Real-time differential tracking of human neutrophil and eosinophil migration *in vivo*

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Background: Hitherto, *in vivo* studies of human granulocyte migration have been based on indiscriminate labeling of total granulocyte populations. We hypothesized that the kinetics of isolated human neutrophil and eosinophil migration through major organs *in vivo* are fundamentally different, with the corollary that studying unseparated populations distorts measurement of both.

Methods: Blood neutrophils and eosinophils were isolated on 2 separate occasions from human volunteers by using Current Good Manufacturing Practice CD16 CliniMACS isolation, labeled with technetium 99m–hexamethylpropyleneamine oxime, and then reinfused intravenously. The kinetics of cellular efflux were imaged over 4 hours.

Results: Neutrophils and eosinophils were isolated to a mean purity of greater than 97% and greater than 95%, respectively. Activation of neutrophils measured as an increase in their CD11b mean fluorescence intensity in whole blood and after isolation and radiolabeling was 25.98 ± 7.59 and 51.82 ± 17.44, respectively, and was not significant ($P = .052$), but the mean fluorescence intensity of CD69 increased significantly on eosinophils. Analysis of the scintigraphic profile of lung efflux revealed exponential clearance of eosinophils, with a mean half-life of 4.16 ± 0.11 minutes. Neutrophil efflux was at a significantly slower half-life of 13.72 ± 4.14 minutes ($P = .009$). The migration of neutrophils and eosinophils was significantly different in the spleen at all time points ($P = .014$), in the liver at 15 minutes ($P = .001$), and in the bone marrow at 4 hours ($P = .003$).

Conclusions: The kinetics of migration of neutrophils and eosinophils through the lung, spleen, and bone marrow of human volunteers are significantly different. Study of mixed populations might be misleading. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: *Granulocytes, eosinophils, neutrophils, kinetics, radiolabeled, lung, liver, spleen, bone marrow, technetium 99m–hexamethylpropyleneamine oxime*

The biodistribution of granulocytes in animals and human subjects has been extensively investigated in both homeostasis and disease states. Most studies have used nuclear medicine methodologies and appear to have established that human granulocytes in homeostasis are distributed in approximately equal proportions in 2 dynamic pools: a marginating and a circulating pool.[1,2] Granulocyte margination is interpreted as the consequence of transient adhesion of these cells to the microvasculature of major organs.[3,4] Shortly after injection of radiolabeled granulocytes, the marginating pool was shown to be distributed predominantly between the spleen (35%), liver (25%), and lung (10%).[5] In these studies the fact that granulocytes are not a pure population of cells has been set aside, and the tacit assumption has been that neutrophils, which are numerically the most common blood granulocytes, at least in healthy subjects, account for most of the signal when assessing biodistribution. This already somewhat dubious proposition has been further undermined by the observation that when a total granulocyte population was radiolabeled with technetium 99m–hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO), eosinophils were labeled with much higher efficiency than neutrophils, such that their mean cellular radioactivity was at least 10-fold higher.[6,7] This implies that eosinophils *in vivo* could be contributing more than half of the overall signal, despite their significantly fewer numbers. Thus the assumption that granulocyte scans essentially reflect neutrophil distribution is not well founded and might be grossly misleading. This is of critical importance because neutrophils and eosinophils have fundamentally different roles in health and in particular disease processes. Whereas neutrophils act as professional phagocytes and home to sites of infection and some forms of inflammation, eosinophils are regarded as key effector cells in other inflammatory diseases, including asthma, eosinophilic pneumonia, eosinophilic colitis, and esophagitis. Furthermore, in patients with some diseases, such as asthma,

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Supported by the Department of Health through the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust and by the Lee Lu Cheung Fund.

