

# A Comparative Study of the FcεRI Molecule on Human Mast Cell and Basophil Cell Lines

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## Key Words

Basophils · FcεRI · KU812 · LAD2 · Mast cells · Truncated β-chain

## Abstract

**Background:** Mast cells and basophils express the high-affinity IgE receptor FcεRI. We have analysed the human mast cell line LAD2 and four subclones of the basophil cell line KU812 in order to reveal possible differences concerning the FcεRI surface regulation, anti-IgE-triggered activation, FcεRIα protein stability and the mRNA level of FcεRIα-, β- and the truncated β-chain (β<sub>T</sub>), and thereby determine the utility of these cell lines in investigations of the FcεRI biology. **Methods:** The surface expression of FcεRI was assessed by flow cytometry, using the monoclonal antibody CRA1. The FcεRI-induced cellular activation (i.e. cross-linking of FcεRI) was determined by changes in the intracellular level of Ca<sup>2+</sup>, which was measured by fluorescence of Fura-2. The level of the FcεRIα protein was determined by a Western blot technique and by a radioimmunoassay. The mRNA level of FcεRIα, β- and β<sub>T</sub>-chain was analysed using real-time PCR. **Results:** Two KU812 subclones and especially LAD2 had FcεRI surface expression which was capable of inducing cellular activation. Both the FcεRI expression and stability of the FcεRIα protein were increased when IgE

was present. All the cell lines expressed mRNA of FcεRIα-, β- and β<sub>T</sub>, with LAD2 tending to have the highest expression. However, a determination of the β/β<sub>T</sub> ratio demonstrated no difference between any of the cell clones. **Conclusion:** These cell lines are important tools in the investigation of both the FcεRI molecule and the effects induced by its activation.

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## Introduction

Mast cells and basophils are effector cells in IgE-associated immune responses, such as type 1 allergic diseases and host resistance to parasites [1]. The high-affinity IgE receptor (FcεRI), which is primarily expressed on mast cells and basophils, arms the effector cells with IgE. FcεRI consists of the IgE-binding α-chain, a β-chain, the function of which depends on its form, and the two γ-chains, which are responsible for intracellular signalling. The full-length classic β-chain contributes to FcεRI surface expression and signaling [2–4], whereas an alternatively spliced truncated form (β<sub>T</sub>) prevents expression [5].

Cross-linking of IgE-armed FcεRI results in activation of the cell and release of preformed mediators (e.g. histamine) and the generation of new mediators (e.g. cytokines

and leukotriene C<sub>4</sub>) [6]. Surface expression of FcεRI is correlated with the serum level of IgE, and this has been used in therapeutic approaches. Anti-IgE treatment decreases the serum IgE level and thereby indirectly lowers the FcεRI expression to a level that affects the effector cell reactivity [7, 8].

We have analysed the FcεRI molecule from the human mast cell line LAD2 and the four human basophilic cell clones KU812, KU812\*, KU812E and KU812F. The LAD2 cell line was established in 2000 (published in 2003) from bone marrow aspirates of a male patient with mast cell sarcoma/leukemia. It is cultured in serum-free medium and is dependent on the cytokine stem cell factor for continued proliferation. LAD2 is described positive for histamine, tryptase and chymase, and to have functional FcεRI and FcγRI receptors [9].

The KU812 clones, which were established from a male patient with blastic crisis of chronic myelogenous leukemia, were published in 1985. These cell lines are cultured with serum and are not dependent on any cytokines [10]. They are described as being positive for histamine and tryptase, and to some extent expression of FcεRI [10–14].

Our aim was to examine the FcεRI molecule from LAD2 and the KU812 clones in order to reveal possible differences concerning the regulation of its surface expression mediated by IgE, the anti-IgE-triggered change in intracellular free Ca<sup>2+</sup>, the FcεRIα protein level and stability, and the mRNA level of FcεRIα-, β- and β<sub>1</sub>-chain, and thereby determine the utility of these cell lines in the FcεRI-related investigation.

## Materials and Methods

### Cell Culture and Stimulation

LAD2 cells were a kind gift from Dr. Dean D. Metcalfe, NIH, Bethesda, Md., USA. KU812 cells were a kind gift from Gunnar Nilsson (Uppsala, Sweden). KU812\* (originally named KU812 by the European Collection of Cell Cultures, ECACC), KU812E and KU812F were bought from ECACC, Salisbury, UK. LAD2 cells were cultured in serum-free media (StemPro-34, Life Technologies, Invitrogen, Taastrup, Denmark) supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin (Life Technologies) and 100 ng/ml recombinant human stem cell factor (PeproTech, Rocky Hill, N.J., USA). The KU812 clones were maintained in RPMI-1640 (Sigma-Aldrich, Brøndby, Denmark) supplemented with 10% FCS (Life Technologies), 50 µg/ml gentamicin and 2 mM L-glutamine (Life Technologies). The cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The LAD2 cell density was maintained between 0.25 × 10<sup>6</sup> and 0.5 × 10<sup>6</sup> cells/ml, and the cell culture was diluted every week. Cell density of the KU812 clones was maintained between 0.25 × 10<sup>6</sup> and 1 × 10<sup>6</sup>

cells/ml, and the cell cultures were diluted twice a week. For stimulation with IgE(ND) (supernatant from the B-cell line U266), KU812 cells were centrifuged, resuspended in culture medium supplemented with 1 µg/ml IgE (ND) and cultured for 24 h under normal conditions.

### Flow-Cytometric Analysis

Cell surface expression of FcεRI, CD32 and CD203c was assessed by flow cytometry. In brief, cells were incubated with 10 µg/ml mouse IgG<sub>2b</sub> anti-human FcεRIα chain antibody CRA1 (Cosmo Bio, Tokyo, Japan) or 10 µg/ml mouse anti-human CD32 antibody (Dako, Horsholm, Denmark) for 30 min on ice. The cells were washed twice in phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and then incubated with 1 µg/ml FITC-conjugated rat anti-mouse IgG<sub>2b</sub>, 25 µg/ml FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse antibody or 10 µl mouse anti-human CD203c-PE antibody (Immunotech, Marseille, France) for 20 min on ice. The cells were washed once in PBS supplemented with 0.5% BSA and analysed by flow cytometry (FACScan, BD Immunocytometry Systems, San Jose, Calif., USA). As a negative control, mouse IgG<sub>2b</sub>, mouse IgG<sub>1</sub> or mouse IgG<sub>1</sub>-RPE (Dako) were used. The extent of receptor expression is represented as the geometric mean of fluorescence intensity (geoMFI). The results are corrected for the isotype control.

