

Original article

Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system

Background: Biochemical and immunochemical methods used for batch control of allergen extracts rely on the binding of IgE molecules to allergens. They do not measure the ability of a protein to induce type I allergic reactions. Therefore, a biological assay was established that is based on the cellular mechanisms of allergies in order to assess the cross-linking capacity of allergens.

Methods: Rat basophilic leukaemia cells were transfected with cDNA coding for the human high affinity IgE receptor chains. The surface expression of the IgE-binding α -chain was detected by FACS analysis and the functional integration of the 'humanized' receptors into the signal transduction cascade was addressed by intracellular calcium mobilization. Mediator release was measured in response to human IgE and a variety of cross-linking allergen preparations.

Results: Several clones were obtained that were able to bind allergen-specific human IgE. The results of the biological assay were compared with those obtained by immunochemical methods. The biological assay was used to determine the potency of allergen extracts, including highly diluted products that cannot be analysed by conventional methods.

Conclusion: A stable 'humanized' basophil cell line was established that will be a useful tool for the standardization and batch control of allergen extracts. Because of its high sensitivity, it can also be used to detect minute quantities of potentially allergenic proteins, e.g. in processed foods. In addition, the test may support the development of novel allergy vaccines, such as recombinant hypoallergenic molecules.

L. Vogel, D. Lüttkopf, L. Hatahet, D. Haustein, S. Vieths

Department of Allergology, Paul-Ehrlich-Institut, Langen, Germany

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Dr L. Vogel
Department of Allergology
Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
D-63225 Langen
Germany

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Experimental quality control of allergen extracts is mainly performed by biochemical and immunochemical methods. The determination of protein concentration is not reliable, as extracts from natural sources may be subject to considerable changes of the protein pattern over time (1, 2). Immunochemical methods depend on the binding of IgE, present in the sera of allergic patients, to molecules that are bound to solid supports. This may change the structural integrity of the allergens and thus destroy the epitopes relevant for inducing the allergic symptoms *in vivo*. Furthermore, results of immunochemical analyses often do not correlate with data obtained from skin testing (3, 4) or may not be easily reproducible (5). In addition, some allergens are present in the extract in such minute quantities that they might escape detection.

Immunochemical methods do not provide any information on the ability of a protein to induce type I allergic reactions as it is the case with *in vivo* tests. Skin tests, however, cannot be used in quality control for ethical reasons and the histamine release assay with fresh blood

from allergic patients is not practical for such routine purposes. The often favoured measurement of the major allergen(s), in order to demonstrate the constant composition of allergen preparations depends on the use of polyclonal or monoclonal antibodies and also does not measure biological activities. Moreover, because not all allergic patients react to the same molecules, extracts adjusted to only the major allergen content might lead to serious side effects in some patients.

In order to overcome these problems, a functional test system, based on the type I allergic reaction, was developed which allows to determine the biological activity of allergen extracts. The genes of the human high affinity IgE receptor (Fc ϵ R1) were transfected into the rat basophilic leukaemia (RBL) cell line RBL-2H3. Stable clones were then investigated for their ability to bind human IgE and release mediators after cross-linking of receptor-bound IgE molecules. The assay was compared with immunochemical methods used for the analysis of allergen extracts and evaluated with respect to its use in the quality control of commercial allergen preparations.

Materials and methods

Cell lines

The RBL cell lines were grown in RPMI1640 supplemented with 5% foetal calf serum and glutamine (Biobrom, Berlin, Germany). The RBL-2H3 cells were selected for maximal mediator release (6). The human α -chain-transfected line RBL-48 (7) was obtained from Dr J. Kochan (Roche, Nutley, NJ, USA). The medium of transfected cell lines contained 1 mg/ml geneticin sulphate (G-418; Invitrogen, Karlsruhe, Germany). Cells were harvested for use when they had reached 85–95% confluence.

