**Fully Automated Assessment of Inflammatory Cell Counts and Cytokine Expression in Bronchial Tissue**

Jacob K. Sont, Willem I. de Boer, W. Annemarie A. M. van Schadewijk, Katrien Grünberg, J. Han J. M. van Krieken, Pieter S. Hiemstra, Peter J. Sterk, and the Asthma Management Project University of Leiden Study Group

Departments of Medical Decision Making, Pulmonology, and Pathology, Leiden University Medical Center, Leiden; University Medical Center St. Radboud, Nijmegen; Erasmus Medical Center, Rotterdam; and Asthma Management Project University of Leiden (AMPUL) Study Group, Leiden, The Netherlands

Automated image analysis of bronchial tissue offers the opportunity to quantify stained area and staining intensity in a standardized way to obtain robust estimates of inflammatory cell counts and cytokine expression from multiple large areas of histopathologic sections. We compared fully automated digital image analysis with interactive digital cell counting and semiquantitative scoring of cytokine expression in terms of repeatability and agreement in bronchial biopsies in 52 patients with mild to moderate atopic asthma. Immunohistochemistry with antibodies against CD3, interleukin (IL)-4, IL-5, and interferon-γ protein was performed on frozen tissue sections, using 3-amino-9-ethylcarbazole as chromogen and hematoxylin as counterstaining. IL-4 and IL-5 messenger RNAs were localized by in situ hybridization without hematoxylin staining. Separation of 3-amino-9-ethylcarbazole and hematoxylin-stained pixels was achieved by linear combination of red- and blue-filtered gray-scale images. Using baseline biopsy specimens, fully automated CD3+ cell counts showed perfect repeatability (r = 1.0) and a strong linear relationship with the interactive procedure (r = 0.98). Automated densitometry showed perfect repeatability (1.0) and a moderate to strong relationship with semiquantitative scoring of protein and messenger RNA expression (r = 0.43–0.89). Relationships between automated and semiquantitative assessments of changes in cytokine expression during 2 years of follow-up were moderate to strong (r = 0.40–0.84). We conclude that fully automated cell counts and automated densitometric analyses in bronchial tissue of patients with asthma are unbiased and help to reduce variability in inflammatory outcomes.

**Keywords**: airway inflammation; asthma; biopsy (bronchial); chronic obstructive pulmonary disease; cytokines; helper T cells, types 1 and 2

Inflammatory cell number and cytokine expression in bronchial biopsies are increasingly considered to be a major outcome in asthma and chronic obstructive pulmonary disease (COPD) research (1). However, the inflammatory infiltrate often shows a patchy focal pattern, sometimes with clusters in which the cells are difficult to count (2). Therefore, there might be considerable within-section, within-biopsy, and between-biopsy variability ("true" variability). To achieve satisfactory stable inflammatory cell counts in bronchial biopsy specimens it is recommended that multiple large areas of airway tissue be examined (3–5).

In addition to this true variability, the number of inflammatory cells that is determined will also be influenced by procedural factors. First, it is labor-intensive to assess cell counts in multiple, large areas of airway tissue. Often it is not feasible to include all available areas in the analysis. Second, it is not always possible to stain all microscopic slides in one batch, especially in large studies. Third, there seems to be an intraobserver learning curve (3). Consequently, some variability in staining intensity might be introduced between staining batches, despite careful standardization of procedures. Fourth, obviously there is variability between observers, which hampers comparison between observers. Therefore, to achieve minimal procedural variability the assessments in biopsy specimens should preferably be done in random order and within a reasonable time span by one observer within a single study. Obviously, this is not always possible in practice.

Automated image analysis might deal with these issues by facilitating the analysis of large areas from multiple bronchial biopsies and by reducing the number of subjective decision moments (1). Therefore, it can be postulated that automated image analysis diminishes the variability in inflammatory cell number from several sources. Furthermore, automated image analysis offers the opportunity to perform densitometric analysis of cytokine expression in bronchial tissue (1). To that end, we developed a fully automated inflammatory cell-counting procedure and densitometric analysis of cytokine expression (interleukin [IL]-4, IL-5, and IFN-γ protein and IL-4 and IL-5 messenger RNA [mRNA]) in histopathologic sections of bronchial biopsy specimens from patients with asthma. In the present study, we compared fully automated true color image analysis with interactive digital cell counting and semiquantitative scoring of cytokine expression in bronchial biopsy specimens in terms of repeatability, and agreement in a cross-sectional and longitudinal design.

