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# FLOW CYTOMETRIC ANALYSIS OF THE BASOPHIL CELL ACTIVATING IMPACT OF POTENTIAL DRUG DELIVERY NANOPARTICLE-CANDIDATE

ÉVA KISS-TÓTH DOJCSÁK[1]*, ZOLTÁN FERENCZI[1], ADRIENN SZALAI JUHÁSZ[1], EMŐKE KISS-TÓTH[2], OLIVÉR RÁCZ[1,3] and BERTALAN FODOR[1]

[1] Department of Nanobiotechnology and Regenerative Medicine, Faculty of Health, University of Miskolc, Miskolc, Hungary
[2] Department of Preventive Health Sciences, Faculty of Health, University of Miskolc, Miskolc, Hungary
[3]Department of Pathological Physiology, Medical Faculty, Šafárik University, Košice, Slovakia

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*Introduction:* Carbon nanotubes – as artificial nano-size ranged materials – have increasing role in the modern biomedical, diagnostic and therapeutic applications. There is a promising option for their use as more potential drug carriers. Despite the favourable properties, their impact (accumulation, elimination, etc.) on biological systems is largely unknown. The main limiting factor of medical use of nanomaterials in most cases is the potential hypersensitive side effect. It can develop in different route, but the activation of basophil granulocytes may play a central role in this process. *Objective:* Our aim was to test the direct activation ability of different, surface modified nanotubes on basophil granulocytes *in vitro*. In parallel we tested the effectiveness of BasoTest planned to use for this study. *Materials and methods:* Using the blood samples of allergic and healthy volunteers we examined the basophil degranulation in the presence of nanotubes and the expression level changes of cell-surface CD63 on FACS Calibur instrument. Our results were compared to positive (fMLP, Mite, Grass) and negative control samples. *Results:* The test we have chosen proved to be sufficiently sensitive and specific for further study. Significant basophil activation was observed in the presence of carbon nanotubes in healthy persons and allergic patients, as well. The activating effect of nanotubes was more prevailed in allergic population. *Conclusion:* Our experiments have proven the fact that nanotubes may play a role in the development of hypersensitive allergic reactions through their basophil granulocyte activator effect.

**Keywords:** carbon nanotubes, allergy, basophil granulocyte

* Corresponding author; E-mail: evakisstoth@gmail.com

## Introduction

The biological and medical applications of artificially prepared ultra-fine particles, like carbon nanotubes (CNT) are a subject to debate since their invention. They may be the choice for *in situ* tumour therapy, as specifically labelled nanoparticles can be delivered to the tumour tissue and through the photo-thermal effect can destroy malignant cells. Doxorubicin and paclitaxel attached to carbon nanotubes were successfully injected into tumour cells of SCID mice [1]. CNTs were also applied for *in vivo* visualization of mouse liver during small-animal Raman imaging, as well as in fluorescence imaging to demonstrate the localization of macrophages. SWCNTs (Single Walled Carbon Nanotubes) were used to display atherosclerotic lesions in the mouse carotid arteries, and also applied in the field of radiology as high-density radio emitting crystals [2–4].

However, CNTs cannot be applied without caution due to their physical-chemical properties. According to the nano-size they have relatively large surface area posing dangerous side-effects after their intravenous and intranasal applications. The smaller, single-walled carbon nanotubes can be easily removed by urine or faces during the body's excretion, but the multi-walled carbon nanotubes showed a short blood circulation time beside accumulation in surrounding tissues causing local inflammation [5–7]. After intravenous application, these particles are easily extracted by the reticuloendothelial system (RES) from the circulation and they accumulate in the lungs, liver and spleen. Functionalization of the carbon skeleton by polyethylene [PEG] coating can ensure longer time in circulation, which is an important aspect of their therapeutic application [8].

The lack of bio-degradability of CNTs is the most important limiting factor of their medical application. Carbon nanotubes are very hard and chemically stable structures and therefore there is a potential risk of their accumulation and ultimately a toxic effect in the cells and tissues. Although, they have only slight direct toxic effects, the possibility of lung, liver and spleen damage in a long-term treatment cannot be excluded [9, 10].