### Measurements of Intracellular Ca<sup>2+</sup> in KU812

Intracellular Ca<sup>2+</sup> measurements were performed using Fura-2/AM as described previously [15]. Briefly, the intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was measured using a digital imaging system consisting of a Zeiss Axiovert 135 microscope equipped with a Zeiss Achrostatigmat ×40 objective with a 1.3 numeric aperture, a CCD 72 video camera and a GenIIsys image intensifier from Dage-MTI (Michigan City, Ind., USA) and MetaFluor software from Universal Imaging (West Chester, Pa., USA). Cells were loaded with 5 µM Fura-2/AM in RPMI-1640 with 0.1% human serum albumin (Statens Serum Institute, Copenhagen, Denmark) for 30 min at 37°C. After washing with PIPES buffer with 0.1% human serum albumin, the cells were transferred to a chamber slide and placed on a microscope stage. [Ca<sup>2+</sup>]<sub>i</sub> was measured according to the equation  $[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times (S_{f2}/S_{b2})$ , where the dissociation constant  $K_d = 224$  nM,  $S_{f2}/S_{b2} = 1.45$ ,  $R_{max} = 2.8$  and  $R_{min} = 0.1$ . Stimulation was done by adding rabbit anti-human IgE or rabbit serum to the cell chamber, using a final concentration of 10 µg/ml anti-IgE or the corresponding amount of rabbit serum.

### Cell Lysates

Cell suspensions were lysed (10<sup>7</sup> total cells/ml) using 1.25 × lysis buffer (62.5 mM Tris HCl, pH 7.4; 1.25% NP-40 (Calbiochem, La Jolla, Calif., USA); 313 mM NaCl; 1.25 mM EDTA; 0.625 mM phenylmethylsulfonylfluoride; 1.25 µg/ml aprotinin; 1.25 µg/ml leupeptin; 1.25 µg/ml pepstatin; 1.25 mM Na<sub>3</sub>VO<sub>4</sub>, and 1.25 mM NaF (all protease inhibitors were bought from Sigma-Aldrich). After adding lysis buffer, the cells were kept on ice for 20 min and then centrifuged (12,000 g, 10 min, 4°C). The supernatants were kept at -80°C until further analysis. Protein contents in the supernatants were measured using the Bio-Rad protein dye according to the manufacturers instructions (Bio-Rad Laboratories, Hercules, Calif., USA).

#### *Detection of FcεRIα Using an Immunoradiometric Assay*

The level of FcεRIα protein in the lysed cell suspensions (lysate from 40,000 cells was used) was determined using a previously described immunoradiometric method [16]. In brief, CRA1-coated Maxisorp tubes (Biotech Line, Slangerup, Denmark) were used to detect the FcεRIα-protein. 100 μl sample diluted in dilution buffer [RPMI-1640, 10% FCS, 0.2% Tween 20, 1 μg/ml IgE(ND)] was added and incubated overnight at 4°C. All samples were adjusted to an IgE concentration of 1 μg/ml before dilution. 100 μl of rabbit anti-IgE-<sup>125</sup>I (Pharmacia Diagnostics, Uppsala, Sweden) diluted 1:7 in PBS containing 0.1% Tween 20, was added to washed tubes, which were incubated overnight at 4°C. γ-Radiation was measured on a 1471 Wizard counter (Perkin Elmer Life Science-Wallac Oy, Turku, Finland) for 3 min. All samples were tested in duplicate. The mean value of 10 buffer measurements plus 2 × SD was used as cutoff. The amount of FcεRIα protein in the standard is validated in arbitrary 'α-chain units'. The standard used (which consists of lysed KU812 cells [16]) was assigned a value of 10,000 α-chain units and could be diluted up to 1,000 times in the immunoradiometric assay.

#### *Western Blot of FcεRIα*

Lysate from 40,000 cells was boiled for 3 min with 6 × Laemmli buffer and run on 10% SDS-polyacrylamide gels, prepared according to the Laemmli protocol [17]. Protein content in the lysates was determined using the Bio-Rad protein dye, to assure that the same protein amount was loaded on the gel. The gels were blotted onto PVDF membranes (Millipore, Bedford, Mass., USA). Excess binding sites were blocked by incubation with 5% BSA in TBST (50 mM Tris base, 150 mM NaCl, 0.5% v/v Tween 20 and 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, pH 7.4) for 2 h at room temperature. To detect FcεRIα protein, 0.14 μg/ml of rabbit anti-human FcεRIα antibody (Upstate Biotechnology, Lake Placid, N.Y., USA) were used as primary antibody, and a 1:7,000 dilution of HRPO-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used as secondary antibody. The blots were visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. Images of the FcεRIα protein bands were obtained using an LAS 3000 imager from Fuji Film Japan (Scandinavian distributor: Science Imaging Scandinavia, Nacka, Sweden).

To control differences in Western blots, each blot included a standard lane that consisted of KU812 cell lysate. Comparisons of scanned blots were made by expressing the digitized band intensities relative to the 50-kDa FcεRIα protein band in the KU812 standard within the same blot and expressed in 'Wb units'.

#### *RNA Isolation and Reverse Transcription*

Total RNA from the cell lines was isolated by RNeasy Mini Kit (Qiagen, Hilden, Germany). For quality and quantity measurements, RNA samples were evaluated using an Agilent 2100 bioanalyser (Agilent Technologies Denmark, Naerum, Denmark) according to the manufacturer's instruction. One microgram of each RNA sample was then submitted to first-strand synthesis. First-strand synthesis was performed as described in earlier work using reverse transcriptase, SuperScript II (Life Technologies, Grand Island, N.Y., USA) [18].

#### *Real-Time PCR*

Expression of the α-, β- and the truncated β<sub>T</sub>-chain was determined using real-time PCR performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). As a consequence of alternative splicing, the β-gene encodes two proteins; the full-length classic β-chain and a truncated form (β<sub>T</sub>). The β<sub>T</sub> transcript retains the 5th intron and in this way it is possible to distinguish between β and β<sub>T</sub> transcripts [5]. For amplification of cDNA, the following primers and probes were used: α-chain (forward) 5' TCT TCA GTG ACT GGC TGC TCC 3', α-chain (reverse) 5' GCT GGC CCT CCA TCA CC 3' and the FAM-marked probe 5' TCA GGC CTC TGC TGA G 3', β-chain (forward, exon 5) 5' CCA CAT CCA CAG TTG CCA GAA AT 3', β-chain (reverse, exon 6) 5' TGA GAA ACA GCA TCA TCA CTA CAA TTT 3', β<sub>T</sub>-chain (forward, exon 4) 5' GTC AAT TAT ATC TGA AAG GAG AAA TGC 3', β<sub>T</sub>-chain (reverse, intron 5) 5' GGA CCC AGA ATC TTA GTC TTC C 3' and the FAM-marked probe used in both β-chain systems 5' TGA GAC CAA GTG CTT TAT GGC TTC CTT 3' (exon 5). The primers were carefully designed to avoid self-complementarity and blasted for similar sequences. The probe and primers were purchased from MWG Biotech (Ebersberg, Germany). The real-time PCR reaction was performed according to the program (50°C for 2 min → 95°C for 10 min → 45 × 95°C for 15 s → 60°C for 60 s) and the following ranges for C<sub>T</sub>-values were found: 19.9–22.3 (α), 21.3–34.4 (β) and 24.3–36.8 (β<sub>T</sub>). The mRNA level was normalized to the endogen control HUP0 (Human Large Ribosomal Protein, Applied Biosystems). C<sub>T</sub>-values for HUP0 were found to be between 15.1 and 16.7.

