

Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients

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ABSTRACT Recently we demonstrated that a high percentage of atopic dermatitis (AD) patients displayed specific immunoglobulin E reactivity to human proteins. Here we show that IgE autoreactivity is found predominantly in AD patients with severe skin manifestations and reveal the molecular nature of four IgE autoantigens. An expression cDNA library constructed from a human epithelial cell line (A 431) was screened with serum IgE from two AD patients. DNA sequence analysis of three IgE-reactive clones identified the α -chain of the nascent polypeptide-associated complex, cytokeratin type II, and the BCL7B oncogen as atopy-related IgE autoantigens (ara). The fourth cDNA coded for an IgE autoantigen containing a typical calcium binding motif that occurred in histogenetically different cells and tissues (keratinocytes, muscle, brain). Recombinant *Escherichia coli*-expressed IgE autoantigens bound IgE from AD but not from patients with other immunologically mediated disorders (graft vs. host disease, systemic lupus erythematosus) and elicited immediate type skin reactions in AD patients. In serum samples collected from an AD patient over a period of 5 years, IgE anti-ara NAC antibody levels peaked during disease exacerbation. Our finding that ara BCL7B was detected in serum bound to IgE antibodies suggests that intracellular IgE autoantigens can become released after tissue damage and may occur as IgE immune complexes. Via binding to antigen presenting cells as well as to effector cells, IgE autoantigen immune complexes may contribute to exacerbation and/or perpetuation of severe atopic diseases even in the absence of exogenous allergens.—Natter, S., Seiberler, S., Hufnagl, P., Binder, B. R., Hirschl, A. M., Ring, J., Abeck, D., Schmidt, T., Valent, P., Valenta, R. Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients. *FASEB J.* 12, 1559–1569 (1998)

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TYPE I ALLERGY represents a genetically determined immune disorder with increasing prevalence that leads to the formation of immunoglobulin E (IgE)² antibodies against innocuous environmental allergens (1, 2). In sensitized atopics, allergen encounter induces cross-linking of high-affinity, Fc ϵ receptor-bound IgE present on effector cells (mast cells, basophils) and, thus, immediate release of anaphylactogenic mediators (e.g., histamine, leukotrienes) (3–5). More chronic manifestations of atopy (e.g., atopic dermatitis (AD) (6–11)) may result from the activation of allergen-specific T cells, a process that is greatly enhanced when allergens are presented to the T cells via receptor-bound immunoglobulins, e.g., IgE (12–14). Whereas the disease-eliciting allergens for many acute manifestations of atopy (i.e., allergic rhinoconjunctivitis, food allergy, allergic asthma) originate from environmental sources such as pollen, mites, animals, or fungi and have been characterized by immunological and molecular biological techniques in great detail (15), the targets for IgE antibodies and the pathogenetic mechanisms operative in the mostly chronic forms of atopy are less clear (16–18). Patients suffering from AD and intrinsic forms of allergic asthma frequently show perpetuation of clinical symptoms or exacerbation of the disease even without obvious contact to environmental allergens (7–9).

It was demonstrated earlier that AD patients showed immediate type hypersensitivity to human

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² Abbreviations: AD, atopic dermatitis; ara, atopy-related IgE autoantigens; Dm, mean wheel diameter; GVHD, graft vs. host disease; IgE, immunoglobulin E; N, nonatopic; NAC, nascent polypeptide complex; ORF, open reading frame; pfu, plaque-forming units; pI, isoelectric point; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; SLE, systemic lupus erythematosus.

protein extracts, suggesting that endogenous proteins may serve as possible targets for IgE autoantibodies (19–22). With reports of IgE cross-reactivity and structural similarity between environmental allergens and human proteins (23–28), these findings gave rise to the idea that IgE autoreactivity could play a pathogenetic role in certain forms of atopy. Recently we demonstrated that IgE autoantibodies can be detected in a large percentage of sera from AD patients (29).

In this study we extend the previous work by analyzing sera from a group of 51 additional AD patients in order to detect the presence of IgE autoantibodies and disease severity. We reveal the molecular nature of four IgE autoantigens by screening of a λ gt 11 expression library constructed from the human epidermal cell line A431 with serum IgE from AD patients. Sequence comparison of the four cDNAs with sequences submitted to the databases identified intracellular proteins with important biological functions such as the α -chain of the nascent polypeptide complex, a protein required for signal sequence specific sorting and translocation (30), cytokeratin type II (31–33), and the BCL7B oncogen (34) as IgE autoantigens. To investigate whether intracellular IgE autoantigens can be released from cells, we raised a rabbit antiserum against recombinant atopy-related IgE autoantigen (ara) BCL7B to detect the autoantigen in serum of an AD patient complexed to IgE autoantibodies. Recombinant IgE autoantigens were expressed in *Escherichia coli* and tested for their IgE binding capacity as well as for their ability to elicit immediate type skin reactions.

MATERIALS AND METHODS

Characterization of patients and sera

AD patients ($n=51$) with or without signs of mucosal atopy were diagnosed according to standard procedures (35). AD patients were also characterized by case history, skin-prick testing with environmental allergens, determination of total serum IgE levels, and IgE with specificity for common aeroallergens by CAP (Pharmacia, Uppsala, Sweden) (Table 1). The clinical severity was graded as mild (localized chronic forms with <10% of the body surface involved), moderate (disseminated lesions over trunk and extremities), and severe forms (e.g., generalized eczema) (Table 2). For control purposes, sera from patients with graft vs. host disease (GVHD) and systemic lupus erythematosus as well as sera from nonatopic individuals were included (29).

Phage, *E. coli* strains, plasmids, and expression cDNA libraries

λ gt 11 phage carrying a unique Eco R I cloning site close to the 3' end of the *lac Z* gene and *E. coli* strains Y1090 [*hsd* ($r_k^- m_k^+$) *lac* U169, *ProA*+, *Ion*-, *araD* 139, *StrA*, *Sup* F *trpC22:Tn10*(pMC9)] and Y1089 [*hsd* ($r_k^- m_k^+$) *lac* U169, *Pro*

A+, *Ion*-, *araD* 139, *StrA*, *hflA150* chr:Tn10 (pMC9)] were purchased from Amersham, Buckinghamshire, U.K. (36). Plasmid pUC18 and pUC19 used for subcloning were purchased from Boehringer-Mannheim, Mannheim, Germany. *E. coli* XL-1 blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^qΔM15 Tn10* (Tet^r)]^c were purchased from Stratagene (La Jolla, Calif.). A λ gt 11 expression cDNA library prepared from the human epithelial cell line A431 was purchased from Clontech (Palo Alto, Calif.).

