed in 41 of 49 ANA⁺ patients (83.7%) and in 19 of 21 AEFA⁺ AD patients (90.5%). These results were significantly higher than those of the ANA⁻ patients (28 of 43, 65.1%, $P < 0.05$, Fisher's exact test). The mean age of the ANA⁺ group (23.6 ± 1.0 years, mean $\pm$ SE, $P < 0.05$, unpaired t -test) was lower than the ANA⁻ group (26.5 ± 0.9). The mean age of the AEFA⁺ group (21.1 ± 1.2 , $P < 0.01$) was also younger than the AEFA⁻ group (25.2 ± 0.6). No significant difference was found between the ANA⁺, the AEFA⁺ and the negative group as to sex.

The white blood cell counts of the ANA⁺ group ($5991.7 \pm 184.8/\text{mm}^3$, mean $\pm$ SE, $P < 0.05$, unpaired t -test) were

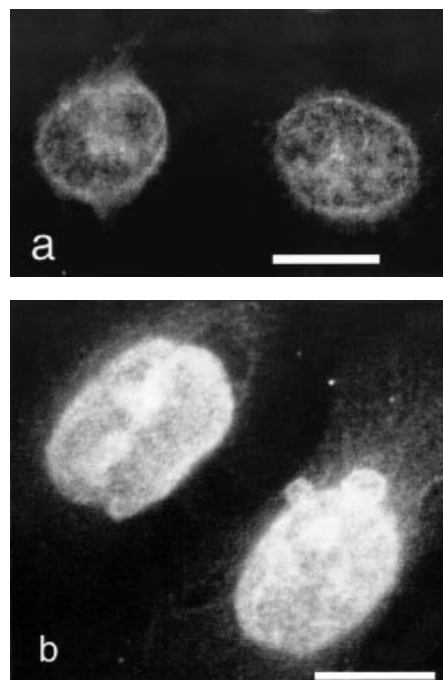


Figure 5. (a) The Hep-2 cell nuclei stained with the anti-p52 antibody-positive serum from an AD patient showed a homogenous and peripheral pattern with scattered cytoplasmic staining (bar: 50 μm). (b) The affinity-purified anti-hEF-1 α IgG stained the Hep-2 cell nuclei in a homogenous and peripheral pattern with scattered cytoplasmic staining (bar: 50 μm).

significantly lower than those counts of the ANA⁻ group (6557.5 ± 257.8), and the AEFA⁺ AD group's (5526.8 ± 267.4 , $P < 0.01$) were also lower than the AEFA⁻ AD group (6384.9 ± 147.3). The serum IgE levels of the ANA⁺ group (4273.2 ± 1139.1 IU/ml) were slightly lower than those of the ANA⁻ group (4717.5 ± 1139.1) and the AEFA⁺ AD group's (3269.0 ± 791.4) were also slightly lower than the AEFA⁻ AD group (4822.3 ± 736.6). However, the lymphocyte counts, eosinophil counts, platelet counts, serum liver enzymes, renal function tests and RAST scores for mites, house dust, candida and pine tree pollen antigens showed no significant difference between the ANA⁺/AEFA⁺ and ANA⁻ groups (unpaired t -test).

Anti-double-strand DNA antibodies, anti-RNP antibodies, anti-Sm antibodies and anti-SS-B(La) antibodies were not detected in any of the samples in this study. Anti-SS-A(Ro) antibodies were detected in one AD patient with ANA. No AD patient in the present study met the SLE criteria of the American College of Rheumatology (ACR) (12,13).

Discussion

In our an earlier report (6) and other reports (5), ANA was identified in severe AD patients and the ligand of the AD autoantibody was speculated to be a nuclear protein. The present study confirmed the presence of ANA in AD patients' sera. ANA in AD revealed several staining patterns and AD patients sera recognizes various nuclear ligands. Because of the close relationship between homogeneous pattern ANA and the anti-p52 autoantibody, we hypothesized that a 52

kDa protein with homogenous nuclear distribution was the ligand. Molecular cloning using anti-p52 antibody-positive sera delineated hEF-1 α , a major member of intracellular elongation factors, as a common IgG autoantibody target in AD. In addition, the purified AEFA showed a homogeneous and peripheral nuclear staining pattern with cytoplasmic staining. Since EF-1 α has been reported to be a ribosomal protein (11), the present data strongly suggest that the major component of ANA in AD is AEFA.

