Lateral Flow Immunoassay Using Europium (III) Chelate Microparticles and Time-Resolved Fluorescence for Eosinophils and Neutrophils in Whole Blood

GERD RUNDSTRÖM,1,2* ANN JONSSON,1,2 OLA MÅRTENSSON,2† IB MENDEL-HARTVIG,2† and PER VENGE3

Background: A simple point-of-care method for measuring leukocyte counts in a doctor’s office or emergency room could be of great importance. We developed a protocol for measuring cell count by disrupting the cell membrane and analyzing specific proteins within the cells and used it to analyze proteins from eosinophils and neutrophils.

Methods: Lateral immunochromatographic (ICR) assays have been developed for eosinophil protein X (EPX) and human neutrophil lipocalin (HNL) as measures of the concentration of eosinophils and neutrophils. The correlation between the lateral ICR assays and cell counting of eosinophils and neutrophils was performed manually and with an automated cell counter. RIA assays measuring the same analytes were also compared with the results from cell counting and lateral ICR assays.

Results: The optimized assays showed analytical detection limits below the clinical ranges of 3.36 μg/L and 2.05 μg/L for EPX and HNL, respectively. The recovery was 114.8%–122.8% for EPX and 94.5%–96.9% for HNL. The imprecision was 3%–17% CV for EPX over the whole range and 5%–16% CV for HNL. The correlation coefficients between manually counted cells and lateral ICR assays were 0.9 and 0.83 for EPX and HNL, respectively.

Conclusion: The numbers of eosinophils and neutrophils in small amounts of blood can be estimated in the point-of-care setting by means of fast lateral ICR assays of EPX and HNL.

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New technologies for point-of-care (POC) analyses include methods based on magnetic particles (1) or lateral flow immunoassays (2). Magnetic bead assays require a strong magnet to perform the separation as well as a detection method. Lateral flow assays may be based on visual detection without detection instrumentation, but if there is a need for quantification, a detection scheme is necessary. Detection schemes may be based on gold particles, colored particles, carbon black, or fluorescence, which is often preferred to obtain the highest sensitivity in an assay. The detector reagents (mainly antibodies) may be directly labeled with fluorophores, such as the Cy family or the Alexa dyes (3). Antibodies can also be coupled to particles incorporated with fluorophores, in which case the fluorescence intensity is amplified. The most sensitive type of fluorescence, time-resolved fluorescence (Tr-FIA), is based on chelates of the lanthanides (e.g., europium, samarium). Incorporating these chelates into particles is an advantageous way to obtain assays with high sensitivity (4–6).

We aimed to develop a simple POC application to measure eosinophils and neutrophils in blood by use of europium (III) chelate microparticles and time-resolved...
fluorescence. We developed assays to measure specific markers, human neutrophil lipocalin (HNL) (7) to measure neutrophil counts and eosinophil protein X (EPX) (8) to measure eosinophil counts.

**Materials and Methods**

**PARTICLES**

Uniform 0.12 and 0.21 μm Fluoromax fluorescent carboxylate-modified europium chelate microparticles (Seradyn) were used as detector reagent in the lateral immunochromatographic (ICR) assays. FluoSpheres, far red (690/720) aldehyde-sulfate, 0.11 μm (Molecular Probes) were used as a detector reagent in the simplified assay.

**ANTIGENS**

We extracted and purified EPX from human whole blood cells as previously described (8). Recombinant HNL (rHNL) was kindly provided by Phadia AB (Uppsala, Sweden). The rHNL showed binding characteristics similar to those of HNL purified from extracts of human neutrophils (7). The proteins were labeled with 125I by the chloramin-T method (9).

**ANTIBODIES**

Monoclonal antibodies (mAbs) against HNL and EPX were produced in mice and cloned for epitope specificity with a Biacore 2000. Binding studies were also performed with the simplified lateral ICR assay. The mAbs were individually deposited on the nitrocellulose membrane and also coupled to far-red fluorescent particles. The chosen mAbs, aEPX cl.616 as capture, aEPX cl.618 for detection, aHNL cl.697 as capture, and aHNL cl.764 for detection, were all of IgG1 subtype. We characterized the specificity of the respective mAbs by Western blot. The antibodies were labeled with 125I by the chloramin-T method (9).