Cell lines, protein extracts, SDS-PAGE, and immunoblotting

Human umbilical vein endothelial cells were cultured and grown as described (37). A human epithelial cell line (A431) established from an epidermoid mamma carcinoma was obtained from American Type Culture Collection (Rockville, Md.). Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 mM β -MET, 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco, Gaithersburg, Md.), and 10% fetal calf serum (Sera Lab, Crawley Down, U.K.). Cells were washed twice in phosphate-buffered saline to remove medium components. Birch (*Betula verrucosa*) pollen extract was prepared from pollen (Allergon, Valinge, Sweden) as described (38). Protein extracts (approximately 200 μ g/cm) were dissolved in sodium dodecyl sulfate (SDS) sample buffer (39), boiled for 5 min, and separated in a 12.5% preparative SDS-polyacrylamide gel (SDS-PAGE). A protein molecular weight marker (Rainbow-Marker, Amersham) was used as a standard. After electrophoretic separation, proteins were electroblotted onto nitrocellulose (Schleicher & Schuell, Dassel, FRG) according to ref 40. For the detection of IgE binding proteins, nitrocellulose strips (5 mm width) were cut from the preparative sheets, blocked twice for 5 min, and once for 30 min in buffer A (50 mM Na phosphate, pH 7.5, 0.5% v/v Tween 20, 0.5% w/v bovine serum albumin, 0.05% NaN₃) at room temperature. Strips were exposed to sera that were diluted 1:10 in buffer A overnight at 4°C and washed as described for blocking; bound IgE was detected with 1:10 in buffer A-diluted, ¹²⁵I-labeled anti-human IgE antibodies (RAST, Pharmacia) as described (41).

IgE immunoscreening of the expression cDNA library

A λ gt 11 expression cDNA library constructed from the human epithelial cell line A431 (Clontech) was screened with serum IgE from two AD patients, one corresponding to #51 in Fig. 1 and Table 1. Both AD patients suffered from severe skin lesions and contained IgE antibodies to a variety of proteins in nitrocellulose-blotted A431 extracts. In brief, 500,000 pfu (plaque-forming units) of the A431 expression cDNA library were plated at a density of 20,000 phage per plate (140 mm diameter) by infecting *E. coli* Y1090 at 43°C. After plaques became visible, the plates were overlaid with nitrocellulose filters (Schleicher & Schuell) that had been soaked in 10 mM IPTG to induce protein synthesis. To reduce serum IgE reactivity to *E. coli*/phage proteins, sera were diluted 1:5 in buffer A and preincubated with nitrocellulose filters containing *E. coli*/ λ gt 11 proteins. After 2 h preadsorption, sera were further diluted in buffer A to a final dilution of 1:10 and exposed overnight at 4°C to nitrocellulose filters containing the plated A431 library. Filter-bound IgE was detected using ¹²⁵I-labeled anti-human IgE antibodies (RAST, Pharmacia). IgE-reactive phage clones were purified to homogeneity by two additional cycles of IgE screening.

TABLE 1. Characterization of atopic dermatitis patients^a

| Patient | Sex | Age | Total IgE | Symptoms | IgE exogenous allergens | IgE human proteins |
|---------|-----|-----|-----------|----------|-------------------------|--------------------|
| 1 | f | 23 | 212 | ad | 0 | - |
| 2 | f | 27 | 3220 | ad rc as | g mi t w f v m | ++ |
| 3 | f | 17 | 1053 | ad rc | g t | - |
| 4 | f | 2 | nd | ad | t g w v | - |
| 5 | f | 36 | 6240 | ad rc | mi t g f a v ab fl | ++ |
| 6 | f | 15 | 1311 | ad | mi a f | +/- |
| 7 | m | 4 | 229 | ad | t g w v | - |
| 8 | f | 63 | 5310 | ad as | mi la a m v | + |
| 9 | f | 40 | 4960 | ad | g v a mi f la t w | ++ |
| 10 | m | 34 | 15,350 | ad | t g la v w | ++ |
| 11 | m | 1 | ,209 | ad | a m v | + |
| 12 | f | 2 | 7 | ad | 0 | - |
| 13 | f | 24 | 12,560 | ad | mi | ++ |
| 14 | m | 28 | 6180 | ad | 0 | ++ |
| 15 | m | 50 | nd | d | 0 | - |
| 16 | f | 56 | 9 | ad | a sh | - |
| 17 | f | 18 | 12,980 | ad rc | a t g mi m w | ++ |
| 18 | f | 27 | 664 | ad | la w v mi f t | - |
| 19 | m | 1 | 110 | ad | a mi | - |
| 20 | f | 59 | 49 | ad | g t a | - |
| 21 | m | 22 | 109,890 | ad rc as | a mi la bla v w | ++ |
| 22 | f | 25 | 984 | ad | a f m mi w v | - |
| 23 | m | 4 | 108 | ad | t g | - |
| 24 | f | 24 | 1099 | ad (p) | g a v | - |
| 25 | f | 30 | 748 | ad | nd | - |
| 26 | f | 22 | nd | ad | nd | + |
| 27 | m | 1 | 14 | ad | a | - |
| 28 | m | 56 | 1416 | ad | mi m a | + |
| 29 | f | 58 | 14 | ad da | 0 | - |
| 30 | f | 23 | 35 | rc hd | g t | - |
| 31 | f | 21 | 5389 | ad eh | g t mi a m | + |
| 32 | f | 24 | 1190 | ad rc as | mi g t v a w | + |
| 33 | m | 19 | 6180 | ad | nd | ++ |
| 34 | f | 22 | 65 | ad | mi | - |
| 35 | f | 34 | nd | d | 0 | - |
| 36 | f | 1 | 415 | ad | g t a mi v | - |
| 37 | m | 57 | 990 | ad rc | mi t g a v f | - |
| 38 | m | 5 | 11,010 | ad | g t a mi m w | ++ |
| 39 | m | 26 | 273 | ad rc as | mi g t a | - |
| 40 | m | 54 | 2019 | ad (p) | v w a | + |
| 41 | m | 4 | nd | ad (s) | mi | - |
| 42 | f | 19 | 2280 | ad | g t w a v mi | + |
| 43 | f | 36 | 1042 | ad | mi g a | +/- |
| 44 | f | 59 | 327 | ad | mi g w a | - |
| 45 | f | 32 | 118 | d rc TIV | a | - |
| 46 | f | 56 | 451 | ad as | mi f g | - |
| 47 | m | 1 | 8 | ad | a | - |
| 48 | m | 13 | 133 | ad rc | g mi a | - |
| 49 | m | 28 | 19 | ad | g | - |
| 50 | f | 24 | 12,560 | ad | mi | ++ |
| 51 | m | 43 | 1756 | ad | t g mi a | ++ |
| N | m | 33 | 26 | 0 | 0 | - |

^a Total IgE is displayed in kU/l; sex: f = female, m = male. Symptoms: ad = atopic dermatitis, ad (p) = atopic dermatitis (prurigo), ad (s) = atopic dermatitis plus seborrhea, da = drug allergy, hd = hand dermatitis, r = rhinitis, c = conjunctivitis, as = asthma, d = dermatitis, eh = eczema herpeticatum, TIV = type IV allergy. IgE to exogenous allergens: g = grass pollen, t = tree pollen, mi = mites, w = weed pollen, f = fungi, v = vegetables and/or fruits, m = milk proteins, a = animal proteins, ab = antibiotics, fl = flour, la = latex, sh = shrimps, bla = cockroach. IgE to human proteins: ++ = strong reactivity, + = positive reactivity, +/- = weak reactivity, - = no reactivity to blotted proteins. Nd = not done; 0 = negative.

