Assessing basophil activation by using flow cytometry and mass cytometry in blood stored 24 hours before analysis

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Background: Basophil activation tests (BATs) have promise for research and for clinical monitoring of patients with allergies. However, BAT protocols vary in blood anticoagulant used and temperature and time of storage before testing, complicating comparisons of results from various studies.

Objective: We attempted to establish a BAT protocol that would permit analysis of blood within 24 hours of obtaining the sample.

Methods: Blood from 46 healthy donors and 120 patients with peanut allergy was collected into EDTA or heparin tubes, and samples were stored at 4°C or room temperature for 4 or 24 hours before performing BATs.

Results: Stimulation with anti-IgE or IL-3 resulted in strong upregulation of basophil CD203c in samples collected in EDTA or heparin, stored at 4°C, and analyzed 24 hours after sample collection. However, a CD63^{hi} population of basophils was not observed in any conditions in EDTA-treated samples unless exogenous calcium/magnesium was added at the time of anti-IgE stimulation. By contrast, blood samples collected in heparin tubes were adequate for quantification of upregulation of basophil CD203c and identification of a population of CD63^{hi} basophils, irrespective of whether the specimens were analyzed by means of conventional flow cytometry or cytometry by time-of-flight mass spectrometry, and such tests could be performed after blood was stored for 24 hours at 4°C.

Conclusion: BATs to measure upregulation of basophil CD203c and induction of a CD63^{hi} basophil population can be conducted with blood obtained in heparin tubes and stored at 4°C for 24 hours. (J Allergy Clin Immunol 2017;139:889-99.)

Key words: *Basophils, CD63, CD203c, anti-coagulants, heparin, EDTA, peanut allergy, cytometry by time-of-flight mass spectrometry, platelets*

Basophils and mast cells are major effector cells of IgE-dependent immune and allergic responses.[1-3] These cells express large numbers of the high-affinity IgE receptor FcεRI on their surfaces, and crosslinking of their FcεRI-bound IgE by bivalent or multivalent allergens induces the secretion of multiple stored and newly synthesized mediators, cytokines, and chemokines.[4-7] Although basophils typically represent less than 1% of peripheral blood leukocytes, analysis of basophil function has become increasingly popular, both because basophils can have certain unique roles in immunity and allergic diseases[8-10] and because blood basophils are much more readily available for analysis than tissue-resident mast cells.

Studies of basophil activation *ex vivo* (ie, basophil activation tests [BATs]) are flow cytometry–based assays to assess basophil activation by various stimuli. The BAT was first developed as a diagnostic test in 1991, and the use of such tests has subsequently increased.[11-13] However, several different BATs are now used, including commercial kits and tests developed and used by research groups. Various BATs differ in the choice of anticoagulant, temperature and duration of blood storage, activation markers measured, consideration of the effects of possible platelet attachment to basophils, reproducibility, whether basophils are studied in whole blood or after various purification steps, and stimulants used to activate the cells.[12,14-21]

However, to compare the most meaningful results of BATs obtained with different patient populations analyzed at various sites, it would be optimal to use standardized methods at all sites. Moreover, such protocols could be used ideally at reference laboratory sites after overnight shipment of samples from widespread clinical sites. We developed a simple protocol that permitted us to perform BATs on whole blood stored for up to 24 hours before analysis and showed that this protocol could be used to analyze basophils both by means of conventional flow cytometry and by means of the newly introduced method of cytometry by time-of-flight mass spectrometry (CyTOF).[22]

METHODS

Outline of experiments

We used blood specimens from both healthy donors and patients with peanut allergy to compare BAT results obtained in blood anticoagulated with EDTA versus heparin and stored before BAT analysis at room (or, for shipped

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Abbreviations used
BAT: Basophil activation test
CMF-PBS: Calcium/magnesium-free PBS
CyTOF: Cytometry by time-of-flight mass spectrometry
DAPI: 4′,6-Diamidino-2-phenylindole
FITC: Fluorescein isothiocyanate
MFI: Mean fluorescence intensity
OIT: Oral immunotherapy
PE: Phycoerythrin
PerCP: Peridinin chlorophyll protein
RT: Room temperature

specimens, ambient) temperature or at 4°C for 4 or 24 hours. See Table E1 in this article's Online Repository at www.jacionline.org for a summary of the design of the experiments depicted in the various regular and supplemental figures.

Blood specimens

Blood from randomly selected anonymous donors (allergy status unknown) was obtained from the Stanford Blood Center (Palo Alto, Calif), and blood from patients with peanut allergy (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org) was obtained as part of enrollment into an institutional review board–approved clinical trial (ClinicalTrials.gov Identifier: NCT02103270). Peanut allergy was defined as having a reaction to a double-blind, placebo-controlled food challenge to peanut (up to 500 mg of peanut protein) and a positive skin prick test response to peanut (≥5 mm).