**ANTIBODY LABELING**

To determine the amounts of antibodies coupled to the particles or to polyethylene glycol (PEG), and to analyze the physical stability in the membrane, we mixed the different mAbs with a small amount (<1% of the total) of radiolabeled antibody (125I-Ab) and then purified the antibodies from the free 125I by gel filtration on prepacked columns containing Sephadex G-25 (PD-10 column, GE Healthcare) in the buffer to be used in the coupling reaction. We measured the concentration of antibody by absorbance at A280 0.1% = 1.38 and calculated the amount of radioactivity per microgram of antibody.

**COUPLING TO CARBOXYL PARTICLES**

We employed a method using 4 different components, originally invented by Ugi (10) and later modified to work with 1 of the components being a solid (11–13), for the immobilization of antibody. The antibody was treated as mentioned above and transferred to a buffer containing 0.25 mol/L Bis-Tris buffer, pH 6.2, in a prepacked gel filtration column containing Sephadex G-25, (PD-10 column, GE Healthcare). We centrifuged the particles with carboxyl groups (CM/EU-particles), and resuspended the pellet in 0.25 mol/L Bis-Tris to a concentration of ~20 g polystyrene/L. We mixed the particles and the antibody solution with 1.5 mol/L acetic aldehyde to a concentration of 20 mmol/L, and finally added 2-(4-morpholinyl)-ethylisocyanid isonitril (Merck) to give a concentration of 1%. The mixture was incubated for 16 h, and the reaction was terminated by the addition of L-glutamic acid and L-aspartic acid, 50 mmol/L each in the final solution, which was left to react for an additional 2 h. The particle conjugate was centrifuged for 20 min at 20 800g at 10 °C. After aspiration we washed the particles 3 times by centrifugation with 0.2% bovine serum albumin (BSA), 20 mmol/L sodium borate, pH 8.0, containing 0.05% sodium azide. Lastly we resuspended the particle conjugate to a concentration of ~20 g polystyrene/L, followed by sonication with a microprobe at 20% amplitude for 3 min.

**COUPLING TO ALDEHYDE PARTICLES**

We treated each antibody as described above and transferred the antibody to a buffer containing 50 mmol/L sodium phosphate, pH 6.5, in a prepacked gel filtration column containing Sephadex G-25. The antibody was mixed with the aldehyde polystyrene particles to a concentration of ~15 g polystyrene/L and 1 g antibody/L. We incubated the mixture for 2.5 h, during which a Schiff’s base adduct was formed. Any excess aldehyde groups were blocked with 50 mmol/L each of L-glutamic acid and L-aspartic acid (Sigma Chemicals), the covalent linkage was reduced with 25 mmol/L sodium cyanoborohydride, NaCNBH3 (Sigma Chemicals), for 30 min, and the particle conjugate was centrifuged for 20 min at 20 800g at 10 °C. We aspirated the supernatant and washed the conjugate by centrifugation and aspiration 3 times with 0.2% BSA and 20 mmol/L sodium borate, pH 8.0, containing 0.05% sodium azide. Finally, we resuspended the particle conjugate to a concentration of ~20 g polystyrene/L, followed by sonication with a microprobe at 20% amplitude for 3 min.

**ANTIGEN BINDING CAPACITY OF IMMOBILIZED ANTIBODIES**

We purified 125I-labeled analyte (EPX or rHNL) from free 125I by gel filtration on a prepacked column containing Sephadex G-25, using 50 mmol/L sodium phosphate, 6% BSA, 0.05% sodium azide, pH 7.5, as elution buffer, and then mixed the 125I-labeled analyte with unlabeled analyte to a concentration of 25 mg analyte/L and ~0.1 mg 125I-analyte/L. The antibody-particle conjugates were sonicated before use and diluted to 20 μg Ab/L in 50 mmol/L sodium phosphate, 0.15 mol/L sodium chloride, 1%BSA, and 0.05% sodium azide, pH 7.5. We added 200 μL analyte solution to 20 μL of antibody-particle conjugate. We then incubated the samples for 2 h on a shaker, followed by 2 washing steps of centrifugation/aspiration.
for 20 min at 20 800g, 10 °C, in 50 mmol/L sodium phosphate, 0.15 mol/L sodium chloride, 1% BSA, and 0.05% sodium azide, pH 7.5. We determined the activity in the pellets with a gamma counter and calculated the capacities of binding EPX and rHNL for the antibodies in the conjugates.

