## huRBL Mediator Release/Degranulation Assay

This assay is based on the use of humanized rat basophilic leukaemia cells (huRBL) for investigating the allergenic response of different antigens (allergens) in allergy research and clinical diagnostics. This cell line is transfected with the human FcεRI, which is the high-affinity receptor for IgE that is introduced into the assay using donor sera derived from allergic patients (RAST class ≥3 for Bet v 1/birch pollen extract). Upon exposure to the allergen the respective IgE is specific for, the cells undergo degranulation resulting in the release of different mediators leading to an allergic response. The release of the mediator β-hexosaminidase can then be measured after addition of a substrate reacting to the released β-hexosaminidase resulting in detectable changes of fluorescence intensity [1].

This assay was used to detect possible changes in the allergenic response of Bet v 1 upon adsorption on our four differently coated $SiO_2$ NPs caused by structural alterations of Bet v 1 and was performed as previously described [2]. For this, huRBL cells (RBL-2H3) were cultured in a 75 $cm^2$ tissue culture flask in huRBL medium (Minimum essential medium Eagle, Sigma Aldrich, Vienna, Austria; supplemented with 4 mM L-glutamine, 5% heat inactivated fetal calf serum, 0.5 mg/mL Gentamycin418) at 37° C in sterile, humidified conditions with 5% $CO_2$ and grown until 80% confluency. Additionally, AG8 cells (ATCC, Wesel, Germany) were cultured in AG8 medium (Opti-MEM I + Glutamax I, reduced serum medium, Gibco™ ThermoFisher Scientific, Paisley, UK; supplemented with 5% heat inactivated fetal calf serum, 1 mg/mL PenStrep) in the same incubator used for the huRBL cells. These cells were used for pre-incubation of the donor serum in order to deplete the patients complement system, which would otherwise attack the huRBL cells. The adherent huRBL cells were harvested by detaching via trypsinisation (1x Trypsin-EDTA incubated for 5 min at 37°C, 5% $CO_2$) after washing the cells twice with 10 mL PBS. The cells where then centrifuged at 250 *g* for 5 min and resuspended in fresh huRBL medium. After counting the cells with a haemocytometer, they were diluted to 1 x $10^6$ cells/mL and 100 µL of the cell suspension were plated to each well of a sterile 96-well cell culture plate (flat bottom with lid, Costar) and incubated overnight at 37°C, 5% $CO_2$. After this, the AG8 cells were harvested (one flask grown for 4 days sufficient for serum incubation needed for two 96-well plates) by centrifugation (250 *g*, 5 min) and resuspended in the required amount of huRBL medium for subsequent pre-incubation of the patients sera for 1 hour, 37°C, 5% $CO_2$. The sera were then centrifuged 5 min at 250 *g* and 100 µL of the supernatants were aliquoted to the sample wells of the 96-well cell culture plate containing the huRBL cells. Serum incubation was conducted overnight at 37°C and 5% $CO_2$. The next day, antigen dilutions were prepared by diluting ready-made coupling samples of Bet v 1 bound to the four differently coated $SiO_2$ NPs and Bet v 1 only with tyrodes buffer ($D_2O$ + $H_2O$ 1:1, 0.95% tyrodes salt, 0.05% $NaHCO_3$, 0.1% BSA). Serial sample dilutions from 1000 ng/mL to 0.0001 ng/mL Bet v 1 were prepared in a second 96-well plate and 100 µL of these antigen dilutions were then added

to the respective wells of the cell culture plate containing the huRBL cells after washing them three times with washing buffer ($H_2O$, 0.95% tyrodes salt, 0.05% $NaHCO_3$, 0.1% BSA) after the overnight serum incubation of the cells. The cells in the sample wells were then incubated for 1 hour at 37°C and 5% $CO_2$ with the antigen dilutions. After this, 10 µL 10% Triton X-100 were added to separate aliquots of the cells (in quintuplicates) to determine the total β-hexosaminidase content of the cells as 100% release control. Then, 50 µL supernatant of the antigen incubated cells and the cells of blank and 100% release control wells were added to 50 µL freshly prepared assay solution (0.1 M citrate buffer pH 4.5, 1.6% 10 mM 4-methyl umbelliferyl-N-acetyl-β-D-glucosaminide, Sigma-Aldrich, St.Louis, MO, USA) and incubated for 1 hour at 37°C and 5% $CO_2$.

Additionally, the remaining supernatant of the huRBL cells in the cell culture plate was discarded and 100 µL 1x MTT solution (1x PBS, 0.05% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the cells and also incubated for 1 hour at 37°C and 5% $CO_2$. The MTT assay was simultaneously performed with the huRBL assay as control experiment in order to confirm the cells viability. The assay was performed as previously described [3]. This colorimetric assay determines the number of viable cells via intracellular reduction of the MTT compound into coloured formazan crystals whose concentration can be determined by measuring the optical density at 570 nm with a plate reader [4].

After incubating both plates (huRBL assay and MTT assay) for 1 hour, the reactions were stopped by adding 100 µL RBL stop solution (Glycine buffer pH 10.7) and 100 µL MTT stop solution (0.1 M glycine solution pH 10.2 in DMSO) to the corresponding plate after discarding the 1x MTT solution from the huRBL cells. Then the fluorescence intensity of the huRBL plate was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm using 25 flashes using a microplate reader (Tecan M200pro). The absorbance of the MTT plate was measured at 570 nm using the same device. Cells were considered as healthy and suitable for RBL assay after determining minimum 70% cell viability with the MTT assay.

The specific release (%) of the huRBL cells induced by the different Bet v 1 samples was calculated using the following equation:

$$\frac{\text{fluorescence of sample} - \text{fluorescence of blank}}{\text{fluorescence of 100 \% release (positive control)} - \text{fluorescence of blank}} \times 100 = \% \text{ release}$$

The specific release in percent was then used to determine the antigen concentration for half maximum release (ng/mL) expressed in a scatter plot with logarithmic scale.

References:


1. Sun, N., et al., *Use of a rat basophil leukemia (RBL) cell-based immunological assay for allergen identification, clinical diagnosis of allergy, and identification of anti-allergy agents for use in immunotherapy.* Journal of immunotoxicology, 2015. **12**(2): p. 199-205.
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4. Van Meerloo, J., G.J. Kaspers, and J. Cloos, *Cell sensitivity assays: the MTT assay*, in *Cancer cell culture*. 2011, Springer. p. 237-245.