**METHODS**

**Subjects**

This study used bronchial biopsy specimens from patients with atopic asthma who participated in two different studies, and was performed to examine automated assessment of cytokine expression (27 patients) (6) and inflammatory cell counts (25 patients) (7). All patients (21 males and 31 females) were nonsmokers at the time of recruitment (nonsmokers for more than 1 year, smokers for fewer than 5 pack-years), were atopic, were between 18 and 50 years of age, and had a history of episodic chest tightness and wheezing in the previous year. Atopy was assessed through a positive skin prick test reaction (wheals greater than 3 mm in diameter) to one or more common airborne allergen extracts (Soluprick; ALK, Copenhagen, Denmark). Prebronchodilator forced expired volume in 1 second (FEV1) was more than 50% of the predicted value and more than 1.5 L, whereas postbronchodilator FEV1 was within the normal range (more than 80% predicted). At the time of patients’ entry into the study, airway hyperresponsiveness was established through a 20% decrease in FEV1 in response to a provocative concentration of inhaled methacholine (PC20) of less than 8 mg/ml. Subjects were eligible when they had used no other medication than regularly inhaled steroids and/or β2 agonists as needed for their asthma during the 6 months before

(Received in original form May 2, 2002; accepted in final form January 29, 2003)

Funded by the Netherlands Asthma Foundation (Project 96.12).

Correspondence and requests for reprints should be addressed to J. K. Sont, Ph.D., Department of Medical Decision Making, 110-S Leiden University Medical Center, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands. E-mail: j.k.sont@lumc.nl


DOI: 10.1164/rcrm.2205003

Internet address: www.atsjournals.org
entry. All subjects gave their written informed consent, and the studies were approved by the local medical ethics committee.

**Design**

With respect to the cell counts, repeatability and agreement were examined in a cross-sectional design, using baseline biopsy specimens (7), by analyzing identical digital images twice (2 weeks apart) by either fully automated or interactive cell-counting procedures. Concerning cytokine expression, we assessed the repeatability and agreement between fully automated true color image analysis and semiquantitative scoring of cytokine expression in a cross-sectional design as well as in a longitudinal design. Therefore, specimens from both baseline and end-point biopsies after 2 years of standardized treatment with inhaled steroids were included in the analysis (6). This also allowed for assessment of the agreement between changes obtained by either technique.

**Bronchoscopic Biopsy Specimens**

Fiberoptic bronchoscopy was done by an experienced investigator (L.N.A.W.), using a standardized protocol according to previous recommendations (8, 9). Each procedure involved detailed explanation, premedication, local anesthesia, bronchoscopy, and sampling according to a previously published protocol (10). Two bronchial biopsy specimens were taken from the right lower lobe subsegments and the middle lobe, using a pair of cup forceps (FB-21C; Olympus, Tokyo, Japan). Biopsy samples were immediately embedded in optimal cutting temperature (O.C.T.) medium (Miles Diagnostics Division, Elkhart, IN) and snap-frozen in isopentane cooled with solid CO₂. Samples were stored at −70°C pending further processing (10).

**Processing of Endobronchial Biopsy Specimens**

In situ hybridization. In situ hybridization was performed on 5-μm-thick frozen sections. We used a 300-bp EcoRI-NheI fragment of the IL-4 cDNA and a 900-bp PstI fragment of the IL-5 DNA, both subcloned into pGEM7Z(+) (Promega, Madison, WI). Both complete cDNAs were obtained from the American Type Culture Collection (Manassas, VA). The specific copy RNA (cRNA) probes were labeled with digoxigenin according to the manufacturer's protocol (Boehringer, Mannheim, Germany). The in situ hybridization was performed as described previously (11, 12). Briefly, frozen sections were fixed with 4% buffered formaldehyde for 30 minutes. After pretreatment, the sections were hybridized with 0.17 ng/μl probe per slide for 16 hours at 42°C. Subsequently, sections were washed in 2× standard saline citrate with 50% formamide at 37°C and then in 0.1× standard saline citrate with 20 mM β-mercaptoethanol at 42°C, and finally were treated with RNase T1 (2 U/ml; Boehringer) in 2× standard saline citrate plus 1 mM ethylenediaminetetraacetic acid at 37°C. The immunodetection of digoxigenin-labeled hybrids was done with nitroblue tetrazolium as chromogen and with 5-bromo-4-chloro-3-indolyl phosphate as coupling agent. The sense riboprobes were included as negative controls and showed only light background staining. Sections for RNA in situ hybridization were not counterstained.