Several studies in rodent models have shown that nanotubes induced local inflammation and fibrosis in their body exposed to carbon nanoparticles via inhalation, and also resulted in granuloma-like lesions in the lungs. The nanoparticles interact with pulmonary alveolar macrophages, and T lymphocytes [11]. Furthermore they provoke allergic reaction in the lung, which promotes the development of asthma or exacerbate existing symptoms of asthma. Beside the increased production of IgE, T cell proliferation and activation of B cells were observed in these processes [10, 11, 13].

Severe acute responses were not observed during intravenous administration of MWCNT (200–400 pg/mouse dose) in the mouse model [12], although this depends on the chemical composition and size of the carbon nanotube.

Allergic response may easily develop during the exposure of the carbon nanotubes. Ryman et al. and Nygaard et al. reported the allergic asthma promoting effect of intranasally or subcutaneously applied multi-walled carbon nanotubes in ovalbumin-sensitized mice [13, 14]. This effect was even more pronounced in animals with pre-existing inflammation process [13].

Bronchial asthma is associated with type I immediate hypersensitivity reaction. The main promoting factor of allergic reactions is the IgE binding on the surface of mast cells and basophil granulocytes. This binding forces the degranulation of these cells. In parallel with this process, the increase of expression of surface CD63 and CD203c is also detectable [15–17]. CD203c is an effective marker for detection of basophil granulocyte activation but not a selective one, because it is also expressed in many other IgE-activated cells [18]. CD63 is a membrane-associated glycoprotein stored in secretory granules, which appears in the cell membrane simultaneously with degranulation of basophil granulocytes. Its expression is fast and restricted only to certain cells following activation with IgE, and therefore it is a useful marker for detection of type I allergic reactions [19].

Basophil activation test (BAT) based on the detection of different antigens is a generally used flow cytometric method in studies of allergic reactions [20]. By this method different allergic reaction – such as action against drugs, hypersensitive reactions elicited by insect bites, food- , latex- or aeroallergens induced allergies – can be predicted with high efficiency [18, 19, 21].

The main purpose of our study was the investigation of direct activating effect of carbon nanotubes on basophil granulocytes by flow cytomteric method.

## Materials and Methods

*Carbon nanotubes*

Multi-walled carbon nanotubes (MWCNT) functionalized with carboxyl (COOH) or hydroxyl (OH) groups were produced at the Department of Applied and Environmental Chemistry, University of Szeged kindly provided by Professor Dr. Zoltán Kónya.

*Patients and controls*

Blood samples were collected from nine volunteers (4 men, 5 women; age: 20–55 years, median age 22 years) having dust mite and/or pollen allergy – verified by Prick skin test. Four of the nine people had only pollen allergy, 3 had dust mite allergy and 2 patients showed positivity for both types of allergies. Two patients suffering from acute pollen and dust mite allergy underwent antihistamine treatment (cetirizine, desloratadine).

The control group included 6 healthy (2 men and 4 women; age: 29–54 years, median 40.5 years) individuals.

*Basophil granulocyte activation*

Four ml of whole blood samples were collected in Li-heparin blood tubes (BD Vacutainer LH68IU) between 8.00–9.00 a.m. Blood samples were stored at room temperature and processed within 1 hour. Samples were further prepared according to instructions of BasoTest (Glycotope Biotechnology, Heidelberg, Germany). Briefly, to 100 ml sample 20 ml stimulating buffer was added and incubated for 10 min at 37ºC. Subsequently, 100 µl activator was added to the samples. The activator contained pollen extract (Grass), dust mite extract (Mite), MWCNT-OH or MWCNT-COOH (100 µg/ml final concentration). N-formyl-Met-Leu-Phe (fMLP) chemotactic peptide was used to activate our positive control samples, while the negative control samples (background) were treated with washing buffer only. After 20 minutes activation at 37ºC, the samples were stained with 20 µl dye mixture anti-hIgE FITC and anti-CD63 PE and incubated in the dark at room temperature for further 20 minutes. The labelling step was followed by addition of 2 ml of lysis buffer, then after 10 minutes of haemolysis the samples were centrifuged (5 min, 250 G, room temperature) and the supernatant was discarded. The non-lysed cells were washed with 3 ml of washing buffer, and the final sample volume was adjusted by the addition of 200 µl washing buffer. Two replicates per sample were performed.