Reagents and human sera

The supernatant of hybridoma IgEL.b4 (ATCC, Code TIB-141) was used as the source of murine IgE. Purified polyclonal human IgE was obtained from Biogenesis (Poole, UK), nonconjugated anti-murine or anti-human IgE and FITC-labelled goat anti-human IgE antibodies were from Nordic Immunology Laboratories (Tilburg, The Netherlands), phycoerythrin-conjugated goat anti-mouse IgE was obtained from Southern Biotechnology (Birmingham, AL, USA). Birch pollen, batch 42991, and timothy grass pollen, batch 33939, extracts (Allergopharma, Reinbek, Germany) were intermediate products from which the diagnostic allergen solutions are prepared. The patients' sera, selected for high titres of birch- or grass-specific IgE and/or a positive skin test and a clear record of seasonal allergic rhinitis, were taken from the serum collection of the Paul-Ehrlich-Institut. Written informed consent was obtained from all serum donors. The serum pools T97 (birch pollen) and G97 (grass pollen) are used in regulatory batch control of commercial allergen products at the Paul-Ehrlich-Institut.

Determination of allergen-specific IgE antibodies

Specific IgE antibodies to birch and grass pollens as well as recombinant birch allergens were measured by the CAP-FEIA system (Pharmacia Diagnostics, Uppsala, Sweden). ImmunoCaps coupled with recombinant Bet v 6 (8) were produced and kindly provided by Dr J. Lidholm (Pharmacia Diagnostics).

Plasmids and transfections

Plasmids containing the genes of the human Fc ϵ R1 chains were the kind gift of Dr U. Blank (Institut Pasteur, Paris). The α -chain cDNA was cloned into the vector pCMV4neo (9), the β -chain was inserted into pBJ1neo (10), and γ into pCDL-SR α 296 (11). Transfections were performed with LipofectamineTM (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. After 48 h, 1 mg/ml G-418 was added to the cell culture medium in order to obtain stable transfectants. The expression of mRNA of rat and human Fc ϵ R1 subunits in RBL-2H3 and transfected clones was studied by RT-PCR with the following primers: human α [GenBank accession number X06948 (12)] forward 5'-TGATGGAGGGC CAGCCCCCTCTCC-3' (bp 365–388), reverse 5'-ATAGTCCAG CTGCCACACTTTGGC-3' (bp 555–532); human β [D10583 (13)] forward 5'-ATATCTCCCCAGGAAGTATCTTCAGGC-3' (bp 88–114), reverse 5'-AATCAATGGGAGGAGACATTTCCCC-3' (bp 738–714); human γ [M33195 (14)] forward 5'-ATGATTCCA GCAGTGGTCTTG-3' (bp 1–22), reverse 5'-CCATCTGATTT CTCATCTAGTGTTATAG-3' (bp 188–161); rat α [M17153 (15)] 5'-GGTCTTAGACAATGGATCCTTTGAC-3' (bp 363–387), reverse 5'-TTACATTCAACCTTGTTCAATAGC-3' (bp 557–533); rat β [M22923 (16)] forward 5'-GCGTCCCCTCCTGCAAAA

GCTCTAC-3' (bp 88–113), reverse 5'-TTATGAAACCACTGG TGCGGACG-3' (bp 743–721); rat γ [L04306 (17)] forward 5'-AT GATCCCAGCGGTGATCTTGT-3' (bp 1–22), reverse 5'-TGA TTTCTACGGCTGGCTATGT-3' (bp 183–161).

FACS analysis

Cells were resuspended in PBS containing 1% BSA and 0.1% NaN₃ and seeded into 96-well microtitre plates at a density of 10⁵ cells/well. They were incubated with 2.5 μ g/ml of either murine (Maine Biotechnology, Portland, ME, USA) or human (Biogenesis) IgE or control antibodies for 30 min at 4°C, and washed three times with 1% BSA in PBS. Goat anti-mouse IgE labelled with phycoerythrin was added at a concentration of 2.5 μ g/ml and FITC-conjugated goat anti-human IgE at 10 μ g/ml. After incubation for 30 min at 4°C, cells were washed three times with 1% BSA in PBS and resuspended in fixation buffer (1% paraformaldehyde in PBS). The expression of Fc ϵ R1 α was determined using a FACScan (Becton Dickinson, Heidelberg, Germany) and LYSIS II software.

Mediator release from RBL cells and mobilization of intracellular calcium

The stimulation of RBL-2H3 and transfected cell lines and the measurement of β -hexosaminidase was performed as described earlier (18) with minor modifications. Human IgE-containing preparations, especially sera, had to be present during the attachment phase of the cells (overnight incubation). To increase and stabilize the secretion of β -hexosaminidase, 50% D₂O was added to the Tyrode's buffer used for the dilution of allergen extracts (19). Total β -hexosaminidase content was determined after lysis of the cells with Triton-X100. Allergen-specific release is given as per cent of total mediator content after correction for spontaneous release.