**Immunohistochemistry.** Immunohistochemistry was performed on frozen tissue sections (5 μm) essentially as described (10, 12, 13). Briefly, frozen sections were fixed with acetone. Subsequently, sections were incubated with appropriate dilutions of the primary antibodies directed against CD3 (Leu4) (BD Biosciences Immunocytemy Systems, Mountain View, CA), and against IL-4, IL-5, or IFN-γ (Genzyme, Cambridge, MA), in conjugated immunoenzyme assays using a secondary biotin-conjugated antibody and a tertiary complex of streptavidin–biotin conjugated to horseradish peroxidase (Dako, Glostrup, Denmark), and 3-amino-9-ethyl-carbazole as chromogen. Finally, sections were counterstained with Mayer's hematoxylin. Incubation with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin instead of the primary antibody served as a negative control (10, 12, 13). For image analysis in general and for automated densitometry in particular, it is a prerequisite that the positive staining be not too saturated to allow differentiation within the darkest staining regions. Furthermore, we advise the use of relatively pale counterstaining to decrease the possible influence of nonspecific background staining on measurements.

**Image Analysis**

Fully automated densitometry of cytokine expression (protein and mRNA) and inflammatory cell-counting procedures were developed with the Zeiss Vision KS400 system (Carl Zeiss, Göttingen, Germany). This system offers broad functionality for digital image analysis for development of a specific application, using a powerful macro language.

**Image Acquisition**

After a 30-minute warming up of the microscope (×200 magnification) the images were digitized with a three-chip color camera, which was coupled to a PC with a 166 MHz Pentium processor and frame grabber with appropriate corrections for nonuniform illumination (shading correction) and settings of the black/white camera signal (white-balance). The size of the captured image was 433 × 10³ pixels (3 × 256 gray values), which corresponded to 660 × 496 μm². Using a motorized microscope table, we were able to merge four of these images into one image, which allows coverage of an area of 7 × 1 bronchial biopsy section. Descriptive data for each compounded image were stored in a corresponding image database.

**Segmentation of Lamina Propria and Epithelium**

The area of lamina propria and epithelium was determined in these images by interactive/manual delineation of the epithelial basement membrane and semiautomated generation of a 125-μm-deep zone beneath the basement membrane (Figure 1), and by semiautomated detection of the delineation of the epithelium toward the airway lumen. The area of the lamina propria and epithelium as well as basement membrane length were automatically measured by the computer and stored in the image database. Lamina propria was defined by the widest possible perimetrically maximal 125 μm deep, beneath the basement membrane excluding damaged tissue, mucous-secreting glands, bronchoalveolar lymphoid tissue, and airway smooth muscle (10). The maximal depth of the area of interest of the 125-μm-deep zone in general includes the lamina propria and excludes the submucosa. Likewise, epithelial area was defined by the area above the basement membrane enclosed by the airway lumen, not excluding damaged epithelium (10).

**Fully Automated Cell-Counting Procedure**

The automated counting of positive cells consists of several image-processing steps to discriminate positive staining from background and the application of an algorithm on positive-staining areas to establish the number of cells. The image processing was based on the red-green-blue (RGB) color model and was developed in a separate set of images from three different study or staining batches. Custom macros were written in KS400 interpreter language to separate the brown-red (B-R) staining (3-aminio-9-ethyl-carbazole) from the blue counterstaining (hematoxylin). There was a high correlation (r > 0.84) between the R- and B-filtered gray-scale values of each pixel. A linear combination of the R- and B-filtered gray-scale images was used to derive a gray-scale image in which the “brown-red” staining of interest is highlighted above a uniform background (14). This first step resulted in a narrow and peaked gray level distribution of background pixels with a longer tail on the left, which represents positive-staining pixels (Figure 2).