Using the β-chain primers facilitated also concomitant measurement of β<sub>T</sub> chain, because they span over the intron that specifies β<sub>T</sub>. However, if the β<sub>T</sub>-chain should be amplified, a product of 491 bp should be made, which is 3–6 times the size of products recommended in real-time PCR. Furthermore, when analysing the amplification reaction on agarose gel, there was no trace of any product around 491 bp representing the β<sub>T</sub>-chain (results not shown). This illustrates that the β-chain primers only amplify the β-chain. Regarding the β<sub>T</sub>-chain measurement, using a primer placed in intron 5 ensured that only the β<sub>T</sub>-chain was amplified.

To determine a relative gene expression level, each real-time PCR reaction plate included a standard consisting of 10-fold dilutions of cDNA made from the human mast cell line LAD2.

## **Results**

### *Surface Receptors*

To detect the basal level of FcεRI expression, the five cell lines were analysed by flow cytometry using the monoclonal anti-FcεRIα antibody CRA1. As shown in figure 1a, LAD2 cells had the highest FcεRI expression with a geometric mean fluorescence intensity (geoMFI) of 23. The expression was more than 4-fold compared with KU812 and more than 10-fold compared with KU812\*. KU812E and KU812F did not have any detectable FcεRI expression.

CD32 and CD203c expression was also determined (fig. 1b, c). In contrast to FcεRI expression, KU812E and

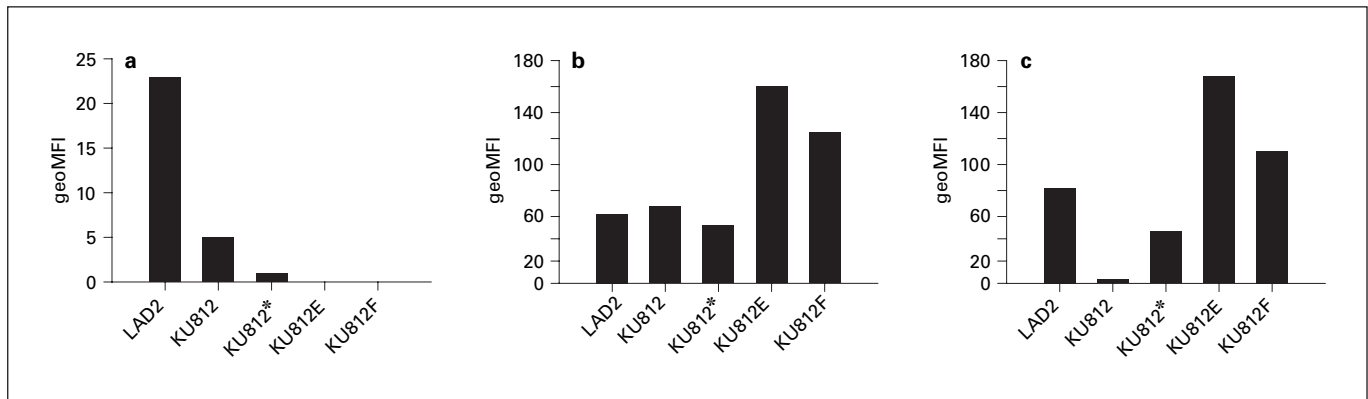


**Fig. 1.** Surface expression of FcεRI (a), CD32 (b) and CD203c (c) on each cell line determined by flow cytometry. The values are given in geoMFI.

KU812F had the highest CD32 (geoMFI of 159 and 122, respectively) and CD203c expression (geoMFI of 167 and 107, respectively). LAD2, KU812 and KU812\* had a comparable, lower CD32 expression (geoMFI between 47 and 62, respectively), but CD203c expression differed. LAD2 and KU812\* demonstrated a CD203c expression with a geoMFI of 77 and 42, respectively, but the expression of CD203c on KU812 was nearly undetectable (geoMFI of 3).

#### *Kinetics of IgE-Induced Upregulation of FcεRI*

To characterise the IgE-induced FcεRI expression, we analysed the IgE effect after 1, 2, 6, 12 and 24 h. The stimulations were made with IgE(ND) obtained from the B-cell line U266. Another isoform, IgE(PS), was also tested, but, the ability to upregulate FcεRI did not differ between the two IgE types (results not shown). For all five cell lines, FcεRI expression and IgE binding were measured at each time interval. Figure 2a shows the FcεRI level and figure 2b the percentage of FcεRI-positive cells. Surface FcεRI expression on LAD2 cells increased during the 24 h from a geoMFI of 26 to 106, and the number of FcεRI-positive cells increased from 74 to 97%. KU812 also showed an increase in FcεRI expression during 24 h, but the increase was not as quick as with LAD2 cells (geoMFI increased from 4 to 12 and the percentage of FcεRI-positive cells changed from 53 to 86%). Furthermore, in contrast to the steady increase in FcεRI expression on LAD2 cells, the FcεRI upregulation on KU812 cells seemed to change kinetics after 2 h after which the upregulation continued at a lower rate. FcεRI expression on KU812\* was just above the detection limit with a geo-

MFI of 1 changing to 2 when stimulated with IgE and only 2% was FcεRI positive. Neither KU812E nor KU812F demonstrated any detectable FcεRI expression.

Figure 2c shows the level of IgE binding and figure 2d the percentage IgE-positive cells. In parallel to the results of FcεRI expression, LAD2 cells showed the greatest IgE binding with a geoMFI of 867 after 24 h and 100% positive cells after just 1 h of incubation. The percentage of positive cells was higher using IgE detection compared to the FcεRIα detection system, due to a better sensitivity. KU812 was found to have a smaller IgE level with a geoMFI of 109 after 24 h, but almost all cells were IgE positive after 1 h of incubation. KU812\* showed IgE-positive cells (35% after 24 h of IgE stimulation) even though FcεRI detection was minimal. KU812E and KU812F had few IgE-positive cells (between 5 and 10%), although FcεRI expression was not measurable.

#### *Anti-IgE-Induced Changes in the Intracellular Free Ca<sup>2+</sup> Concentration*

FcεRI on the five cell lines was further functionally analysed regarding their anti-IgE-triggered activation. We examined the intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in cells (incubated with IgE for 24 h) stimulated with anti-IgE. Both LAD2 and KU812 cells demonstrated a comparable rise in [Ca<sup>2+</sup>]<sub>i</sub> (300–350 nM). However, as seen in figure 3a and 3b, the [Ca<sup>2+</sup>]<sub>i</sub> profile differed between the two cell types. More oscillations in the [Ca<sup>2+</sup>]<sub>i</sub> were found among the KU812 cells. Few of the KU812\* cells reacted when stimulated with anti-IgE (fig. 3c), but none of the KU812E or KU812F cells became activated