Determination of intracellular calcium mobilization from the endoplasmic reticulum was carried out using FURA-2 as before (20), with the exception that 50% D₂O was present in the Tyrode's buffer in which the allergen extracts were diluted. Measurements were carried out with the LS50B spectrometer (Perkin-Elmer, Rodgau, Germany) and the calcium concentrations were calculated using the FL-WinLab software provided with the spectrometer.

SDS-PAGE and Western blotting

Electrophoresis and blotting were essentially performed as described earlier (21). After blocking, the blotting membranes were incubated with patients' sera, diluted 1 : 10 in tris-buffered saline with 0.05% Tween20 and 0.1% BSA (600 μ l/lane) in a multiscreen apparatus (BioRad Laboratories, München, Germany). Bound serum IgE was visualized with a horseradish peroxidase-coupled rabbit anti-human IgE antibody (DAKO, Hamburg, Germany) using the ECL detection system (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions.

Results

Generation of transfected RBL clones expressing humanized Fc ϵ R1

Transfections were performed either with the plasmid containing the cDNA of the α -chain alone or by co-transfection of three plasmids encoding the α -, β -, and γ -chains, respectively. Stable clones were tested for

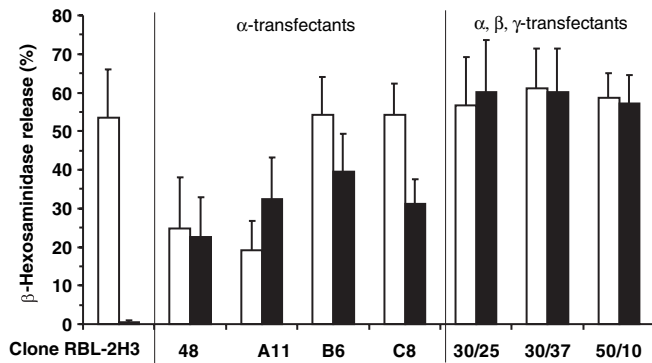


Figure 1. Comparison of transfected clones. Cells were sensitized with murine IgE and cross-linked with goat anti-mouse IgE (open columns). In the same way, the cells were incubated with human myeloma IgE and goat anti-human IgE (black columns).

their ability to release mediators in response to polyclonal human IgE and cross-linking by anti-human IgE antibodies. Transfecting the α -chain alone yielded only 14 of 81 clones with a mediator release above 30%. The co-transfection of all three human receptor chains produced 61 clones, of which 45 showed a release above 40% with a maximum at 55–60%. The release of β -hexosaminidase for some clones from various transfections is shown in Fig. 1 and is compared with the nontransfected cell line RBL-2H3 and the clone RBL-48. As the clone RBL-30/25 consistently proved to be superior to most other transfectants, it was used for all further studies.

The surface expression of the human Fc ϵ R1 α -chain was further demonstrated by FACS analysis (Fig. 2). The endogenous rat α -chain was detected to about the same extent on RBL-2H3, RBL-48, and RBL-30/25 cells (Fig. 2A, C and E). Human IgE did not bind to RBL-2H3 cells (Fig. 2B) whereas RBL-48 seemed to be a mixture of cells that express the human α -chain at low and intermediate levels (Fig. 2D). Only clone RBL-30/25 consisted of a nearly homogeneous population of cells expressing high levels of the human α -chain (Fig. 2F). Calcium mobilization assays demonstrated that the 'humanized' IgE receptors were functionally integrated into the signalling cascade of the rat cells (Fig. 3). Only the transfected clones RBL-B6 and RBL30/25 showed a substantial increase in the intracellular calcium concentration induced by human IgE (Fig. 3A), whereas all cell lines responded to murine IgE (Fig. 3B).