COUPLING OF PEG TO ANTIBODIES

We mixed the antibodies with a small amount (<1% of the total amount) of radiolabeled antibody and transferred this mixture to a buffer containing 10 mmol/L of sodium phosphate, pH 7.5, as described earlier. The SPA-PEG (Shearwater Polymers, Mw 5000 D) was dissolved in dimethyl formamide to 100 mg PEG/L. We then added SPA-PEG to the antibody solution in a 5-fold molar excess. The mixture was incubated for 1.5 h on a rotating wheel, and the unreacted SPA-PEG separated on a Sephadex G-200 column. We calculated the concentration of Ab-SPA-PEG by measuring the radioactivity.

LATERAL ICR ASSAYS FOR HNL AND EPX

The mAbs (aEPX and aHNL) coupled to PEG were both deposited onto the nitrocellulose membrane (Millipore) using a Biodot dispensing apparatus (Biodot XYZ 3000 1419) to form the reaction zone (RZ). We cut the nitrocellulose membrane into 5 mm wide strips, and mounted the strips in the assay device with an adsorbent filter mounted in the rear end of the device. The ICR assay device (Fig. 1) had a sample port and an upstream buffer port.

We diluted whole blood 1/40 in 20 mmol/L Tris-HCL, 0.2% N-cetyl-N-N-N-trimethylammonium bromide (CTAB), and 1% BSA, pH 8.4, and then applied 20 μL to the sample well. Immediately after the sample addition, we added 20 μL of aHNL and aEPX antibodies conjugated to Europium (III) chelate particles (CM/EU) to the buffer well and allowed the solution to flow toward the RZ. We washed the excess reagents from the nitrocellulose membrane and the RZ with 80 μL of buffer (20 mmol/L Tris-HCL, 54 mmol/L NaCl, 3% BSA, 1% sucrose, 0.05% bovine gamma globulin, and 0.05% NaN₃, pH 8.4). A scanning LED (light emitting diode, 385 nm) was used to excite the fluorophores, and a photo diode used to collect the emitted light from the bound conjugate particles for calculation of raw data, relative fluorescence units in a specially designed reader.

SIMPLIFIED LATERAL ICR ASSAY

We deposited the aEPX and/or aHNL mAbs on the nitrocellulose membrane using the Biodot dispensing apparatus to form the RZ. The nitrocellulose membrane was cut into 5-mm wide strips. We applied 30 μL HNL and/or EPX, diluted in buffer (20 mmol/L Tris-HCL, 0.2% CTAB, 1% BSA, pH 8.4), or whole blood diluted 1/40 in the same buffer, to the strip. We then added 20 μL of mAb (aEPX and/or aHNL) conjugated to the fluorescent particles (far red) to the strip. After a wash with 20 mmol/L Tris-HCL, 54 mmol/L NaCl, 3% BSA, 1% sucrose, 0.05% bovine gamma globulin, and 0.05% NaN₃, pH 8.4, we analyzed the strips in a specially designed scanning fluorometer, with a red diode laser at 670 nm. The raw data, expressed as relative fluorescence units, was used for calculation of bound conjugate.

PHYSICAL STABILITY OF MAB IN THE NITROCELLULOSE MEMBRANE

We mixed the mAbs (aEPX and aHNL) with 125I Ab and deposited this mixture onto the nitrocellulose membrane. The radioactivity was measured in a gamma counter. The simplified lateral ICR assay was performed with a blood sample diluted 1/40. After the assay we again measured the radioactivity.

