Innovative robust basophil activation test using a novel gating strategy reliably diagnosing allergy with full automation

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Abstract

Background: Allergy is one of the most common chronic diseases in Europe. Therefore, an increased need for specific and sensitive diagnostic tests that truly detect allergy exists. This study aimed at establishing a highly specific high-throughput and automated basophil activation test (BAT) that proves the existence of an allergy with utmost probability.

Methods: BAT from 1104 samples was analyzed; a novel gating strategy with three antibodies (FcεRIα, CD203c, CD63) was established and compared with our published protocol (12 antibodies). Based on the novel gating strategy, storage conditions, automated measurement, and analyses using R (1376 samples out of 1389) were optimized to set up a high-throughput BAT.

Results: No differences in sensitivity and specificity were found between the novel three antibody (FcεRIα, CD203c, CD63) and the 12 antibody gating strategy or between automated and manually analyzed samples (saving up to 90% of labor time). The time frame for basophil activation measurement after blood donation has been extended considerably (whole blood storage ≤7 days (RT) and 17 days (4°C) prior to BAT preparation and measurement). Respective storage conditions were optimized for samples after stimulation, staining, and preparation (≤7 days (RT) and 28 days (4°C)). These achievements were confirmed by a nationwide ring trial showing robustness and applicability of our BAT on a variety of flow cytometers.

Conclusion: Our considerable optimizations overcame the hurdles that until now prevented the BAT from being used as high-throughput allergy diagnostic test in routine laboratories and shall allow for collaborative studies between clinics and research centers.

KEYWORDS
allergy diagnostic, clinical trials, oleosins, peanut allergy, sensitization

Abbreviations: APC, Allophycocyanin; BAT, basophil activation test; CCR3, C-C chemokine receptor type 3; CD, cluster of differentiation; CV, coefficient of variation; Cy7, Cyanine7; EAACI, European Academy of Allergy and Clinical Immunology; Emmax, emission maximum; FMLP, formyl-methionyl-leucyl-phenylalanine; FSC, forward scatter; IL, interleukin; MFI, median fluorescence intensity; MQ10, Miltenyi Biotec MACSQuant 10; PBS, phosphate-buffered saline; PE, R-phycocerythrin; pps, percentage points; ROC, receiver operating characteristic; RT, room temperature; SOP, standardized operating procedure; SSC, side scatter; TS, time series.

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INTRODUCTION

Allergic disorders are one of the most common chronic diseases in Europe, and many patients are not adequately treated due to misdiagnosis. Skin prick test (SPT) and immunoglobulin (Ig)E antibody assays detect only sensitization but do not prove allergy. In order to prove allergy in unclear cases, challenge test with the allergen source is currently diagnostic gold standard.

However, the basophil activation test (BAT), which mimics the allergic reaction in vitro, has manifold advantages as diagnostic test: good safety profile, high sensitivity/specificity, and the potential to predict the severity of an allergic reaction. Importantly, it allows discrimination between sensitized asymptomatic and truly allergic individuals. Thus, the BAT has the potential to replace expensive and risky allergen challenge tests. Yet, no consensus has been found as to which of the many BAT protocols should be applied to obtain comparable results through their harmonization. Minimalistic selection of identification markers, for example, causes high cutoffs, due to contaminations with other cells. So far, only the use of two activation markers (CD63, CD203c) is widely accepted.

In our previously published BAT protocol using 12 antibodies, high sensitivity and specificity (100%), discriminating between allergic and sensitized but asymptomatic individuals, with a low cutoff for positivity were achieved. This protocol, too expensive and complex for routine diagnostic settings, forced us to simplify and expand our BAT protocol without affecting its excellent diagnostic performance. Together with an automation of sample preparation, measurement, data analysis, and elongation of the time span between blood donation and sample processing/measurement we enabled the feasibility of our protocol in a nationwide ring trial.

