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Research Article

CD63 vs. CD203c for the diagnosis of neuromuscular blocking agents immediate-type hypersensitivity

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Abstract. Background: Basophil activation test (BAT) using CD63 marker was extensively studied for in vitro diagnosis of perioperative immediate-type hypersensitivity reactions while CD203c marker was studied to a lesser extent. Methods: BAT using CD63 and CD203c markers was performed in two groups of patients: patients with positive history of perioperative hypersensitivity reaction and positive skin tests to neuromuscular blocking agents (NMBA) (group 1) and a control group which included patients exposed to NMBA with negative skin tests (group 2). Results: We have selected 8 patients with positive history of perioperative hypersensitivity reaction and positive skin tests to NMBA. 10 BAT using CD63 with 7 positive results were performed in this group while BAT 203c presented identification issues in 6 patients and unspecific basophil activation in 4 patients. 10 control patients were included in group 2. We have performed 10 BAT CD63 with negative results while BAT 203c presented 5 cases of identification issues and unspecific basophil activation issues in 5 cases. Conclusions: Use of CD203c as an activation marker did not showed promising results in our study.

Key Words: Basophil activation test; CD63; CD203c; Hypersensitivity reaction; Neuromuscular blocking agents

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Introduction

The diagnosis of peri-anesthetic hypersensitivity reactions is based on clinical features, skin tests (considered as gold standard tests for neuromuscular blocking agents), serum IgE measurements and drug challenge tests. All in vivo tests carry the risk of a new acute event during testing, which is not negligible (Ene-Cociș et al 2018). In vitro tests avoid patient's re-exposure and can identify the culprit drug and safe alternatives, being included in testing algorithms for anesthetic anaphylaxis (Tacquard et al 2016).

Basophil activation tests (BAT) imply the in vitro exposure of the basophils with the culprit drugs. Basophil activation test analyses using flow cytometry changes in the molecules expressed at the cell surface. CD63 marker is anchored in the basophilic granule membrane. Its expression outside the cell reflects cell degranulation after the fusion between the granules and cell membrane. Thus, CD63 expression has been proposed as a marker of basophil degranulation (Sudheer et al 2005, Boumiza et al 2005,Ebo et al 2011).

CD203c corresponds to a surface antigen expressed on human basophils, belonging to the type II transmembrane protein family. Among leukocytes CD203c appears to be selectively expressed on the basophil/mastocyte lineage. To date, no other cells from human peripheral blood have been reported to express this marker. Its expression on basophils is rapidly upregulated after allergen stimulation being proposed as a new tool for immediate type hypersensitivity diagnostic. The use of CD203c marker could improve basophil recognition during flow- cytometry and its higher expression during activation compared with CD63 marker and could increase basophil activation test's sensitivity (Boumiza et al 2005).

For neuromuscular blocking agents (NMBAs), which in some French series are reported to be responsible of 60.6% of intra-anesthetic hypersensitivity events (Tacquard et al 2017), BATs were performed using different activation markers on the surface of the basophils. Most of the published studies have tried to validate BAT using CD63 as activation marker, demonstrating variable sensitivity (36-92%) and good specificity (93-100%) depending on the studied population and the testing protocol (Sudheer et al 2005, Abuaf et al 1999, Monneret et al 2002, Kvedariene et al 2006, Ebo et al 2006, Sainte-Laudy et al 2006, Petrișor et al 2014 , Petrișor et al 2015). For a life-threatening

event like anaphylaxis, the sensitivity of the tests needs improvement and other cellular markers could be of use. Few studies have tested patients with NMBAs anaphylaxis with BAT using CD203c and one study revealed a sensitivity of 64% and specificity of 100% (Sudheer et al 2005).

Our aim was to conduct a comparative study for two different BAT protocols, one in which CD63 is used as activation marker (CD63 BAT) and one using CD203c (CD203c BAT), in order to characterize the differences between the two methods, in a well-defined cohort of patients with past NMBAs-induced immediate-type hypersensitivity reaction.

Material and methods

The ethical approval was obtained from the Research Ethics Committee of the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca (307/02.06.2015) and the Clinical Emergency County Hospital of Cluj. We included in our study 8 adult patients with previous intra-anesthetic immediate-type hypersensitivity reactions which had NMBAs as triggers for anaphylaxis, as identified by positive skin tests conducted according to current guidelines (group 1) (Brockow et al 2013). Control group included 10 surgical patients with negative NMBA skin tests who underwent uneventful general anesthesia with exposure to the tested NMBA (group 2). All patients and controls signed the informed consent forms. Patients with corticosteroid, antidepressant, anti-histamine 1 and anti-histamine 2 treatments, pregnant women and children were excluded.