Characterization of cDNA clones coding for human IgE autoantigens

Twenty primary IgE-reactive phage clones were obtained by IgE immunoscreening of 500,000 phage. Phage DNA was sub-

jected to restriction analysis using Eco R I and Kpn I/Sac I (42). According to the restriction analysis (insert sizes; internal Sac I or Eco R I sites), different types of clones coding for ara were distinguished. Phage DNA of four clones that differed from each other in their restriction patterns was di-

TABLE 2. Total IgE levels, severity of atopic eczema, and atopic manifestations in patients without and with moderate and intensive IgE autoreactivity^a

| Patient group | Average total IgE | Mild eczema | Moderate eczema | Severe eczema | Resp. atopy (asthma) |
|-----------------|-------------------|-------------|-----------------|---------------|----------------------|
| 0 (n = 29) | 353 | 13 | 12 | 4 | 2 |
| +/-, + (n = 10) | 2084 | 4 | 5 | 1* | 2 |
| ++ (n = 12) | 16,907 | 0 | 8 | 4 | 2 |

^aTotal IgE levels (kU/l) were determined with the CAP system (Pharmacia). Group 0: no Western blot: detectable IgE autoantibodies; group +/-, +: moderate IgE autoreactivity; group ++: intensive IgE autoreactivity. Mild eczema: localized chronic (e.g., flexural) or hand and feet eczema (<10% of the body surface); moderate eczema: disseminated lesions over trunk and extremities; severe eczema: generalized eczema, severe excoriations. *Eczema herpeticum.

gested with Kpn I and Sac I to excise the cDNA inserts flanked by λ gt 11 sequence. This allowed subcloning of the fragments in known orientation and determination of the correct open reading frame (ORF). The restriction fragments were isolated by preparative agarose gel electrophoresis and subcloned into plasmid pUC18/19. The ligation products were transformed into *E. coli* XL-1 blue and positive clones were identified by DNA restriction analysis. The DNA sequence of both strands was determined using λ gt 11 forward and λ gt11 reversed sequencing primers (Clontech) as well as internal sequencing primers (MWG, Ebersberg, Germany), a T7 polymerase sequencing kit (Pharmacia), and ³⁵S dCTP (NEN, Stevenage, U.K.) (43). The correct transfer of the phage cDNA inserts into the plasmids was confirmed by hybridization of the original immunopositive phage clones with the plasmid-derived inserts. All molecular biological methods followed established protocols (42). The cDNA and deduced amino acid sequences of the ara clones were compared with the EMBL/SwissProt library and GenBank using the BLAST program. All DNA and amino acid sequences were analyzed with the MacVector program (Kodak, Rochester, N.Y.) to calculate the ORF, the isoelectric point (pI), secondary structure, presence of protein subsequences, and antigenicity.

RNA isolation and Northern blot analyses

RNA was isolated from human umbilical vein cells and the human epithelial cell line, A431, using the guanidine isothiocyanate method followed by cesium chloride density gradient centrifugation (44). Approximately 20 μ g total RNA was separated by denaturing agarose gel electrophoresis and blotted

onto nitrocellulose (Schleicher & Schuell). The cDNAs coding for IgE autoantigens were isolated by preparative agarose gel electrophoresis and radiolabeled using ³²P-dCTP according to ref 45. Northern blots were hybridized with the ³²P-labeled fragments and washed to a stringency of 0.75 \times SSC, 0.1% SDS, 65°C. Blots were exposed to Kodak X-OMAT films using intensifying screens for 24–48 h.

IgE binding capacity of recombinant IgE autoantigens

The IgE binding capacity of recombinant IgE autoantigens was tested by a non-denaturing plaque assay (46). Nitrocellulose filters containing phage expressing β -gal-fused IgE autoantigens (ara NAC, ara BCL7B, ara CALC, ara KER) and, for control purposes, λ gt11 phage expressing β -gal, were cut into sectors and probed with the two AD sera used for screening of the library; for control purposes, with sera from two graft vs. host, two systemic lupus erythematosus patients, a nonatopic individual, and buffer alone. The total IgE levels (kU/l) in the sera were as follows: 1 (AD): 1756; 2 (AD): 360; 3 (GVHD): 56; 4 (GVHD): 33; 5 (SLE): 1500; 6 (SLE): 521; N: 22. Bound IgE was detected as described for immunoscreening.

Expression and purification of β -gal-fused recombinant IgE autoantigens: production of a rabbit anti-ara BCL7B antiserum

Recombinant β -gal-fused IgE autoantigens and β -gal alone were expressed in lysogenic *E. coli* Y1089. Lysogenic *E. coli* Y1089 were grown in LB medium containing 100 mg/l am-

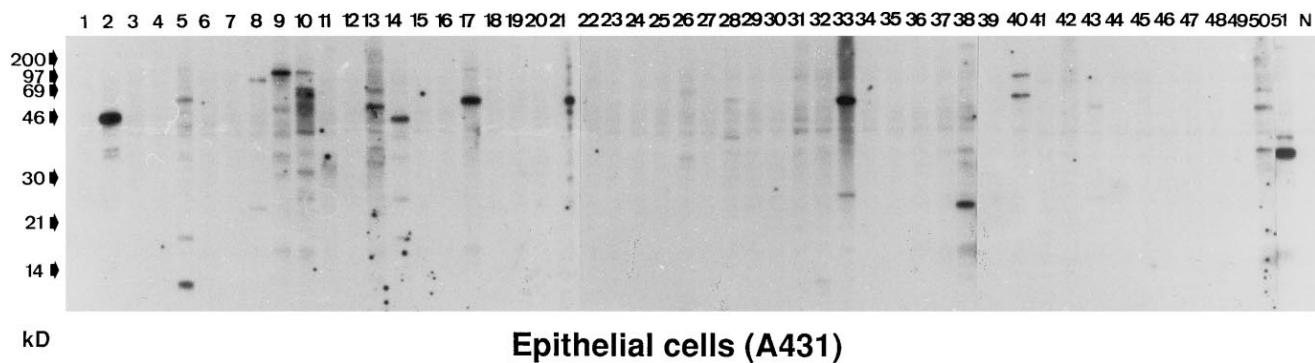


Figure 1. Serum IgE reactivity of AD patients with nitrocellulose-blotted A-431 proteins. Nitrocellulose-blotted human epithelial cell line (A431) proteins were probed with sera from 51 AD patients (lanes 1–51) and a nonatopic control individual (N). The molecular masses are shown in kilodaltons (kD) on the left.

picillin, 0.4% w/v maltose, and 10 mM MgCl₂ at 32°C until an OD 600 of 0.5 was reached. The cultures were then shifted to 42°C for 30 min, and expression of the β-galactosidase fusion proteins was induced at 37°C for an additional hour by adding IPTG to a final concentration of 5 mM. *E. coli* cells containing the recombinant β-gal fusion proteins or β-gal alone were collected by centrifugation at 3000 rpm for 10 min and stored at -20°C. Recombinant ara β-gal fusion proteins or β-gal were purified by affinity chromatography using Sepharose-coupled mouse anti-β-gal antibodies (Protosorb, Promega, Madison, Wis.). Fractions containing purified recombinant proteins were dialyzed against Aqua bidest. and analyzed for protein content and purity by SDS-PAGE.