The second step was a normalization of the staining intensity range to adjust for batch specific background and maximal staining intensity by the chromogen. This was achieved by setting the darkest 25th pixel (batch/staining specific) to gray value 0 (black) and the modus of the background peak to gray value 255 (white) (Figure 2). This step was included to eliminate differences in staining intensity between batches that are separately processed.

The third step consisted of setting a cutoff between positive and no staining. Because the distribution is normalized in the second step, this allowed us to use the same cutoff at gray value 170 for all images irrespective of the processing batch (Figure 2). After implementing this step results in an image with only black and white (positive) pixels.

The next step was to reduce noise by deleting isolated pixels and regions of up to three connected pixels. Subsequently, a closing procedure consisting of a dilation followed by an erosion of two pixels was applied. This closing procedure preserves nearly the original size of the positive-staining regions while allowing the generation of connections between near regions and holes and concave bulges to be flattened.
The algorithm to determine the number of positive cells was based on the area and morphometric characteristics (maximum length, maximum width, and fiber length) of positive regions. The algorithm is applicable only for lymphocytes (CD3⁺, CD4⁺, CD8⁺ cells) and needs to be slightly different for other cell types. The computer then provides the user with an image present positive cell clusters labeled in green and projected on the original image (Figures 1 and 3) and a corresponding database with the number of positive cells established by the computer algorithm (Table 1).

**Interactive Cell Counting**
The interactive cell counting procedure was performed on exactly the same digital images as were used for the fully automated procedure. This consisted of placing a cross-mark on a cell in the image by pressing the mouse button. Subsequently, the computer registered in a database how many times the mouse button was pressed (10). Furthermore, after the interactive cell-counting procedure was performed, we applied a macro that was developed to assess the agreement between the number of interactively placed cross-marks within a region and the number of cells established by the fully automated procedure (within-cell cluster agreement). It is necessary to realize that this widely accepted method does not yield a true numerical density and might be subject to a volume bias, that is, larger cells will be counted more frequently.

**Fully Automated Densitometric Analysis**
The fully automated densitometric analysis of cytokine expression differed slightly for protein and mRNA expression. With respect to mRNA expression the intensities of the three color channels of the RGB image were combined and directly converted into a gray value image. The computer then provided the user with a bar graph of the gray level distribution and the average gray level was stored into a corresponding database.

To assess cytokine protein expression on slides counterstained with hematoxylin, similar image-processing steps are required as for the cell-counting procedure. These steps result in a gray level image of staining for cytokine protein. Again, the computer produces a gray level bar graph and the average gray value is stored in the corresponding database.

**Semi-quantitative Scoring**
Semi-quantitative scoring of cytokine expression was done in four categories ranging from no staining to very intense staining (categories: −, ±, +, ++) (12). Examples of cytokine expression in epithelium and lamina propria in bronchial biopsies are shown in Figure 4.

**Statistical Analysis**
The number of CD3⁺ cells was log-transformed to allow for heteroscedasticity and expressed per 0.1 mm² of lamina propria (15). Repeatability of both automated and interactive cell count procedures was assessed by intraclass correlation coefficient (R) for the number of cells within a positive-staining cluster as well as for the total number of positive cells expressed per area of lamina propria. Agreement between cell count procedures was assessed on the average of multiple areas of bronchial biopsy specimens and expressed by the intraclass correlation coefficient (R).

The repeatability of cytokine expression was assessed by weighted \( \kappa (\kappa_w) \) for the semi-quantitative method and by intraclass correlation coefficient (R) for the automated method. The agreement (automated versus semi-quantitative) was expressed as the Pearson’s correlation coefficient (r). In addition, we assessed the agreement between changes in cytokine expression during 2 years of standardized treatment with inhaled steroids, obtained with both the automated and semi-quantitative techniques. Agreement was expressed by Pearson’s correlation coefficient.

All statistical analyses were done with the statistical software package STATA 6.0 (StataCorp, College Station, TX). Values of \( p < 0.05 \) were considered significant.