Testing was performed more than 6 weeks after the hypersensitivity reaction and up to a maximum of 1 year. For patients with history of perianesthetic hypersensitivity reaction of more than 1 year needing immediate surgery, retesting with skin tests and basophil activation test was performed.

Each patient was tested using BAT CD63 and BAT CD203c for the culprit NMBA and for controls for the NMBA tolerated during surgery. Blood was sampled in a K3EDTA medium without using the tourniquet and needles.

For the CD63 BAT we used six tubes for analysis. The first tube contained 50 microliters (µl) of stimulation buffer (containing calcium, heparin and interleukin 3) as a negative control. The next 2 tubes, positive controls, contained 50 µl of anti- FcεRI (a highly specific monoclonal antibody for the IgE receptor) and 50 µl of FMLP (a non-specific cell activator - the chemotactic peptide N-FormylMet-Leu) each. The remaining tubes contained the culprit NMBA in the following concentrations: rocuronium- 500 µg/ml, 50 µg/ml, 5 µg/ml; atracurium- 2.5 µg/ml, 0.25 µg/ml, 0.025 µg/ml; succinilcholine 5 µg/ml, 0.5 µg/ml, 0.05 µg/ml. 50 µl of patient's blood was added in each tube and 100 µl of stimulation buffers was added afterwards. Anti-CCR3 (CD193)-PE (monoclonal antibodies to human chemokine receptor CCR3 labeled with phycoerythrin) (Miltenyi Biotec, order no: 130-108-888) - 20 µl and anti-CD63-FITC (monoclonal antibodies to human CD63 labeled with fluorescein isothiocyanate) (Exbio Antibodies- clone MEM-259) - 20 µl were used as staining reagents. After a 15 minutes incubation at 37°C in a water bath, 2 milliliters (ml) of pre-warmed lysing solution was added to each tube and incubated 10 minutes at room temperature. After centrifuging and washing, the cells were suspended in 300µl wash buffer.

For the CD203c BAT the first tube, the negative control contained 50 µl of patient's blood, the two positive controls contained 50 µl of anti- FcεRI and 50 µl of FMLP and the next 3 tubes contained the culprit NMBA in the same concentrations as previously mentioned. The staining reagents for this technique were anti-CCR3 (CD193)-FITC (monoclonal antibodies to human chemokine receptor CCR3 labelled with fluorescein isothiocyanate) (Miltenyi Biotec, order no: 130-108-888) - 20 µl and anti-CD203c-PE (monoclonal antibodies to human CD203c labeled with phycoerythrin) (Exbio Antibodies- clone NP4D6) - 20 µl added in each tube. No stimulation buffer was used for this technique. After a 15 minutes incubation at 37°C in a water bath, 2 milliliters (ml) of pre-warmed lysing solution was added to each tube and incubated 10 minutes at room temperature. After centrifuging and washing, the cells were suspended in 300µl wash buffer.

The increase of the CD63 marker and CD203c on basophils was quantified using CellQuest software (FACSCalibur Becton Dickinson San Jose California USA Analyzer 2001). Our flow cytometer is equipped to detect Forward Scatter, Side Scatter and the two fluorochromes FITC and PE. Laboratory limit of basophilic cell analyzed for allergies is set to 600. We set the gate by including the entire basophil population CCR3 with low Side Scatter (SSC low) and calculated the percentage of CD63/CD203c positive cells compared to the total amount of basophilic cell gated. The result was considered positive when the percent of activated basophils was 5% or more over spontaneous activation observed for the negative control and the stimulation index calculated as the ratio between the percentage of activated basophils with the allergens and the negative control was ≥2 (Hagău et al 2010).

Results

We have selected 8 patients with positive history of perioperative hypersensitivity reaction and positive skin tests to at least one neuromuscular blocking agent (NMBA) (group 1).

4 of our patients presented with more than 1 year history and they were retested using skin tests and basophil activation test as needing immediate surgery and the rest of 4 patients were tested at 2, 5, 6 and respectively 9 months after the hypersensitivity reaction.