RADIOIMMUNOASSAY FOR EPX AND HNL

We performed the Pharmacia EPX RIA according to the manufacturer’s instructions. We performed the RIA for HNL as described previously (7).

DILUTIONS OF EPX AND HNL

We diluted standard preparations for both EPX and rHNL to 0.13–200 μg/L in the sample buffer (20 mmol/L Tris-HCL, 0.2% CTAB, 1% BSA, pH 8.4). We also diluted a blood sample (1/40) and further diluted it in the same buffer. We then analyzed both the standards and the sample dilutions in the assay device.

STATISTICS

Multicalc software (PerkinElmer) was used for calculation of concentration and imprecision.

RESULTS

EFFECT OF PEGYLATION

In an attempt to make the mAbs more active and physically stable in the membrane, we conjugated them to PEG.
The physical stability in the nitrocellulose membrane for aEPX is shown in Fig. 2. When the mAb was not conjugated to PEG it was easily washed away by the flow of sample and buffer, and <50% was left after the physical stability assay. The fluorescent signal from the assay showed that the activity for the mAb increased when the mAb was conjugated to PEG at low excess (5-fold) of PEG at the time of conjugation. With higher excess of PEG, ≈10 times, the activity decreased, probably as a result of blocked active sites on the mAb. To have >80% of the mAb left in the membrane with desired activity, the 10-fold excess of PEG, in proportion to aEPX, was chosen for deposition onto the membrane.

The mAb aHNL showed better physical stability in the membrane when it was not conjugated to PEG, and only 5-fold excess of PEG was needed to achieve the same stability and activity as for the aEPX mAb.

**OPTIMIZATION OF DETECTION CONJUGATES**

We chose antibody pairs with the mAbs conjugated to fluorescent particles (far red) functionalized with aldehydes. In the assay device we conjugated the mAbs to fluorescent microparticles (Europium chelate) modified with carboxyl groups (CM/EU).

The conjugates were prepared with different labeling densities of antibodies in the coupling method. Two different diameters for the CM/EU particles were also investigated for the aHNL mAb. All conjugates were characterized by a method measuring the capacity of binding the antigen, and also the performance in the immunoassay used in the assay device. The method for determination of the capacity to bind the antigen measured both high and low affinity Abs, because a large excess of antigen was used. The results from the capacity method indicated rather high consistency between the aHNL conjugates for both particle sizes and for variation in labeling densities. Approximately 0.5 mol antigen/mol antibodies were bound to the particles. For the aEPX conjugate, the binding capacity for its antigen showed inferior results (approx. 0.1 mol antigen/mol antibodies).

The results from the HNL immunoassay showed that the conjugates prepared with the CM/EU particle with the larger diameter (0.21 μm) showed less sensitivity and higher nonspecific binding compared with the conjugates prepared with the smaller diameter particle (0.12 μm). The dose–response curves from the immunoassay, performed with the conjugates prepared with 0.12 μm CM/EU, demonstrated similar results for the 3 highest excesses of antibodies during conjugation. The lowest excess (44 μg Ab/mg particle) showed high nonspecific binding and poor sensitivity.

**DOSE–RESPONSE**

We evaluated the amount of mAb deposited onto the nitrocellulose membrane, and the amount of mAb in the fluorescent conjugate. The evaluation was performed in the simplified assay, with the mAbs conjugated to fluorescent particles (far red) for EPX assay and to CM/EU particles for the HNL assay. The results for EPX indicated that the amount of mAb deposited on the membrane should be 250 ng/strip, and the amount of mAb in the conjugate solution should be 0.4 μg/strip to avoid excessively high signal in the detector and to optimize sensitivity. For the HNL assay, the optimal amount of conjugate mAb was the same as for EPX (0.4 mg Ab/L), but the best sensitivity was achieved with a lower amount dispensed to the nitrocellulose membrane (62.5 ng/strip). In the correlation study between the RIA assays, cell counting, and the lateral ICR assays, 250 ng aHNL/strip was chosen to make the assays more comparable.