*Analysis of activated basophil granulocytes*

The analysis of activation was performed on FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. The 50,000 leukocytes per

sample were analysed. CellQuest Flow Cytometry analysis software was used (BD Biosciences Pharmingen) for the evaluation of the results. In the first step, we determined the population of basophil granulocytes (gate R1), where the cells resulted low side scatter ($SSC^{low}$) with additional high IgE ($hIgE^{high}$) positivity. Second, the proportion of activated cells was determined in this population as the ratio of hIgE and CD63 double-positive cells (Fig. 1).

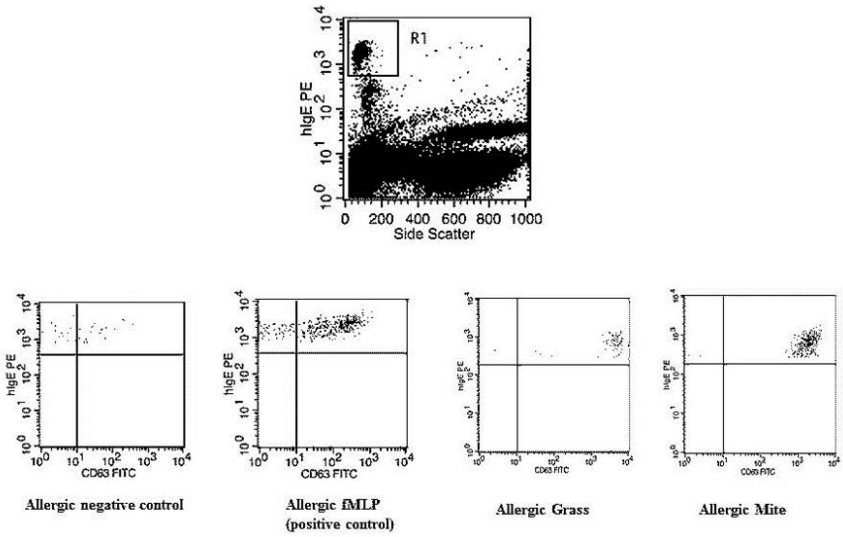


**Figure 1.** Basophil granulocyte population was determined on the basis of side scatter (SSC) and hIgE positivity, which is shown in the gate R1. Quadrant dot plots show the considerably shifted cloud images after different treatments at the bottom

The degree of activation was expressed as a percentage after subtracting the background value (negative control) from treated sample values.

Cut-off values, sensitivity and specificity of allergens provided by the test were determined using Receiver Operating Characteristic (ROC) analysis (SigmaPlot, Systat Software Inc.) and were compared with the 15% threshold value recommended by the manufacturer.

*Statistical analysis*

Statistical analysis of the data was performed using GraphPad Prism 5.02 statistical software. Non-parametric Mann–Whitney test was used for statistical

evaluation of the data. The difference between the test groups were considered statistically significant when P value was <0.05, respectively.

## Results

*Calculation the effectiveness of BasoTest*

The activation mean values of negative control (washing buffer treated) samples have proven to be similar to those achieved in healthy and allergic groups, numerically 1.18% for healthy controls (min.: 0%, max.: 20%) and 1.42% for allergic samples (min.: 0%, max.: 3.48%).

fMLP chemotactic protein – used as positive control – resulted intense CD63 expression in both healthy (median 75.57%, min.: 25.0%, max.: 90.62%) and allergic (median 45.77%, min.: 36.61%, max.: 87.50%) [P = 0.1447] test groups. fMLP stimulation showed 100% sensitivity (where the 95% coincidence interval [CI] 0.6637 to 1.000), and 16% specificity (where 95% [CI] 0.004211 to 0.6412) according to ROC analysis. The positive predictive value was 54.55% and the negative predictive value was 100%. The positive and negative likelihood ratios were 1.2 and 0.0. We considered the sample activated wherein the activation was higher than the 30.8% cut-off value (Figs 2 and 3). In our case, this limit was higher than 25.2% recommended by the manufacturer.