Purification and analysis of IgE anti-ara BCL7B immune complexes

IgE was affinity purified from serum of an AD patient with (see Fig. 4: AD 1) and a nonatopic individual without recombinant ara BCL7B-specific IgE antibodies using CNBr-Sepharose-coupled monoclonal anti-human IgE antibodies (Pharmacia). Comparable amounts (20 μg/cm gel) of anti-IgE precipitates purified from the two sera were separated by SDS-PAGE and blotted onto nitrocellulose. Nitrocellulose strips were probed with a rabbit anti-ara BCL7B antiserum and, for control purposes, with the rabbits preimmune serum, both diluted 1:1000 in buffer A. Bound rabbit antibodies were detected with a 1:2000 diluted ¹²⁵I-labeled donkey anti-rabbit antiserum (Amersham) and visualized by autoradiography.

Skin-prick testing with recombinant IgE autoantigens

Skin-prick test experiments were performed in two AD patients with (MD, HP) and three nonatopic individuals without (SN, VR, CD) ara-reactive IgE antibodies. Patient MD corresponds to AD 1 in Fig. 4 and #51 in Fig. 1. Purified recombinant β-gal-fused IgE autoantigens and β-gal were diluted to a concentration of 20 μg/ml in 0.9% NaCl for skin testing. Individuals were skin-prick tested on their forearms with 20 μl of each of the recombinant IgE autoantigens β-gal, 0.9% NaCl, histamine (ALK, Horsholm, Denmark), and Timothy grass pollen extract (ALK) as described (47). Skin reactions were recorded 20 min after prick testing by surrounding of the wheal reaction and transfer with Scotch tape to paper. The mean wheal diameter (Dm) of the wheal area was calculated as follows: $Dm = (D1 + D2) / 2$. D1 and D2 represent the maximal transversal and longitudinal diameter of the wheal, respectively.

Detection of ara NAC- and Bet v 1-specific IgE antibodies in serum samples collected from an AD patient over a period of 5 years

Serum samples were obtained from an AD patient (MD) containing ara NAC- and Bet v 1-specific IgE antibodies over 5 years (February 1992–June 1997) by venipuncture. Sera were stored until use at -20°C. Nitrocellulose strips containing equal amounts of blotted recombinant β-gal-fused ara NAC and birch pollen extract were exposed to 1:10 in buffer A-diluted serum samples overnight at 4°C. Bound IgE antibodies were detected with ¹²⁵I-labeled anti-human IgE antibodies (Pharmacia) and visualized by autoradiography.

RESULTS

Atopic dermatitis patients with severe skin lesions display intensive IgE reactivity to nitrocellulose-blotted human epithelial proteins

We tested sera from 51 atopic dermatitis patients with well-documented clinical background for IgE auto-reactivity to nitrocellulose-blotted human epithelial cell extracts (Fig. 1; Table 1). A high percentage of the AD sera (22/51) contained Western blot-detectable IgE autoantibodies. Sera (*n*=12) with the most intensive IgE reactivity to blotted human proteins (++) comprised a group of patients with high average total IgE levels (16907 kU/l); the group of patients (*n*=10) with weaker reactivity (+, +/-) had a lower average total IgE (2084 kU/l) and those AD patients (*n*=29) without Western blot detectable IgE autoantibodies had the lowest average total IgE level (353 kU/l) (Table 2). As observed previously in another group of AD patients (29), IgE reactivity to nitrocellulose-blotted human epithelial proteins was frequently associated with elevated total serum IgE levels but represented a specific and distinct reactivity to certain human proteins. In the previous study (29), the specificity of IgE autoreactivity was confirmed by the fact that hyper-IgE sera from patients without signs of chronic atopy and IgE myeloma supernatants containing high levels of total IgE failed to bind to Western-blotted human proteins as well as by the demonstration that IgE antibodies to blotted human proteins could be specifically preadsorbed with human proteins (29). Results obtained in our blotting experiment (Fig. 1) further support the contention that AD patients display specific IgE auto-reactivity because 1) certain sera with elevated total IgE levels (e.g., serum # 3) failed to bind to human proteins, 2) certain sera with lower total IgE levels (e.g., serum # 2, 51) displayed more intensive binding compared to sera containing much higher levels of total IgE (e.g., serum # 8, 50), and 4) sera reacted individually with bands of various molecular weights (Fig. 1; Table 1).

All 12 patients with intensive IgE reactivity to human proteins suffered from moderate to severe forms of atopic eczema, whereas 13 of the 29 patients without IgE autoreactivity suffered from mild, 12 from moderate, and only 4 from severe atopic eczema (Table 2). We also noted that those AD patients with strong IgE autoreactivity frequently exhibited IgE antibodies against many different environmental allergens (Table 1).

Isolation of cDNA clones coding for IgE autoantigens: sequence analysis of IgE autoantigens reveals identity with intracellular human proteins

An expression cDNA library constructed from human epithelial (A431) cells in phage λgt 11 was

screened with serum IgE from two AD patients with severe skin lesions and IgE autoantibodies detectable by Western blot. Twenty primary IgE-reactive phage clones were obtained after screening 500,000 pfu of the A431 library. According to DNA restriction analysis and sequencing, several atopy-related IgE autoantigens (aras) were identified, of which four are characterized in detail. **Table 3** shows that the ara clones code for fragments of four different intracellular human proteins with important biological functions. Ara NAC displayed sequence identity with the carboxyl-terminal portion of the human α -chain of the nascent polypeptide complex (NAC) (Table 3), a protein required for signal sequence-specific sorting and translocation of intracellular proteins (30). It contained an ORF of 279 nucleotides coding for a carboxyl-terminal polypeptide of 10.3 kDa and a predicted pI of 9.55. The sequence identity of ara NAC with cDNA clones that had been isolated during random sequencing projects from skeletal muscle and hepatocytes indicates that ara NAC is expressed in histogenetically unrelated tissues (Table 3).

The ara BCL7B cDNA showed sequence identity with the human BCL7B protein, possibly representing an oncogene (34) and several human cDNAs isolated from gall bladder, skeletal muscle, placenta, liver, and the ocular ciliary body (Table 3). The ORF of ara BCL7B comprised 546 nucleotides coding for a polypeptide of 20.1 kDa and a predicted pI of 4.22 (Table 3).