Disclosure of potential conflict of interest: P. J. Blower has grants/grants pending with various funding agencies and has received travel/accommodations/meeting expenses for academic invited talks. C. J. Corrigan has consultant arrangements with Chiesi, Novartis, and Allergy Therapeutics; has received payment for lectures, including service on speakers' bureaus, from GlaxoSmithKline; has received payment for development of educational presentations from Henry Stewart Talks; and has received travel/accommodations/meeting expenses from Novartis. G. E. D. Mullen has received grants from Department of Health/Medical Research Council. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication November 7, 2012; revised June 18, 2013; accepted for publication June 28, 2013.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2013.06.031

Abbreviations used
FITC: Fluorescein-5-isothiocyante
HSA: Human serum albumin
MFI: Mean fluorescence intensity
^{99m}Tc-HMPAO: Technetium 99m hexamethylpropyleneamine oxime
PE: Phycoerythrin
ROI: Region of interest
TAC: Time activity curve

heterogeneity has been proposed according to the relative contributions of neutrophils and eosinophils. Clearly, the ability to differentiate eosinophil and neutrophil migration is of fundamental importance.

In this study we used the Current Good Manufacturing Practice CD16 CliniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), which allows separation of blood neutrophil and eosinophil granulocytes according to expression or nonexpression of CD16 by using anti-CD16–conjugated magnetic beads. This allowed cellular separation, labeling, and reinfusion to be performed in a completely sterile environment in sufficient numbers and in a workable time frame with minimal stimulation of the cells that can modify their migration. We hypothesized that the time courses of efflux of neutrophils and eosinophils into the major organs of healthy adult subjects are fundamentally different.

METHODS

Participants and study design

Four healthy volunteers were each scanned twice (2 male subjects aged 26 and 35 years and 2 female subjects aged 27 and 32 years). All volunteers had blood neutrophil and eosinophil counts within the laboratory's normal range and no clinical signs or symptoms whatsoever, particularly no signs or symptoms of upper or lower respiratory tract infection or inflammation, at the time of the study. The study was approved by an independent United Kingdom national research ethics committee and by the Administration of Radioactive Substances Advisory Committee of the United Kingdom. All subjects provided written informed consent to participate and were recruited at King's College London. On their first visit, volunteers were imaged after reinfusion of autologous radiolabeled neutrophils and returned within 11 weeks to be imaged again after reinfusion of autologous radiolabeled eosinophils.

Good Manufacturing Practice neutrophil and eosinophil isolation and radiolabeling

All open manipulations were carried out in a grade A environment in a pharmaceutical isolator located in a grade D background Good Manufacturing Practice facility in the Department of Nuclear Medicine at Guy's Hospital (London, United Kingdom). Each volunteer donated one 110-mL sample of venous blood on 2 separate occasions. At each visit, 105 mL of blood was collected in a heparinized syringe (sodium heparin at 1000 units per 105 mL of whole blood) of which 15 mL was centrifuged to retain a plasma sample and the remainder was diluted 1:1 with CliniMACS buffer (Miltenyi Biotec) and then layered over 95 mL of Ficoll Plus (GE Healthcare, Buckinghamshire, United Kingdom) and centrifuged at 450*g* for 25 minutes at room temperature. In addition, 4 mL of blood was collected into a Vacutainer EDTA tube (BD Biosciences, Oxford, United Kingdom) for cell counting, and 1 mL of blood was collected into 1 mL of 0.4% formaldehyde/PBS for flow cytometric analysis. After Ficoll separation, the diluted plasma was retained, whereas the mononuclear layer and Ficoll were discarded. The erythrocyte/granulocyte pellet was resuspended in autologous diluted plasma to a final volume of 95 mL, 20 mL of 6% Hespan (Grifols, Cambridge, United Kingdom) was added, and the mixture was centrifuged at 8*g* for 15 minutes at room temperature (to separate the granulocytes from the red cells). The granulocyte layer was washed once with 100 mL of CliniMACS buffer containing 0.5% human serum albumin (HSA; Octapharma, Manchester, United Kingdom; CliniMACS-HSA buffer) and then resuspended in 10 mL of HSA buffer to which 0.8 mL of CD16 reagent (Miltenyi Biotec) had been added. After incubation at room temperature for 30 minutes on a slow cycle of a rotary mixer, the granulocytes were washed once with 50 mL of HSA buffer and then resuspended in the same buffer before being transferred to a sterile, nonpyrogenic blood collection bag (Miltenyi Biotec). The bag was then connected to the automated CliniMACS system, where the passage of the granulocytes in HSA buffer through thesystem and column eliminated $CD16^-$ cells (predominantly eosinophils), which were then collected in a sterile collection bag. After removal of the magnetic field by using the CliniMACS program, the $CD16^+$ fraction (neutrophils) was eluted into another sterile bag attached to the CliniMACS tubing set. Eosinophils (approximately 1×10^7) or neutrophils (approximately 10×10^7) were incubated with up to 700 MBq of ^{99m}Tc-HMPAO (Ceretec, GE Healthcare) in accordance with the manufacturer's instructions. After incubation at ambient temperature for 15 minutes, the labeled cells were washed and resuspended in 2.5 mL of autologous plasma. Radiolabeling efficiency was measured by dividing the pellet activity (in MBq) by the total activity (pellet and supernatant) times 100.