The results of the RT-PCR for the α -, β -, and γ -subunits of human and rat Fc ϵ R1 in RBL-30/25 and RBL-2H3 cells are shown in Fig. 4. The transfectants were positive in the assay for fragments of the human α -chain and the three rat chains of the receptor, but no mRNA of the human β - and γ -chains could be detected (Fig. 4A, lanes 1–6). The parent cell line RBL-2H3 exclusively was positive for the three rat chains (Fig. 4A,

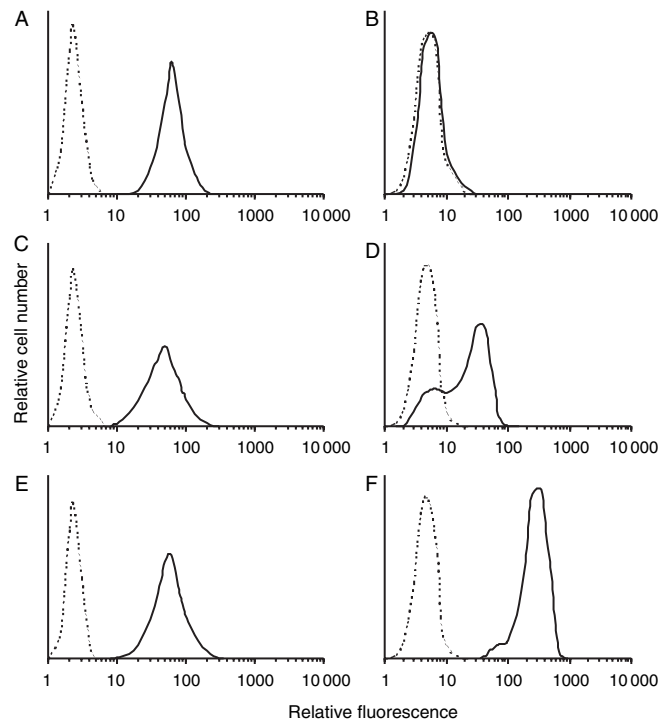


Figure 2. The FACS analysis of transfected cell lines. RBL-2H3 (A, B), RBL-48 (C, D) and RBL-30/25 (E, F) were incubated with mouse IgE (A, C, E) or human IgE (B, D, F), respectively. IgE molecules bound to Fc ϵ R1 were detected with goat anti-mouse IgE coupled to phycoerythrin (left panels) and goat anti-human IgE coupled to FITC (right panels). Broken lines represent the isotype controls with irrelevant antibodies.

lanes 7–12). Primers annealed specifically with human or rat α -, β - and γ -chains as demonstrated in Fig. 4B with the plasmids used for transfection.

Transfectants with a humanized Fc ϵ R1 respond to sera from allergic patients

The original RBL-30/25 cells have been recloned and several clones were established that routinely reach mediator release levels of 80–100% using human polyclonal IgE antibodies. In order to demonstrate the applicability as a human model system, RBL-30/25 cells were sensitized with allergen-specific IgE present in the sera of allergic patients. Cross-linking of receptor-bound IgE molecules was induced with serial dilutions of birch (*Betula verrucosa*) or timothy grass (*Phleum pratense*) pollen, chosen as examples for clinically important allergens. In addition, the pollen extracts were separated by SDS-PAGE, transferred to nitrocellulose and probed with the same sera. Table 1 summarizes the data of various sera containing IgE against birch allergens at the optimal protein concentration of the extract (approx. 0.1 μ g/ml). The maximum mediator releases were obtained with serum dilutions between 1 : 20 and 1 : 40.