ANA has been pointed out as the specific marker antibody for some collagen diseases (14), especially the anti-U1-RNP antibody in mixed connective tissue disease, anti-DNA antibody in SLE and anti-topoisomerase I antibody in scleroderma. The identified ANA antigens are mostly housekeeping gene products, and are abundantly and ubiquitously expressed proteins. EF-1 α is a family member of EF-1 α , β and γ , and is an abundantly expressed ubiquitous gene. The functions of EF-1 α are not fully understood. In its known major role, EF-1 α binds to aminoacyl-tRNA and contributes to the right codon-anticodon selection in the ribosome (15). Thus, EF-1 α may fulfill conditions as the ANA ligand. Until now, AEFA has been identified in dermatomyositis (DM), but the reported incidence of AEFA in DM was <1% and no specific relationship with specific clinical manifestations has been reported (16,17). AEFA was undetectable in normal sera and was encountered in 65% of the ANA⁺ AD patients and 18.8% of the tested AD patients. The prevalence of AEFA in total AD was not so high, but was far more frequently detected in AD than DM. In addition, the prevalence of the anti-Sm antibody, a well-known autoantibody for SLE, was limited to ~20–30% of SLE patients (18) and that of the anti-La (SS-B) autoantibody in Sjögren's syndrome is 10–40% (19). Therefore, the prevalence of AEFA in AD is not much lower than that of SLE-specific autoantibodies. In addition, AD is not a single gene mutant disease and may be due to various immunological abnormalities. The prevalence of AEFA in ANA⁺ AD was as high as 65%, which may indicate a different biological role for AEFA in AD. These data also raise the possibility that AEFA is a marker antibody for AD.

There are several hypotheses regarding the nature of the eliciting antigens in collagen diseases. Microbial antigens, idiotypic networks and exposure of the altered autoantigens have been implicated in the generation of autoantibodies. Repeated exposure of specific antigens to the immune system, under autoantibody-producing conditions, may enhance the generation of specific autoantibodies. The lesional epidermis of AD is hyperproliferating with EF-1 α expression when patients are recovering from dermatitis. AD facial lesions are exposed to UV. UV is known as a potent inducer of ANA (20). UV, scratching and dermatitis injure the facial lesional keratinocytes, and expose cytoplasmic EF-1 α to the immune system. The cytotoxicity of AEFA to the normal epidermis has not been determined. Once acquired, AEFA can bind any injured keratinocytes' EF-1 α and exacerbates and prolongs the inflammatory reactions.

Viral infection is still a potent inducer of autoantibody production. EF-1 α was also identified as a cellular cofactor that stimulated the binding of RNA polymerase II and TRP-185 to the HIV-1 long terminal repeat RNA, which is critical for increasing the gene expression in response to the trans-

activator protein Tat (21). In non-HIV AD lesions, immunoreactive HIV-1 Tat was clearly detected in Langerhans cells, keratinocytes, dendritic cells and blood vessel endothelium, and the expression of Tat was significantly increased by immunological stimulation with antigen patch testing (22). Thus, it is possible that EF-1 α is involved in the immunological inflammatory responses of AD.

AEFA⁺ patients were characterized by marked facial exanthema, significantly low leukocyte counts and low serum IgE levels. While no patient in the present study met the ACR SLE criteria (12,13), leukopenia and facial exanthema are also characteristic of SLE. Taken together with the lower levels of serum IgE, AEFA⁺ AD may be a subgroup with an immunological background related to SLE.

The mechanism involved in the acquisition of hypersensitivity to endogenous antigens, as well as to exogenous environmental antigens, remains to be clarified. However, autoreactivity to self-antigens may be a potent driving force in the long-lasting AD inflammatory reactions of adulthood. The present data cannot show the biological roles of AEFA except as a marker antibody of severe adult AD. T and B cell responses to recombinant hEF-1 α is in progress.

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Abbreviations

p52	52 kDa protein
ACR	American College of Rheumatology
ANA	antinuclear antibody
AEFA	anti-hEF-1 α autoantibody
AD	atopic dermatitis
DM	dermatomyositis
hEF	human elongation factor
GST	glutathione S-transferase
HO	homogenous
IPTG	isopropylthio- β -D-galactoside
NU	nucleolar pattern
PCNA	proliferating cell nuclear antigen pattern
SP	speckled
SLE	systemic lupus erythematosus

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