Viability, phenotype, and purity of purified radiolabeled neutrophils and eosinophils

Aliquots of 1×10^6 cells collected from whole blood after CD16 CliniMACS isolation and after radiolabeling were analyzed by means of flow cytometry. Cells were fixed with 0.4% formaldehyde in PBS at 37°C for 4 minutes and then washed in HSA buffer and resuspended in CliniMACS-HSA buffer. Cells were analyzed by using a FACSCalibur machine (BD Biosciences) with the following antibodies and fluorochromes, according to the manufacturer's instructions: CD16-phycoerythrin (CD16-PE) and CD15– fluorescein-5-isothiocyanate (CD15-FITC; Miltenyi Biotec), CD66-PE, CD11b-FITC, CD69-FITC, IgG-FITC/IgM-PE (isotype controls), and a mononuclear FITC cocktail of CD3, CD19, CD56, and CD80 (BD Biosciences). Staining of CD11b on neutrophils was performed on fixed cells at 4°C for no more than 10 minutes because it has been previously shown that staining for longer periods and at room temperature induces activation of CD11b on neutrophils.[8] Possible activation of the $CD66^+CD16^+$ neutrophil population during *ex vivo* manipulation and radiolabeling was investigated by examining changes in the mean fluorescence intensity (MFI) of $CD11b$[9] staining in whole blood and after isolation and radiolabeling and based on the percentage of cells coexpressing CD11b above a fluorescence threshold ($CD11b^{High}$) observed after incubating unfixed whole blood from healthy volunteers (n = 10) with GM-CSF (500 ng/mL, BD Biosciences) at 37°C for 2 hours.[8] This was used to define a $CD16^+CD11b^{low}$ and $CD16^+CD11b^{high}$ population (see Fig E1 in this article's Online Repository at www.jacionline.org). Quadrants were based on negatively stained cells and the isotype control. Possible activation of $CD66^+CD16^-$ eosinophils was investigated by examining changes in CD69 MFI by staining cells from whole blood and after radiolabeling and based on the percentage of cells expressing $CD69$[10] fluorescence compared with unstained cells and cells stained with isotype control with the stated reagents. Cellular viability was assessed with an automated cell counter (Countess; Invitrogen, Paisley, United Kingdom) based on Trypan blue exclusion.

Imaging protocol and data analysis

With the volunteer positioned in the dual-headed gamma camera (Symbia; Siemens Medical Solutions, Surrey, United Kingdom), 100 MBq (96 ± 27 MBq) of labeled autologous eosinophils or neutrophils suspended in 2.5 mL of autologous plasma was injected intravenously as a bolus. Dynamic anterior and posterior views were acquired with low-energy general purpose collimators as a sequence of 1-second frames for the first 2 minutes, followed by 30-second frames up to 32 minutes after injection. Further images (5 minutes per frame) were acquired at 1, 2, and 4 hours after injection. An outline/localization image with a ^{57}Co flood source positioned between the camera