

Serum IgE Autoantibodies Target Keratinocytes in Patients with Atopic Dermatitis

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Previous studies have shown that sera of patients with severe atopic dermatitis (AD) contain IgE specific for self-proteins, supporting the hypothesis of autoreactivity as a pathogenic factor in AD. In this study, we screened a large panel of AD patients ($n=192$) by western blotting (WB) for IgE reactivity not only against the human epithelial cell line A431 but also against primary keratinocytes (KCs). To investigate autoantigenic cell structures in detail, normal human skin and primary KCs were incubated with sera from both WB-reactive patients and, for control purposes, healthy individuals, and analyzed by immunohistology, confocal laser microscopy, and flow cytometry. Our analysis revealed that 28% of AD patients, but not healthy individuals, display serum IgE autoreactivity by WB analysis. The individual IgE reaction patterns of the sera pointed to the existence of unique as well as common specificities against epidermal or A431-derived proteins. Immunostainings identified cytoplasmic and, occasionally, also cell membrane-associated moieties as targets for autoreactive IgE antibodies. Interestingly, in certain autoreactive patients, the surface-staining pattern was accentuated at cellular contact sites. We conclude that IgE autoreactivity is common, particularly among severe AD patients, and that non-transformed primary cells are needed for characterization of the entire spectrum of IgE-defined autoantigens.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease occurring in 5–20% of children and 1–3% of adults. Allergen studies indicate that approximately 80% of AD patients have elevated levels of total and exoallergen-specific IgE, which is directly associated with the severity of disease (Tan *et al.*, 1996). Moreover, there exists a small proportion of patients with the typical signs of AD in which sensitization to a broad panel of exoallergens is not detectable by *in vitro* or *in vivo* tests (Schmid-Grendelmeier *et al.*, 2001). This finding supports the involvement of either a non-immunological or an autoreactive pathomechanism in the perpetuation of the clinical manifestations.

In fact, in the past decade autoreactive IgE antibodies against self-proteins have been identified in AD (Valenta *et al.*, 1996; Natter *et al.*, 1998; Fluckiger *et al.*, 2002). Studies

concerning endogenous antigens as triggers in AD have shown that sera from atopic patients, particularly those from patients with severe AD, contain IgE antibodies against human proteins expressed in histogenetically different cell types, most importantly in epithelial cells (Valenta *et al.*, 1996; Natter *et al.*, 1998; Seiberler *et al.*, 1999a,b). Some of the IgE-defined epithelial autoantigens (for example, Hom s 1–5) have been identified and characterized at the molecular level (Mossabeh *et al.*, 2002). These autoantigens are intracellular, mostly cytoplasmic proteins (Natter *et al.*, 1998), suggesting that their release from damaged tissue would trigger responses mediated by IgE or T cells (Valenta *et al.*, 1998) in AD patients.

Recombinant human autoallergens characterized so far result from immunoscreenings of an epithelial carcinoma cell line (A431) (Seiberler *et al.*, 1999a,b), which may not perfectly reflect the physiological repertoire of proteins expressed in the epidermis. Skin-derived autoantigens potentially relevant for the disease could thus have been overlooked. Therefore, we wanted to analyze the range of IgE autoreactivities against primary epidermal cell-derived proteins and to compare them with those against A431-derived proteins. Furthermore, we wished to discover against which cell type, subcellular structure, or non-cellular formations of the skin the IgE-autoantibodies were directed. By doing so, we sought to build a basis for future investigations of their molecular nature.

RESULTS

Serum IgE autoreactivity is present in one fourth of AD patients

In a first set of experiments, we analyzed the prevalence of epithelial cell-reactive serum IgE autoantibodies in a large

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Abbreviations: A431, epithelial tumor cell line; Ab, antibody; AD, atopic dermatitis; EASI, eczema area and severity index; HRP, horseradish peroxidase; IGA, Investigators' Global Assessment Score; IH, immunohistology; KC, keratinocyte; WB, western blotting

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study population of AD patients. A total of 192 patient sera were screened by western blotting (WB) with protein extracts derived from the epithelial cell line A431 and with epidermal extracts from the skin of one healthy donor. A serum was considered “autoreactive” if the IgE contained in it recognized at least one band in one of the protein extracts used. Healthy individuals completely lacked serum IgE reactivity against the above-mentioned protein extracts, whereas approximately one-fourth (28%, $n=54$) of all AD patients exhibited IgE reactivity against both epithelial extracts (Figure 1). IgE autoreactivity against proteins derived from normal dermis was absent in all tested sera (data not shown), probably due to the cellular paucity of this tissue. IgE autoreactivity against primary epidermal extracts was irrespective of the healthy skin donor, indicating ubiquitous occurrence of autoantigens in healthy human skin. (Figure S1).

IgE autoreactivity against epidermis-derived proteins is clearly associated with elevated total serum IgE and the severity of disease

Next, we set out to identify common clinical features of autoreactive AD patients. Statistical analysis of the clinical data revealed that autoreactive AD patients exhibit significantly ($P<0.001$) higher total serum IgE levels when compared with non-autoreactive AD patients. Despite this significant correlation between IgE autoreactivity and high total serum IgE levels ($>1,000\text{ IU ml}^{-1}$), there also existed several AD patients with elevated serum IgE levels ($<1,000\text{ IU ml}^{-1}$) who did not show autoreactivity against epithelial proteins. These patients provide an internal control and indicate that IgE autoreactivity does not occur as a non-specific phenomenon due to high IgE levels.

Disease activity, as determined by the Investigators' Global Assessment (IGA) Score and the Eczema Area and Severity Index (EASI), was also significantly ($P<0.001$) higher

in autoreactive patients than in non-autoreactive ones (Figure 2). These data are in agreement with earlier observations showing that AD patients with serum IgE autoreactivity form a distinct subpopulation of patients with severe disease and considerably higher total serum IgE (Natter *et al.*, 1998). In contrast to this observation, we were not able to find a significant correlation within the group of autoreactive AD patients between the intensity of the WB reaction or a special recognized moiety on the one hand and the EASI and IGA on the other (data not shown), indicating that IgE autoreactivity is an important but not the only relevant factor in this complex skin disease.

Interestingly, all of the IgE-autoreactive patients met the criteria of the extrinsic-type AD (that is, increased serum IgE and/or sensitization to environmental allergens; Wollenberg *et al.*, 2000), whereas in the group of the non-autoreactive AD patients 17% had a characteristic intrinsic-type AD (Schmid-Grendelmeier *et al.*, 2001; Novak and Bieber, 2003).