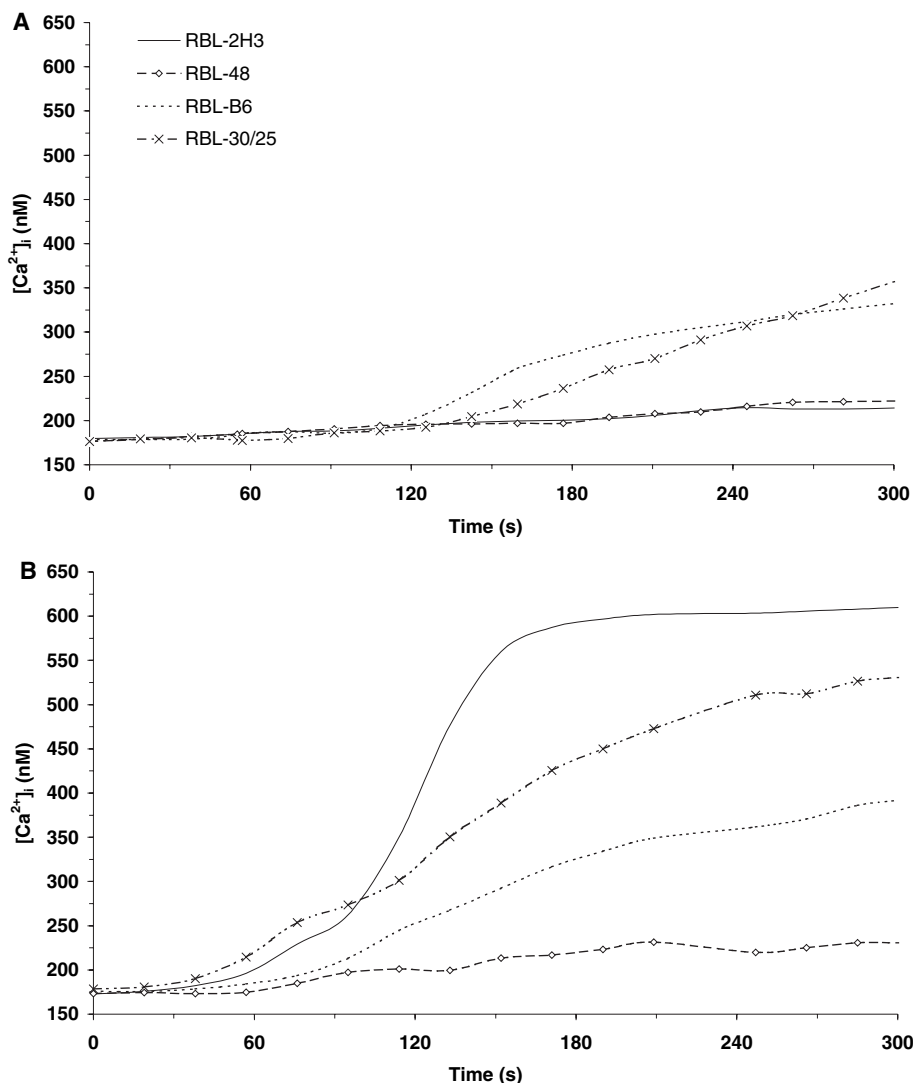


Figure 3. Measurement of calcium mobilization from the endoplasmic reticulum. RBL-2H3 and the transfected clones RBL-48, -B6 and -30/25 were sensitized with human IgE and loaded with FURA-2/AM. After hydrolysis of the ester and equilibration of the cells at 37°C in the spectrometer, IgE bound to the receptors was cross-linked with goat anti-human IgE (A). In the same way, cells were sensitized with murine IgE and cross-linked with goat anti-mouse IgE (B).

In general, all sera detected the most important major and minor allergens, i.e. Bet v 1 and Bet v 2, albeit to various extents. The allergen-specific IgE content (measured by CAP and verified by immunoblotting) and the ability to release mediators from cells carrying humanized FcεR1 are in good accordance. In a few cases, sera that contained high amounts of allergen-specific IgE induced a rather low mediator release. This means that the sera used for the analysis of allergen extracts have to be chosen carefully. Similar results were obtained with sera from patients allergic to timothy grass (Table 2) showing that the sum of IgE specific for the main grass pollen allergens correlates well with the observed mediator release levels.

Evaluation of commercial allergen extracts with the biological assay using the transfectant with humanized FcεR1

To evaluate the biological assay for its use in the analysis of commercial allergen extracts, RBL-30/25 cells were sensitized with the serum pool G97 that was used in routine batch control of grass pollen allergens. Mediator release was induced by the addition of serial dilutions of *P. pratense* pollen skin prick, provocation, and intracutaneous test solutions, respectively. The intermediate product, from which these solutions were prepared, was also included in the analyses. Controls where cells were sensitized with polyclonal IgE and cross-linked with anti-IgE antibodies were present in each microtitre plate to