In group 1, 7 positive skin prick test (SPT) patients and 3 positive intradermal tests (IDT) patients were identified as highlighted in Table No.1. Patient 1 presented positive SPT to both rocuronium and atracurium and Patient 4 presented positive IDT to succinilcholine and rocuronium.

10 BAT CD 63 were performed in our patients with 7 positive results (70%) and no technical problems identified. BAT CD63 for Patient 1, with positive results for rocuronium and a stimulation index of 10.95 is presented in Figure No.1A.

10 BAT using CD203c were performed in group 1 patients with two types of technical issues identified. Basophil identification and cell separation was inappropriate in 6 tests while we observed non-specific basophil activation without exposing the cells to the culprit drug in 4 tests. BAT CD203c for Patient 1 with rocuronium anaphylaxis in which the isolation of the basophil plot was difficult is presented in Figure No.1B.

Table No.1- Patients and controls' results for CD63 BAT and CD203c BAT for different neuromuscular blocking agents (NMBAs)

	Drug tested	Skin test	BAT-CD63	BAT-CD203c
Patient 1.	Rocuronium	SPT- positive	SI 3.69/3.69/10.95 positive	Inappropriate basophil separation
Patient 1.	Atracurium	SPT- positive	SI 11.17/3.87/13.8 positive	Non-specific Ba marking after staining
Patient 2.	Rocuronium	IDT- positive	SI 6.03/ 8.30/4.05 positive	Inappropriate basophil separation
Patient 3.	Atracurium	SPT- positive	SI 0.97 /5.22/1.52 positive	Inappropriate basophil separation
Patient 4.	Succinicholine	IDT-positive	SI 0.73/5.96/8.21 positive	Inappropriate basophil separation
Patient 4.	Rocuronium	IDT-positive	SI 14.11/8.04/7.47 positive	Inappropriate basophil separation
Patient 5.	Atracurium	SPT- positive	SI 0.48/0.24/0.11 negative	Inappropriate basophil separation
Patient 6.	Atracurium	SPT- positive	SI 0/0/0.14 negative	Non-specific Ba marking after staining
Patient 7.	Atracurium	SPT-positive	SI 3.39/0.45/0.45 positive	Non-specific Ba marking after staining
Patient 8.	Atracurium	SPT-positive	SI 0.86/0.53/0.24 negative	Non-specific Ba marking after staining
Control 1.	Rocuronium	Negative	SI 0.24/1.19/ 0.42 negative	Non-specific Ba marking after staining
Control 2.	Rocuronium	Negative	SI 1.87/1.21/1.24 negative	Inappropriate basophil separation
Control 3.	Atracurium	Negative	SI 1.13/1.8/0.51 negative	Non-specific Ba marking after staining
Control 4.	Atracurium	Negative	SI 0.92/0.34/1.84 negative	Inappropriate basophil separation
Control 5.	Atracurium	Negative	SI 0.57/0.54/0.79 negative	Inappropriate basophil separation
Control 6.	Rocuronium	Negative	SI 0.68/0/ 0.68 negative	Non-specific Ba marking after staining
Control 7.	Atracurium	Negative	SI 0.43/ 1.32/0.94 negative	Non-specific Ba marking after staining
Control 8.	Rocuronium	Negative	SI 0.86/ 0.65/0.60 negative	Non-specific Ba marking after staining
Control 9	Atracurium	Negative	SI 0.51//0.35/0.96 negative	Inappropriate basophil separation
Control 10	Atracurium	Negative	SI 0.96/1.7/1.21 negative	Inappropriate basophil separation

BAT- basophil activation test, Ba- basophil, SI- stimulation index, Ba- basophil, CN- negative control