Comparison of the reactivity against epidermal protein extracts and A431 extracts

To investigate which protein extract is best suited for the detection of autoreactivity in AD patients, we compared reactivity against A431 with that against proteins extracted from freshly isolated keratinocytes (KCs) by WB. Of the 54 patients with IgE autoantibodies, 45 (=83%) recognized primary epidermis-derived proteins and 42 (=78%) recognized A431-derived proteins. Although most AD sera (61%) reacted with antigens in both the extracts (Table 1), 12 AD patients (=22%) reacted exclusively with extracts from epidermal tissue and nine (=17%) exclusively with A431 extracts. These data indicate that potentially autoreactive patients could have been overlooked if the screening was solely performed with protein extracts derived from the cell line A431 and that a maximum detection can be achieved by

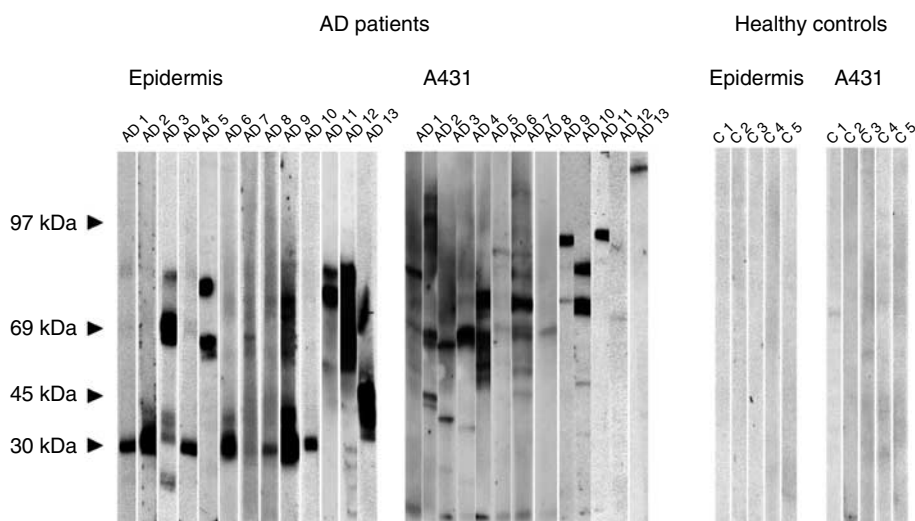


Figure 1. Representative WB with sera of AD patients and healthy controls. Protein extracts of primary human epidermis or the epithelial cell line A431 were separated by SDS-PAGE, blotted onto nitrocellulose-membranes, cut into strips, blocked, and incubated with individual sera of patients (AD1–13) or healthy control individuals (C1–5); serum IgE reactivity was detected using HRP-conjugated mouse anti-human IgE mAb.

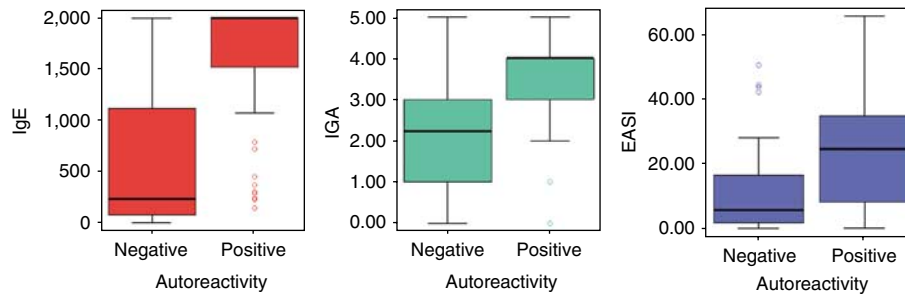


Figure 2. Box plot of serum autoreactivity and total serum IgE, IGA score, and EASI score. Autoreactive and non-reactive AD patients were correlated with the total serum IgE and clinical scores IGA and EASI. The black bar indicates the median score or IgE level; the margins of the colored boxes indicate the 25th and 75th percentile; the whiskers indicate 1.5 interquartile distance; and the open circles show mavericks (outside 1.5 interquartile distance). All three statistical analyses revealed $P < 0.001$ as defined by the Mann-Whitney-U-test and are thus statistically significant.

Table 1. Statistical analysis of the autoreactive AD patients

Autoreactivity against	% of autoreactive AD patients (n=54)	Number of tested persons (n)	Intrinsic type of AD (n)	Mean serum IgE (IU ml ⁻¹) (SD)	Mean IGA (SD)	Mean EASI (SD)
A431 and epidermis	61	33	0	1,788* (± 463.6)	3.4* (± 1.2)	22.2* (± 17.2)
A431 alone	17	9	0	1,551* (± 775.2)	3.5* (± 1.4)	25.9* (± 16.3)
Epidermis alone	22	12	0	1,265* (± 710.2)	3.6* (± 1.0)	28.9* (± 9.5)
Non-autoreactive	0	138	23	681 (± 835.2)	2.4 (± 1.2)	10.0 (± 10.6)

AD, atopic dermatitis; EASI, eczema area and severity index; IGA, Investigators' Global Assessment score.

*No statistically significant differences between the three subgroups of AD patients.

a combination of both protein extracts. There was no difference in the clinical scores and the IgE levels within the autoreactive AD patients detected by the two distinct antigenic sources (Table 1).

A detailed analysis of the band pattern in the western blots revealed that the IgE-autoreactive moieties in epidermal extracts differed in their molecular weights from those identified in A431 extracts by the very same sera. When analyzing interindividual serum reactivity against cell line A431, we could not detect prevalently recognized moieties among autoreactive AD patients. In contrast, when using primary epidermal extracts as an antigenic source, we were able to identify a common band of approximately 30 kDa in 60% of all autoreactive AD patients (Figure 1).

Autoantigens are expressed by KCs in the skin

Next, we sought to determine which cells of the skin express IgE-defined autoantigens. For this purpose, frozen sections of normal human skin were incubated with the sera of 15 autoreactive AD patients or five healthy controls. Serum IgE that bound to the sections was detected with an anti-IgE antibody. We found that six of 15 patients, but none of the healthy individuals, showed detectable IgE reactivity against normal human epidermis. Figure 3 shows representative examples of such reaction patterns. Similar to the results obtained by WB, we detected an interindividually heterogeneous IgE-binding pattern. For example, serum IgE of AD patient I showed predominantly nuclear labeling, whereas that from patient II was preferentially bound to membrane-associated structures. In total, two of the 15 AD patients had

clearly detectable serum IgE reactivity against the cell periphery of KCs.