The cDNA sequence of ara CALC (**Fig. 2**; Table 3) displayed identity with expressed human sequence tags that were isolated from keratinocytes, brain, lung, breast, liver, and heart. Although to date no biological function has been described for this protein, we found a typical calcium binding domain (DLNGDGEVDMEEF) in the amino-terminal portion of its deduced amino acid sequence. The ara CALC cDNA contained an ORF of 939 nucleotides and coded for a 36 kDa polypeptide with a pI of 8.1 (Table 3).

The ara KER cDNA coded for the central helical portion of human cytokeratin type II (Table 3), a prominent component of the mammalian cytoskele-

ton that is responsible for the formation of intermediate filaments in epithelial tissues (31–33). An ORF of 1119 nucleotides coding for a 42.6 kDa polypeptide with a pI of 4.96 was found in the ara KER encoding cDNA. The amino acid sequence deduced from the ara KER ORF contained a helix-loop-helix motif (KAATLTDEINFLRAL), an intermediate filament motif (IATYRKLLE), and a leucine zipper motif (LKDAKNKLEGLEDALQKAKQ).

Transcripts for IgE autoantigens are detected in human epithelial and endothelial cells

RNA blots prepared from the human epithelial cell line A431 and from human umbilical vein cells were probed with the ara cDNAs (ara NAC, ara BCL7B, ara CALC, ara KER) (**Fig. 3**). The ara NAC cDNA hybridized with epithelial and endothelial cell RNA at approximately 900 nucleotides. The ara BCL7B cDNA hybridized with both RNA preparations at approximately 1900 nucleotides. Bands of approximately 1800 and 3500 nucleotides, the latter presumably representing a pre-mRNA form, could be detected in A431 cells with the ara CALC cDNA. No clear signal was obtained when endothelial cell RNA was probed with the ara CALC cDNA (data not shown). The ara KER cDNA coding for the conserved central helical portion of cytokeratin type II hybridized with epithelial as well as endothelial cells of approximately 2100 nucleotides. An additional band of approximately 1500 nucleotides was detected in epithelial RNA, representing a possible degradation product or a homologous mRNA. A similar hybridization pattern was reported earlier for human type II mesothelial keratin (32). With the exception of ara CALC, all ara cDNAs hybridized to mRNA of epithelial and endothelial cells with comparable intensity. The sequence identity of ara CALC with cDNAs isolated from human keratinocytes, brain, lung, liver, and heart (Table 3) indicates, however, that ara CALC is also expressed in a variety of histogenetically unrelated tissues.

TABLE 3. Characterization of ara clones^a

| Clone | ORF | Mol wt | pI | Sequence identity with |
|-----------|------|--------|------|--|
| ara NAC | 279 | 10.3 | 9.55 | α -chain NAC (X80909); skeletal muscle (Z28479); hepatocyte (D12194) |
| ara BCL7B | 546 | 20.1 | 4.22 | BCL7B (X89985); BCL7A (X89984); gall bladder (T32620); skeletal muscle (Z19236 Z19237); placenta (R78288 R63145 R78289 R31642 R32359); fetal liver/spleen (T86806 R93746 H49818 T86711 H49562); ocular ciliary body (R88359) |
| ara CALC | 939 | 36 | 8.1 | Keratinocytes (D29420); brain (Z43480 T03435 H24195 H14111 R67168 R172270, R41944); lung (T90587); breast (R69536); fetal liver (R87799 R87736); heart (T32765); unknown tissue (T57172, T57104) |
| ara KER | 1059 | 42.6 | 4.96 | Cytokeratin type II (P02538) |

^a GenBank accession numbers and sources of cDNA clones with significant sequence identity are listed. The lengths of the ORFs are given in nucleotides and the molecular masses of the deduced proteins in kilodaltons. The pI was calculated for the IgE binding fragment encoded by the ORF.

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gaa ttc CGG GGT CTG GAT CAA TAT ATA ATA AAA CGC TTT GAT GGA AAG AAA ATT TOC CAG GAA CGA GAA AAA TTT GCT GAT GAA 84
Arg Gly Leu Asp Gln Tyr Ile Ile Lys Arg Phe Asp Gly Lys Lys Ile Ser Gln Glu Arg Glu Lys Phe Ala Asp Glu

GGC AGT ATA TTT TAC ACC CTT GGA GAA TGT GGG CTC ATA TOC TTT TCA GAC TAC AIT TTC CTC ACA ACT GTT CTT TOC ACT CCT 168
Gly Ser Ile Phe Tyr Thr Leu Gly Glu Cys Gly Leu Ile Ser Phe Ser Asp Tyr Ile Phe Leu Thr Thr Val Leu Ser Thr Pro

CAG AGA AAT TTT GAA AIT GCC TTC AAG ATG TTT GAT TTG AAT GGA GAT GGA GAA GTA GAT ATG GAA GAA TTT GAA CAG GTT CAG 252
Gln Arg Asn Phe Glu Ile Ala Phe Lys Met Phe Asp Leu Asn Gly Asp Gly Glu Val Asp Met Glu Glu Phe Glu Gln Val Gln

AGC ATC ATT CGC TOC CAA ACC AGT ATG GGT ATG CGC CAC AGA GAT CGT CCA ACT ACT GGC AAC ACC CTC AAG TCT GGC TTG TGT 336
Ser Ile Ile Arg Ser Gln Thr Ser Met Gly Met Arg His Arg Asp Arg Pro Thr Thr Gly Asn Thr Leu Lys Ser Gly Leu Cys

TCA GCC CTC ACA ACC TAC TTT TTT GGA GCT GAT CTG AAG GGA AAG CTG ACA ATC AAA AAC TTC CTC GAA TTT CAG CGT AAA CTG 420
Ser Ala Leu Thr Thr Tyr Phe Phe Gly Ala Asp Leu Lys Gly Lys Leu Thr Ile Lys Asn Phe Leu Glu Phe Gln Arg Lys Leu

CAG CAT GAT GTT CTG AAG CTT GAG TTT GAA CGC CAT GAC CCT GTG GAT GGG AGA AIT ACT GAG AGG CAG TTT GGT GGC ATG CTA 504
Gln His Asp Val Leu Lys Leu Glu Phe Glu Arg His Asp Pro Val Asp Gly Arg Ile Thr Glu Arg Gln Phe Gly Gly Met Leu

CTT GCC TAC AGT GGG GTG CAG TOC AAG AAG CTG ACC GCC ATG CAG AGG CAG CTC AAG AAG CAC TTC AAA GAA GGA AAG GGT CTG 588
Leu Ala Tyr Ser Gly Val Gln Ser Lys Lys Leu Thr Ala Met Gln Arg Gln Leu Lys Lys His Phe Lys Glu Gly Lys Gly Leu

ACA TTT CAG GAG GTG GAG AAC TTC TTT ACT TTC CTA AAG AAC ATT AAT GAT GTG GAC ACT GCA TTG AGT TTT TAC CAT ATG GCT 672
Thr Phe Gln Glu Val Glu Asn Phe Phe Thr Phe Leu Lys Asn Ile Asn Asp Val Asp Thr Ala Leu Ser Phe Tyr His Met Ala

