BRIEF REPORT: PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Use of 111-Indium-labelled autologous eosinophils to establish the

in vivo kinetics of human eosinophils in healthy subjects

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Abstract

Eosinophils are the major cellular effectors of allergic inflammation and represent an important therapeutic target. Whilst the genesis and activation of eosinophils has been extensively explored, little is known about their intravascular kinetics or physiological fate. This study was designed to determine the intravascular lifespan of eosinophils, their partitioning between circulating and marginated pools, and sites of disposal in healthy individuals. Using autologous, minimally manipulated 111-Indium-labelled leukocytes with blood sampling, we measured the eosinophil intravascular residence time as 25.2 hours (compared to 10.3 hours for neutrophils) and demonstrated a substantial marginated eosinophil pool. Gamma camera imaging studies using purified eosinophils demonstrated initial retention in the lungs, with early re-distribution to the liver and spleen, and evidence of re-circulation from a hepatic pool. This work provides the first in vivo measurements of eosinophil kinetics in healthy volunteers and shows that 111-Indium-labelled-eosinophils can be used to monitor the fate of eosinophils non-invasively.

Introduction

Eosinophils play a key role in allergic inflammation [1] and represent an important therapeutic target in asthma and other allergic diseases. They have the capacity to release histotoxic substances, including granule proteins, inflammatory cytokines and reactive oxygen metabolites, which cause bronchoconstriction, epithelial damage, hyper-responsiveness and airway remodelling [2-6].

Much is known about the cellular mechanisms regulating the development and maturation of eosinophils, their release from the bone marrow, and the processes involved in their recruitment, activation and clearance during allergic inflammation [7-11]. By contrast, very little is known about the physiology of circulating eosinophils in humans. Due to the relative scarcity of eosinophils in the blood of healthy individuals (range: 0.0-0.4 ×10^9/l), previous attempts to study eosinophil kinetics have been restricted to patients with hypereosinophilia [12-14], hampered by label re-utilisation following pulse injection of 3-H-thymidine [15], or relied on autoradiographs developed >500 days [16]. We have used 111-Indium-labelled mixed leukocytes with post-injection isolation of eosinophils to ascertain their intravascular lifespan, and subsequently purified 111-Indium-labelled autologous eosinophils with gamma camera imaging to assess organ-specific trafficking in vivo. We have demonstrated an intravascular lifespan for circulating eosinophils exceeding 24 hours and revealed extensive intravascular margination of these cells, together with evidence of re-circulation from a hepatic pool.

Study design

Participants
Healthy male and female adults with normal lung function and eosinophil counts (range: 0.02-0.38 ×10^9/l) gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by Cambridgeshire Research Ethics Committee (09/H0308/119) and the Administration of Radioactive Substances Advisory Committee of the United Kingdom (83/3130/25000).
**Leukocyte Labelling**

Mixed leukocytes isolated from blood by hetastarch sedimentation [17] were labelled with 111-Indium tropolonate and re-injected into an antecubital vein. Administered activities ranged from 8.3-13.0 MBq. Venous blood (40 ml) was sampled at 0.75, 2, 4, 6, 9, 12, 24, 48 and 72 hours post re-injection. From each sample, neutrophils and eosinophils were isolated in parallel to ≥99% purity using the RoboSep® system (StemCell Technologies), and the radioactivity measured using a gamma counter.

**Eosinophil Labelling**

Eosinophils were isolated from autologous venous blood using plasma-Percoll gradients [17] followed by immunomagnetic separation with clinical grade anti-CD16 microbeads (CliniMACS, Miltenyi Biotec). Isolated eosinophils (91 ± 1% pure) were labelled with 111-Indium tropolonate (0.73 ± 0.15 MBq/10⁶ cells).

**Eosinophil Activation Status**

The activation status of the 111-Indium-labelled eosinophils was assessed by cell surface marker expression (CD69, CD44, CD81 and CD66b), eosinophil shape change, transmission electron microscopy analysis of cell morphology and eosinophil-derived neurotoxin (EDN) granule release (Supplemental Methods).

**Analysis**

Percentage granulocyte recovery was calculated using the formula:

\[ \text{Neutrophil or eosinophil recovery} \% = \frac{\text{Neutrophil or eosinophil-associated activity (cpm/ml)} \times \text{blood volume (ml)}}{\text{Injected neutrophil or eosinophil-associated activity (cpm/ml)}} \]  

Blood volume was estimated as described [19]. An automated differential leukocyte count was obtained (LH750, Beckman Coulter) and used to correct for the efficiency of the isolation for each individual recovery. Intravascular lifespan was determined by dividing the area under the time-activity curve by the 45 minute recovery. Values are expressed as mean ± SEM and ‘\( p < 0.05 \) was considered significant.