Next, primary undifferentiated KCs were incubated with sera of the six AD patients, which had shown epidermal IgE binding by immunohistology (IH), and sera of two healthy controls. Using immunofluorescence microscopy, we were able to confirm the results derived from IH and showed that serum IgE binding is most pronounced at sites of cellular contact in the two patients with IgE reactivity against membrane structures (Figure 3). This indicates that junctional structures may be targets for autoreactive IgE of certain patients. Anti-IgE immunostainings of KCs incubated with sera from healthy persons gave no detectable signal.

Autoantigens can occur on various subcellular structures of KCs, including cell surface

To determine whether the serum IgE of our patients exhibiting peripheral staining by immunofluorescence binds to extracellular or intracellular membrane structures of KCs, we performed FACS analysis with viable KCs that were non-enzymatically detached and probed with autoreactive AD serum. Surface-bound IgE was detected with mouse anti-human IgE mAb and Alexa488-labeled goat anti-mouse IgG antibody (Ab). Using this method, we found serum IgE binding to detached viable primary KCs in three of 15 autoreactive AD patients (Figure S2). This binding of the autoreactive IgE in the three AD sera was almost completely prevented when the KCs were trypsin-treated before incubation with patient sera (data not shown), demonstrating that freely accessible surface proteins are the targets of

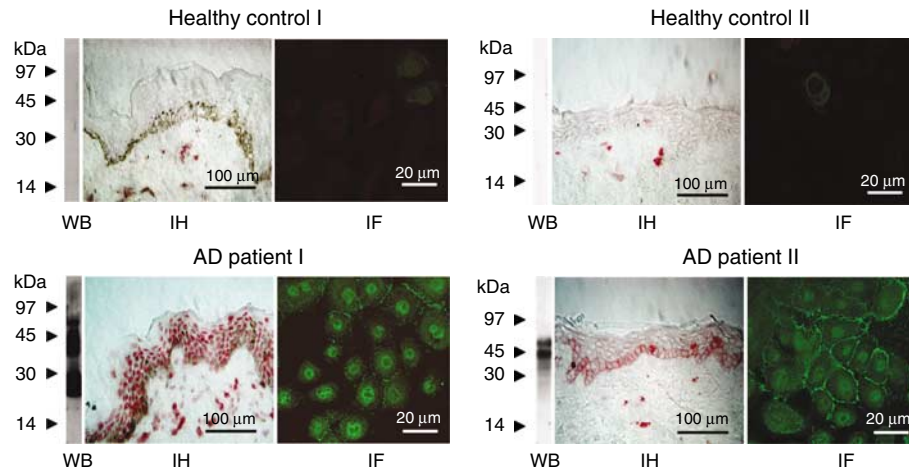


Figure 3. IH and immunofluorescence stainings of tissue sections of normal skin and cultured primary KCs. WB: Protein extract of primary human epidermis was separated by SDS-PAGE, blotted onto nitrocellulose-membranes, cut into strips, blocked, and incubated with individual sera of patients or healthy control individuals; serum IgE reactivity was detected using HRP-conjugated mouse anti-human IgE mAb. IH: Original magnification $\times 40$. Frozen skin sections of normal human skin were acetone-fixed and incubated with patient serum, and bound IgE was detected with mouse anti-human IgE-HRP mAb and AEC reagent (red staining). Melanocytes are visible in the basal layer of the epidermis as brown cells. Immunofluorescence: Original magnification $\times 100$. Primary human KCs were grown on chamber slides (low calcium, basal cell-like phenotype), fixed, incubated with patient serum, and washed, and cell-bound IgE was detected with a self-labeled mouse anti-human IgE-A488 mAb (staining shown in green). The magnification is presented as scale bar within each microscopic picture.

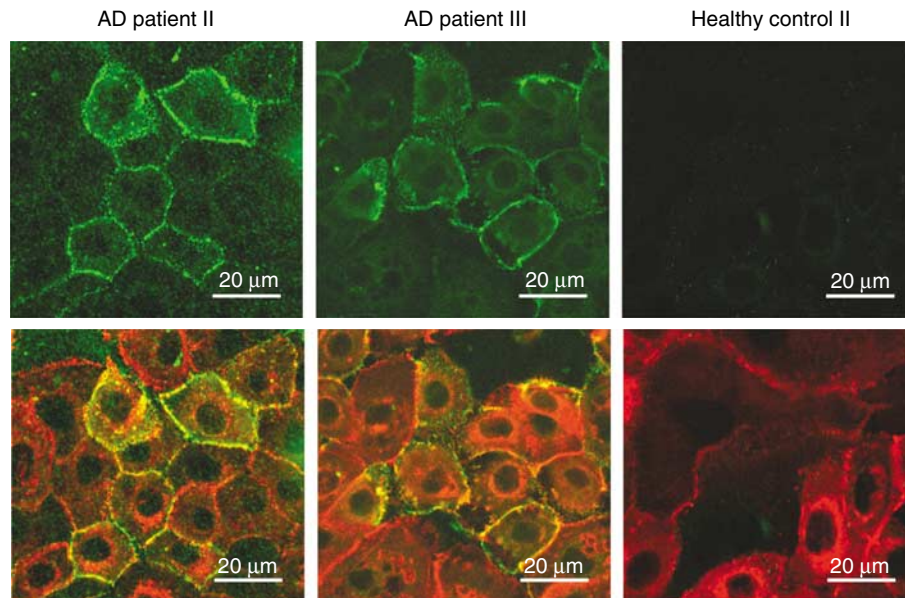


Figure 4. Immunofluorescence stainings of viable cultured primary KCs. Original magnification $\times 100$: Primary human KCs were grown on chamber slides (low calcium, basal cell-like phenotype) and incubated with patient serum or healthy control serum, and cell-bound IgE was detected with a mouse anti-human IgE Ab followed by goat anti-mouse-A488 Abs. Propidium iodide was added during the first antibody-incubation step to visualize dead cells. Finally, cells were fixed, permeabilized, and stained with rhodamine-phalloidin to enable visualization of the cytoskeleton of the KCs. The upper panel shows IgE staining (green), and the lower one depicts the results of an IgE (green)/phalloidin staining (red) double staining. None of the cells exhibited nuclear propidium iodide positivity. Serum IgE of patient II binds to the surface of KCs in a dotted manner, whereas in patient III IgE seems to bind more homogeneously to the outer membrane. The magnification is presented as scale bar within each microscopic picture.

autoreactive serum IgE. Surface IgE binding was limited to epithelial cells, with a significant preferential binding to primary KCs, and could not be detected on other tumor or primary cell types (Table S1), suggesting the recognition of KC-specific autoantigens.