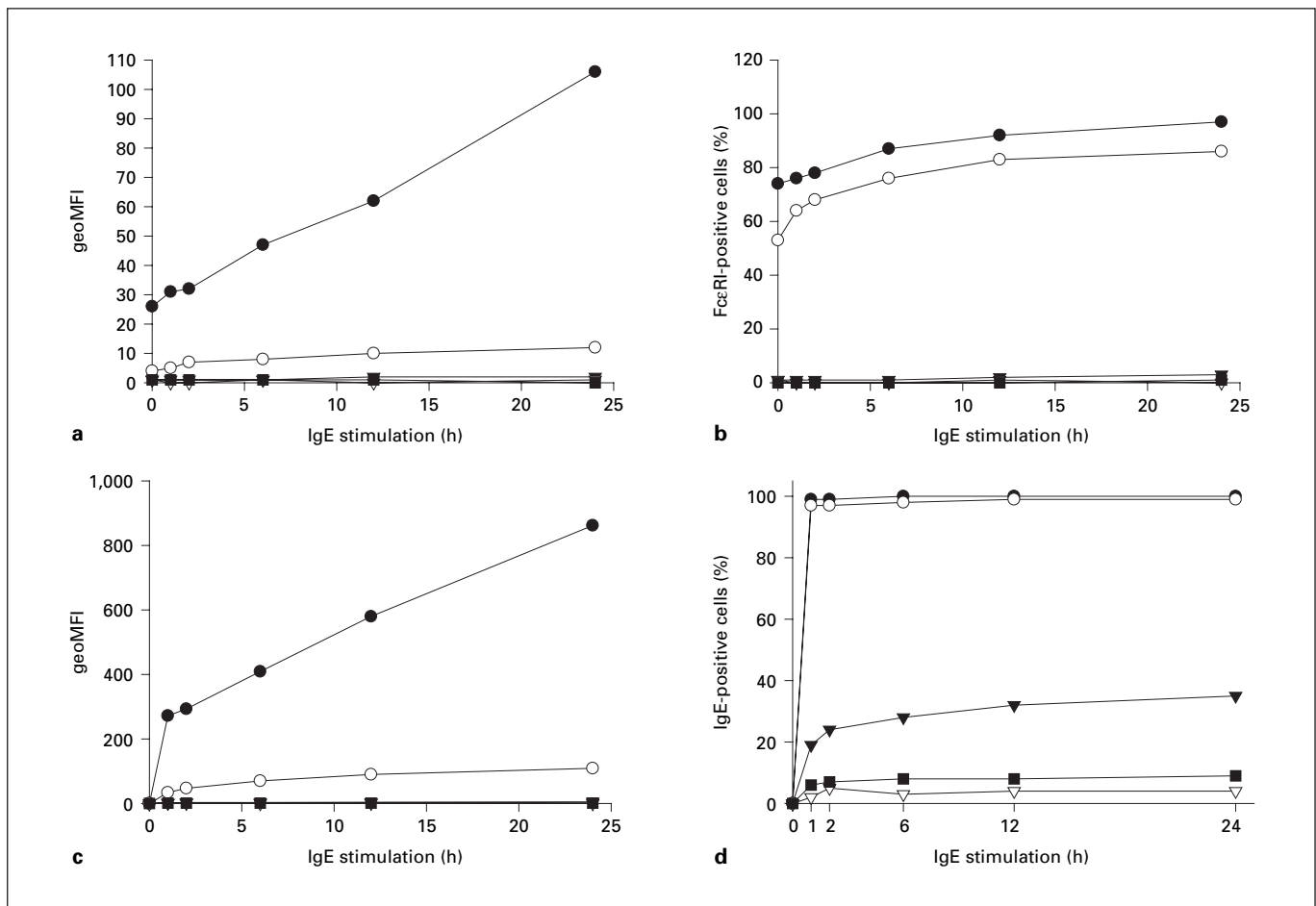


**Fig. 2.** IgE-induced FcεRI expression during 24-hour stimulation with 1 µg/ml IgE. **a** FcεRI expression (geoMFI). **b** The percentage of FcεRI-positive cells. **c** The amount of IgE bound (geoMFI). **d** The percentage of IgE-positive cells. ● = LAD2, ○ = KU812, ▼ = KU812\*, ▽ = KU812E, ■ = KU812F.

(fig. 3d, e). Anti-IgE-stimulated cells without prior IgE incubation did not cause any changes in  $[Ca^{2+}]_i$  (results not shown). Rabbit serum has been shown to induce changes in  $[Ca^{2+}]_i$ , and was used to confirm the reactivity of the cell lines not responding to anti-IgE stimulation (fig. 3d, e).

#### *FcεRIα Protein Level*

To investigate possible quantitative and qualitative differences regarding the FcεRIα protein, the cell lines were analysed before and after 24 h of IgE stimulation, using a radioimmunoassay (quantitative) and Western blot (qualitative). The results obtained by the radioimmunoassay are shown in figure 4. After IgE stimulation, the FcεRIα protein level in LAD2 increased more than

2-fold. In contrast, none of the KU812 clones displayed any significant increase in FcεRIα protein.

The Western blot analysis of FcεRIα protein enabled us to distinguish between the extracellular 66-kDa protein and the intracellular 50-kDa FcεRIα protein [19]. As seen in figure 5a, LAD2 was the only cell line with a detectable 66-kDa protein (lanes 1 and 2). After 24 h of IgE stimulation, the intensity of the 66-kDa band increased as seen in lane 2 (fig. 5b). These results are comparable with the flow-cytometric results. The 50-kDa protein was also increased in the LAD2 cells after IgE stimulation (fig. 5c); however, to a much lesser extent than the 66-kDa protein. All the KU812 clones expressed the 50-kDa protein, but IgE stimulation did not increase the FcεRIα protein level.

