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Intra- and inter-laboratory validation of an innovative huFcεRIα-RBL-2H3 degranulation assay for *in vitro* allergenicity assessment of whey hydrolysates

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ABSTRACT

Cow's milk-derived whey hydrolysates are milk substitutes for cow's milk allergic infants. Safety assessment of these hydrolysates is crucial. Currently, huFcεRIα-RBL-2H3 cells, sensitized with serum IgE from cow's milk allergic patients, are used to assess *in vitro* residual allergenicity. However, limited availability and high inter-lot variation of sera impede the standardization of safety testing. Recently, we generated an oligoclonal pool of chimeric human (chu)IgE antibodies against bovine β-lactoglobulin (BLG) as an alternative for human serum. These antibodies demonstrated increased sensitivity, specificity and reproducibility. An inter-laboratory ring trial using our new degranulation assay with different whey-based hydrolysates was performed at four independent laboratories to investigate the robustness and reproducibility. RBL-2H3 cells expressing huFcεRIα were sensitized with our oligoclonal pool of anti-BLG chuIgE antibodies. The cells were subsequently incubated with an amino-acid based formula (AAF), two extensively hydrolyzed formulas (eHF) and three partially hydrolyzed formulas (pHF) to assess the degranulation upon challenge. Results demonstrated a very strong inter-laboratory correlation and the intra- and inter-laboratory variations were acceptable. The AAF and both eHFs showed no degranulation, whereas all pHFs demonstrated degranulation. The study showed that this degranulation assay is robust and reproducible within and between laboratories. This new *in vitro* degranulation assay seems predictive for allergenicity outcome and might therefore be considered as a relevant substitute for animal models.

1. Introduction

Allergic responses to cow's milk proteins (CMP) leading to cow's milk allergy (CMA) are characterized by a T helper 2 response that result in the production of allergen-specific IgEs. These IgEs bind to high affinity IgE receptors (FcεRI) on mast cells or basophils, followed by cross-linking of these IgEs by allergens, eliciting degranulation and thus the release of mediators such as histamine, leukotrienes, and inflammatory cytokines. The optimal conditions for this release depend on the concentration of membrane-bound allergen-specific IgEs, the concentration of the allergen and the affinity of the IgE for the allergen (Knol 2006; Christensen et al. 2008). Resulting clinical symptoms may present on the skin, in the gastrointestinal tract, and in the airways and may even include life threatening anaphylactic shock.

CMA is the most common allergy in early childhood, with an estimated prevalence of 3% in the pediatric population (Hudson 1995). While breastfeeding is considered to be the gold standard for infant nutrition, hypoallergenic (HA) formulas are a good alternative for infants at risk of developing allergy or for infants diagnosed with CMA. HA formulas contain hydrolyzed CMP which are processed by the enzymatic treatment, heat treatment and/or ultrafiltration of CMP. These

Abbreviations: AAF, amino acid-based formula; ALA, α-lactalbumin; BLG, β-lactoglobulin; CM, cow's milk; CMA, cow's milk allergy; eHF, extensively hydrolyzed formula; FcεRI, high affinity IgE receptor; HA, hypoallergenic; IgE, immunoglobulin E; RBL, rat basophilic leukemia; pHF, partially hydrolyzed formula.