**Dynamic Imaging**

Volunteers lay supine above a double-headed gamma camera (Elscint, Apex SPX Helix) fitted with a medium-energy, parallel-hole collimator. After bolus injection of labelled eosinophils, activities in the chest and abdomen were recorded by imaging with a frame time of 1 second for 2 minutes followed by 20 seconds for 38 minutes. At later time-points there was a single frame time of 10 minutes. To generate organ time-activity curves, regions of interest were drawn over the right and left lungs, right ventricle, bone marrow (pelvis), liver and spleen using Xeleris software (GE Healthcare). Counts per MBq injected in these regions were recorded and corrected for 111-Indium physical decay.

**Results and discussion**

To determine the kinetics of minimally manipulated eosinophils, mixed leukocytes (Supplemental Figure S1) were isolated from peripheral blood, labelled with 111-Indium and re-injected. These experiments were considered essential, given the
potential for *ex vivo* cell activation to affect the subsequent behaviour of cells when re-injected [20]. The 45 minute recovery value is indicative of the proportion of labelled cells remaining in the freely-circulating blood pool (rather than sequestered in tissue vascular beds). We obtained a 45 minute recovery for neutrophils of $57 \pm 10\%$ (n=7), which is consistent with previous values [20] and thus validates our methodology. This was followed by mono-exponential removal from the circulation (Figure 1A). Interestingly, eosinophils isolated in parallel from the same blood samples displayed markedly different kinetics (Figure 1B). Firstly, 45 minute eosinophil recovery ($15 \pm 2\%$), was lower than the corresponding value for neutrophils suggesting a larger pool of marginated eosinophils. Secondly, after an initial decline in labelled eosinophils between 45 minutes and 2 hours the number in the circulation recovered at 4 and 9 hours (suggesting possible re-circulation) before final mono-exponential removal. Thirdly, the intravascular lifespan of eosinophils was $25.2 \pm 3.8$ hours compared to $10.3 \pm 0.1$ hours for neutrophils. Plasma radioactivity was $\leq 2\%$ indicating stable cell-association of the 111-Indium label. Whilst even the minimally manipulated labelled mixed leukocyte population may be susceptible to *ex vivo* activation, considerable efforts were made to minimize this possibility; namely, blood withdrawal was performed using a 19G needle to prevent perturbation of the granulocytes, acid-citrate dextrose was used as an anticoagulant as it results in less activation than heparin or EDTA [21], and hetastarch was used to remove red blood cells as sensitivity reactions have been reported following dextran use [17].

Having established the kinetics of minimally manipulated eosinophils, we next imaged sites of eosinophil margination and uptake/disposal. Eosinophils were purified and labelled *ex vivo* prior to re-injection. Autoradiographs confirmed that the incorporated 111-Indium label was cell-associated (Supplemental Figure S2). There were no significant differences in the expression of CD44, CD69, CD81 or shape change between the two cell populations, although CD66b was more highly expressed on the fully purified eosinophils (Figure 1D-E), as previously described [22]. Furthermore, there was no alteration in the granule morphology of the 111-Indium-labelled purified eosinophils compared to whole blood eosinophils (Figure 1F and Supplemental Figure S3). The purified eosinophils had a 45 minute recovery of $15 \pm 2.6\%$ and intravascular lifespan of $30 \pm 2.7$ hours (Figure 1C), which was highly comparable with the values obtained using labelled mixed leukocytes, again providing evidence that the purified eosinophils were non-activated. A comparison of the peripheral eosinophil count and the labelled eosinophil recovery revealed a close correlation between the kinetics of labelled and non-labelled cells, suggesting that the labelled eosinophils behave physiologically (Supplemental Figure S4A). Correction of labelled eosinophil counts to the peripheral eosinophil numbers (Supplemental Figure S4B) shows a near mono-exponential decline in the number of circulating labelled eosinophils and suggests that the physiological nocturnal eosinophilia reflects mobilisation of eosinophils from the marginated blood pool.