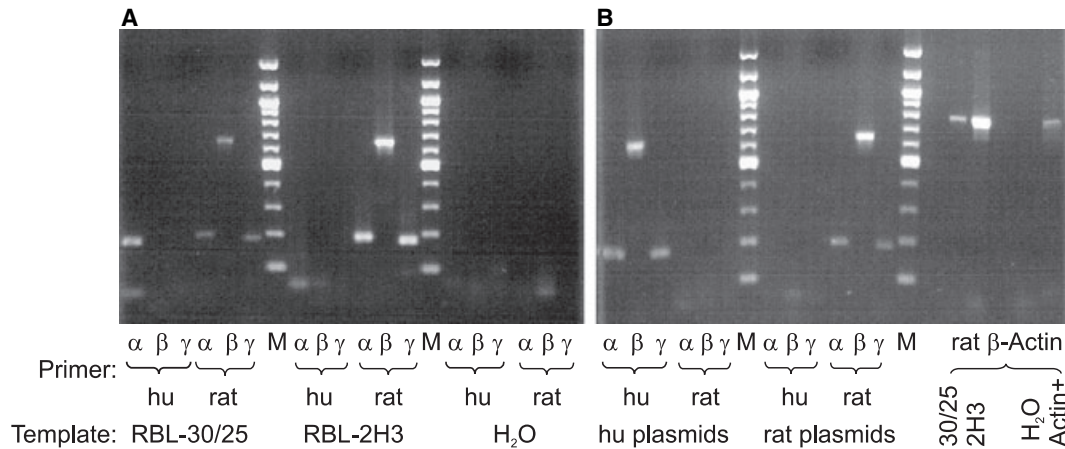


Figure 4. Identification of mRNA coding for the α -, β - and γ -subunits of human and rat Fc ϵ R1 of transfected RBL-30/25 cells and the nontransfected cell line RBL-2H3 (A) as well as from plasmids (B). M represents the 100-bp DNA marker.

Table 1. Comparison of assays for birch pollen extracts

Serum*	Birch-spec. IgE (kU/l)	Release (%)†	Bet v 1		Bet v 2		Bet v 6	
			Blot	CAP (kU/l)	Blot	CAP (kU/l)	Blot	CAP (kU/l)
Control serum	<0.35	0.4 ± 0.2	–	<0.35	–	<0.35	–	<0.35
PEI127	41.7	28.1 ± 5.6	+	37.1	+	2.6	–	<0.35
PEI131	43.5	24.0 ± 11.2	++	31.2	++	14.6	–	<0.35
PEI077	49.7	7.1 ± 3.9	+	29.5	+/-	<0.35	–	<0.35
PEI063	52.0	12.3 ± 2.2	++	35.4	–	<0.35	–	0.66
PEI061	54.1	33.4 ± 12.1	++	37.5	++	32.0	–	<0.35
PEI079	60.9	23.9 ± 7.0	++	77.6	–	<0.35	–	<0.35
PEI090	94.8	34.7 ± 8.6	+++	>100	–	<0.35	–	<0.35
PEI017	>100	39.3 ± 7.5	+++	>100	+/-	<0.35	–	<0.35
PEI062	>100	27.5 ± 7.8	+++	66.8	+/-	<0.35	–	<0.35
pool T97	84.8	44.0 ± 14.8	+	n.d.	+	n.d.	–	n.d.

n.d., Not determined.

*All patients had positive skin tests and/or clinical symptoms to the relevant allergens.

†Maximal release (mean of at least five independent measurements ± SD) induced by birch pollen extract for each serum at optimal dilution (1 : 20–1 : 40).

ensure the consistency of the test. Figure 5A shows that the obtained mediator release curves had the same slopes. The allergen concentrations that induced half maximal release, differ by a factor of about ten in the following order: prick > provocation > intracutaneous. This correlates well with the allergen content given by the manufacturer, which is 50 000 BU/ml for skin prick, 5000 BU/ml for provocation and 500 BU/ml for intracutaneous test solutions, respectively.

Finally, various lots of timothy grass skin prick test solutions from a single manufacturer were analysed for batch-to-batch consistency. As described above, RBL-30/25 cells were sensitized with serum pool G97 and receptor-bound IgE cross-linked with serial dilutions of skin prick test solutions manufactured in three consecutive years. From Fig. 5B it can clearly be seen that the three batches of the grass prick test give almost identical mediator release curves. This shows that the allergen

compositions and concentrations of the batches are very similar as it is demanded by national and international regulations.