In a next step, we also performed immunostainings with autoreactive sera on primary KCs cultured on slides (Figure 4). By this method, we detected membrane labeling of the viable cells in five of 15 autoreactive AD patients. Compared with FACS analysis, two additional, but rather weak, KC

surface-reactive AD patients were identified by immunofluorescence, possibly due to the higher sensitivity of the latter method.

DISCUSSION

Although serum IgE reactivity against human proteins had been reported to occur in AD sera some 10 years ago (Valenta *et al.*, 1996), our knowledge about the significance of this phenomenon is still incomplete. The recombinant human autoallergens characterized so far (Hom s 1–5) result from immunoscreenings of the epithelial carcinoma cell line A431 with sera of autoreactive AD patients (Natter *et al.*, 1998). Results obtained previously show that IgE reactivity of AD sera against these structures is a rather infrequent event (Natter *et al.*, 1998; Valenta *et al.*, 1998; Mossabeh *et al.*, 2002; Aichberger *et al.*, 2005). As the phenomenon of serum IgE autoreactivity occurs in approximately a quarter of our study population, Hom s 1–5 are likely to represent only a small fraction of the target antigens recognized by autoreactive IgE, suggesting that important autoreactive targets still await identification.

To address this issue, we decided to use primary epidermal cell extracts as a screening tool for IgE autoreactivity and to compare the results with the previously used extracts derived from A431. Our screening revealed that a considerable portion of AD patients (28%) exhibit serum IgE autoreactivity against epidermal extracts and extracts from the epithelial cell line A431. This autoreactivity in AD patients was significantly correlated with the severity of the disease, defined by the amount of total serum IgE and the clinical scores on the EASI and IGA. This relationship suggests that IgE autoantibodies are the result of repetitive and severe injury of the skin. Thus, it is conceivable that environmental exoallergens initiate the acute phase of the allergic cutaneous disease (Valenta *et al.*, 2000) and thereby destruction of KCs and release of autoantigens into the circulation. IgE autoimmunity may consequently contribute to chronicity of the disease even in the absence of environmental allergens. This hypothesis is supported by longitudinal studies showing that IgE responses to autoallergens occasionally increase during periods of disease exacerbation, and by the discovery that some environmental allergens share structural and functional similarities with human proteins (Cramer *et al.*, 1996; Meyer *et al.*, 1999; Kinaciyan *et al.*, 2002).

Interestingly, all the autoreactive AD patients were of the extrinsic type (total serum IgE > 100 IU ml⁻¹ and/or sensitization against common allergens). Three autoreactive patients displayed elevated total serum IgE but no sensitization against common allergens. These patients may be sensitized to exoallergens not tested by us, or, alternatively, the elevated total serum IgE may result from a “pure” reactivity against autoallergens. The latter explanation is in accordance with a report of two intrinsic-type AD patients with IgE autoimmunity against an exoallergen-crossreactive human protein (Schmid-Grendelmeier *et al.*, 2005).

An explanation for the significant correlation of autoreactivity and severity of the disease is that elevated

autoreactive serum IgE levels themselves account for the worsening of disease. IgE immune complexes could target effector cells like mast cells and basophils (Valenta *et al.*, 1996; Natter *et al.*, 1998), initiating the release of pre-formed mediators (that is, histamine, proteoglycans, tryptase, chymase), the production of cytokines (that is, IL-4, IL-5, IL-13), and, consequently, an allergic tissue response (Irani *et al.*, 1989). Support for this idea comes from the finding that purified recombinant IgE autoantigens caused histamine release from basophils and induced immediate type-I skin reactions (Valenta *et al.*, 1991, 1996; Cramer *et al.*, 1996; Natter *et al.*, 1998; Appenzeller *et al.*, 1999).

When we compared the moieties recognized in epidermal extracts and extracts derived from A431, we found incoherent band patterns in most autoreactive patients, indicating the presence of distinct autoantigens in the two protein extracts. Based on these results, we propose that extracts of freshly isolated skin cells reflect the *in vivo* antigen situation better than the A431 tumor cell extracts, and are thus indispensable for screening for autoreactive AD patients. In addition, a substantial proportion of autoreactive AD patients showed serum IgE reactivity selectively against proteins derived from epidermal and not A431 extracts, suggesting that autoreactive AD patients might have been overlooked when the screening was performed solely with cell line A431. We cannot definitely rule out the possibility that exogenous microbial antigens or allergens deposited in the skin are also recognized by serum IgE (Cramer *et al.*, 1996; Schmid-Grendelmeier *et al.*, 2005). The fact that we detected IgE serum reactivity against several sterile epidermal cell line preparations (A431, HeLa) and normal epidermal extracts strongly suggests that IgE autoreactivity is a major component of the IgE self-reactivity observed.

The comparative analysis of the moieties recognized revealed that every patient’s serum IgE was directed against an individual, very diverse spectrum of proteins in the respective extracts. This diverse reaction pattern may explain the low prevalence of serum IgE reactivity against the known autoantigens (Hom s). Interestingly enough, and unlike the situation with A431 extracts, we were able to identify a presumably commonly recognized moiety at around 30 kDa in extracts derived from epidermal sheets. Such a common target protein is of great interest, as more ubiquitous proteins are needed to analyze the pathogenetic relevance of these IgE-defined autoantigens.

To determine against which structures of the skin the serum IgE antibodies of the autoreactive patients are directed, we performed IH stainings of normal human skin sections that had been incubated with autoreactive serum IgE of AD patients. We found an individually variable staining pattern reaching from a predominant nuclear labeling throughout the whole epidermis to a specific labeling of the membranes of the basal layer cells. Flow cytometry and cytological analysis of viable cultured KCs revealed that the latter results from the staining of KC surfaces. Thus, the reported intracellular localization of autoantigens in AD (Cramer *et al.*, 1996; Valenta *et al.*, 1998; Fluckiger *et al.*, 2002) is not an obligatory feature. Moreover,

cytological analysis of cultured KCs revealed diverse forms of surface reactivity, indicating the existence of a variety of surface-reactive target molecules for IgE in the individual patients. It is particularly tempting to speculate that recognition of surface structures on KCs by serum IgE could result in a direct crosstalk between IgE and epidermal KCs, leading to perturbation and functional modulation of KC, similar to what has been described for IgG autoantibodies in diseases such as pemphigus vulgaris. In AD it is thus conceivable that autoreactive IgE antibodies are directly responsible for epidermal spongiosis.