**RESULTS**
The areas of lamina propria and epithelium per patient examined were at least 0.2 and 0.075 mm², respectively. The geometric mean
number of CD3-positive cells was 70.1 cells/0.1 mm² (1.9-fold SD) and 19.9 cells/0.1 mm² (3.8-fold SD).

The repeatability of cell counting on identical images of both the newly developed (fully automated) and traditional methods (interactive cell counting) of the number of cells per 0.1 mm² lamina propria is shown in Table 2. It appeared that the repeatability was almost perfect ($R_i = 0.96$) for semiautomated and perfect ($R_i = 1.0$) for fully automated cell count procedures. The repeatability of fully automated densitometric analysis and semiquantitative scoring of cytokine expression on identical images is depicted in Table 3. Repeatability of semiquantitative scoring ranged from moderate ($\kappa = 0.59$) to very good ($\kappa = 0.95$), whereas repeatability of automated densitometric analysis was perfect ($R_i = 1.0$) for the fully automated procedure.

The agreement between the fully automated procedures and traditional methods was first assessed by cross-sectional analysis (Tables 2 and 3). The agreement between fully automated and interactive cell counting of the number of cells per 0.1 mm² of lamina propria as well as of the number of cells per cluster was almost perfect ($R_i = 0.97$) (Table 2 and Figure 5). With respect to fully automated densitometric analysis and semiquantitative nondigitized scoring of cytokine expression the agreement between the procedures ranged from moderate to very good for all markers in the airway epithelium ($r = 0.43–0.89$) as well as in the lamina propria ($r = 0.59–0.80$) (Table 3). Assessment of mRNA expression seemed to show better agreement ($r = 1.0$) for fully automated cell count procedures.

The repeatability of fully automated densitometric analysis and ($r = 0.65–0.89$) than the corresponding protein expression ($r = 0.43–0.69$). Furthermore, longitudinal analyses were performed on the pooled data of the two treatment strategies for the 17 patients who were available for follow-up. Baseline and end-point cytokine levels, as well as their correlation determined by semiquantitative scoring and fully automated densitometry, are shown in Table 4. After 2 years of follow-up, there was a positive and significant correlation between baseline and end-point levels of IL-4 protein and mRNA in the lamina propria, and of IL-5 mRNA in the
epithelium, using automated densitometric analysis. Agreement between changes from baseline after a 2-year follow-up in fully automated densitometric analysis and semiquantitative scoring was moderate to good for cytokine expression in the airway epithelium \((r = 0.40–0.84)\) (Table 5 and Figure 6) and fair to good for the lamina propria \((r = 0.28–0.77)\) (Table 5).

**DISCUSSION**

This study shows that fully automated cell counts and densitometric analyses in bronchial biopsy specimens of patients with asthma help to reduce within- and between-study variability. The novel methods outperform both the interactive cell count procedure and the semiquantitative scoring of cytokine expression in terms of repeatability. Furthermore, they seem to be unbiased and show a strong linear relationship with these traditional methods. This indicates that fully automated procedures improve the feasibility of assessment of inflammatory cell numbers in large areas from multiple bronchial biopsies. This will further reduce the variability in estimates of inflammatory cell number and cytokine expression. As we demonstrated, this allows a more sensitive detection of differences in cytokine expression. Therefore, it is likely that the introduction of automated digital image analysis procedures in biopsy studies of patients with asthma or COPD will increase the statistical power to detect longitudinal changes as well as between-group differences and it will improve comparability between studies.

This is the first study comparing fully automated true color image analysis with interactive digital cell counting and semiquantitative scoring of cytokine expression in terms of repeatability and agreement in biopsy specimens of patients with asthma. In contrast to interactive computerized counting, fully automated true color image analysis has been applied in only one study to assess the inflammatory cell number in biopsy studies of patients with asthma (7). Obviously, the principles of true color image analysis do not uniquely apply to this setting.