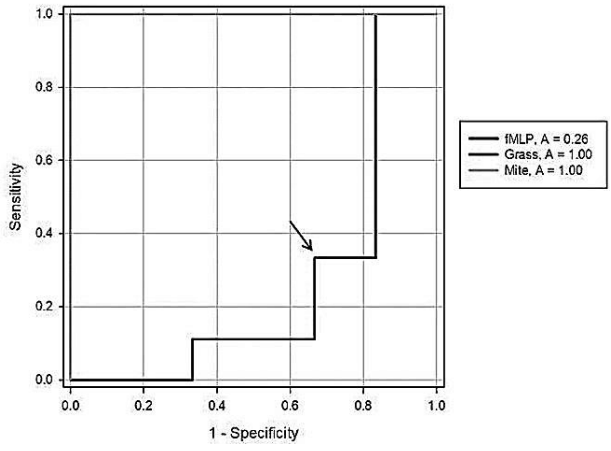


**Figure 2.** The Receiving Operating Characteristic (ROC) curve. Based on the ROC curve it is established that the effectiveness of the diagnostic test for allergies to pollen or dust mite allergens is maximal (A = 1:00). Area under the curve is 0.26 for fMLP activator (95% CI –0.06630 –0.5848). The optimal cut-off value is 30.8% (black arrow) for the positive control

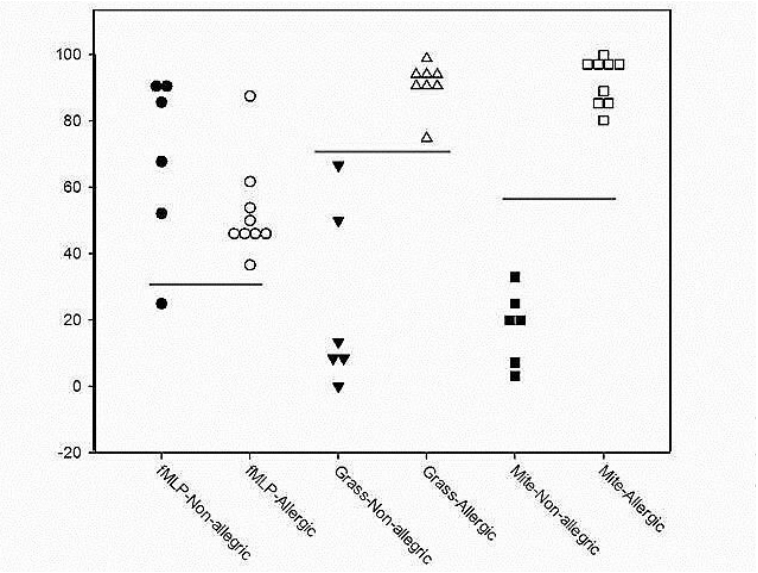


**Figure 3.** Determined cut-off values (horizontal lines) for fMLP, Grass pollen and dust mite activators

We managed to prove the existence of pollen allergy through the BasoTest for those individuals who were certified by Prick test previously. The median activation of their basophil granulocytes was 91.34% (min.: 74.86%, max.: 98.81%). In contrast the healthy control group showed only 10.03% activation rate (min.: 0.0%, max.: 66.67%) [P = 0.0007]. The sensitivity and specificity of test proved to be 100% for pollen specific responses (where 95% [CI] 0.6306 to 1.000 and 0.5407 to 1.000). Positive and negative predictive value of the test was 100%. The cut-off value was defined as 70.8% by ROC analysis.

In dust mite allergic individuals a marked CD63 expression was observed after dust mite exposition (mean value of activation: 93.77%, min.: 80.23%, max.: 98.4%), while only a moderate 18.82% activation was seen in the control group (min.: 3.14%, max.: 33.33%) [P = 0.0010] at the same time. Based on the obtained results, the sensitivity and specificity of the test was also 100% for dust mite activation (where 95% [CI] 0.6637 to 1.000 and 0.5407 to 1.000). Positive and negative predictive value of the test was 100%. The cut-off limit of the activation was defined as 56.6% (Figs 2 and 3).

*Carbon nanotubes have direct basophil granulocyte activation effect*

Applying the above-mentioned test we extended our investigations to test the direct basophil granulocyte activating ability of carbon nanotubes, as well.

The basophil granulocyte activating effect of MWCNT functionalized with hydroxyl group did not show significant difference between healthy (median: 18.82%, min.: 12.5%, max.: 50%) and allergic (median: 36.22%, min.: 21.17%, max.: 50, 0%) persons [P = 0.1271].

Basophil granulocytes showed activation in both healthy (median: 29.59%, min.: 7.17%, max.: 100%) and allergic (median: 40.25%, min.: 9.24%; max.: 75.00%) groups as a result of the carboxyl group functionalized carbon nanotubes. Significant difference [P = 0.7763] was not found between these two groups.