MATERIAL AND METHODS

Study approval

The study was approved by the local ethics committee of the University of Lübeck, Germany (approval nos. 10–126, 16–268, and 13–136). All study participants gave written informed consent.

Study populations

Patients with a convincing history of peanut allergy and controls (A, sensitized but asymptomatic as well as B, non-allergic individuals) were continuously recruited between 2015 and 2020 in Borstel and Lübeck (Germany). After a thorough clinical history supported by a standardized questionnaire, the status of the study population was confirmed by SPT, serology, and/or basophil activation test (Tables S1-S3). Heparinized whole blood from the study population was used to perform the basophil activation test (BAT). Detailed demographic characteristics of study subjects included in BAT experiments are listed in Tables S1-S3.

Allergens and stimulants

The following allergens were either isolated and purified from natural (n) sources or recombinantly expressed (r) in E. coli BL21(DE3) and purified at the Research Center Borstel (Borstel, Germany): peanut oleosins (n) and defensins (n), Der p 2 (r/n), Bet v 1 (n), Ara h 8 (r), Ara h 14 (r), Ara h 15 (r). The identity of each allergen was verified on a...
Indoor Biotechnology Ltd. (Cardiff, UK): Ara h 1 (n), Ara h 2 (n), Ara h 6 (n). Peanut extract was prepared according to the protocol of Boldt et al. The stimulants formyl-methionyl-leucyl phenylalanine (FMLP 1 µM, Sigma-Aldrich, Steinheim, Germany), anti-IgE (1:1 mixture of goat anti-human IgE (1 µg/ml, Sigma-Aldrich, Steinheim, Germany), and goat anti-human IgE (1 µg/ml, Abcam, Cambridge, UK) were used as positive controls for the BAT, PBS as negative control.

2.3 | Flow cytometric instruments and antibodies

Measurements were conducted on an LSRII instrument (BD Biosciences, San Jose, California, USA), of which 3 lasers and 7 (12 antibody gating strategy) or 3 (three antibody gating strategy) detection bandpass (BP) filters were used for manual data acquisition (configurations listed in Table S4). Automatic sample preparation and measurement were performed on a MACSQuant10 (Miltenyi Biotec, Bergisch Gladbach, Germany). For the inter-laboratory testing, 10 further flow cytometers (BD Biosciences) were involved: 3× FACSCantoll, 2× LSRII, 2× Fortessa, 1× Symphony, 1× FACSLyric, and 1× FACSCalibur (configurations listed in Table S4). A detailed description of used antibodies for different protocols (3 antibody protocol, 12 antibody protocol, and CCR3 protocol) is documented in Table S5.

2.4 | Basophil activation test (BAT)

The 12 antibody BAT and the identification of basophils (gating) was performed as previously reported. Although interleukin (IL)-3 is often used as priming agent, we refrained from using it. For the evaluation of the three antibody gating strategy (three antibody BAT), the acquired data of the 12 antibody BAT were taken and cells gated as shown in Figure S1A. Analysis was conducted with the same fixed quadrant for the CD203c vs. CD63 gating for the 12 and the three antibody evaluation (Figure S1B). The work-up of the BAT with CCR3 was the same as for the 12 antibody protocol with the exception that a reduced set of 4 anti-human antibodies (Biolegend, Fell, Germany) was used (CCR3-BV421, CD45-BV510, CD203c-PE, and CD63-APC). Basophils were identified as shown in Figure 1A and their activation status analyzed. The total numbers of detected basophils were recorded.

2.5 | Automation process

For the automation process, a sufficient amount of lysis buffer was prepared first, and the tubing of the storage solution was moved into the lysis buffer reservoir. After that, the flow cytometer was flushed extensively with lysis buffer. Furthermore, the anti-human antibody mixture was prepared (three antibody mixture: FcεRIα-PE/
Comparison between basophil markers (Figure 1C) and flow cytometric instruments (Figure 5) was performed using the Mann-Whitney U test. Calculation of the optimal CD63 cutoff value was done using ROC (receiver operating characteristic) analysis (Figure 6C). Comparison of the activation levels and the total number of basophils (Figure 6A,B) was performed using the Wilcoxon signed-rank test.