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hydrolyzed formulas are generally categorized as either partial or extensive hydrolysates based on the degree of hydrolysis of the CMP, and can be characterized by assessing the molecular weight distribution of the residual proteins or peptides. However, allergenicity of HA formulas cannot be ascertained solely based on the peptide size distribution of the hydrolyzed proteins contained therein. Several clinical studies have been performed to address the sensitizing capacity of partial and extensive HA formulas in high risk children (Zeiger and Heller 1995; Halken et al. 2000; Chan et al. 2002; von Berg et al. 2003). Differences in peptide size, variations in protein sources and hydrolysis methods can modify the hypoallergenic nature of these formulas (Hays and Wood, 2005) stressing the importance of adequate pre-clinical testing of HA formulas. Animal models to assess the allergenicity of HA formulas have been developed but have never been fully validated (Fritsché 2003; van Esch et al. 2013). Therefore, an assessment of residual allergenicity of hydrolyzed formulas by peptide size distribution analysis, residual allergen detection by ELISA and SDS-PAGE/western blotting and immune incubation with specific antibodies, and *in vitro* cellular degranulation assays is proposed as a strategy for the screening of HA formulas. (van Esch et al., 2011). One of these proposed *in vitro* cellular degranulation assays uses the rat basophilic leukemia cell line RBL-2H3 stably transfected with the α-chain of the human FcεRI (RBL-huFcεRI) as target cells. The α-chain of human FcεRI associates with one β-chain and two γ-chains of rat FcεRI, and forms functional high affinity IgE receptor complexes on the surface of RBL-huFcεRI cells. Several investigators have extensively tested and established the usefulness of RBL-huFcεRI cell degranulation after exposure to IgE containing serum from allergic patients, and subsequent cross-linking with either anti-human IgE antibodies (Takagi et al. 2003) or with multiple allergens (Dibbern et al. 2003; Marchand et al. 2003; Ladics et al. 2008). Although these studies clearly demonstrated that RBL-huFcεRI cells form the basis of a relevant *in vitro* model system for the detection of allergens and for the study of IgE-allergen interactions (Takagi et al. 2003; Dibbern et al. 2003), it was also demonstrated that serum contained factors that blocked IgE binding to FcεRI or induced cross-linking of membrane-bound IgE by anti-human IgE antibodies (Takagi et al. 2003). Furthermore, Marchand et al. found that different serum IgEs trigger the release of mast cell mediator with variable efficacy and with a poor correlation to their immunochemically determined allergen-specific IgE content (Marchand et al. 2003). Ladics et al. (2008) thus concluded that (i) there was no consistent RBL-huFcεRI cell degranulation assay for a potential food allergen, and that (ii) the RBL-huFcεRI cell degranulation assay's utility in safety assessment, predictive value and reproducibility for evaluating the cross-reactivity of proteins with allergens needed further investigation with additional proteins and well-characterized sera (Ladics et al. 2008).

Recently, we developed a pool of six β-lactoglobulin (BLG)-specific chimeric human IgE monoclonal antibodies (MAb) covering the allergenic epitopes of BLG to improve the use of the RBL-huFcεRI cell degranulation assay (Knipping et al. 2014). The optimal concentration of our pool of BLG-specific chimeric IgE monoclonal antibodies for use in the RBL-huFcεRI degranulation assay was earlier established and tested with whey hydrolysates with different degrees of hydrolysis to assess sensitivity and specificity (Knipping et al. 2014). The ultimate goal for the development of the huFcεRIα-RBL-2H3 degranulation assay is to use it to predict residual allergenic potential in modified food proteins, in particular CMP hydrolysates. Currently, it is obliged to test such hydrolysates, mostly in animal models. The degranulation assay is proposed to assess the potential allergenicity of food proteins specifically for already allergic individuals. Degranulation assays are already in use for this purpose but unreliable sources of specific antibodies (from human sources) limit the implementation and acceptance of such assays. As a first step to acceptance of the huFcεRIα-RBL-2H3 degranulation assay, we have conducted this ring trial to assess the transferability to other laboratories. In this study, we have assessed the robustness and reproducibility of the degranulation assay at four different laboratories: Nutricia Research; Utrecht University of Applied Sciences (UAS); Bioceros; and the Institute for Risk Assessment Sciences (IRAS), all situated in the Netherlands.