BATs

Blood specimens were gently rotated at room temperature (RT) or 4°C for 4 or 24 hours after blood collection. Immediately before starting BAT assays, samples were put into a water bath at 37°C for 30 seconds. One hundred microliters of whole blood was mixed with 100 μL of medium only or each stimulant. More details about the BAT protocols and the reagents used can be found in the Methods section in this article's Online Repository at www.jacionline.org.

CyTOF

Metal-labeled antibodies used for CyTOF analysis are shown in Table E4 in this article's Online Repository at www.jacionline.org. Other details can be found in the Methods section in this article's Online Repository. In addition, details on basophil quantification with fluorescence beads, basophil and platelet analysis by means of confocal microscopy, and preparation of peanut extract can be found in the Methods section in this article's Online Repository.[23]

Statistical analysis

Mann-Whitney *U* tests were performed (the groups analyzed are described in the figure legends), and the results are reported in figure legends. We considered a *P* value of less than .05 to be statistically significant.

RESULTS

BATs can be performed 24 hours after collection of heparin-anticoagulated blood stored at 4°C

We sought to identify conditions of blood collection and storage that would permit conducting BATs with specimens stored as long as 24 hours before analysis. This interval would permit shipping specimens obtained at one location to another for analysis. We performed anti-IgE or IL-3 stimulation of basophils in whole blood and used changes in CD203c[24-26] and CD63[11,27,28] values as basophil activation markers. Basophils were gated as $CD123^+$ and $HLA\text{-}DR^-$ cells,[29] and expression of CD203c and CD63 in gated basophils was shown in histograms (Fig 1).

Basophils exhibited upregulation of both CD203c and CD63 on anti-IgE or IL-3 stimulation in samples from normal blood donors that were collected in either EDTA or heparin, although the CD63 upregulation in EDTA was minimal (Fig 1). CD203c was uniformly upregulated in both EDTA and heparin specimens. In heparin, but not EDTA, specimens anti-IgE stimulation induced a strongly bimodal upregulation of CD63, yielding a basophil population with high levels of fluorescence intensity (in Fig 1, the "$CD63^{hi}$" basophil population represented 0.02% in EDTA and 22% in heparin specimens, respectively). In subsequent experiments we compared the intensity of responses under different protocols of testing by using mean fluorescence intensity (MFI) to quantify CD203c and the percentage of $CD63^{hi}$ basophils to quantify CD63.

We first compared results obtained 4 or 24 hours after blood storage at 4°C or RT. When blood samples in EDTA were stimulated with anti-IgE or IL-3, the most significant and largest differences in basophil CD203c expression (ΔCD203c) were in specimens stored for 24 hours at 4°C (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). In heparin specimens, ΔCD203c was similar under all 4 conditions after anti-IgE stimulation, but as with EDTA specimens, the most significant and substantial ΔCD203c after IL-3 stimulation was in specimens stored for 24 hours at 4°C (see Fig E1, *A*). $CD63^{hi}$ basophils were observed only in specimens collected with heparin and stimulated with anti-IgE, but the results obtained in the 4 conditions of storage were very similar (Fig 1 and see Fig E1, *B*). Absolute MFI values for CD203c without stimulation (RPMI media) were low under all conditions, with values in EDTA specimens being higher in samples stored for 24 hours at either temperature, whereas the opposite was the case for specimens collected in heparin (see Fig E1, *C*). No obvious $CD63^{hi}$ populations were observed without anti-IgE or IL-3 stimulation in any of the conditions (see Fig E1, *D*). However, in tests of heparin specimens, 3 of 11 donors barely responded to anti-IgE stimulation (both ΔCD203c and percentage of $CD63^{hi}$ basophils were nearly zero), but the cells responded to IL-3 stimulation. Therefore we considered these 3 subjects to be nonreleasers.[30-34]

Importance of exogenous or added calcium/magnesium for BAT measurements

In tests of blood collected in either EDTA or heparin tubes from the same healthy donors, no significant difference was observed in ΔCD203c between EDTA and heparin specimens ($P = .0627$) on anti-IgE stimulation, but anti-IgE significantly induced $CD63^{hi}$ basophils only in the heparin specimens ($P < .0001$; Fig 2, *A*). Absolute levels of CD203c MFI were higher in basophils in heparin than in EDTA specimens (Fig 2, *B*) both without stimulation and after anti-IgE stimulation (data not shown), which resulted in similar values for ΔCD203c in the EDTA and heparin specimens.

The addition of calcium/magnesium to blood collected in EDTA tubes just before anti-IgE stimulation resulted in induction of a $CD63^{hi}$ population in response to anti-IgE that was similar to that induced in heparin specimens (Fig 3, *A* and *B*; EDTA vs heparin; $P = .0079$ without calcium/magnesium, $P > .05$ with