2.9 Further Methods

Inter-laboratory survey and Evaluation of the BAT using an R script are described in detail in Appendix S1.

3 RESULTS

3.1 Choice of basophil identification markers

At first, we focused on specific basophil markers (CD203c, FcεRIα) from our original protocol. Although CD203c is used manifold as activation marker to distinguish allergic from healthy individuals, this marker is also constitutively expressed on basophils. Therefore, we used CD203c in our set-up as marker for identification and not as a marker for activation. Further, we were looking for markers, which expression intensities remained relatively constant to achieve a robust and clear distinction between basophils and other cells. We tested CD203c, FcεRIα, and CCR3 (C-C chemokine receptor type 3),
the latter marker already used in commercially available BAT kits and used here as a benchmark.

When following the gating strategy CCR3 vs. side scatter (SSC) used by many investigators, a discrete basophil population appeared for PBS (phosphate-buffered saline, Figure 1A) to set up a proper basophil gate. However, a markedly reduced contamination with cells, which did not express the basophil marker already used in commercially available BAT kits and used here as a benchmark.

To evaluate the reliability of our new protocol, we compared it to our high-performance 12 antibody protocol (Figure S1B, and cited literature). For this purpose, 1104 samples were analyzed by both protocols with regard to the number of basophils and their activation. No significant difference was found for the total number of basophils (Figure 2C, allergic patients), in which median was 0.5% (5% quantile: −10.3 percentage points (pps) and 95% quantile: 9.9 pps). Comparing basophil activation, we found no significant differences between the results of both strategies, indicated by high correlation coefficient ($R^2 = 0.997$, Figure 2D) and a median of 0 (5% quantile: −1.56 pps and 95% quantile: 1.82 pps). The three antibody protocol showed the same high diagnostic sensitivity and specificity as the 12 antibody protocol, but saved 86% of expenses (as calculated using list prices).

3.3 | Elongated time frame for measurements

BAT samples should be analyzed <24 h after blood donation, but first data indicate that short-term storage of blood did not compromise subsequent basophil activation. We analyzed prepared samples (time series (TS1)) or whole blood (TS2) after different storage periods.

To analyze the reactivity of basophils in prepared samples over time (TS1), we took blood samples from 16 individuals (10 patients allergic to either birch pollen, house dust mite, or peanut, respectively; 3 sensitized but non-allergic individuals and 3 non-allergic subjects), stimulated them (PBS, allergen, and anti-IgE) once at day 0, and analyzed these samples over a period of 4 weeks, for at least twice a week (see Figure 3A and Figure S3). In order to find out whether storage conditions have an influence on the BAT results, we split the initially prepared samples and stored one part at 4°C and the other at constant room temperature (RT, 19°C) under exclusion of light to prevent fluorochrome degradation.

TS1: Activation of basophils changed slightly (4°C, Figure 3A) over the course of 28 days, whereas it changed after 7 days in samples stored at RT (Figure S3). The median increase of activation (allergic individuals, 4°C) by allergen was below 5 pps over a period of 28 days. A continuous rise of activation over the first days to 32 pps at day 14 was seen (RT). Non-allergic subjects and only sensitized individuals showed a slight increase (mean 1.4 pps after 28 days) in activation (4°C) and an increasing starting at day four after allergen stimulation (RT, mean 6.3 pps after 14 days). Background signal (PBS control) was unaltered (4°C) or increased slightly over time (RT).

Storage at RT was accompanied by a continuous dying of the basophil population that forced us to discontinue the measurements of the samples stored at RT after day 14.