2. Methods

2.1. Materials

All materials were purchased by the same supplier, and for the human IgE and anti-human IgE also the same lot number was used by all four laboratories. RBL-hεIa-2B12 cells were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin–streptomycin solution (Invitrogen) and 50 mg/ml Geneticin selective antibiotic (Invitrogen). For the 100% release, human purified IgE (Millipore AG30P; lot number2195995) and rabbit anti-human IgE antibodies (DakoCytomation A0094; lot number 00066372) were used. The pool of six β-lactoglobulin (BLG)-specific chimeric human IgE MAb (Bioceros) was made by combining 1 μg/ml of each MAb. Tyrode's buffer (pH 7.4) consists of 130 mM NaCl (Merck), 5 mM KCl (Merk), 1.4 mM $CaCl_2$ anhydrous (Merck), 1 mM $MgCl_2$ hexahydrate (Sigma), 5,6 mM D-(+)- glucose anhydrous (Sigma-Aldrich), 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; Merck) and 0.1% HSA (Sigma). Cells were lysed with 10% Triton X-100 (BDH Chemicals Ltd). The substrate 4-methyl umbelliferyl-N-acetyl-ß-D-glucosaminide (Fluka) was made in citrate buffer (0.1 M citric acid; Merck). For stimulation of degranulation the following CMP or HA formulas were used: BLG (Davisco Foods International), casein (Sigma-Aldrich), amino-acid-based formula (AAF; Neocate®, Nutricia), extensively hydrolyzed formula (eHF; Nutrilon pepti®, Nutricia) and four blinded whey-based hydrolysates (H1–H4; FrieslandCampina). After finalization of all degranulation experiments (see below), H1, H3 and H4 proved to be partially hydrolyzed formulas, whereas H2 was an extensively hydrolyzed formula.

2.2. BLG protein ELISA

For the determination of residual BLG proteins in the four hydrolyzed formulas (H1–H4), the IN VITRO TEST™ Beta-Lactoglobulin ELISA Kit (ELISA Systems; ESBLG-48 or ESBLG-96) was performed according to the manufacturer's protocol.

2.3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The pattern of the residual proteins in the four hydrolyzed formulas (H1–H4) was analyzed with SDS-PAGE. A Bio-Rad Mini-PROTEAN II system was used with a 20% TGX gel (Bio-Rad). Molecular weight markers (Bio-Rad) were used as a reference. The proteins were diluted in a 1:3 ratio with reducing sample buffer (6.05 g Tris (Roche), 8.0 g SDS (Boehringer Mannheim), 3.2 g dithiothreitol (ICN), 20 mg bromophenol blue (Merck) in 60 ml H_2O and 40 ml glycerol 87% (Merck), pH 6.8) and 100 μg of protein was loaded on the gel. The marker is a standard broad-range marker with a range of 250–10 kDa (Precision Plus Protein Standards; Bio-Rad). The proteins were stained in the gel using silver staining according to Wray et al. (1981).

2.4. Molecular weight distribution of peptides by gel permeation chromatography (GPC)

Hydrolysate samples were dissolved in 5 mg/ml HPLC-eluent (eluent composition is dependent on the column to be used and is specified below for the appropriate column), and undissolved particles were removed by centrifugation (5 min at 13000 rpm), if necessary followed by filtration (0.45 μm cellulose acetate filter).

Size exclusion chromatography for the eHF (H2) was performed with a Superdex Peptide 10/300 column (GE Healthcare) at 30 °C,

with a flow rate of 0.9 ml/min using an injection of 20 µl of a 5 mg/ml HPLC-eluent (7.4 g trifluoro acetic acid and 1173 g acetronitrile in 3500 g H_2O) solution. The column was calibrated with 10 peptide standards: Cytochrome C ($M = 12327$), Aprotinin ($M = 6500$), Adrenocorticotropic hormone from porcine pituitary gland ($M = 4567$), Insulin A-chain oxidized ammonium salt from bovine pancreas ($M = 2532$), Angiotensinogen 1–14 renin substrate porcine ($M = 1759$), Bradikinin salt ($M = 1060$), Bradykinin fragments 1–7 ($M = 757$), Bradykinin fragments 1–5 ($M = 573$), Ala-Ala-Ala-Ala-Ala ($M = 373$) and Gly-Leu ($M = 188$) all from Sigma-Aldrich. The eluate was monitored at 200 nm.