It is also conceivable that KC surface-bound IgE facilitates contact formation with FcεRI⁺ positive cells, such as LCs or IDECs (Sary *et al.*, 2005). This direct interaction may lead to enhanced antigen transfer from KCs to antigen-presenting cells, and, as a consequence, to IgE-facilitated autoantigen presentation (Maurer *et al.*, 1998; Sary *et al.*, 2005), which may contribute to the chronicity of the disease (Mittermann *et al.*, 2004).

The frequent occurrence of IgE autoimmunity and its strong correlation with severity of the disease suggests a pathophysiologically important role of these autoantibodies in AD, although we cannot definitively exclude the possibility that they occur as a “bystander” phenomenon (Mittermann *et al.*, 2004). However, approximately a quarter of the AD patients exhibit serum IgE autoreactivity and the detection of autoreactive serum IgE is a useful serological marker for the disease activity in these patients. It defines a new subgroup of AD patients who may benefit from a treatment with an anti-IgE mAb (Lane *et al.*, 2006) and, perhaps, from a specific immunotherapy. In this context, it also seems imperative to subject such individuals to effective antipruritic therapy, thus interrupting the vicious circle of scratching, release of autoantigen, and, ultimately, development of acute eczematous lesions. Such an intervention should probably be implemented early in the patient's life (Mothes *et al.*, 2005).

MATERIALS AND METHODS

Patients

We collected sera from 192 AD patients who had been diagnosed according to the criteria of Hanifin and Rajka (1980). All individuals were tested for their total serum IgE level and allergen-specific serum IgE to common inhalant allergens, including grass pollen, birch pollen, ragweed, house dust mites, cats, dogs, and molds (ImmunoCAP system; Pharmacia, Uppsala, Sweden). Additionally, they were scored on the IGA, the EASI, and other clinical instruments (Hanifin *et al.*, 2001; Eichenfield *et al.*, 2005). Table S2 gives an overview of the age, sex, mean total serum IgE, mean IGA, and mean EASI of the patients tested. All patients ceased atopy-specific therapy, such as steroids, topical calcineurin inhibitors, and antihistamines, at least 2 weeks before enrollment. Sera of non-allergic volunteers ($n=26$), without allergic personal history, served as controls. Sera were stored at -20°C until investigation.

Tissue specimens

Whole skin: frozen tissue sections from normal adult human skin were purchased from BioChain (Hayward, CA).

Epidermal sheets and dermis: normal adult human skin from non-atopic individuals was obtained *ex vivo* from reduction surgery and split-thickness samples were prepared with a manual dermatome. Epidermal sheets were separated from the dermis after 1-hour dispase (Becton Dickinson, Franklin Lakes, NJ) digestion of the basement membrane as described previously (Kriehuber *et al.*, 2001).

Cell lines

The human epithelial cell line A431 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 10% fetal calf serum (heat-inactivated) at 37 °C, under 5% CO₂, in a humidified incubator.

Primary human foreskin KCs were purchased from PromoCell (Heidelberg, Germany) and grown in EpiLife medium (Cascade Biologics, Portland, OR) supplemented with 0.006 mM Ca²⁺.

Western blot

Cultured cells (A431) and freshly isolated epidermal and dermal sheets were dissolved in 2 × reducing Laemmli buffer (120 mM Tris pH 6.8, 4% SDS, 20% glycerol, 2.5% β-mercaptoethanol) by sonication on ice. The protein extracts were cleared by high-speed centrifugation, boiled for 5 minutes at 96 °C, and stored at -20°C until use.

WB was performed as described previously (Kriehuber *et al.*, 2001). Briefly, proteins were separated by 12% SDS-PAGE, transferred by semi-dry electroblotting onto nitrocellulose membranes (0.2 μm; Biorad, Hercules, CA), and blocked with 5% dry milk (blocking grade; Biorad)/phosphate-buffered saline (PBS). The blots were then incubated with serum (1:5 diluted in blocking milk) at 4 °C for at least 12 hours and washed with PBS-T (PBS + 0.1% Tween 20; Biorad). Binding of serum IgE was detected using the anti-human IgE mAb B3102E8 (horseradish peroxidase (HRP)-conjugated; Southern Biotechnology, Birmingham, AL) (1:1,000). The immune reaction was visualized with ECL or ECL-plus (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's directions.

Immunohistochemistry

Frozen sections of normal human skin were acetone-fixed, chloroform-treated, rehydrated in PBS, blocked in 2% BSA/PBS, and exposed to serum overnight at 4 °C. Afterward, bound IgE was detected with anti-human IgE mAb B3102E8 (HRP-conjugated; Southern Biotechnology) (1:200) and visualized with AEC reagent (3-amino-9-ethylcarbazole; Sigma-Aldrich, St Louis, MO) according to the manufacturer's directions.

Immunofluorescence

Cytological analysis: culture slides. Primary KCs were grown on culture slides (Becton Dickinson) until 70% confluence, fixed with ice-cold acetone, blocked with 1% BSA/PBS + 1% normal mouse serum (DakoCytomation, Glostrup, Denmark), and incubated with patient serum (1:5) for 1 hour on ice. After being washed with PBS buffer, bound IgE was detected with A488-labeled (mAb labeling kit; Invitrogen) mouse anti-human IgE-mAb B3102E8 (Southern Biotechnology). Slides were examined with a confocal

microscope (LSM510; Carl Zeiss MicroImaging GmbH, Jena, Germany).

Cytological analysis: culture slides of living KCs. Primary KCs were grown on culture slides (Becton Dickinson) until 70% confluence, blocked with 1% BSA/PBS + 1% normal goat serum (DakoCytomation), and incubated with patient serum (1:3) for 1 hour on ice. After being washed with PBS buffer, bound IgE was detected with mouse anti-human IgE-mAb B3102E8 (Southern Biotechnology) and goat anti-mouse IgG A488 (Invitrogen). Propidium iodide (final concentration, 1 µM; Invitrogen) was added to visualize dead cells. Consequently, cells were fixed and permeabilized (cell permeabilization kit; An der Grub, Kaumberg, Austria) according to the manufacturer's protocol and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Slides were examined with a Zeiss Axiophot fluorescent microscope and images were obtained from areas where no propidium iodide-positive cells were visible by light microscopy.