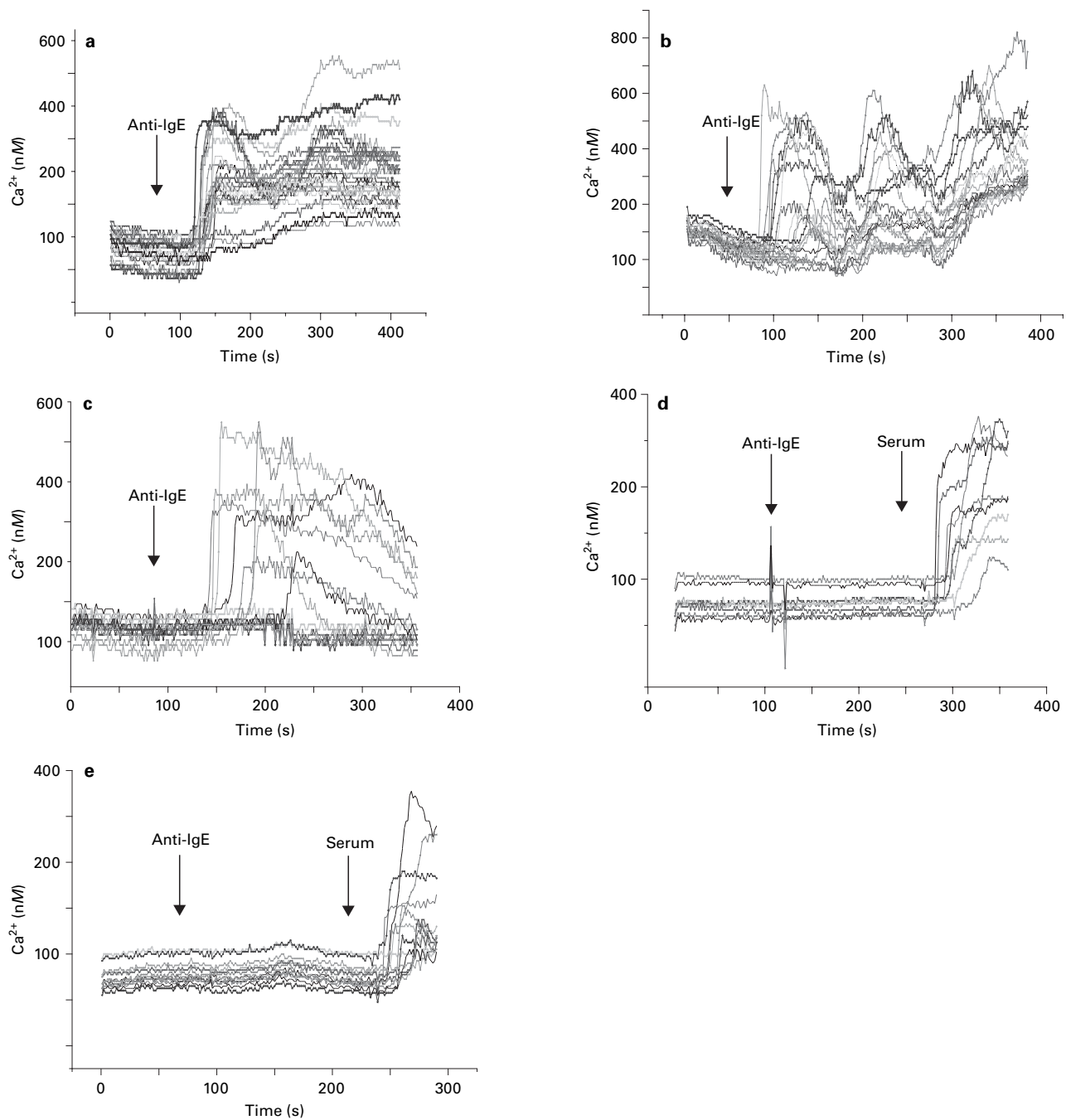


**Fig. 3.** Changes in  $[Ca^{2+}]_i$  by anti-IgE stimulation. All cell cultures were incubated with IgE for 24 h before stimulation with anti-IgE. Each curve represents the  $Ca^{2+}$  level in a single cell. **a** LAD2. **b** KU812. **c** KU812\*. **d** KU812E. **e** KU812F.

**Fig. 4.** Determination of FcεRIα protein (α-chain units) in cell lysates from cells with or without 24-hour IgE stimulation. + = IgE stimulation

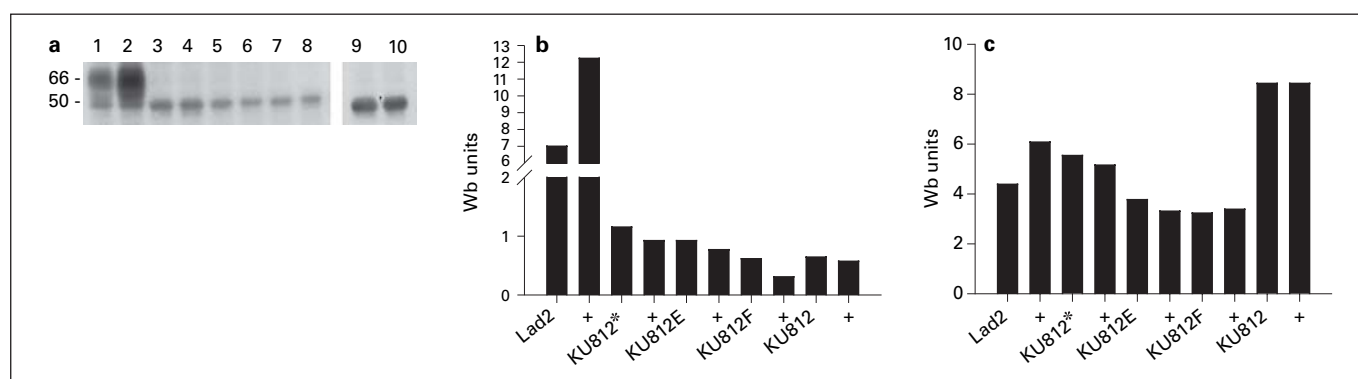
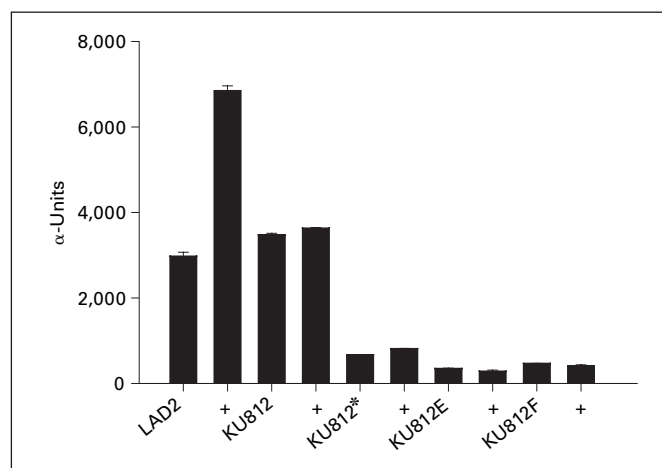


**Fig. 5.** Western blot analysis of the FcεRIα protein. **a** Western blot illustrating the 66- and 50-kDa FcεRIα protein in each cell line stimulated with or without IgE for 24 h. Lane 1 = LAD2; lane 2 = LAD2 + IgE; lane 3 = KU812; lane 4 = KU812 + IgE; lane 5 = KU812\*; lane 6 = KU812\* + IgE; lane 7 = KU812E; lane 8 = KU812E + IgE; lane 9 = KU812F; lane 10 = KU812F + IgE. **b, c** The intensity of the 66- (**b**) and 50-kDa FcεRIα protein band (**c**) from each cell line with or without 24-hour IgE stimulation given in Wb-units (the protein band intensity is expressed relative to a standard). + = IgE stimulation.

#### IgE Protection of FcεRIα Protein Turnover

The stability of the FcεRIα protein from each cell line was tested. FcεRIα protein was obtained by lysing the cells, and the lysate was then incubated at 37°C with or without 1 μg/ml of IgE for 5 h and analysed quantitatively. Figure 6a shows the percentage of α-chain unit left in the samples after incubation. The α-chain unit in lysate from each cell line before incubation is given a value of 100%. The largest reduction in FcεRIα protein was seen in LAD2 lysate, where the α-chain unit level decreased from 100 to 0.57%. The α-chain unit level decreased only to 10% when IgE was present. All the KU812 clones had a comparable pattern regarding the FcεRIα protein turn-

over. The 37°C incubation decreased the α-chain unit level to 3–6%, and when IgE was present the α-chain unit level only decreased to around 30%, suggesting a better protection of the FcεRIα protein than in LAD2 cells.

The FcεRIα protein turnover in LAD2 was further analysed by Western blot to examine any difference in stability between the 66- and the 50-kDa protein (fig. 6b). We determined the ratio between the intensity of the 66- and 50-kDa protein (R66/50) and found a R66/50 value of 1.22 in untreated lysate, of 1.08 in 37°C-treated lysate and of 1.25 in 37°C-treated lysate with IgE. This indicates that the stability and IgE protection of the 66- and 50-kDa protein are comparable.