**DETECTION LIMIT**

Standard samples were diluted to concentrations close to zero and analyzed in 5 replicates. The SD was calculated, and 3 SD above the zero concentration was accepted as the detection limit in the assays. The lowest detection limits in the assay, 0.082 and 0.05 μg/L for EPX and HNL respectively, were achieved with the lowest labeling density. Conjugates with aHNL mAb and the larger particles (0.21 μm) were also investigated. Because of the high

![Fig. 2. The right y axis shows the physical stability of the binding between aEPX and the nitrocellulose membrane with or without conjugation to PEG, shown as percentage left after extensive washing. The left y axis shows the relative fluorescent units (RFU) from a sample for different excess amounts of PEG relative to aEPX (x axis).](image)
nonspecific binding, the results showed poor detection limits compared with conjugates prepared with 0.12-µm diameter particles.

**RECOVERY**

Analyses were performed after addition of known concentrations of EPX and HNL. The observed values, relative to the expected values, were 94.5%–96.9% for HNL and 114.8%–122.8% for EPX.

**PARALLELISM**

Dilutions of standard EPX and HNL, and a diluted whole blood sample (1/41), behaved in a similar manner and had similar slopes (Fig. 3).

**IMPRECISION**

We investigated assay imprecision with 13 clinical samples diluted 1/40 in the sample buffer. The samples were run in 5 replicates each. The concentrations ranged from 1–37 µg/L for EPX and 1–25 µg/L for HNL. The CVs were 2%–17%, with the lowest imprecision in the range 6–10 µg/L.

**RELATIONSHIP BETWEEN LATERAL ICR ASSAYS AND BLOOD EOSINOPHIL AND NEUTROPHIL COUNT**

We performed eosinophil and neutrophil counts in blood, and compared the results to the number of EPX and HNL in whole blood extracts as measured by the lateral ICR assays. In one set of samples eosinophils and neutrophils were calculated as percentages of 400 leukocytes counted manually under the microscope by 2 experienced technicians. The results showed a correlation to eosinophil counts of \( r = 0.90 \) (\( P < 0.001 \)) for EPX, and a correlation to neutrophil counts of \( r = 0.83 \) (\( P < 0.001 \)) for HNL (Table 1). In a 2nd set of samples we compared the number of EPX and HNL in extracts to results from a cell counter. The results, presented in Fig. 4 and 5, show linear correlations between eosinophil counts and EPX in extracts (\( r = 0.82, P < 0.001 \)) and between neutrophil counts and HNL in extracts (\( r = 0.88, P < 0.001 \)).

**Discussion**

We previously identified several candidate proteins as specific markers of eosinophils and neutrophils (15), and found that eosinophil peroxidase showed the best relationship to blood eosinophil counts. In some populations, however, eosinophil peroxidase deficiencies would produce false-negative results. Here we chose EPX as our eosinophil marker, although small amounts of EPX can be derived from neutrophil granulocytes. The production of reagents for an EPX assay was easy and economical. We chose HNL as our neutrophil marker because this protein is entirely specific to neutrophil granulocytes. An alternative candidate, myeloperoxidase (16), shows a close relationship with the number of blood neutrophils but also has the disadvantage of known deficiencies, which would

### Table 1. Correlation between the newly developed lateral ICR assays for HNL and EPX, and correlation between RIA for the same analytes and 2 methods for counting cells.

<table>
<thead>
<tr>
<th>Correlation between methods</th>
<th>Neutrophils/HNL</th>
<th>Eosinophils/EPX</th>
<th>Neutrophils/HNL</th>
<th>Eosinophils/EPX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICR–manually counted</td>
<td>0.88</td>
<td>0.82</td>
<td>0.83</td>
<td>0.9</td>
</tr>
<tr>
<td>ICR–cell count</td>
<td>0.9</td>
<td>0.86</td>
<td>0.95</td>
<td>0.91</td>
</tr>
<tr>
<td>RIA–manually counted</td>
<td>0.9</td>
<td>0.99</td>
<td>0.99</td>
<td>0.94</td>
</tr>
<tr>
<td>RIA–cell count</td>
<td>0.93</td>
<td>0.93</td>
<td>0.93</td>
<td>0.93</td>
</tr>
<tr>
<td>Manually counted–cell count</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
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</tr>
</tbody>
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![Fig. 3. Analysis of dilution of standard preparations of EPX and HNL, 0.13–200 mg/L, and dilution of a blood sample.](image-url)
produce false-low neutrophil numbers. Lactoferrin (17) is also specific to neutrophils but showed a poorer correlation to neutrophil counts than HNL.