Statistics

Statistical analysis was performed using box-plot analysis and the Mann-Whitney *U*-test (SPSS 12; SPSS Inc., Chicago, IL). *P*-values <0.05 were considered statistically significant.

Ethical considerations

All patients gave informed written consent for participating in this study. The investigational protocol was approved by the ethics committee of the Medical University of Vienna (EK 188/2005) and followed the Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Epithelial cells (A), primary cells (B), and tumor cell lines (C).

Table S2. Subjects' statistics.

Figure S1. WB with serum of one autoreactive AD patient and five different epidermal protein extracts.

Figure S2. Three of 15 AD patients revealed serum IgE binding to the surface of vital KCs.

REFERENCES

- Aichberger KJ, Mittermann I, Reininger R, Seiberler S, Swoboda I, Spitzauer S *et al.* (2005) Hom s 4, an IgE-reactive autoantigen belonging to a new subfamily of calcium-binding proteins, can induce Th cell type 1-mediated autoreactivity. *J Immunol* 175:1286–94
- Appenzeller U, Meyer C, Menz G, Blaser K, Cramer R (1999) IgE-mediated reactions to autoantigens in allergic diseases. *Int Arch Allergy Immunol* 118:193–6
- Cramer R, Faith A, Hemmann S, Jaussi R, Ismail C, Menz G *et al.* (1996) Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus*. *J Exp Med* 184:265–70
- Eichenfield LF, Lucky AW, Langley RG, Lynde C, Kaufmann R, Todd G *et al.* (2005) Use of pimecrolimus cream 1% (Elidel) in the treatment of atopic dermatitis in infants and children: the effects of ethnic origin and baseline disease severity on treatment outcome. *Int J Dermatol* 44:70–5
- Fluckiger S, Scapozza L, Mayer C, Blaser K, Folkers G, Cramer R (2002) Immunological and structural analysis of IgE-mediated cross-reactivity between manganese superoxide dismutases. *Int Arch Allergy Immunol* 128:292–303
- Hanifin JM, Rajka G (1980) Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 92:44–7
- Hanifin JM, Thurston M, Omoto M, Cherill R, Tofte SJ, Graeber M (2001) The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. *Exp Dermatol* 10:11–8
- Irani AM, Sampson HA, Schwartz LB (1989) Mast cells in atopic dermatitis. *Allergy* 44(Suppl 9):31–4
- Kinaciyan T, Natter S, Kraft D, Stingl G, Valenta R (2002) IgE autoantibodies monitored in a patient with atopic dermatitis under cyclosporin A treatment reflect tissue damage. *J Allergy Clin Immunol* 109:717–9
- Kriehuber E, Breiteneder-Geleff S, Groeger M, Soleiman A, Schoppmann SF, Stingl G *et al.* (2001) Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med* 194:797–808
- Lane JE, Cheyney JM, Lane TN, Kent DE, Cohen DJ (2006) Treatment of recalcitrant atopic dermatitis with omalizumab. *J Am Acad Dermatol* 54:68–72
- Maurer D, Fiebigler E, Reininger B, Ebner C, Petzelbauer P, Shi GP *et al.* (1998) Fc epsilon receptor 1 on dendritic cells delivers IgE-bound multivalent antigens into a cathepsin S-dependent pathway of MHC class II presentation. *J Immunol* 161:2731–9
- Meyer C, Appenzeller U, Seelbach H, Achatz G, Oberkofler H, Breitenbach M *et al.* (1999) Humoral and cell-mediated autoimmune reactions to human acidic ribosomal P2 protein in individuals sensitized to *Aspergillus fumigatus*. *J Exp Med* 189:1507–12
- Mittermann I, Aichberger KJ, Bunder R, Mothes N, Renz H, Valenta R (2004) Autoimmunity and atopic dermatitis. *Curr Opin Allergy Clin Immunol* 4:367–71
- Mossabeh R, Seiberler S, Mittermann I, Reininger R, Spitzauer S, Natter S *et al.* (2002) Characterization of a novel isoform of alpha-nascent polypeptide-associated complex as IgE-defined autoantigen. *J Invest Dermatol* 119:820–9
- Mothes N, Niggemann B, Jenneck C, Hagemann T, Weidinger S, Bieber T *et al.* (2005) The cradle of IgE autoreactivity in atopic eczema lies in early infancy. *J Allergy Clin Immunol* 116:706–9
- Natter S, Seiberler S, Hufnagl P, Binder BR, Hirschl AM, Ring J *et al.* (1998) Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients. *FASEB J* 12:1559–69
- Novak N, Bieber T (2003) Allergic and nonallergic forms of atopic diseases. *J Allergy Clin Immunol* 112:252–62
- Schmid-Grendelmeier P, Fluckiger S, Disch R, Trautmann A, Wuthrich B, Blaser K *et al.* (2005) IgE-mediated and T cell-mediated autoimmunity against manganese superoxide dismutase in atopic dermatitis. *J Allergy Clin Immunol* 115:1068–75
- Schmid-Grendelmeier P, Simon D, Simon HU, Akdis CA, Wuthrich B (2001) Epidemiology, clinical features, and immunology of the “intrinsic” (non-IgE-mediated) type of atopic dermatitis (constitutional dermatitis). *Allergy* 56:841–9
- Seiberler S, Bugajska-Schretter A, Hufnagl P, Binder BR, Stockl J, Spitzauer S *et al.* (1999a) Characterization of IgE-reactive autoantigens in atopic dermatitis. 1. Subcellular distribution and tissue-specific expression. *Int Arch Allergy Immunol* 120:108–16
- Seiberler S, Natter S, Hufnagl P, Binder BR, Valenta R (1999b) Characterization of IgE-reactive autoantigens in atopic dermatitis. 2. A pilot study on IgE versus IgG subclass response and seasonal variation of IgE autoreactivity. *Int Arch Allergy Immunol* 120:117–25
- Sary G, Bangert C, Stingl G, Kopp T (2005) Dendritic cells in atopic dermatitis: expression of Fc epsilon RI on two distinct inflammation-associated subsets. *Int Arch Allergy Immunol* 138:278–90