GGA GCA TCT CTT GAT AAA GTG ACC ATG CAG CAG GTG GCC AGG ACA GTG GCT AAA GTG GAG CTC TCA GAC CAC GTG TGT GAT GTG 756
Gly Ala Ser Leu Asp Lys Val Thr Met Gln Gln Val Ala Arg Thr Val Ala Lys Val Glu Leu Ser Asp His Val Cys Asp Val

GTG TTT GCA CTC TTT GAC TGT GAT GGC AAT GGC GAA CTG AGC AAT AAG GAA TTT GTT TOC ATC ATG AAG CAA CGG CTG ATG AGA 840
Val Phe Ala Leu Phe Asp Cys Aso Gly Asn Gly Glu Leu Ser Asn Lys Glu Phe Val Ser Ile Met Lys Gln Arg Leu Met Arg

GGC CTG GAA AAG CCC AAA GAC ATG GGT TTC ACT CGC CTC ATG CAG GCC ATG TGG AAA TGT GCA CAG GAA ACT GCC TGG GAC TTC 924
Gly Leu Glu Lys Pro Lys Asp Met Gly Phe Thr Arg Leu Met Gln Ala Met Trp Lys Cys Ala Gln Glu Thr Ala Trp Asp Phe

GCT TTA CCC AAA CAG TAA 942
Ala Leu Pro Lys Gln *
ccc cac act gca aga ggg gac ccc tcc acc ccc agt acc ctg gac ccc ctc cgc aga gtc tog gca gag ccc ttt gtg ctg ctg 1026
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ctg ctg ggc tct gat tct gcc caa tga gta tcc coa tag gtt ctc aaa aac atg aac aag tog tgt aaa gct cag aca ttt gtc 1194
agc ctc aac agc acc acc cat tca agc atc ctg tgg ata aag aat tca ggg aac cat cca cac acc tgc caa ccc tgg gaa gca 1278
tcc agt tct caa atc gtt ttt gct atg gat tta tac taa caa gaa cat tcc ttg act tcc ctc ctg ctg gtg ttt taa agc cac 1362
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gag aag cct gog ccc cag gaa gga tct gtg tta gtc cct ggg atg gct oca agg oct gct cta gga agg cag cat gct cag tgg 1530
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ctc cag tgt gac cct gtg ctt agt gag caa tag tga ttg agc tca tgt tcc ctg caa tgt gcc att tcc tct cca gga tgg gcc 1698
tct aaa gct gag gcc tgg ctc aga gcc tgt ttg ccc tct gtc tta aac aat tgt aaa tat cac tta aat tat aac cat ttg caa 1782
taa aca tcc cca aag tta aaa aaa aaa aaa aaa cga att c 1822

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Figure 2. cDNA and deduced amino acid sequence of ara CALC. The DNA sequence of the open reading frame is printed in capital letters and has been aligned with the deduced amino acid sequence. The Eco R I cloning sites are printed in italics and the stop codon is indicated by an asterisk. A calcium binding motif is underlined. The cDNA sequence of ara CALC has been submitted to the EMBL database under the accession number Y17711.

Recombinant β -gal-fused IgE autoantigens specifically bind IgE antibodies from AD patients

Sera from two AD patients (AD: 1, 2) and, for control purposes, from two patients with GVHD (3, 4), two with systemic lupus erythematosus (SLE: 5, 6), and a nonatopic individual (N), were tested for IgE reactivity to filter-bound recombinant β -gal-fused IgE autoantigens (ara NAC, ara BCL7B, ara CALC, ara KER) or β -gal (β -gal) alone (**Fig. 4**). Sera from the two AD patients displayed IgE reactivity of varying intensity to recombinant IgE autoantigens. Serum 1 reacted most strongly with recombinant ara NAC and much more weakly with ara BCL7B, ara CALC, and ara KER. Serum 2 bound most intensively to ara CALC and weakly to the other three autoantigens. No IgE reactivity of the AD sera with phage expressing β -gal only was

observed. Sera from the GVHD, SLE patients, and the nonatopic person failed to display IgE reactivity to the recombinant IgE autoantigens or β -gal, although total IgE levels in the two SLE sera were comparable to those of the AD sera (Materials and Methods; Fig. 4).

Ara BCL7B is released into the circulation and can occur in serum complexed to IgE autoantibodies

To investigate whether cell-bound intracellular IgE autoantigens could be released into the circulation, we raised a rabbit antiserum against purified recombinant ara BCL7B. IgE antibodies were isolated from the serum of an AD patient (**Fig. 5A**) with and a nonatopic person (**Fig. 5B**) without recombinant ara BCL7B-reactive IgE by using Sepharose-coupled monoclonal anti-human IgE antibodies. Comparable

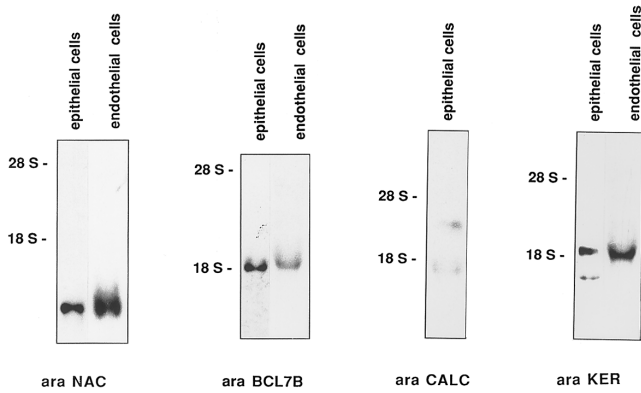


Figure 3. Expression of transcripts coding for IgE autoantigens in A 431 cells and human umbilical vein cells determined by Northern blotting. The position of the 28S and 18S ribosomal RNAs is indicated.

amounts of anti-IgE precipitates obtained from both individuals were separated by SDS-PAGE and blotted onto nitrocellulose. The rabbit anti-ara BCL7B anti-serum specifically bound to a 22–23 kDa moiety in nitrocellulose-blotted anti-IgE precipitates of the AD but not of the nonatopic individual (Fig. 5, lanes I). The molecular weight of the rabbit anti-ara BCL7B-reactive product corresponded to the molecular weight described for the complete BCL7B protein (34). The rabbit preimmune serum failed to react with the anti-IgE-precipitates (Fig. 5, lanes P).