Variation in the cellular content of specific granule protein may affect their use as markers of cell numbers. Such variation could have genetic causes but could also be the consequence of activation of the cells to release their granule proteins. Indeed, the deviations from the regression lines seen in the figs. may be explained by such circumstances, in addition to the actual contribution from the imprecision of the assays. Previous results using RIAs with very low imprecision have shown, however, that this has minor bearing on the variation of the results. Thus, the deviations may, to a large extent, be explained by the mean imprecision of 9% for the lateral ICR assay. The relationship between analytical imprecision and clinical requirements for eosinophil and neutrophil counts must be considered, however. The biological variation of eosinophil numbers in a nonallergic, healthy population is 50–500 × 10⁶/L and of neutrophils is 1.3–5.4 × 10⁹/L. As estimated by Statland (18), the intraindividual variation of eosinophil counts is 21.1% (CV) and the interindividual variation 76.4% (CV), whereas the intraindividual variation for neutrophil counts is 24.6% (CV) and the interindividual variation 39.6% (CV). Adopting the Tonk formula of an imprecision requirement of <25% of the interindividual biological variation, the lateral ICR assays of eosinophil and neutrophil numbers seem to fulfill these requirements.

The proteins to be analyzed are present within the cell, and the cell membrane has to be disrupted to measure the concentrations. We used the cationic detergent N-cetyl-N-N-N-trimethylammonium bromide (CTAB), known to work for solubilization of cell membranes. CTAB is also needed for optimal protein activity from the proteins in the eosinophil. Detergent concentration and blood dilution were optimized to immediately solubilize the cell membrane and to work in the lateral ICR assay. The concentration of 0.2% CTAB in 20 mmol/L Tris-HCL, 1% BSA, was optimal for extraction of the proteins within the eosinophils and neutrophils.

To quantify the analyte concentration we used fluorescent microparticles (Europium chelate) modified with carboxyl groups (CM/EU) as detector reagents, and de-
developed a reader to measure the time-resolved fluorescence from the excited chelate in the particles. The reader uses a motorized mechanism to move the assay device in the focal plane of an ultraviolet LED and collects the emitted light for calculation of raw data. The smallest amount detectable in the reader was $3.3 \times 10^2$ particles/mm$^2$.

The solid phase in the assay device was a microporous nitrocellulose membrane backed with a polyester film. To optimally quantify the concentration of the analytes in the assays, we would prefer a more standardized material that would be easier to produce, but with the same capillary flow characteristics as for the nitrocellulose membrane, such as a membrane made of synthetic polymers.

The capture antibodies in the RZ in the ICR assays for EPX and HNL were more stable in the nitrocellulose membrane if they were first conjugated to PEG, as reported in the literature (14). Some of the antibodies had increased activity after the pegylation with a low excess of PEG, possibly from the altered interfacial contacts between the nitrocellulose membrane and the protein. PEG conjugation is known to extend in vivo proteins or peptides, and the excess of PEG in relation to the Ab, must be optimized for each Ab. We found 5000 D to be optimal for the Abs investigated, and a molar excess of 5–10 was generally best to increase the physical stability in the membrane and retain the activity.

The 2nd antibody in the sandwich assay was conjugated to the detector particle, in some methods an aldehyde-functionalized particle (far red fluorescence), and in the assay device the antibodies were conjugated to carbamate modified particles (CM/EU) incorporated with europium chelate. The far red particles were used in the evaluation of reagents for practical reasons, before the reader detecting EU-chelate was fully optimized.

In conclusion, this fast lateral ICR assay with the use of EPX and HNL enables the estimation of numbers of eosinophils and neutrophils in small amounts of blood, a method that can be performed in the POC setting.

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