Size exclusion chromatography for the pHF (H1, H3 and H4) was performed with a Superdex 75 10/300GL column (GE Healthcare) at 25 °C, with a flow rate of 0.5 ml/min using an injection of 20 µL of a 5 mg/ml HPLC-eluent (8.76 g NaCL and 7.35 g $CaCl_2{}^*2H_2O$ in 1 L H_2O; mix 900 g of this solution with 78.0 propanol-2) solution. The column was calibrated with 9 peptide standards: Bovine serum albumin ($M = 66000$), Ovalbumin ($M = 43000$), BLG ($M = 18400$), Myoglobin ($M = 17800$), ALA ($M = 14200$), Cytochrom C ($M = 12327$), Aprotinin ($M = 6500$), Adrenocorticotropic hormone from porcine pituitary ($M = 4567$) and Insulin A-chain ($M = 2532$) all from Sigma-Aldrich. The eluate was monitored at 214 nm.

2.5. Degranulation of RBL-huFcεRI cells (RBL-hεIa-2B12 cells)

The cell-line RBL-hεIa-2B12, which was transfected with the α-chain of human Fcε receptor type 1 (hu) FcεRI complex (Takagi et al. 2003), was used for the RBL-huFcεRI degranulation assay. Degranulation of RBL-huFcεRI cells was performed as described previously (Knipping et al. 2014). Confluent growing RBL-huFcεRI cells (1×10^5/well) in 96-wells flat bottom culture plate were sensitized overnight with 3 µg/ml commercially available human purified IgE and stimulated with 10 µg/ml rabbit anti-human IgE antibodies in Tyrode's buffer for 1 h. This release served as maximum release (100% degranulation). The sensitized cells with a pool of BLG-specific chimeric IgE monoclonal antibodies (Bioceros) were stimulated with anti-human IgE (10 µg/ml Tyrode's buffer), BLG, casein, AAF, eHF or blinded whey-based hydrolysates (1 µg/ml in Tyrode's buffer/HSA) for 1 h. The minimal degranulation (min) indicates the spontaneous degranulation of untreated cells and should always be below 20%. β-Hexosaminidase activity was determined by a fluorescence assay using 4-methylumbelliferyl-N-acetyl-α-D-glucosamine as a substrate (Linko-Lopponen and Makinen 1985). The β-hexosaminidase activity released into the medium was expressed as the percentage of maximum release observed after cross-linking with anti-human IgE antibodies (set as 100% degranulation, see above). Individual samples were tested in triplicate, and each degranulation assay was performed 10 times by each lab on different days.

2.6. Statistical analysis

Samples were scored positive for degranulation when the percentage of degranulation was above min (spontaneous degranulation) + 20% (calculated variation of the assay in this study). This variation is the average (20%) of the variation in % per condition (tested sample n = 10) per lab which ranged from 17 to 23%. Comparison of degranulation was done by using a two-tailed Pearson Chi-Square test using the statistical software package SPSS 19.0. Intra- and inter-laboratory precision and reproducibility analysis (performance characteristics) were performed by 10 independent replicate analysis and results were reported as mean % degranulation, standard deviation (SD), and coefficients of variation (CV; acceptable 0.2–0.5 for *in vivo* and cell based assays http://www.bsrlabs.com/Assay-Development.html). Correlation of results between the different laboratories was performed by linear regression analysis and Pearson's correlation coefficient (r).

3. Results

3.1. Characterization of hydrolysates H1–H4 using BLG protein ELISA, molecular weight distribution of peptides by gel permeation chromatography (GPC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The BLG ELISA showed that pHF H1, H3 and H4 contained around 0.4 ppm BLG, whereas the eHF H2 was below the detection limit of 0.2 ppm (Fig. 1A). Results of the SDS-PAGE are depicted in Fig. 1B.