After having obtained these promising results, we also wanted to know whether donated blood could be stored for a prolonged period of time without affecting the classification of the tested individuals (TS2). Therefore, we sampled blood from 5 individuals (3 patients allergic to house dust mite, 1 non-allergic subject, and 1 sensitized but non-allergic individual), prepared (stimulation and staining), and analyzed the stored blood multiple times over a period of 3 weeks. To study the impact of the storage temperature, the initially donated blood samples were split into two parts, which were stored analogously to the prior experiment (at 4°C and RT, respectively).

TS2: Although activation varied over the investigated period (patient #1 (Figure 3B) and #2 (Figure S4)), it steadily declined for patient #3 (Figure S4). However, diagnosis of allergic patients was possible within 17 days (blood storage at 4°C). No difference in the activation levels of the basophils was observed for the control individuals. As for the previous experiment (TS1), we had to discontinue the measurements of blood that was stored at RT after day 7, because of the fast decline of viable basophils that could respond to the allergen stimulation.

To verify our observations, we initiated a field experiment in which the basophil activation in samples from 3 allergic blood
donors was investigated prior and after postal shipment (Figure 3C). Fresh donor blood was split into 6 parts, whereas one part was prepared (stimulation and staining) and analyzed on the same day and the other 5 parts were handed over to 5 different local post offices. After submission of the samples to the local distribution center, samples were delivered back to our laboratory, prepared (stimulation and staining), and analyzed as quintuple determinations 3 days after initial blood donation. We observed slight disparities in activation levels, but the background signal (PBS) was low, and activation induced by allergen was always distinguishable from background.
Therefore, allergic study participants could be correctly identified, despite the fact that the blood has not been processed prior to the shipment via different post offices. Therefore, the results of our BAT protocol demonstrate the feasibility of whole blood sample shipment to other laboratories allowing them to perform the BAT with reproducible results.

3.4 | Inter-laboratory testing (ring trial)

We wanted to assess the robustness of our new protocol in cooperation with 9 other laboratories (six different flow cytometers, Table S4). For this purpose, we divided prepared samples (PBS, anti-IgE, or allergen) and sent them via conventional mail to different core facilities within Germany (Figure 4A). A detailed protocol (Appendix S1, Protocol S1) with calibration beads to set up different instruments was provided together with the samples. Overall, the desired number of basophils (800) was recorded at all instruments, and activation of basophils was detectable. With regard to the results (Figure 4B), minor differences for basophil activation were observed, comparing our data with those from our cooperation partners. The mean difference for samples incubated with PBS, anti-IgE, and allergen were 0.43 pps (range 0.01–2.33 pps), 0.63 pps (range 0.01–5.28 pps), and 1.60 pps (range 0.05–11.07 pps), respectively. Moreover, we generally observed a very low background for all investigated individuals (mean 0.28%).

3.5 | Automatic sample processing and measurement to reduce hands-on time

Integration of the BAT into routine diagnostics is largely hampered by enormous laboratory work as all commercially available BAT kits need to be performed in tubes, which impedes a high-throughput measurement of samples. Therefore, we established an automatic sample processing and measurement using a flow cytometer with integrated robotic functions (MACSQuant10 (MQ10), Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After having manually performed incubation of blood and stimulants in a 96-deep well plate, the plate was transferred to the 96-well plate holder on the instrument. Here, the automated protocol (Appendix S1) was conducted by the MQ10. To assess the feasibility of this approach, we compared our published manual sample preparation to automated work-up using the same blood samples in parallel (Figure 5). Although measurements were conducted on two different flow cytometers (manual: LSRII; automated: MQ10, according to our standardized operating procedure (SOP)), there was no difference using the manual or automated sample preparation. However, using the automated protocol reduced average hands-on time for 96 samples from about 9 h to 1.5 h, which saved more than 80% of manual work-up.