Berger and coworkers demonstrated that image analysis is valuable for quantifying inflammatory cells in bronchi of patients with COPD undergoing resection for pulmonary carcinoma (17). However, their algorithm is suitable only for the identification of solitary positive inflammatory cells in the bronchial tissue and does not allow assessment of the number of inflammatory cells that are situated in clusters, which can be frequently found in bronchial tissue of patients with asthma (5). Others have employed image analysis in the detection of growth factor expression in airway epithelium (18, 19). In contrast to the current study, expression in these studies was quantified as the percentage of positively stained area relative to total epithelial area, which differs from densitometry that results in a mean gray value or optical density (14). Most previous research studies in asthma or COPD adopted, in contrast to the current method, the hue-saturation-intensity (HSI) method for color detection (17–19). The hue signal represents the wavelength of a color, and the saturation describes the amount of white light added to a pure color. As is outlined below, the intensity signal generated by the HSI method may not be the most appropriate measure to express the density of the chromogen in counterstained microscopic slides. Furthermore, by adopting the HSI model none of these studies were using fully automated image analysis.

**TABLE 1. NUMBER OF POSITIVE CELLS IDENTIFIED BY THE COMPUTER ALGORITHM PER CLUSTER**

<table>
<thead>
<tr>
<th>Cluster Label</th>
<th>Positive Cluster Cell Count ((\mu m^2))</th>
<th>Cluster Area ((\mu m^2))</th>
<th>Cluster Label</th>
<th>Positive Cluster Cell Count ((\mu m^2))</th>
<th>Cluster Area ((\mu m^2))</th>
<th>Cluster Label</th>
<th>Positive Cluster Cell Count ((\mu m^2))</th>
<th>Cluster Area ((\mu m^2))</th>
<th>Cluster Label</th>
<th>Positive Cluster Cell Count ((\mu m^2))</th>
<th>Cluster Area ((\mu m^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1</td>
<td>29</td>
<td>1</td>
<td>28</td>
<td>37</td>
<td>1</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>30</td>
<td>24</td>
<td>1</td>
<td>29</td>
<td>38</td>
<td>4</td>
<td>235</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>48</td>
<td>26</td>
<td>8</td>
<td>546</td>
<td>39</td>
<td>1</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>86</td>
<td>27</td>
<td>1</td>
<td>51</td>
<td>40</td>
<td>1</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>28</td>
<td>28</td>
<td>2</td>
<td>92</td>
<td>41</td>
<td>1</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>95</td>
<td>29</td>
<td>3</td>
<td>111</td>
<td>42</td>
<td>8</td>
<td>563</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>34</td>
<td>30</td>
<td>1</td>
<td>52</td>
<td>44</td>
<td>1</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>44</td>
<td>31</td>
<td>1</td>
<td>59</td>
<td>45</td>
<td>1</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>253</td>
<td>34</td>
<td>1</td>
<td>34</td>
<td>46</td>
<td>4</td>
<td>202</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>28</td>
<td>36</td>
<td>1</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cluster label corresponds to the positively staining cell clusters that are labeled in green and projected on the original image depicted in Figure 3.
Figure 4. Examples of cytokine expression (categories: –, ±, +, ++) in epithelium (Ep) and lamina propria (LP) in bronchial biopsies of a patient with asthma: IL-4 protein (Ep, +; LP, ++) (top left), IL-5 protein (Ep, –; LP, –) (top right), IFN-γ protein (Ep, +; LP, +) (bottom left), and IL-4 mRNA expression (Ep, +; LP, –) (bottom right).

<table>
<thead>
<tr>
<th>TABLE 2. REPEATABILITY OF FULLY AUTOMATED AND SEMIAUTOMATED COUNTING PROCEDURES OF CD3+ CELLS AND AGREEMENT BETWEEN BOTH PROCEDURES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>d (SDd)</td>
</tr>
<tr>
<td>r</td>
</tr>
<tr>
<td>R</td>
</tr>
</tbody>
</table>

Definition of abbreviations: d = mean fold difference between repeated procedures: count 1/count 2; r = Pearson’s correlation coefficient; R = intraclass correlation coefficient; SDd = SD of differences between repeated procedures. n = 25.