Detection of increased IgE anti-ara NAC antibody levels in an AD patient during periods of disease exacerbation

To determine whether IgE autoreactivity may vary during the course of the disease, we analyzed 12 serum samples from an ara NAC-reactive AD patient that had been collected over a period of 5 years for the presence of ara NAC-specific IgE antibodies. Serum samples were exposed to comparable amounts of nitrocellulose-blotted recombinant β -gal-fused ara NAC and a seasonal exogenous allergen (Bet v 1, the major birch pollen allergen) (48) (Fig. 6). Although IgE anti-Bet v 1 reactivity was increased after the birch pollen seasons (October 1992; May, August 1993; June 1997), most pronounced IgE anti-ara NAC antibody reactivity was observed by the end of winter (February 1993; February 1995; January 1997), when the AD patient had experienced exacerbations of the disease (Fig. 6). Overall, IgE anti-ara NAC reactivity appeared to be stronger in serum samples obtained during the last 4 years of disease (Fig. 6).

Purified recombinant IgE autoantigens elicit immediate type skin reactions

Recombinant β -gal-fused IgE autoantigens (ara NAC, ara BCL7B, ara CALC, ara KER) as well as β -gal alone were expressed in *E. coli* Y1089 and affinity purified

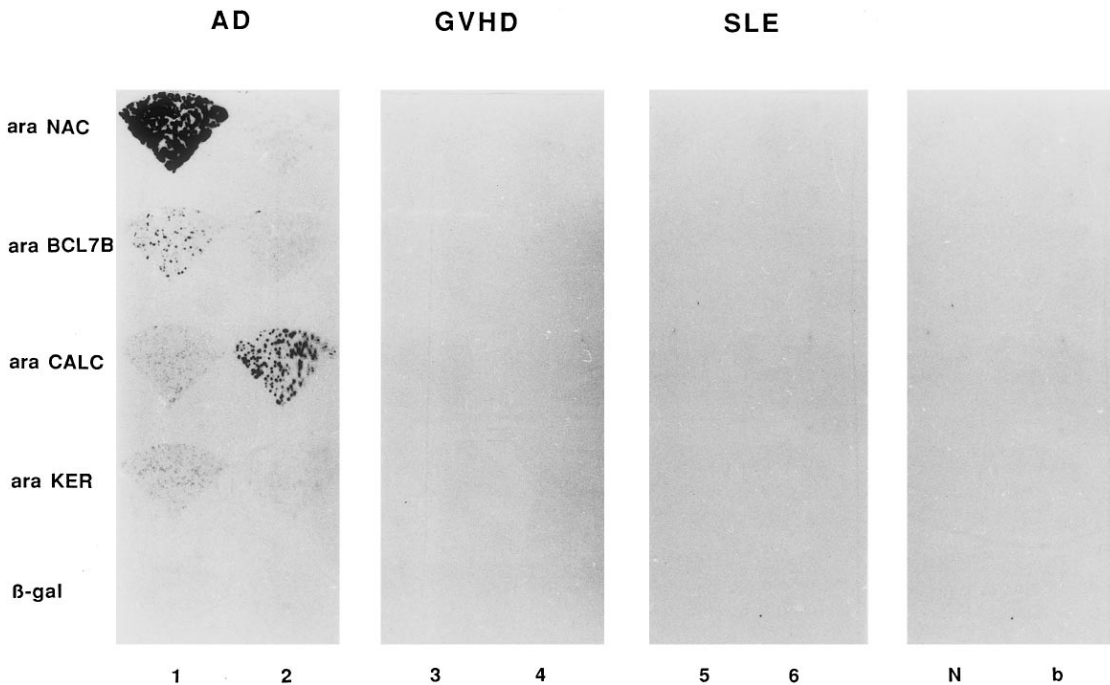


Figure 4. IgE binding capacity of recombinant β -gal-fused IgE autoantigens (ara NAC, ara BCL7B, ara CALC, ara KER), and β -gal. Nitrocellulose sectors containing λ gt11 phage expressing β -gal-fused recombinant human IgE autoantigens and β -gal were incubated with serum IgE from two AD (1, 2), two GVHD (3, 4), two SLE patients (5, 6), and a nonatopic individual (N) or buffer (b) without addition of serum. The total IgE levels (kU/l) in the sera were as follows: 1: 1756; 2: 360; 3: 56; 4: 33; 5: 1500; 6: 521; N: 22. Bound IgE antibodies were detected with 125 I-labeled anti-human IgE antibodies.

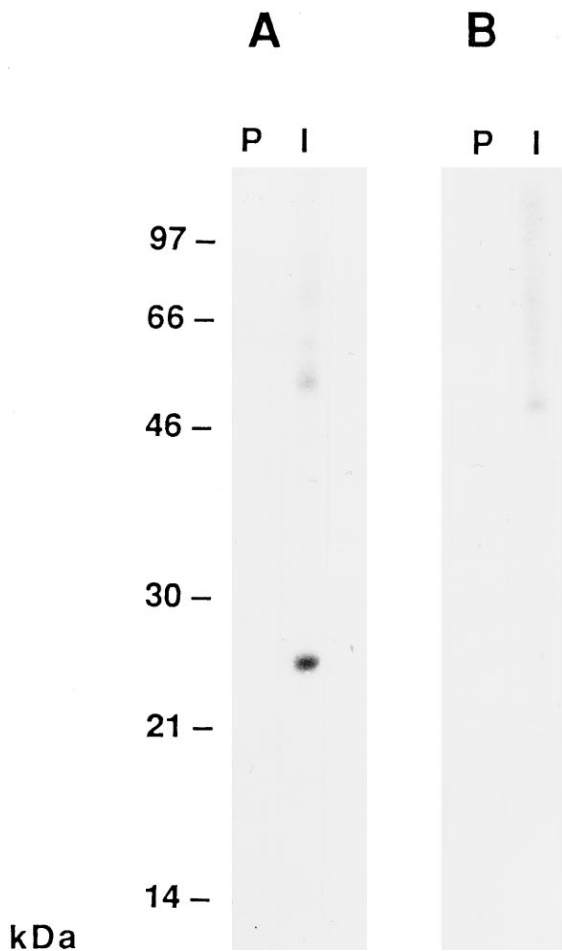


Figure 5. Presence of IgE anti-ara BCL7B immune complexes in serum from an AD patient. Comparable amounts of Western-blotted anti-IgE precipitates from an AD patient containing anti-ara BCL7B-reactive IgE (A) and a nonatopic individual (B) were exposed to a rabbit anti-recombinant ara BCL7B antiserum (lanes I) or rabbit preimmune serum (P). Bound rabbit antibodies were detected with ^{125}I -labeled donkey anti-rabbit antiserum and visualized by autoradiography.

using Sepharose-coupled anti- β -gal monoclonal antibodies. Recombinant ara NAC, ara BCL7B, ara CALC, and ara KER elicited immediate type skin reactions in an atopic dermatitis patient (MD) containing ara-specific IgE antibodies (Table 4). Recombinant ara NAC and ara BCL7B induced skin reactions