**Fig. 6.** Determination of FcεRIα protein turnover in lysate from each cell line. **a** The FcεRIα protein was determined by radioimmunoassay, and the results are given as percentage (mean of 2 experiments) of α-chain unit left in the samples after incubation. The α-chain unit in lysate from each cell line before incubation is given a value of 100%. Black box = Cell lysate incubated at 37°C for 5 h; white box = cell lysate + IgE (1 μg/ml) incubated at 37°C for 5 h. The α-chain unit in each sample is given as the percentage of units left after incubation compared to the non-incubated sample. **b** Western blot of the 66- and 50-kDa FcεRIα protein in LAD2 cell lysate incubated with or without IgE at 37°C for 5 h. Lane 1 = LAD2 lysate; lane 2 = LAD2 cell lysate incubated at 37°C for 5 h; lane 3 = LAD2 cell lysate + IgE (1 μg/ml) incubated at 37°C for 5 h.

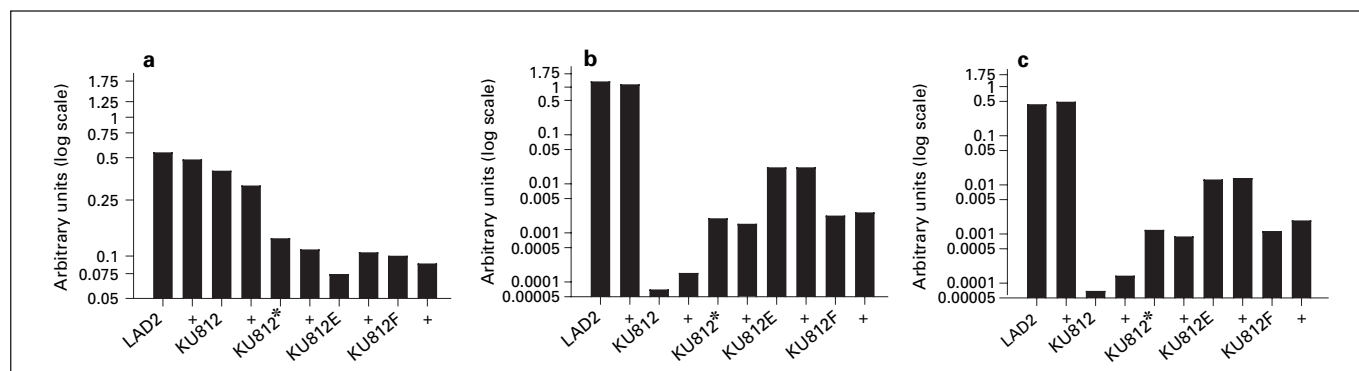
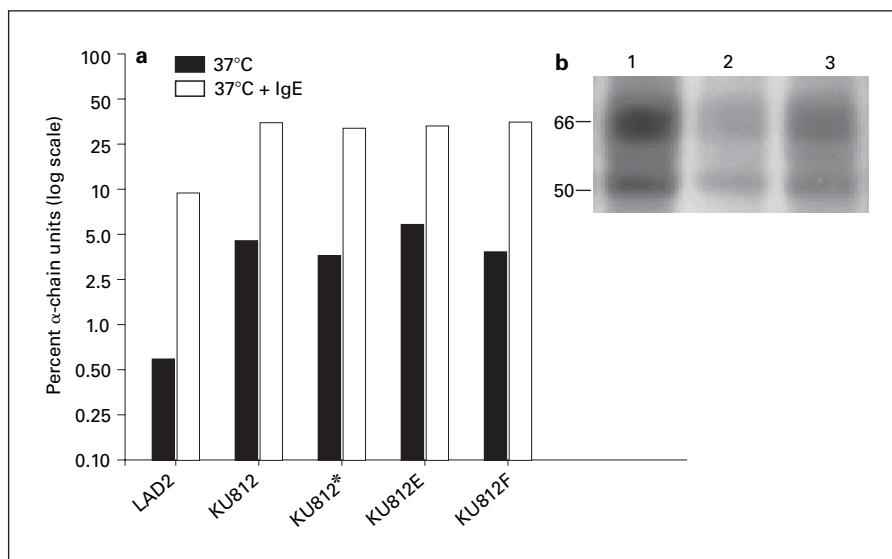


**Fig. 7.** The mRNA expression of FcεRIα (**a**), FcεRIβ (**b**) and FcεRIβ<sub>T</sub> (**c**) in cells incubated with or without IgE for 24 h. The mRNA expression was normalised to HUPO and given in arbitrary units extrapolated from a standard curve. + = IgE stimulation.

#### *FcεRIα, β and β<sub>T</sub> mRNA Level*

The FcεRIα-, β- and the truncated β<sub>T</sub>-chain mRNA were analysed using real-time PCR. The relative FcεRIα, β and β<sub>T</sub> mRNA levels in the five cell lines before and after 24 h of IgE stimulation are shown in figure 7. Regarding FcεRIα mRNA expression, LAD2 cells were found to have a higher expression compared to KU812\*, KU812E and KU812F. The difference between LAD2 and KU812 was not significant.

The β and β<sub>T</sub> mRNA was found to be expressed at a very high level in LAD2 cells compared with all the KU812 clones. Especially KU812 had a very low β and β<sub>T</sub> mRNA level (between  $2 \times 10^{-4}$  and  $7 \times 10^{-5}$  arbitrary

units). However, a determination of the β/β<sub>T</sub> ratio illustrated no difference in any of the cell clones, which were found to have a ratio >1 (between 1.01 and 2.80), illustrating more β than β<sub>T</sub>. Neither FcεRIα, β nor β<sub>T</sub> mRNA levels were affected by IgE stimulation in any of the cell lines.

#### **Discussion**

The study of human mast cells and basophils has been hampered by the facts that mast cells are difficult to isolate and purify from tissue, and it is difficult to get pure

basophils in a substantial amount, even though they are found in the blood. Cell lines have therefore been a useful supplement in the study of these cell types. We have compared two human cell lines, the recently established mast cell line LAD2 and the basophil cell line KU812.

LAD2 is reported to express FcεRI [9]; however, the amount of FcεRI and the number of positive cells is not described. We found that 74% of the LAD2 cells were FcεRI positive with a geoMFI of 23. In comparison, only two of the four KU812 clones displayed FcεRI, but they were still less positive than LAD2. The expression of FcεRI on KU812 and/or KU812\* is reported either by IgE binding or by the use of specific FcεRI antibodies [14, 16, 20–23]. Nevertheless, one study shows that KU812 is without FcεRI, but that IL-4 treatment is capable of inducing FcεRI expression [12]. In a previous study, results of FcεRI expression in KU812F agree with our results [24]. KU812E has not been analysed for FcεRI expression previously.

All FcεRI-positive cells were capable of binding IgE and, with some variation, upregulating FcεRI expression (fig. 2). LAD2 had the highest FcεRI expression during the 24-hour IgE stimulation and did not seem to decrease afterwards. The IgE-induced FcεRI expression on KU812 was not as impressive as the one found on LAD2 and it was slower. Furthermore, the results indicate that FcεRI expression reached a plateau at 24 h, which we confirmed performing a 14-day IgE incubation study (results not shown). This is contradictory to results obtained with purified human basophils where FcεRI expression increased over 7 days [25]. The differences could be explained by the fact that KU812 divides every 24 h, and if the FcεRI molecules are equally divided between the two daughter cells, as published for RBL-1 cells [26], the IgE-induced number of FcεRI would be halved every 24 h.