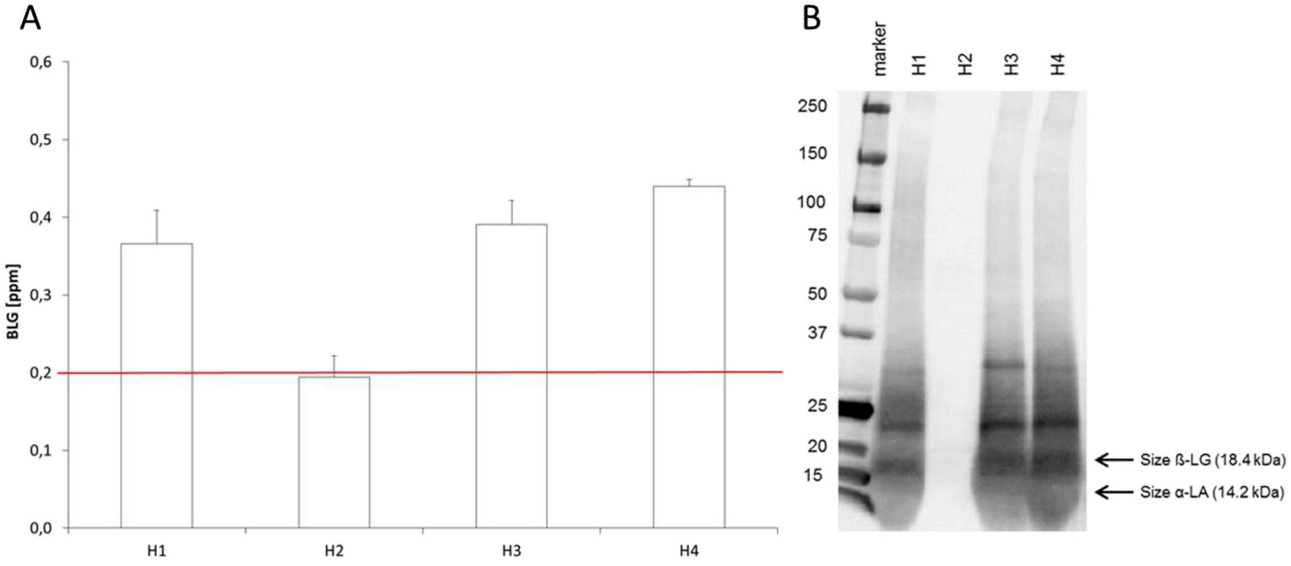

Fig. 1. The residual concentrations (ppm) of BLG of partially hydrolyzed formulas H1, H3 and H4 and extensively hydrolyzed formula H2 (A). Data are expressed as mean + SD (n = 3). The line indicates the detection limit (0.2 ppm). Protein patterns by SDS-PAGE of partially hydrolyzed formulas H1, H3 and H4 and extensively hydrolyzed formula H2 (B). The marker is a standard broad-range marker.

Table 1
Molecular weight distribution in % of the hydrolyzed formulas H1–H4.

Molecular weight (Da)	H1 (%)	H2 (%)	H3 (%)	H4 (%)
>20,000	14.9		6.9	6.7
10,000–20,000	5.8	0.1	4.9	5.8
5000–10,000	17.6	2.2	24.3	23.3
3,000–5000	17.8	7.0	19.6	20.2
1,000–3,000	43.9	36.8	44.4	43.9
500–1,000		24.6		
<500		29.3		

There were no (residual) proteins detected in the eHF H2, whereas distinct bands, especially BLG at 18.4 kDa, were visible in the pHF H1, H3 and H4. The molecular weight distribution of the peptides is shown in Table 1. These results clearly showed that a much higher percentage of >10 kDa peptides (ranging from 20.7–12.5%) were present in pHF H1, H3 and H4 than in eHF H2 (0.1%).

3.2. Inter- and intra-laboratory variation and robustness of the degranulation assay

During the set-up phase of the assay at the different laboratories it was found that some of the materials were very crucial for obtaining comparable results. Culturing of the RBL-huFcεRI cell was optimal in culture flasks from NUNC (cat no 156499) and transparent flat bottom 96-wells plates from BD Falcon (cat no 353072). In previous experiments when comparing different batches of FBS (unpublished data), it was found that some batches from different suppliers showed higher background degranulation. While performing the degranulation assay in the current and previous studies, especially the human IgE and anti-human IgE used for the 100% degranulation from different suppliers proved to be crucial for the reproducibility of the results (data not shown).