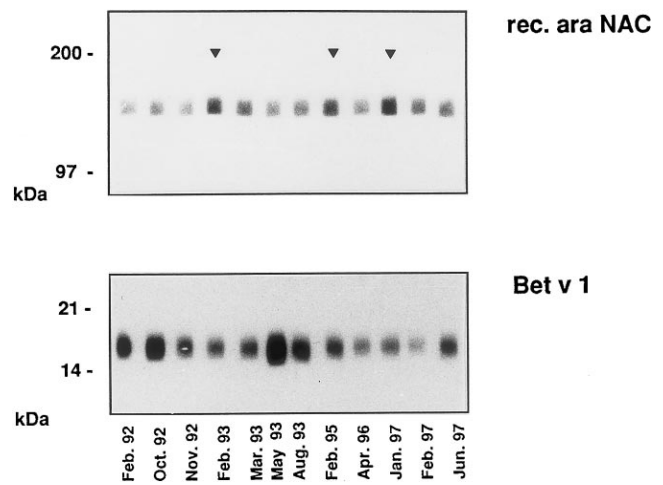


Figure 6. Monitoring of IgE anti-recombinant ara NAC and anti-Bet v 1 antibodies in serum samples of an AD patient collected over a period of 5 years. Equal amounts of nitrocellulose-blotted recombinant β -gal-fused ara NAC (rec. ara NAC) and Bet v 1 (Bet v 1) were exposed to serum samples collected between February 1992 and June 1997. Bound IgE antibodies were detected with ^{125}I -labeled anti-human IgE antibodies and visualized by autoradiography. Molecular masses (kDa) are displayed in the left margin. Peaks of IgE anti-ara NAC reactivity in February 1993, February 1995, and January 1997 were indicated by arrows.

in another AD patient (HP) containing ara NAC and ara BCL7-specific IgE, whereas β -gal and sodium chloride failed to elicit skin reactions in the AD patients (MD, HP) and nonallergic persons (SN, VR, CD) (Table 4). Timothy grass pollen extract induced wheal reactions only in patients allergic to Timothy grass (MD, HP). Histamine led to immediate type skin reactions in all individuals tested (Table 4).

DISCUSSION

Recently we demonstrated that sera from AD patients, but not from patients suffering from other immunologically mediated diseases, contained IgE autoantibodies against a variety of human proteins that were expressed in histogenetically unrelated cell types (29). In the present study we extend the hy-

TABLE 4. Induction of immediate skin reactions with purified recombinant IgE autoantigens^a

| Individual | ara NAC | ara BCL7B | ara CALC | ara KER | β -gal | NaCl | Histamine | Timothy |
|------------|---------|-----------|----------|---------|--------------|------|-----------|---------|
| MD | 5.5 | 8 | 7 | 5 | 0 | 0 | 7.5 | 14.5 |
| HP | 4 | 5.5 | 0 | 0 | 0 | 0 | 5 | 18.8 |
| SN | 0 | 0 | 0 | 0 | 0 | 0 | 7.5 | 0 |
| VR | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |
| CD | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 |

^a Results represent the mean wheal diameters in mm (Dm). The initials of the AD patients (MD, HP) and nonallergic control persons (SN, VR, CD) are listed.

pothesis that IgE autoimmunity may play a pathogenetic role in AD. By clinical and serological characterization of 51 AD patients, we demonstrate that AD patients with intensive IgE reactivity to human proteins tended to suffer from more severe manifestations of atopy. Next, we screened a human epithelial expression cDNA library with serum IgE of two AD patients to reveal the molecular nature of IgE autoantigens. The sequence analysis of four IgE-reactive clones identified intracellular human proteins with important biological functions as atopy-related IgE autoantigens. The IgE autoantigens included 1) ara NAC, a protein belonging to the nascent polypeptide-associated complex (30), which is involved in signal sequence-specific sorting of proteins, 2) ara BCL7B, a potential oncogene (34), 3) ara CALC, a protein containing a typical calcium binding motif that is expressed in a variety of tissues, and 4) ara KER, cyto-keratin type II, a component of the cytoskeleton and intermediate filament system (31–33). Common features of the IgE autoantigens described in our study were their mostly intracellular occurrence, the fact that they shared no significant sequence homology with known exogenous allergens, and their expression in histogenetically unrelated human cells (e.g., epithelial, endothelial cells) and tissues.

Although recognized occasionally by IgG from atopic as well as nonatopic individuals (data not shown), recombinant IgE autoantigens specifically bound IgE antibodies from AD patients but not from sera of patients suffering from other immunologically mediated disorders with partially elevated IgE levels (systemic lupus erythematosus). Results obtained with the recombinant IgE autoantigens thus confirm our earlier observation based on Western blotting (29) that AD patients show specific and distinct IgE reactivity to a variety of human proteins. The IgE autoantigens defined, though mostly representing intracellular components, may become available to IgE antibodies after tissue damage. Those abundantly expressed in skin (e.g., ara KER) may be liberated at the sites of tissue damage (skin lesions), whereas others (ara NAC, ara BCL7B, ara CALC) that occur in a variety of tissues may be transported complexed to IgE autoantibodies to sites containing Fc ϵ receptor-expressing cells. This assumption gained support from our demonstration that ara BCL7B, besides being cell bound, was detected in serum from an ara BCL7B-reactive AD patient complexed to IgE autoantibodies. Binding of IgE autoantigen immune complexes to effector cells (e.g., mast cells, basophils, eosinophils) may then lead to release of biological mediators and immediate type symptoms. The latter is supported by our finding that recombinant β -gal-fused IgE autoantigens, but not β -gal alone, induced immediate type skin reactions in two AD patients containing ara-specific IgE autoantibodies. In addition to

the elicitation of immediate type symptoms, IgE autoantibodies may also contribute to the typical delayed type skin manifestations in AD if IgE autoantigen immunocomplexes are presented to T cells by professional antigen-presenting cells (e.g., dendritic cells). For exogenous allergens, it has been shown that T cells are more efficiently activated when antigen is presented via Fc receptors (12–14), but experiments with IgE autoantigens will have to await the availability of monoclonal human IgE autoantibodies and specific T cell clones.

Evidence for the association of IgE autoantibody formation and disease severity came from the analysis of serum samples collected from an AD patient over a period of 5 years. IgE anti-ara NAC antibody levels increased during disease exacerbation, whereas IgE against an exogenous allergen (Bet v 1) peaked after seasonal allergen exposure. Recombinant IgE autoantigens may serve as paradigmatic tools to study the role of IgE autoimmunity in severe forms of atopy and allow us to monitor the course of the autoimmune-mediated component of tissue damage and, perhaps, design immunological strategies of disease intervention. FJ

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Note added in proof: Another IgE autoantigen recently described (Valenta, R., Natter, S., Seiberler, S., Wichlas, S., Maurer, D., Hess, M., Pavelka, M., Grote, M., Ferreira, F., Szepfalusi, Z., Valent, P., and Stingl, G. Molecular characterization of an autoallergen, Hom s 1, identified by serum IgE from atopic dermatitis patients. *J. Invest. Dermatol.*, in press) was designated Hom s 1 according to the International Allergen Nomenclature. Accordingly, ara NAC, ara BCL7B, ara CALC, and ara KER are submitted to the Allergen Nomenclature as Hom s 2–Hom s 5.

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