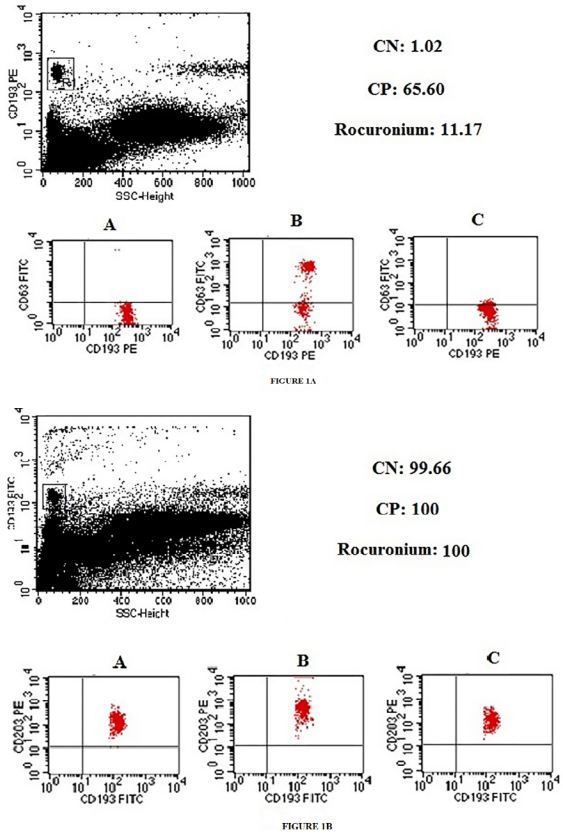

Figure 1A -CD63 basophil activation test in Patient 1. A- Negative control; B- Positive control; Stimulation with rocuronium 50mcg/mL; Results- the percentage of activated basophils

Figure 1B- CD203c basophil activation test in Patient 1; A- Negative control; B- Positive control; Stimulation with rocuronium 50mcg/mL Results- the percentage of activated basophils

All group 2 patients presented negative skin tests and successful exposure to the tested NMBA. 10 BAT CD63 were performed with negative results. In the 10 BAT 203c performed for this group we have found 5 patients in whom basophil could not be identified and separated and 5 patients for whom non-specific activation was observed in the negative control as evidenced in the Table No.1.

Discussion

In patients with suspected intraoperative immediate-type hypersensitivity reactions, diagnosis requires a complex allergological workup for culprit drugs and safe alternatives identification (Tacquard et al 2016). Functional assays are needed to improve diagnosis and find alternatives (McGowan et al 2013).

Our patients with old history of hypersensitivity reaction needing surgery were retested in order to avoid false- negative results as Mertes et al. states that the sensitivity of skin tests may decrease over time, being rather stable for muscle relaxants and decreased for antibiotics (Mertes et al 2011).

In the study of Sudheer et al. which included 14 patients with suspected NMBAs anaphylaxis (from which 9 patients had positive skin tests), the sensitivity of CD203c BAT was 36% and the specificity 100% (Sudheer et al 2005). Other comparative studies are lacking, even if the search for new techniques with higher sensitivity for anesthetic drugs are lacking.

Chirumbolo et al. used fluorescence intensity in an in vitro investigation of basophil activation using a two laser multiparametric flowcitometry and three activation agents. In this study basophil activation was studied after stimulation with the agonist by examining changes in the mean fluorescence intensity

assessment to specific membrane marker fluorochrome. Resting basophils behaved as non-express cells for CD63 but expressing CD203c at a low level (approximately 3000 fluorescence units). After stimulation with fLMP, 41.4% of cells showed a CD63 bright phenotype and increase in fluorescence while almost all basophils up-regulated CD203c membrane expression (the increase in fluorescence intensity was most evident for CD203c marker). The study concluded that CD203c was less sensitive as an activation marker having an important expression during the resting state but, on the other hand, being up-regulated in all cells following activation, could be used in individuals previously classified as "non-responders" when using BAT CD 63 (Chirumbolo et al 2008).

Sturm et al. studied the use of IL3 in BAT CD203c for wasp allergy and reported no benefits as it significantly reduced the difference between baseline and stimulated responses and in contrast, for BAT CD63 protocol enhanced the responsiveness to basophil activating substances. In our small study we used stimulation buffer containing IL3 for BAT CD 63 and no stimulation buffer for the CD203c technique. Identification and activation issues were present during BAT CD203c (Sturm et al 2010).

Conclusion

In our study BAT CD203c used as an activation marker did not showed promising results in the diagnosis of NMBA hypersensitivity reactions. One study limitation is our small number of patients with positive skin tests for NMBAs. We also could not differentiate well the basophils from other cells. The basophils all were stained with anti-CD203c in most patients and controls, suggesting that CD203c is a constitutively expressed marker on the cellular surface. Considering this, only a method that could quantify the density of the molecules on basophils' surface or fluorescence intensity could differentiate between activated and non-activated basophils after challenging with the culprit allergen.

The test could help optimization of the diagnostic methods for perioperative anaphylaxis, but further studies are needed in this direction in order to establish the utility of CD203c as an activation marker or as an identification marker.

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