Gamma camera images of re-injected 111-Indium-labelled eosinophils demonstrated initial transit through the lungs, clearing to baseline by 40 minutes, with early accumulation in the liver and progressive accumulation in the spleen and bone marrow (Figure 2). Of note, the 9 hour blood recirculation peak coincided with a significant reduction of activity in the liver, and to a lesser extent, in the bone marrow signal. In contrast to neutrophils [23, 24], very little signal was seen within the axial skeleton at any time-point (Figure 2C). This implies that the bone marrow is not a
major site for eosinophil disposal, with the principal sites being the liver and spleen (Figure 2D). Interestingly, following re-injection of eosinophils in a single splenectomised volunteer (Supplemental Figure S5) there was increased eosinophil uptake in both the liver and also in the bone marrow; the distribution at 48 hours was 64% activity in the liver and 31% activity in other sites (principally comprising the bone marrow).

These studies reveal for the first time the kinetics of eosinophil circulation in healthy human subjects, and demonstrate their physiological uptake in, and release from, the reticulo-endothelial system. These studies also provide the basis for future non-invasive quantification of eosinophil accumulation in tissues.

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Authorship Contributions

N.F. performed experiments, analyzed the data, and wrote the manuscript, with contributions from the other authors as appropriate; N.R.S. performed experiments; S.H. and C.S. analyzed data; C.L. performed experiments; C.K.S., K.S. and K.K.B. coordinated the labelling and imaging study; P.R. performed experiments; A.M.C. analyzed data and wrote the manuscript; E.R.C. and A.M.P. conceived and designed the study and wrote the manuscript.

A.M.P., A.M.C. and E.R.C. are joint senior authors.

All authors discussed the results and commented on the manuscript.

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References


**Figure Legends**

**Figure 1. Granulocyte blood kinetics and activation status of 111-Indium-labelled eosinophils.**

Following re-injection of 111-Indium labelled mixed leukocytes, 40 ml blood samples were taken and (A) Neutrophils (NEU) and (B) eosinophils (EO) were isolated from 40 ml blood samples using RoboSep® before measurement of cell-associated radioactivity. (C) Kinetics of eosinophils isolated following re-injection of purified 111-Indium labelled eosinophils. Data represent the mean ± SEM of 7 independent experiments (A and B) (*p < 0.05 compared with 2 h and 6 h values using a one-way ANOVA test) and mean ± SEM of 6 independent experiments (C). Non-cell associated radioactivity was measured by collecting plasma samples (open circles). Where error bars are not visible the SEM lies within the symbols. (D) Representative flow cytometry and quantification of CD69, CD44, CD66b and CD81 expression in
freshly isolated whole blood eosinophils (WB), mixed leukocyte eosinophils (ML) ± 111-Indium tropolonate and CD16 purified eosinophils (EO) ± 111-Indium tropolonate. Histograms represent WB (red line), ML (blue line) or EO (black line). Isotype-matched controls (grey fill) for WB, ML or EO are shown from left to right. (E) Eosinophil shape change. Freshly isolated WB, and CD16 purified EO ± 111-Indium tropolonate were incubated with 10 ng/ml GM-CSF (GM) before assessment of shape change as described in Supplemental Methods. The means of cell size in forward scatter signal are shown. Data represent the mean ± SEM of at least 3 independent experiments (*p < 0.05 compared with whole blood values using a one-way ANOVA test). (F) Representative TEM images of eosinophils in WB, ML ± 111-Indium tropolonate and EO ± 111-Indium tropolonate incubated with or without IL-5 (10 ng/ml). IL-5 treated eosinophils display signs of vacuolation (black arrows) and loss of granule integrity (white arrows). Original magnification ×3500.

Figure 2. Gamma camera quantification of 111-Indium-labelled eosinophils. (A) Anterior gamma camera images at 5 min and 40 min following re-injection, showing accumulation in the right lung (RL), left lung (LL), liver (L) and spleen (S). (B) The distribution of radioactivity over 72 h for the right lung (blue), liver (green), spleen (black), bone marrow (purple) and eosinophil recovery (red) following re-injection of 111-Indium-labelled eosinophils. (C) The distribution of radioactivity over 40 min for lungs, liver, spleen, bone marrow and right ventricle following re-injection of 111-Indium-labelled eosinophils. (D) The final distribution of eosinophils within the liver and spleen at 48 h. Radioactivity in ‘other sites’ was calculated by subtracting the liver, spleen, lung and blood activity values from the total radioactivity injected. The value obtained may reflect regions such as the bone marrow which are difficult to fully quantify on images of the upper body alone. Data represent the mean ± SEM of 6 independent experiments (*p < 0.05 compared with 40 min liver values using a one-way ANOVA test).
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