Discussion

The transfection of RBL-2H3 cells with the genes of the human Fc ϵ R1 yielded clones that can be sensitized with human IgE and degranulate after cross-linking of receptor-bound human IgE. Transfections with the human α -chain alone have been performed earlier (7, 9, 22), but the mediator release induced by human IgE and allergens was not sufficient for our purposes. As RBL-2H3 cells, selected for high-level mediator release in the murine system (6), were available this cell line was used for transfections. Introducing all three human genes into RBL-2H3 led to a number of stable clones that were high

Table 2. Comparison of assays for timothy grass pollen extracts

Serum*	Timothy grass-spec. IgE (kU/l)	Release (%)†	Immunoblot		
			Phl p 1/5	Phl p 4	Phl p 11
Control serum	<0.35	0.4 ± 0.2	—	—	—
PEI114	9.3	20.4 ± 2.7	+/-	—	+
PEI107	43.1	27.9 ± 3.8	+/-	—	++
PEI061	45.6	31.0 ± 6.7	++	+/-	++
PEI062	75.7	14.9 ± 3.5	+	+/-	+
PEI017	81.8	15.9 ± 3.8	+	+/-	+
PEI077	97.7	30.9 ± 9.0	+	+	+
PEI039	>100	45.9 ± 10.9	+++	+	++
PEI048	>100	50.5 ± 16.2	+++	+	+/-
PEI127	>100	34.9 ± 5.8	++	++	+
pool G97	97.4	48.2 ± 9.1	++	+/-	+

*All patients had positive skin tests and/or clinical symptoms to the relevant allergens.

†Maximal release (mean of at least five independent measurements ± SD) induced by timothy grass pollen extract for each serum at optimal dilution (1 : 20–1 : 40).

releasers in the human system. A possible explanation could be that very high amounts of DNA (30–50 µg) were used in the transfections, resulting in the insertion of multiple copies of the α -chain gene at various genomic sites. The clone RBL-30/25 (and others) has been maintained in culture for several years now and still expresses the human α -chain at a high level.

The transfection had to be performed in order to obtain suitable cells because no permanently growing human cell line was available that met all the requirements. Neither the human mast cell line HMC-1, which does not express Fc ϵ R1 (23, 24) nor the leukaemia cell lines KU812 (25) and LAMA-84 (26), resembling basophilic granulocytes, release mediators in response to cross-linking IgE. Although it had been reported that KU812 cells could differentiate in response to cytokines (27), this did not lead to an enhanced production and release of mediators (L. Vogel, unpublished observations). Very recently, cell lines LAD-1 and -2 with characteristics of human mast cells have been described (28), but they have the disadvantage of a very long doubling time (2–3 weeks). Nevertheless, it will be interesting to study how they compare in the mediator release assay with transfected cell lines.

The biological assay for allergen extracts in the human system was developed to supplement the available biochemical and immunochemical methods. The latter can only measure the binding of IgE to proteins, whereas the cell-based test gives information about the biological activity of an allergen. In the present study it is shown that the biological activity of a variety of diagnostic allergen preparations can be measured with the 'humanized' mediator release assay. The allergen content of different test solutions was well reflected by the results of the biological assay. Even the biological activity of highly diluted, e.g. intracutaneous, test solutions could be determined which is not possible with immunochemical