- Tan BB, Weald D, Strickland I, Friedmann PS (1996) Double-blind controlled trial of effect of housedust-mite allergen avoidance on atopic dermatitis. *Lancet* 347:15–8
- Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P, Bettelheim P *et al.* (1991) Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253:557–60
- Valenta R, Maurer D, Steiner R, Seiberler S, Sperr WR, Valent P *et al.* (1996) Immunoglobulin E response to human proteins in atopic patients. *J Invest Dermatol* 107:203–8
- Valenta R, Natter S, Seiberler S, Wichlas S, Maurer D, Hess M *et al.* (1998) Molecular characterization of an autoallergen, Hom s 1, identified by serum IgE from atopic dermatitis patients. *J Invest Dermatol* 111:1178–83
- Valenta R, Seiberler S, Natter S, Mahler V, Mossabeh R, Ring J *et al.* (2000) Autoallergy: a pathogenetic factor in atopic dermatitis? *J Allergy Clin Immunol* 105:432–7
- Wollenberg A, Kraft S, Oppel T, Bieber T (2000) Atopic dermatitis: pathogenetic mechanisms. *Clin Exp Dermatol* 25:530–4

[Figure S1 \(jpeg 59K\)](#)

WB with serum of one autoreactive AD patient and 5 different epidermal protein extracts:

Protein extracts of 5 different ex-vivo human epidermal preparations (lane 1-5), one extract of cultured primary human fore-skin KC (lane 6), one extract of the cell line A431 (lane 8) and buffer control (lane 7) were separated by SDS-PAGE-electrophoresis, blotted onto nitrocellulose-membranes, cut into strips, blocked and incubated with the autoreactive serum of AD patient V (non surface-reactive) or with healthy control serum I; serum IgE-reactivity was detected using HRP-conjugated mouse anti-human IgE mAb. A representative example out of three autoreactive sera is shown. Each autoreactive serum recognized identical numbers and molecular weights in the different protein extracts.

[Figure S2 \(jpeg 37K\)](#)

3 out of 15 AD patients revealed serum IgE binding to the surface of vital KC: KC were non-enzymatically detached (Cell Dissociation Buffer™ enzyme-free, Invitrogen), washed, blocked in 1% PBS/BSA + 1% goat serum and incubated on ice for 1h with patient serum (1:5). Surface-bound IgE was detected with the mouse anti-hu-IgE mAb B3102E8 (Southern Biotech) and a goat anti-mouse-IgG-A488 Ab (Invitrogen). To gate out the dead cells, PI (Invitrogen, final conc.: 1µM) was added. Samples were analyzed using a FACS-Scan™ flow cytometer (Becton Dickinson). Only viable, PI negative cells are shown. Positive surface stainings are depicted in colors: AD patient II is shown in red, AD patient III in green and AD patient IV in yellow.

[Table S1 \(doc 127K\)](#)

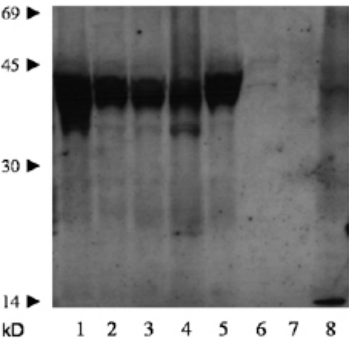
epithelial cells

[Table S2 \(doc 28K\)](#)

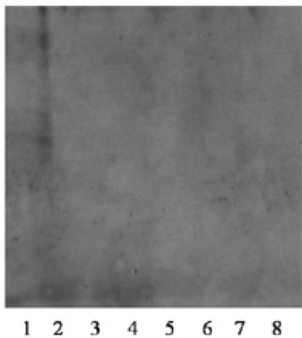
Subjects' statistics

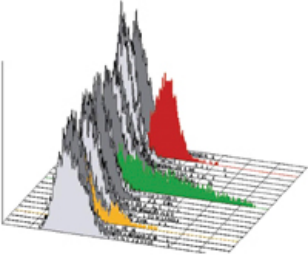
[Supplementary Table Legends \(doc 38K\)](#)

AD patient V



healthy control I





Suppl. Table 1

Table 1A.: epithelial cells:

	A431 fix and perm (intracellular)	A431	HeLa	KC
healthy control 1	47	7.7	8.1	4.9
healthy control 2	48	11	8.3	7.9
healthy control 3	48.7	8.6	8.3	6.8
healthy control 4	44.5	7.5	7.6	3.9
healthy control 5	59.3	14.8	7.6	3.4
mean MFI	49.5	9.9	8.0	5.4
SD	5.7	3.1	0.4	1.9
MEAN+3SD	66.6	19.1	9.0	11.1
AD patient 1	62	8	7.5	5.6
AD patient 2	72.9	8.8	7.3	4.9
AD patient 3	61.6	9.6	7.4	5.8
AD patient 4	86.1	11.1	8.1	20.7
AD patient 5	93.2	10.5	9.7	17.9
AD patient 6	39.4	7.4	6.7	2.8
AD patient 7	38	7.4	7.5	2.8
AD patient 8	63.8	9.4	8.6	10
AD patient 9	80.8	7.6	6.7	2.5
AD patient 10	72.1	11.8	10.8	11.1
AD patient 11	50.2	8.6	6.9	3.6
AD patient 12	46.7	9.3	6.9	4.1
AD patient 13	54.8	10.6	7	3.2
AD patient 14	54.8	8.4	6.6	4.1
AD patient 15	47.6	8.1	6.8	2.6

Table 1B.: primary cells:

	Monocytes	B cells	pDC	CD8 ⁺ T cells	CD4 ⁺ T cells
healthy control 1	514	345	779	18	64
healthy control 2	432	318	944	14	69
healthy control 3	310	182	1107	12	42
healthy control 4	446	244	733	16	61
healthy control 5	378	431	877	15	45
mean MFI	416.0	304.0	888.0	15.0	56.2
SD	76.6	95.5	147.6	2.2	12.0
MEAN+3SD	645.7	590.5	1330.8	21.7	92.2
AD patient 1	438	242	922	17	44
AD patient 2	562	260	1247	18	46
AD patient 3	635	269	1152	20	52
AD patient 4	442	339	978	17	27
AD patient 5	514	252	927	14	49
AD patient 6	639	401	688	16	41
AD patient 7	586	239	842	15	29
AD patient 8	491	423	877	15	61
AD patient 9	355	295	959	15	73
AD patient 10	478	232	872	17	27
AD patient 11	444	345	1303	13	38
AD patient 12	437	296	1005	16	54
AD patient 13	426	186	920	14	43
AD patient 14	425	233	1134	14	54
AD patient 15	392	377	973	13	40

Table 1C.: tumor cell lines:

	Jurkat	K562	Huvec
healthy control 1	1.5	14.5	28.4
healthy control 2	1.7	8.4	18.6
healthy control 3	1.4	12.9	16.0
healthy control 4	1.4	11.2	14.8
healthy control 5	1.9	11.4	14.6
mean MFI	1.6	11.7	18.5
SD	0.2	2.3	5.8
MEAN+3SD	2.2	18.5	35.9
AD patient 1	1.6	12.3	14.8
AD patient 2	1.5	12.3	17.5
AD patient 3	1.4	11.8	19.4
AD patient 4	1.6	13.5	24.1
AD patient 5	1.5	13.1	18.0
AD patient 6	1.5	14.3	17.7
AD patient 7	1.9	14.6	15.9
AD patient 8	1.4	15.3	18.2
AD patient 9	1.4	17.6	14.4
AD patient 10	1.5	16	15.7
AD patient 11	1.4	15.4	12.6
AD patient 12	1.3	13.9	15.8
AD patient 13	1.8	13.5	16.4
AD patient 14	1.4	13.6	18.3
AD patient 15	1.3	12	15.1

Suppl. Table 2: Subjects' statistics

	AD patients	healthy persons
number of patients	192	26
male/female	59/133	13/13
mean age [years]	39 (18-80)	31 (25-64)
mean serum IgE [IU/ml]	938.3 (25-2000)	11.5 (<2-20)
mean IGA	2.7 (0-5)	0
mean EASI	13.1 (0-64)	0

Supplementary Materials Legend for Online Production

Suppl. Table 1

Table 1A: epithelial cells

HeLa cells (cervical carcinoma cells) were obtained from ATCC and grown in DMEM high-glucose medium (Invitrogen) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin and 10% fetal calf serum (heat inactivated) at 37°C, 5% CO₂, in a humidified incubator. A431 and primary human foreskin KC were grown as described before.

A431 fix and perm:

Cells were non-enzymatically detached (Cell Dissociation Buffer™ enzyme-free, Invitrogen), washed, fixed and permeabilized using cell Cell Permeabilization Kit (An der Grub) according to the manufacturers brochure, blocked in 1% PBS/BSA + 1% goat serum and incubated on ice for 1h with patient or control serum (1:5). Bound IgE was detected with the mouse anti-hu-IgE mAb B3102E8 (Southern Biotech) and a goat anti-mouse-IgG-A488 Ab (Invitrogen). Samples were analyzed using a FACS-Aria™ and Diva™ software (Becton Dickinson).

A431, HeLa and KC:

Cells were non-enzymatically detached (Cell Dissociation Buffer™ enzyme-free, Invitrogen), washed, blocked in 1% PBS/BSA + 1% goat serum and incubated on ice for 1h with patient or control serum (1:5). Surface-bound IgE was detected with the mouse anti-hu-IgE mAb B3102E8 (Southern Biotech) and a goat anti-mouse-IgG-A488 Ab (Invitrogen). To gate out the dead cells, PI (Invitrogen, final conc.: 1µM) was added. Samples were analyzed using a

FACS-Aria™ and Diva™ software (Becton Dickinson). It was gated for viable, PI negative cells.

As negative control served cells incubated without serum. Numbers indicate the mean fluorescence intensity (MFI) of gated cells. The mean MFI and the standard deviation (STDDEV) based on the results of the healthy controls. IgE binding was considered as positive, when their MFI was $\geq$ mean MFI of healthy controls + 3 STDDEV (significance $p \leq 0,001$) and such positive reactions are highlighted in grey.

Table 1B: primary cells

Freshly isolated PBMC of a healthy person were stained for surface IgE after incubation with serum as described before. Cellular subsets were identified using mouse anti-hu CD19 mAb-APC (Becton Dickinson), mouse anti-hu CD3 mAb-ECD (Immunotec, Quebec, Canada), mouse anti-hu CD4 mAb -PerCp (Becton Dickinson), mouse anti-hu CD8 mAb-PE (Becton Dickinson), mouse anti-hu CD14 mAb-PE (Becton Dickinson), mouse anti-hu CD11b mAb-Pc5 (Becton Dickinson) and mouse anti-hu BDCA-2 mAb-APC (Miltenyi, Bergisch Gladbach, Germany). Samples were analyzed using a FACS-Aria™ and Diva™ software (Becton Dickinson). Analysis and interpretation was performed as described above. No significant positive IgE surface binding was detectable.

Table 1C: tumor cell lines

Jurkat (leukemia cell line), K562 (erythroleukemia cell line) and Huvec (Human umbilical vein endothelial cell line) were obtained from ATCC.

Jurkat and K562 were grown in RPMI 1640 medium plus supplements, Huvec

cells in DMEM high glucose plus supplements as mentioned before. Cells were stained and analyzed as described for A431, HeLa and KC in Table 1A. No significant positive IgE surface binding was detectable.

Since AD sera were reactive with A431 lysates in WB experiments, A431 cells whose intracellular protein contents were rendered accessible for IgEs by fixation and subsequent permeabilization are used as positive controls. In our experiments 8 out of the 15 patients displayed IgE reactivity to fixed and permeabilized A431 cells, 5 out of them with a high statistical significance ($p \leq 0,001$). Differences between the percentages of sera tested positive in WB (100%) versus FACS (~ 50%) are explained by different denaturing procedures thus affecting different epitopes in each experimental setting. Interestingly, none of the 15 autoreactive AD patients showed surface reactivity with non-epithelial primary cells or tumor cell lines. In contrast, 3 out of the 15 patients showed IgE-surface binding on primary KC with high statistical significance ($p \leq 0,001$). 2 out of these patients also had noticeable IgE-surface binding to HeLa cells, an epithelial cervical carcinoma cell line, but no AD patient showed IgE-reactivity to A431 cell surface.

These results suggest that IgE-surface binding, if occurring in AD, is rather specific for epithelial cells, with a superior sensitivity to primary KC. Earlier publications suggested exclusive intracellular binding of autoreactive IgE when using A431 cells (Seiberler *et al.*, 1999a; Seiberler *et al.*, 1999b) as antigenic source. Our experiments now indicate that these previous data do not reflect the whole spectrum of IgE autoreactivity. The fact that autoreactive serum IgE binds to primary KC and in a different reaction pattern compared to tumor cell

78 lines support that primary KC are better qualified for studying pathogenetic
79 relevant mechanisms in AD.

80

81 Suppl. Table 2

82 Subjects' statistics