3.6 | Development of a robust automated analysis template using R

Based on 1389 individual BAT samples, we created a data-driven automatic analysis algorithm using Bioconductor tools in R (Appendix S1). Although the automatic approach was able to identify even small numbers of basophils correctly, we implemented a threshold of 100 basophils as minimum requirement for analysis by the algorithm (Figure 5). Thus, 1376 out of 1389 samples (99.1%) could be analyzed automatically. The comparison of automatic assessment to manual evaluation by an expert showed a very good agreement with respect to the activation level (Figure 6A) and total number of basophils (Figure 6B), resulting in a correlation coefficient of 0.952 and 0.967 (p < .001), respectively. Automatic analysis resulted in the same diagnostic sensitivity (100%), but a slightly lower specificity (97%) for peanut-allergic patients (Figure 6C) compared to our previous results. Using our automated assessment, all patients at risk are identified correctly, and only one out of 30 non-allergic controls will be advised to avoid peanuts due to an exaggeration of activated proportion of basophils by the algorithm (1.04 pps above the cutoff, Figure 6C). However, results of the algorithm were almost identical to that of manual gating using the 12 or three antibody protocol conducted by an expert (Figure 6D). Additionally, for automatic analysis, we implemented a visual quality control and quality checks to identify technical errors/problems (Figure S5 and Appendix S1) derived from the sample preparations.

4 | DISCUSSION

The dramatic increase of allergic diseases has led to a growing need for reliable high-throughput diagnostic tests that do not simply
detect the presence of allergen-specific IgE but also reveal its biological consequences.\textsuperscript{2,6,8,17,35} The basophil activation test (BAT) has an enormous diagnostic potential, but its application in routine diagnostics is largely hampered by many hurdles (e.g., robustness), which we addressed here. Many BAT protocols claim to be sufficient with only one single surface marker exploited.\textsuperscript{9,10,12,14,22,26} For example, CCR3 can be sufficient for non-stimulated samples (Figure 1A), but due to stimulation-induced decrease of CCR3 expression, the basophil population is contaminated with non-basophil cells with a subsequent underestimation of their activation level. Therefore, the value of CCR3 is still a matter of debate.\textsuperscript{36-39}

We addressed this problem. Our novel protocol together with our novel gating strategy has been shown to be a robust approach that allows identification of pure basophil populations (CD203c and FceRI\alpha) and the evaluation of their activation (CD63 expression). Our gating strategy includes doublet exclusion that is not yet used by any of commercially available BAT kits. Using bright fluorochromes (PE, PE-Cy7, APC) with an optimized low spillover further advances...
Most flow cytometers can be addressed with our protocol as they operate with the required lasers (488 nm, 633 nm). There are minor spillover effects caused by the combination of PE/PE-Cy7, but these neither influence the analysis nor the outcome of measurements. In fact, there was no need for compensation, which normally has to be done carefully.

To prove diagnostic reliability of our new protocol, a data set of more than 1104 samples (51 study patients) was reanalyzed with regard to number and activation level of basophils and then compared to the results of our highly specific and sensitive antibody protocol. The obtained data were almost identical to those already published, resulting in similar ROC curves and demonstrating high diagnostic sensitivity (100%) and specificity (97%) of our test (Figure 6C and cited literature). This further demonstrates that our protocol dramatically reduces complexity and costs (86% saving) of BAT without affecting its superior diagnostic performance. Moreover, the results were achieved without using any extrinsic stimulants such as interleukin (IL)-3, which have been denoted as expendable by the European Academy of Allergy and Clinical Immunology (EAACI).

A major hurdle for implementation of the BAT into routine diagnostics is the requirement to analyze blood samples within a few hours after donation a limitation that has already been addressed by other BAT-specialists. Their results showed that a delayed analysis of samples (after stimulation, staining, and preparation) even after 5 days of storage may be feasible if using CD63 expression as a marker for basophil activation.