The percentage of IgE-positive cells was equal between LAD2 and KU812 even though there was a 8-fold difference in FcεRI expression, indicating that the difference in FcεRI expression was not an effect of insufficient IgE binding. KU812\* did also show some IgE-positive cells but minimal FcεRI expression. The reason for the small IgE-induced change in FcεRI expression could be that FcεRI synthesis is very slow in KU812\* cells, and even though IgE stabilizes and thereby prevents degradation of FcεRI molecules on the cell surface, expression of new FcεRI on the surface is slow on KU812\* cells. Few IgE-positive KU812E and KU812F cells were found despite the cells not displaying any FcεRI expression, and the staining was therefore considered unspecific.

Interestingly, cells with a low FcεRI expression (KU812E and KU812F) had the highest CD32 and CD203c expression. It has been described that cross-linking of FcεRI induces upregulation of CD203c [27]. Surprisingly, our results evidence that cells expressing FcεRI (KU812) had the lowest, nearly undetectable level of CD203c, while cells with no detectable FcεRI expression (KU812E and KU812F) expressed the highest level. Additionally, KU812E and KU812F seem to be more IgG sensitive because of their high CD32 expression compared to KU812 and KU812\*, which in contrast appear more IgE sensitive. It is not known whether there is an association between CD32 and CD203c expression, but this distinctive receptor expression clearly demonstrates some difference in the four KU812 subclones that could reflect different degrees of differentiation.

Functionality of FcεRI was measured by anti-IgE-induced changes in  $[Ca^{2+}]_i$ . We did not examine the release of mediators such as histamine, because anti-IgE-triggered histamine release did not appear in the KU812 clones, even though they contain histamine (results not shown). LAD2, KU812 and KU812\* had a comparable rise in  $[Ca^{2+}]_i$ , despite different FcεRI expression. This illustrates that although KU812\* nearly had an undetectable FcεRI expression, the level of FcεRI was high enough to obtain cross-linking and cell signalling. The  $[Ca^{2+}]_i$  profile in the KU812 cells displayed more oscillations than those seen in the other cells. These oscillations could be due to a feed-back inhibition from the  $Ca^{2+}$ -activated protein kinase C preventing the Ins(1,4,5)P<sub>3</sub>-induced  $Ca^{2+}$  release, as described in RBL-2H3 cells [28, 29]. All responding cells had a lag period of about 30–50 s before the rise in  $Ca^{2+}$  was observed. This may be explained by the diffusion time of the antibody or the time lag for the onset of the signalling events. Interestingly, we found that rabbit serum could change  $[Ca^{2+}]_i$  in all the cells, but the mechanism was not elucidated.

LAD2 was the only cell line which demonstrated an increase in the total FcεRI protein amount after IgE stimulation. Western blot analysis further illustrated that this change in FcεRIα protein was in particular due to an increase in the 66-kDa protein (fig. 5). It was possible to detect the 50-kDa protein in all the cell lines, indicating that the KU812 clones without detectable FcεRI surface expression do possess FcεRI protein. In the KU812 cell clones, the 50-kDa protein level was not affected by IgE stimulation, which is in comparison to results obtained with human basophils [19]. However, the constitutive expression of the 50-kDa protein among the KU812 clones is in contrast to that in basophils, since

they lose their 50-kDa protein pool after 1 day of culturing [19]. The difference in the protein expression could be a result of human basophils having a higher turnover of FcεRI.

The FcεRI protein was analysed regarding its stability. LAD2 lysate had the lowest FcεRIα protein level and the poorest IgE protection, which could illustrate a more unstable FcεRI protein in these cells. Previously, we have demonstrated that the instability in the FcεRIα protein in KU812 was not induced by proteases [16], but since protease inhibitors have not been tested on the LAD2 lysate, except the ones in the lysis buffer, it is possible that some mast-cell-specific protease activity also could contribute to the degradation seen in LAD2. Additionally, the difference in FcεRIα protein stability among the cell lines could also reflect the difference in the loss of epitope/ligand binding sites involved in CRA1 and/or IgE binding.

The 66- and 50-kDa protein stability was additionally examined in LAD2 using Western blot analysis, which revealed that there is no difference in the stability between the fully glycosylated surface 66-kDa FcεRI protein or the intracellular pre-Golgi 55-kDa protein. Studies on the FcεRIα protein have demonstrated that glycosylation is not necessary for folding and IgE binding [30]. According to our results, glycosylation is neither important for the stability.

The IgE-induced upregulation of FcεRI (surface and total protein) did not affect the mRNA level in any of the cell lines. These results are comparable with results obtained in transfectants, mouse mast cells and human basophils [31–33]. LAD2 and KU812 differed markedly in the surface and total protein expression of FcεRIα, but the mRNA level did not differ significantly.

Regarding the β-chain mRNA expression, the KU812 clones, especially KU812, had an interestingly low ex-

pression. This could indicate that the FcεRI complexes on the cell surface of KU812 clones are expressed both as αγ<sub>2</sub> and as αβγ<sub>2</sub>. The β<sub>T</sub>-chain is reported to prevent FcεRI surface expression in transfectants by inhibiting α-chain maturation and competes with the classic β-chain to control FcεRI expression [5]. We found that the β<sub>T</sub>-chain mRNA expression was also low among the KU812 clones. Therefore, the different FcεRI expression found among these clones cannot be explained by β<sub>T</sub>. However, this does not necessarily mean that β<sub>T</sub> does not affect FcεRI expression. The study by Donnadieu et al. [5] on β<sub>T</sub> was done in αβ<sub>T</sub>γ<sub>2</sub> transfectants, where there was no competition from the classic β-chain, but because the cell lines have both β and β<sub>T</sub>, our results could simply be an effect of β overruling β<sub>T</sub>. This hypothesis is further supported by the β/β<sub>T</sub> ratio, which was above unity in all the cell lines, illustrating a larger expression of β compared to β<sub>T</sub>.

We have analysed LAD2 and the four KU812 subclones regarding their FcεRI molecule. We found that LAD2 and two of the KU812 clones (KU812 and KU812\*) express a functional FcεRI protein, with expression and stabilization being inducible by IgE stimulation. Especially LAD2 has a very dynamic FcεRI expression that is rapidly increased by IgE compared to the KU812 clones. Furthermore, besides the expression of FcεRIα and β mRNA, which is expressed at a very high level in LAD2, all the cell lines express β<sub>T</sub> mRNA. However, none of the cell lines analysed revealed FcεRI expression, which appeared to be influenced by the β<sub>T</sub> chain.

We believe that these cell lines provide a new means for mast cell and basophil research concerning the FcεRI molecule, and they present an important tool in the investigation and improvement of new therapeutic approaches of allergic diseases.