Results of the huFcεRIα-RBL-2H3 degranulation assay (10 individual experiments) from all four laboratories are shown in Table 2 and Fig. 2. 'Min' indicates the spontaneous degranulation of the cells. 'hIgE + anti-hIgE' was set at 100% representing IgE-dependent degranulation. The positive controls, MAb pool stimulated with anti-hIgE or intact BLG, showed a degranulation of ≈80%. The negative controls, MAb pool stimulated with casein, AAF or eHF, showed no degranulation. pHF H1, H3 and H4 showed degranulation of 60–80% whereas the eHF H2 did not show any degranulation. The most important finding is that with the Y/N score for degranulation (assessment whether the hydrolysate is able to evoke an allergic reaction), all the laboratories evaluate the tested hydrolysates in the exact same manner. Intra-laboratory (CV: Nutricia 0.07–0.43, UAS 0.10–0.88, Bioceros 0.07–0.45 and IRAS 0.13–0.40) and inter-laboratory (CV: 0.03–0.40) variations of degranulation results were acceptable (0.2–0.5 for cell based assays; only 2 outliers of 0.81 and 0.88 by UAS). Moreover, a very strong correlation (r = 0.857–0.934) was found between the degranulation results determined by all participating laboratories (Table 3).

4. Discussion

Growing concerns about food safety have increased the attention for legislation around health claims on food products. These health claims need to be substantiated with experimental data, and for HA formulas, the elimination of potential allergic epitopes needs to be confirmed. For safety assessment of CM hydrolysates, a robust and reproducible functional assay for determination of (non)allergenicity is essential. For this purpose we recently developed a pool of six BLG-specific chimeric human IgE monoclonal antibodies, covering the same allergenic BLG epitopes as identified by epitope mapping using sera of CMA individuals, for the use in a mast cell degranulation assay using RBL-cells (Knipping et al. 2014). This *in vitro* RBL degranulation assay is one of many tests used for the assessment of residual allergenicity of whey hydrolysates. Other assays includes peptide size distribution analysis, and residual allergen detection by ELISA and SDS-PAGE/western blotting. The hydrolysates H1, H3 and H4 tested in this ring trial showed residual levels of BLG in the ELISA and SDS-PAGE, and the peptide size distribution demonstrated a higher percentage of >10 kDa peptides, whereas H2 tested negative for residual (BLG) proteins in all assays.

The developed chimeric human IgE antibodies specifically recognize BLG, which is the major allergen (≈55–65%) in bovine whey. ALA is another allergen (≈15–25%) in bovine whey. In previous experiments in which whey protein degradation was followed during the hydrolysis process, a concurrent degradation of BLG and ALA was shown (Knipping et al. 2012). Therefore, this degranulation assay with our pool of BLG-specific chimeric human IgE monoclonal antibodies, could be a highly relevant and sensitive *in vitro* model to assess the safety of hydrolyzed formula for infants diagnosed with CMA, regardless of whether it concerns BLG or ALA.

The aim of the current study was to assess the robustness and reproducibility of the degranulation assay at four different laboratories; Nutricia Research, UAS, Bioceros and IRAS. During the set-up phase of the assay at the different laboratories it was found that consistency in some of the materials was crucial in order to obtain comparable results and for this ring trial it was decided to use the same materials, and if applicable (antibodies) the same lot numbers, at all laboratories as indicated in the Method section. The hydrolysates H1, H3 and H4 tested in this ring trial showed degranulation of 60–80% whereas the H2 hydrolysate did not show any degranulation, which is completely in agreement with the results from the BLG ELISA, SDS-PAGE and peptide size distribution analysis. The degranulation results demonstrated a very strong correlation between all laboratories (Pearson's $r > 0.85$), and the inter- (CV: 0.03–0.40) and intra-laboratory variations (CV 0.03-0.88) were even

Table 2
Degranulation as percentage of maximum degranulation induced with hIgE + anti-hIgE (=100%) obtained from four different laboratories; Nutricia Research, Utrecht University of Applied Sciences (UAS), Bioceros and Institute for Risk Assessment Sciences (IRAS). Mean, SD and CV (within and between laboratories (overall)) from 10 independent experiments (samples tested in triplicate), and degranulation judgment Y/N (*i.e.*, above min + 20%) are indicated.