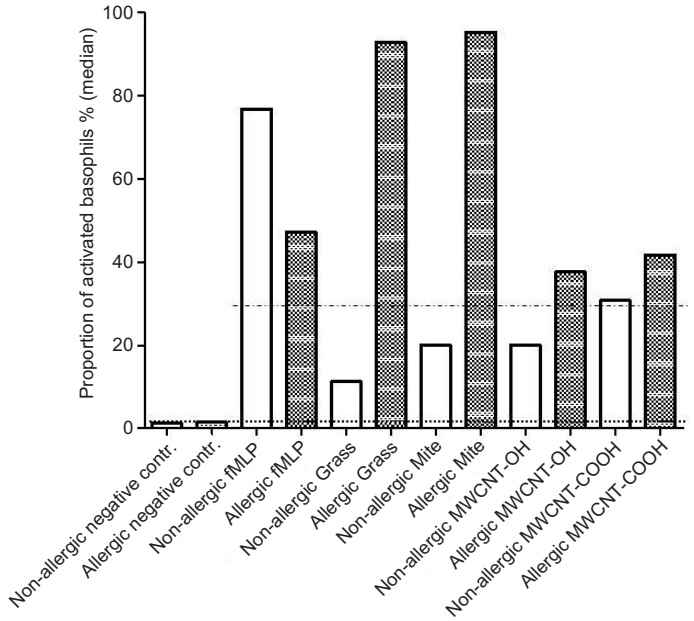


**Figure 4.** The extent of the basophil granulocyte activation changes due to various activators. The lower dotted line (· · ·) indicates the upper limit of background activation. The upper dashed line (· – · – ·) denotes the cut-off value determined for the positive control fMLP

We suppose that the basophilic granulocytes of allergic persons were more sensitive for both carbon nanotube treatments. The activation was determined in allergic groups as a result of the two carbon nanotubes exceeded the cut-off value of the positive control fMLP (30.8%).

Compared to the negative control group (background) significant CD63 expression changes were seen in the case of MWCNT-OH [P = 0.0047, P = 0.0040] and MWCNT-COOH [P = 0.0042, P = 0.0040] treated allergic and healthy group (Fig. 4).

## Discussion

There are a number of natural or synthetic allergenic agents, which can trigger immediate hypersensitivity type I allergic reaction. Provocation tests, as skin Prick test and autologous serum skin tests make easy to predict such allergic reactions in advance [22]. Blood tests usually show the amount of total hIgE. However, this is not always reliable, as in the case of other diseases (haematologic diseases and parasitic infections) the level of hIgE can increase in the circulation.

In recent years a number of literatures reported that the capability of various allergens triggering hypersensitivity reactions can be reliably tested and predicted by basophil granulocyte markers used in flow cytometer [23, 24]. Said and his team demonstrated or predicted the allergy triggering effect of drugs – derivatives of corticosteroids and fluoroquinolones – in advance for some individuals using BAT [25–27]. The basophil activation test was also used in special cases, such as the verification of allergy triggering effects of vaccines and contrast media [28, 29].

The selected BasoTest primarily used to detect pollen- and dust mite allergies. Our studies have shown that the test was absolutely sensitive and specific for both allergens, however, subjects responded differently for fMLP chemotactic peptide used as positive control. Therefore, 25.2% cut-off value specified by the manufacturer was raised up to 30.8% in our experiments.

In this study two allergic individuals participated anti-allergic medication that does not affected our measurements. This observation confirms Medrala's previous finding that BAT is a useful and accurate measurement methodology which is not influenced by cetirizine therapy [21].

In presence of CNTs the carbon nanotubes functionalized with hydroxyl or carboxyl groups caused significant basophil granulocyte activation in peripheral blood samples of healthy and allergic individuals compared to the background.

Our results suggest that carbon nanotubes may have direct activation effect on basophil granulocytes *in vitro* at 100 μg/ml final concentration and this effect is more pronounced in persons with already existing allergic condition.

Further studies needed on a larger number of healthy probands and in patients with different disturbances of the immune system to clear the possible role of nanoparticles on the promotion of IgE-independent basophil granulocyte activating mechanisms.

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