## References

- Galli SJ: Mast cells and basophils. *Curr Opin Hematol* 2000;7:32–39.
- Donnadieu E, Jouvin MH, Kinet JP: A second amplifier function for the allergy-associated Fc(ε)RI-β subunit. *Immunity* 2000;12:515–523.
- Lin S, Cicala C, Scharenberg AM, Kinet JP: The Fc(ε)RIβ subunit functions as an amplifier of Fc(ε)RIγ-mediated cell activation signals. *Cell* 1996;85:985–995.
- Dombrowicz D, Flamand V, Miyajima I, Ravetch JV, Galli SJ, Kinet JP: Absence of FcεRI α chain results in upregulation of Fc γRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between FcεRI and Fc γRIII for limiting amounts of FcR β and γ chains. *J Clin Invest* 1997;99:915–925.
- Donnadieu E, Jouvin MH, Rana S, Moffatt MF, Mockford EH, Cookson WO, Kinet JP: Competing functions encoded in the allergy-associated Fc(ε)RIβ gene. *Immunity* 2003;18:665–674.
- Turner H, Kinet JP: Signalling through the high-affinity IgE receptor FcεRI. *Nature* 1999;402:B24–B30.
- MacGlashan DW Jr, Bochner BS, Adelman DC, Jardieu PM, Togias A, McKenzie-White J, Sterbinsky SA, Hamilton RG, Lichtenstein LM: Down-regulation of Fc(ε)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol* 1997;158:1438–1445.

- 8 Lin H, Boesel KM, Griffith DT, Prussin C, Foster B, Romero FA, Townley R, Casale TB: Omalizumab rapidly decreases nasal allergic response and FcεRI on basophils. *J Allergy Clin Immunol* 2004;113:297–302.
- 9 Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, Rao VK, Metcalfe DD: Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res* 2003;27: 677–682.
- 10 Kishi K: A new leukemia cell line with Philadelphia chromosome characterized as basophil precursors. *Leuk Res* 1985;9:381–390.
- 11 Blom T, Huang R, Aveskogh M, Nilsson K, Hellman L: Phenotypic characterization of KU812, a cell line identified as an immature human basophilic leukocyte. *Eur J Immunol* 1992;22:2025–2032.
- 12 Hara T, Yamada K, Tachibana H: Basophilic differentiation of the human leukemia cell line KU812 upon treatment with interleukin-4. *Biochem Biophys Res Commun* 1998;247: 542–548.
- 13 Blom T, Nilsson G, Sundstrom C, Nilsson K, Hellman L: Characterization of a human basophil-like cell line (LAMA-84). *Scand J Immunol* 1996;44:54–61.
- 14 Magnusson CG, Haard J, Matsson P, Karlsson T, Nilsson K, Johansson SG: Demonstration of specific high-affinity Fc epsilon-receptors on the human basophil-like leukemia cell line KU812 by flow cytometry. *Allergy* 1995;50: 72–77.
- 15 Dissing S, Nauntofte B, Sten-Knudsen O: Spatial distribution of intracellular, free Ca<sup>2+</sup> in isolated rat parotid acini. *Pflugers Arch* 1990; 417:1–12.
- 16 Jensen BM, Hansen JB, Dissing S, Gerwien J, Skov PS, Poulsen LK: Monomeric immunoglobulin E stabilizes FcεRIα from the human basophil cell line KU812 by protecting it from natural turnover. *Clin Exp Allergy* 2003;33: 655–662.
- 17 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- 18 Glue C, Hansen JB, Schjerling P, Jinquan T, Poulsen LK: LPS-induced cytokine production in the monocytic cell line THP-1 determined by multiple quantitative competitive PCR (QC-PCR). *Scand J Clin Lab Invest* 2002;62: 405–412.
- 19 Saini SS, Richardson JJ, Wofsy C, Lavens-Phillips S, Bochner BS, Macglashan DW Jr: Expression and modulation of FcεRIα and FcεRIβ in human blood basophils. *J Allergy Clin Immunol* 2001;107:832–841.
- 20 Almlof I, Nilsson K, Johansson V, Akerblom E, Slotte H, Ahlstedt S, Matsson P: Induction of basophilic differentiation in the human basophilic cell line KU812. *Scand J Immunol* 1988;28:293–300.
- 21 Fischkoff SA, Kishi K, Benjamin WR, Rossi RM, Hoessly MC, Hoxie JA, Seals C, Anderson L, Ishizaka T, Hakimi J: Induction of differentiation of the human leukemia cell line, KU812. *Leuk Res* 1987;11:1105–1113.
- 22 Fujimura Y, Tachibana H, Yamada K: A tea catechin suppresses the expression of the high-affinity IgE receptor Fc epsilon RI in human basophilic KU812 cells. *J Agric Food Chem* 2001;49:2527–2531.
- 23 Kraft S, Novak N, Katoh N, Bieber T, Rupec RA: Aggregation of the high-affinity IgE receptor Fc(ε)RI on human monocytes and dendritic cells induces NF-κB activation. *J Invest Dermatol* 2002;118:830–837.
- 24 Fukuda T, Kishi K, Ohnishi Y, Shibata A: Bipotential cell differentiation of KU-812: Evidence of a hybrid cell line that differentiates into basophils and macrophage-like cells. *Blood* 1987;70:612–619.
- 25 MacGlashan D Jr, McKenzie-White J, Chichester K, Bochner BS, Davis FM, Schroeder JT, Lichtenstein LM: In vitro regulation of FcεRIα expression on human basophils by IgE antibody. *Blood* 1998;91:1633–1643.
- 26 Furuichi K, Rivera J, Isersky C: The receptor for immunoglobulin E on rat basophilic leukemia cells: Effect of ligand binding on receptor expression. *Proc Natl Acad Sci USA* 1985;82: 1522–1525.
- 27 Boumiza R, Monneret G, Forissier MF, Savoye J, Gutowski MC, Powell WS, Bienvenu J: Marked improvement of the basophil activation test by detecting CD203c instead of CD63. *Clin Exp Allergy* 2003;33:259–265.
- 28 Miura K, MacGlashan DW Jr: Expression of protein kinase C isozymes in human basophils: Regulation by physiological and nonphysiological stimuli. *Blood* 1998;92:1206–1218.
- 29 Ozawa K, Yamada K, Kazanietz MG, Blumberg PM, Beaven MA: Different isoenzymes of protein kinase C mediate feedback inhibition of phospholipase C and stimulatory signals for exocytosis in rat RBL-2H3 cells. *J Biol Chem* 1993;268:2280–2283.
- 30 Robertson MW: Phage and *Escherichia coli* expression of the human high affinity immunoglobulin E receptor α-subunit ectodomain. Domain localization of the IgE-binding site. *J Biol Chem* 1993;268:12736–12743.
- 31 MacGlashan D Jr, Xia HZ, Schwartz LB, Gong J: IgE-regulated loss, not IgE-regulated synthesis, controls expression of FcεRI in human basophils. *J Leukoc Biol* 2001;70:207–218.
- 32 Borkowski TA, Jouvin MH, Lin SY, Kinet JP: Minimal requirements for IgE-mediated regulation of surface Fc epsilon RI. *J Immunol* 2001;167:1290–1296.
- 33 Kubo S, Matsuoaka K, Taya C, Kitamura F, Takai T, Yonekawa H, Karasuyama H: Drastic up-regulation of FcεRI on mast cells is induced by IgE binding through stabilization and accumulation of FcεRI on the cell surface. *J Immunol* 2001;167:3427–3434.