Samples	Nutricia				UAS				Bioceros				IRAS				Overall
	Mean %	SD	CV	Y/N	Mean %	SD	CV	Y/N	Mean %	SD	CV	Y/N	Mean %	SD	CV	Y/N	CV
Min	11.7	2.2	0.19	N	9.0	7.3	0.81	N	19.5	8.8	0.45	N	10.6	3.9	0.37	N	0.37
MAb pool + a-hIgE	77.2	5.3	0.07	Y	82.7	8.2	0.10	Y	79.2	11.4	0.14	Y	80.1	10.8	0.13	Y	0.03
BLG	61.7	6.6	0.11	Y	81.4	10.9	0.13	Y	70.3	5.1	0.07	Y	81.2	11.5	0.13	Y	0.13
Casein	23.9	7.5	0.43	N	19.9	9.6	0.88	N	25.3	8.5	0.42	N	24.1	9.6	0.28	N	0.40
AAF	13.7	5.9	0.32	N	8.8	7.8	0.48	N	20.2	8.4	0.34	N	9.7	2.9	0.38	N	0.10
eHF	21.3	6.6	0.31	N	27.5	10.3	0.38	N	24.8	9.5	0.38	N	16.2	6.6	0.39	N	0.22
H1	58.5	6.7	0.11	Y	72.5	10.4	0.14	Y	59.3	10.3	0.17	Y	73.7	15.2	0.20	Y	0.13
H2	20.6	5.9	0.29	N	23.6	12.2	0.52	N	26.2	9.2	0.35	N	18.7	7.9	0.40	N	0.15
H3	63.5	6.3	0.10	Y	75.7	10.0	0.13	Y	59. 7	10.9	0.18	Y	75.3	14.4	0.18	Y	0.12
H4	64.3	6.1	0.10	Y	76.4	8.6	0.11	Y	63.1	14.7	0.23	Y	75.7	13.1	0.16	Y	0.10
Min + 20%	31.7				29.0				39.5				30.6				