To examine the limits of prepared samples and the storability of blood, respectively, we initiated two independent time series (TS1, TS2). Our results (TS1) show that measurement of stored samples after stimulation, staining, and preparation is possible for several days (RT) and up to at least 28 days (4°C). Furthermore, we have shown that there is no need to analyze whole blood within 24 h (TS2). A correct classification of study participants was still possible after 7 days (RT), or after 17 days (4°C). A small real-world pilot study (shipping blood samples prior to BAT preparation and measurement) revealed comparable tendencies as in the TS2, namely only a small alteration in basophil activation level. This issue might be, in parts, attributed to daily preparation of stimulants and buffers, which underlie unavoidable variations. Our observations confirm findings.
of others and stress the fact that the currently propagated limited time span of 24 h until sample measurement (stored samples after preparation or whole blood) needed to be re-evaluated.\textsuperscript{30-32,42} A nationwide ring trial evaluated the robustness of our protocol and verified practical usefulness of our findings on the prepared samples. Overall, an inter-assay coefficient of variation (CV)
of 7.82% for IgE and 6.71% for allergens was calculated. It is of note that the samples were transported without cooling and had been measured between three and seven days after blood donation and preparation. This outcome provides substantial data that transfer of samples to external institutions is possible without a negative influence on data quality, so that our protocol provides a beneficial approach for scientists and clinicians without direct access to flow cytometers, opening up new dimensions of cooperative studies, clinical trials, and routine measurements in analytical laboratories.

BATs routine application in medical care units and analytical laboratories is further hampered by time-consuming and laborious handling of samples in single tubes (using commercial BAT kits). Therefore, we transferred our novel protocol to a flow cytometer with robotic functions. Only preparation of stimuli and addition of blood have to be done manually. All other steps are fully automated, which is, to best of our knowledge, the first approach to realize a high-throughput BAT. In direct comparison with our manual protocol, we observed no differences in activation levels of basophils (Figure 5), but saved over 80% of labor time.

Besides automated sample processing, we aimed to automatically analyze acquired BAT data. This is the second attempt to implement a data-driven algorithm for BAT—the first is based on a CCR3 protocol, which has been shown to be less robust compared to our new gating protocol. Poor basophil identification by the algorithm might be one reason for the almost 10 times higher number of experiments requiring manual gating compared to our results (8.5% vs. 0.9%), which are obtained by robust basophil identification markers. Several conditions changed during the study period of our three antibody protocol (eg photomultiplier tubes, staff members), but the results of the data-driven programmatic analysis are comparable with the results obtained by manual gating of an expert (Figure 6). This automated data analysis template will reduce analysis time of approximately 1 h down to the transfer of acquired data to analysis server. Ideally, such an algorithm could be integrated in the instruments’ software in future to provide an all in one solution for operators.

As with every study, there are some limitations to the research presented here. First, the number of investigated allergic participants and controls is relatively small, but the ones included are well-characterized. Second, experiments were mainly performed on the same flow cytometer at our facility. However, the data obtained from our ring trial indicate that variations between flow cytometers can be minimized by thorough calibration of the instrument. Investigations including more and other single allergens in BAT with samples from patients with different allergy severity grades (class I and class II food allergy) are already underway.

In conclusion, based on a robust novel gating strategy we have developed an automated high-throughput highly discriminative basophil activation test protocol that drastically reduces hands-on time as well as analysis time without a flow cytometric expert, opening up possibilities for multicenter studies and the routine use of BAT as allergy diagnostic test.

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CONFLICT OF INTEREST
None of the authors had any financial relationships for themselves and their immediate family/significant others.

AUTHOR CONTRIBUTIONS
JB, CS, TS, SK, and UJ designed the study, JB, CS, MH, and SK performed experiments and acquired data, JB, CS, MH, SK, and TS analyzed data. JB, CH, TS, and UJ wrote the manuscript. UJ obtained the positive votes from the ethics committee, recruited, and characterized the patients and control individuals and raised the third party funding.

PATENT APPLICATION
Determination of basophil activation is subject of a recently filed EP patent application No. 20191666.5.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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