First, none of the gray level images produced by the red-green-blue (RGB) or HSI method contains sufficient information to provide reliable separation of the colors brown and blue (20). Therefore, multiple threshold settings are needed, which introduce subjective decision moments and subsequent observer bias. Furthermore, positive descriptions of color must be broad enough to include all the features of interest as well as strict enough to exclude background. This scenario creates a color description that is often specific for each image, exhibiting impaired accommodation of color hues among different images (21).

Second, many staining procedures demonstrate various structures in different hues (20). Deposition of chromogen on top of structures that stain positive by the counterstaining (e.g., hematoxylin) results in a different color that is compounded of coun-
TABLE 3. REPEATABILITY OF FULLY AUTOMATED AND SEMIQUANTITATIVE DENSITOMETRIC PROCEDURES AND THEIR AGREEMENT (CROSS-SECTIONAL RELATIONSHIP)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Epithelium (n = 27)</th>
<th>Lamina Propria (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Semi-quantitative (κw)</td>
<td>Automated (R)</td>
</tr>
<tr>
<td>IL-4 (mRNA)</td>
<td>0.87</td>
<td>1</td>
</tr>
<tr>
<td>IL-4 (protein)</td>
<td>0.66</td>
<td>1</td>
</tr>
<tr>
<td>IL-5 (protein)</td>
<td>0.77</td>
<td>1</td>
</tr>
</tbody>
</table>

Definition of abbreviations: IL = interleukin; κw = weighted κ for semiquantitative scores; R = Pearson’s correlation coefficient; R = intraclass correlation coefficient for continuous variables.

TABLE 4. BASELINE LEVELS AND CORRELATION BETWEEN BASELINE AND END-POINT LEVELS OF CYTOKINE PROTEIN AND mRNA EXPRESSION ASSESSED BY SEMIQUANTITATIVE AND AUTOMATED DENSITOMETRIC PROCEDURES IN AIRWAY LAMINA PROPRIA AND EPITHELIUM

<table>
<thead>
<tr>
<th>Procedure/Cytokine</th>
<th>Baseline</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean*</td>
</tr>
<tr>
<td>Semi-quantitative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 protein</td>
<td>20</td>
<td>1.9</td>
</tr>
<tr>
<td>IL-5 protein</td>
<td>24</td>
<td>1.0</td>
</tr>
<tr>
<td>IFN-γ protein</td>
<td>23</td>
<td>1.6</td>
</tr>
<tr>
<td>IL-4 mRNA</td>
<td>26</td>
<td>0.9</td>
</tr>
<tr>
<td>IL-5 mRNA</td>
<td>24</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Definition of abbreviations: IL = interleukin; mRNA = messenger RNA; r = Spearman rank correlation coefficient.

TABLE 5. RELATIONSHIP BETWEEN LONGITUDINAL CHANGES IN FULLY AUTOMATED DENSITOMETRY AND SEMIQUANTITATIVE SCORING

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Agreement (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (mRNA)</td>
<td>0.84</td>
</tr>
<tr>
<td>IL-5 (mRNA)</td>
<td>0.77</td>
</tr>
<tr>
<td>IL-4 (protein)</td>
<td>0.52</td>
</tr>
<tr>
<td>IL-5 (protein)</td>
<td>0.71</td>
</tr>
<tr>
<td>IFN-γ (protein)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Definition of abbreviations: IL = interleukin; r = Pearson’s correlation coefficient.
Figure 6. Relationship between changes from baseline after 2-year follow-up in fully automated densitometric analysis and semiquantitative scoring for IL-4 mRNA expression in the airway epithelium. The regression line has been drawn.

methods does not necessarily indicate unsatisfactorily agreement, because this might be due to lack of between-subject variability in the parameter under study. For example, the only moderate correlation between automated and semiquantitative measurements of IL-5 protein expression in the epithelium seemingly contrasts with the good correlation between automated and semiquantitative measurements of longitudinal changes in IL-5 expression.

In conclusion, we validated the current fully automated methods in biopsies of patients with mild to moderate asthma. They agree well with both the interactive cell count procedure and the semiquantitative scoring of cytokine expression and show optimal repeatability of the analysis of cell counts and cytokine expression.

References


11. de Boer WI, Schuller AG, Vermey M, van der Kwast TH. Expression scoring for IL-4 mRNA expression in the airway epithelium. The regression line has been drawn.