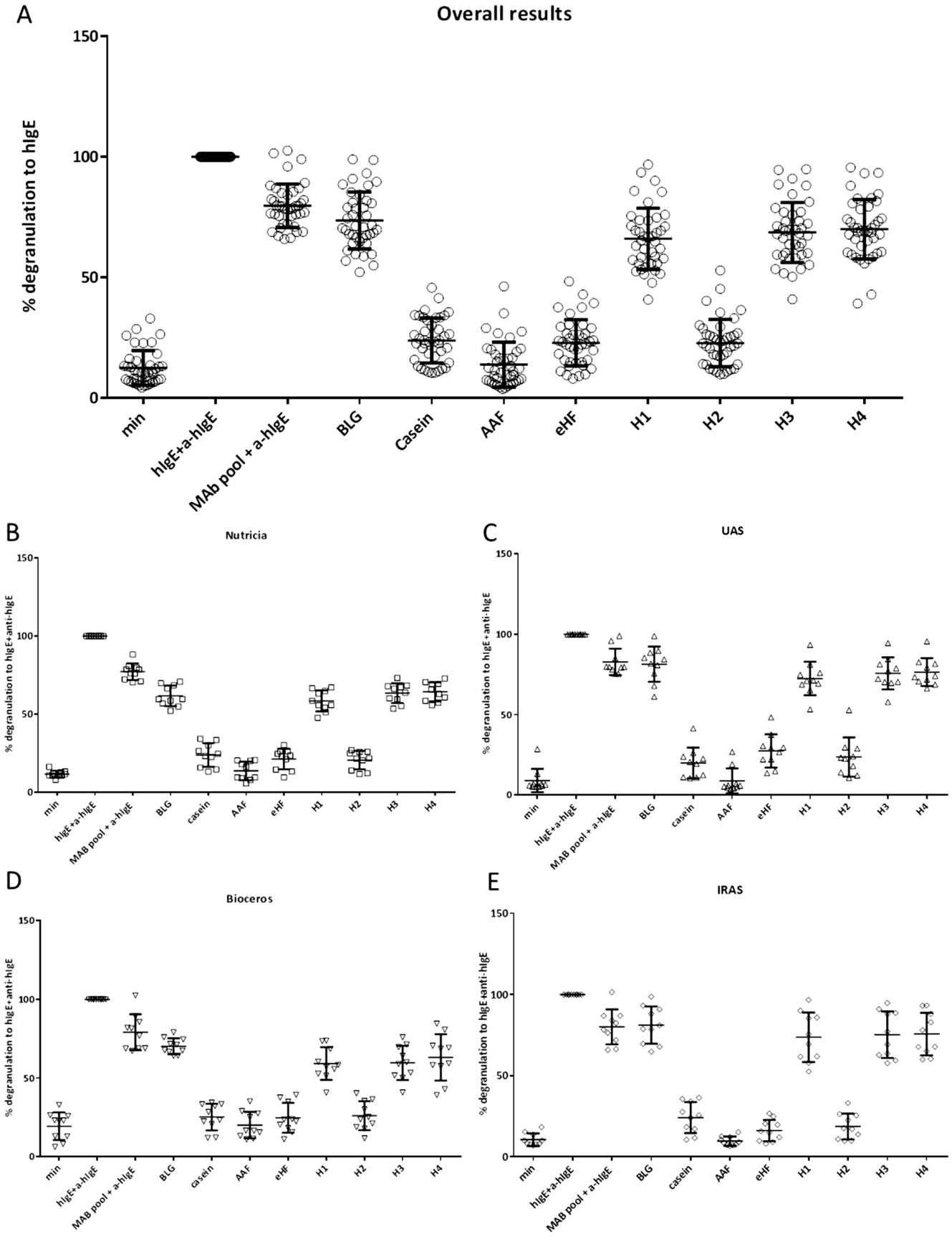

Fig. 2. RBL-huFceRI assay with the pool of BLG-specific chimeric human IgE monoclonal antibodies. Human FceRI a chain expressing RBL cells were incubated with the pool of chimeric huIgE anti-BLG antibodies, and subsequently cross-linked with anti-hIgE antibodies (10 μg/ml), BLG, casein, AAF, eHF and whey-based hydrolysates H1–H4 (all 1 μg/ml). The percentage of degranulation from each examined preparation is indicated: compiled results from all participating labs (A) and results from Nutricia Research (B), UAS (C), Bioceros (D) and IRAS (E). Data are expressed as mean ± SD of 10 independent experiments.

Table 3
Correlation (*r*) analysis between the different laboratories; Nutricia Research, Utrecht University of Applied Sciences (UAS), Bioceros and Institute for Risk Assessment Sciences (IRAS).

	UAS	Bioceros	IRAS
Nutricia	0.934	0.907	0.930
UAS		0.857	0.929
Bioceros			0.879

below or within the acceptable range of 0.2–0.5 except for 2 outliers of 0.81 and 0.88.

The control hydrolysates AAF (Neocate, Nutricia) and eHF (Nutrilon pepti, Nutricia), as well as extensively hydrolyzed formula H2 (FrieslandCampina) used in this ring trial, are commercially available infant formulas for the management of cow's milk allergy. According to legislation, these hydrolysates have been tested in *in vivo* and clinical studies (de Boissieu and Dupont 2002; Giampietro et al. 2001) before being launched onto the market and demonstrated good outcome on allergenicity.

In conclusion, the ring trial showed that the *in vitro* degranulation assay is robust and reproducible within and between laboratories and that this assay seems predictive for *in vivo* allergenicity outcome. The huFcεRIα-RBL-2H3 degranulation assay may therefore be considered as a relevant substitute for animal models after full validation.

Transparency document

The Transparency document associated with this article can be found, in online version.

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