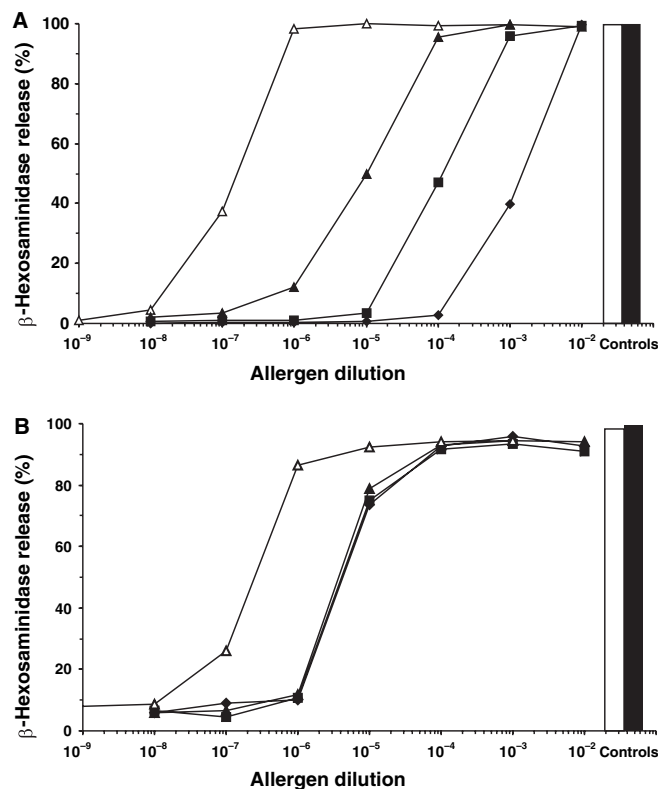


Figure 5. Titration of various test allergens (A). Cells of the clone RBL-30/25 were sensitized with human grass-specific serum pool G97 and then cross-linked with serial dilutions of skin prick ($\blacktriangle$), provocation ($\blacksquare$), and intracutaneous ($\blacklozenge$) test solutions as well as with the intermediate extract ($\triangle$). As controls, the cells were sensitized with murine (open columns) or human (black columns) IgE (polyclonal) and cross-linked with anti-IgE antibodies. Comparison of SPT batches 1 ($\blacklozenge$), 2 ($\blacksquare$), and 3 ($\blacktriangle$) and the intermediate product ($\triangle$) of timothy grass pollen (*Phl p*) from one manufacturer (B). Cells were sensitized as given above. The prick test solutions used for cross-linking had been produced in three consecutive years.

assays. Furthermore, the evaluation of the test with different batches of one allergen preparation has shown that it can be used to control batch-to-batch consistency. We have extended these analyses to the comparison of the biological assays in the mouse (18, 21) and human systems with a variety of allergen extracts. Among these were more 'complex' extracts, such as house dust mite and cat dander. In each case, it could be demonstrated that the assays in the murine and human systems on the one hand as well as cellular and CAP inhibition assays on the other hand lead to very similar results in the majority of experiments (29). This is remarkable, above all with respect to the fact that the assay systems can complement each other. On the one hand, for some allergens it is hard to find appropriate sera from human donors. On the other hand, other allergens are very poor IgE inducers in mice, even in so-called high-responder strains. In these

cases, one can switch between the systems and therefore analyse the whole variety of allergen extracts. For birch and grass pollen allergens, where the availability of appropriate sera is not a serious problem, we have in the meantime changed the serum pools several times with no change in the results whatsoever.

As the cell line RBL-30/25 has a very high sensitivity towards human IgE, which exceeds that of any other published transfectant so far (30–32), it can be envisaged that it will be a valuable tool in studies of IgE-mediated degranulation. The observation that some sera with high allergen-specific IgE contents do not result in a correspondingly high mediator release has been unexpected. In addition, with some sera the mediator release dropped drastically when the cells were sensitized with very high concentrations of serum. Although readily imaginable, this is not because of high total IgE amounts in these sera. The serum with the highest total IgE content (PEI107 > 15.000 kU/l) did not show this effect. Furthermore, no reduction of mediator release was observed after addition of up to 10 µg/ml polyclonal human IgE (data not shown). It seems that very low levels of FcεR1-occupancy is sufficient to trigger a considerable response. This view is also supported by early data on the interaction of FcεR1, IgE and 'allergens' [reviewed in Ref. (33)] showing that even the aggregation of only 1–10% of the receptors present on mast cells results in mediator release.

The newly developed 'humanized' mediator release assay will be a valuable tool in the analysis of allergen extracts. In contrast to immunochemical methods, this assay can give additional information on the allergenicity of the extract components and allergen extracts can be standardized on the basis of their biological activity. It

can be envisaged that the biological assay will also support the development of certified reference materials, as it is the scope of the CREATE project (34), and the formulation of in-house references for allergens (35). In both cases, the assay could circumvent the need for extensive *in vivo* skin tests because it directly measures the biological activities of the allergens. In addition, the cell line-based assay can show whether single recombinant (or natural) allergens are really representative of a whole extract and, if not, which additional components are needed for an individual patient. Apart from that, activities that escape detection by measuring single allergens, are easily determined with this functional assay. This will lead to an increased level of safety for the patients. Other potential applications of the test are the analysis of recombinant hypoallergenic allergens and the detection of minute amounts of allergenic proteins in complex mixtures, such as foods, as this assay directly addresses the allergenicity of the proteins in contrast to available methods.

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