Flow Cytometry in Impression Cytology Specimens
A New Method for Evaluation of Conjunctival Inflammation
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Purpose. To investigate feasibility and potential uses of flow cytometry in impression cytology as a new procedure to assess and quantify conjunctival inflammation.

Methods. Specimens for cytology were collected by impression from 30 patients with various chronic ocular surface disorders and from 10 normal subjects. Two specimens were obtained in each eye: One was transferred onto a glass slide and processed by immunofluorescence with antibodies to human leukocyte antigen (HLA)–DR antigens; cells from the other were suspended in phosphate-buffered saline for flow cytometry. Monoclonal antibodies to HLA–DR antigens and CD23, the low affinity receptor to immunoglobulin E, were used.

Results. Abnormal expression of HLA–DR and CD23 by conjunctival cells was found in 13 of 18 dry eyes and in 20 of 22 eyes with chronic conjunctivitis, whereas specimens remained almost negative (less than 10% of cells were positive) in normal eyes. Percentages of positive cells ranged between 20% and 70% in normal eyes. Percentages of positive cells ranged between 20% and 98% of all conjunctival cells. Correlation between the two methods, immunocytology and flow cytometry, was highly significant (coefficient of correlation 0.77, P = 0.0001). Moreover, HLA–DR positivity, at its strongest intensity, was observed in a minority of cells (1% to 12%), most of which were resident class II–expressing dendritic cells. Percentages of those cells expressing high levels of HLA–DR were 3 ± 1.2% in normal eyes, 5.8 ± 4% in dry eyes (P = 0.05), and 5.9 ± 3.5% in eyes with chronic conjunctivitis (P = 0.02).


The immunologic status of the ocular surface is often very difficult to assess accurately in some conjunctival disorders, including chronic conjunctivitis, allergic keratoconjunctivitis, or dry eyes. Clinical criteria lack specificity and in most cases do not allow a precise diagnosis. Dosage of lacrimal proteins may be valuable but is often difficult to realize, especially in dry eyes, or to interpret, as in some complex inflammatory diseases. One of the best ways to investigate the ocular surface could be by conjunctival biopsy, which offers specimens of epithelial layers and conjunctival stroma to be examined by light or electron microscopy, conventional cytology, and immunohistochemical analyses. These techniques may allow identification and counting of inflammatory cells and analysis of cell membrane markers, intracytoplasmic molecules, or extracellular matrix components. However, biopsy remains a traumatic technique that cannot be undertaken in patients simply for research purposes or in benign conditions.

We therefore developed immunocytologic procedures to analyze conjunctival cells using impression cytology, a nontraumatic way to collect cells from the ocular surface.1–3 With this method, we looked for inflammatory changes in conjunctival cells and found an abnormal expression of class II antigens HLA–DR and CD23, the low affinity receptor to immunoglobulin E, in cases of chronic inflammation.4 We could identify antigen-positive dendritic cells in the most superficial layers of conjunctival epithelium and count precisely the proportion of epithelial cells expressing immune markers. This was of special interest in patients with clinically noninflamed eyes—for example, in patients with dry eyes or in those receiving chronic topical therapy.4,5

To improve the quality of our investigations with an objective and reliable method, we developed a new technique of analyzing conjunctival cells derived from impression cytology, using flow cytometry. We present our preliminary results to validate this method, discuss technical issues, and compare morphologic and cyometric patterns.

METHODS. Patients. Cytologic specimens were taken by impression from 30 patients with chronic ocular surface disorders: 14 with dry eyes (including three with documented Sjögren’s syndrome) and 16 with chronic conjunctivitis of allergic origin (four with perennial allergic conjunctivitis), secondary to contact lenses (seven patients wearing contact lenses for more than 5 years), or topical antiglaucoma treatments (five patients treated for more than 1 year with two drugs). All patients had been symptomatic for more than 3 months when they were included. Ten other subjects, with eyes considered healthy after complete ophthal-
mologic examination, were also investigated. This study was done according to the tenets of the Declaration of Helsinki and the advice of the ethical committee of Ambroise Pare Hospital. All patients gave their informed consent for collecting cytologic specimens by impression processing them by immunocytochemistry to look for eventual conjunctival inflammation. None of them had received steroid or antiinflammatory drugs for at least 2 months. Clinical diagnoses were made on the basis of subjective symptoms, Schirmer’s test, and slit lamp examination, including fluorescein and rose bengal staining. Only eyes with typical clinical features were considered for immunologic investigations. Patients with doubtful or mixed diagnoses were not included. The fluorescein test and rose bengal staining were performed after impression cytology to avoid nonspecific fluorescence.

Cell Collection and Processing. Conjunctival cytologic specimens were collected by impression from both eyes of each study participant using 20 µm polye-ether sulfone filters (Supor Membranes, Gelman Sciences, MI), as previously described. In each eye, two filters were applied successively, without exerting any pressure, onto the superior and superotemporal bulbar conjunctiva, in two different but neighboring areas. Care was taken to collect specimens only in nonexposed regions of the conjunctiva. Membranes were removed immediately after contact. Areas of collected cells could be seen easily on the filters: they covered approximately 50% to 70% of the total surface. If a specimen was not readily visible, a new one was collected in another area. One membrane from each eye was moistened in phosphate-buffered saline (PBS, pH 7.4), and firmly pressed onto a gelatin-coated slide to transfer as many conjunctival cells as possible onto a transparent surface, useful for immunocytochemistry. The other specimen was left in 2 ml of PBS, gently agitated for 30 minutes, and centrifuged (200g, 5 minutes) before processing for flow cytometry.

Immunostaining Procedures. On glass slides, specimens were reacted with a monoclonal antibody directed against a common determinant of HLA-DR conjugated to fluorescein isothiocyanate (Immunotech, Luminy, France). The antibody, in a 1:50 dilution was incubated for 30 minutes. After washing in PBS, specimens were counterstained with propidium iodide to identify nuclei and show cells negative for antigens. They were mounted in AF1 mounting medium (Citifluor, London, UK) before examination. Specimens with damaged cells or with fewer than 200 cells were not considered. Percentages of positive epithelial cells were determined by counting at least 200 cells throughout different areas of the slide.

Cells processed for flow cytometry were reacted for 30 minutes with fluorescein isothiocyanate-conjugated anti-HLA-DR and anti-CD23 antibodies (Immunotech), and fluorescein isothiocyanate-conjugated antimouse immunoglobulin G1 (Immunotech), as a negative control. Cell suspensions were then centrifuged in PBS (200g, 5 minutes), resuspended in 400 µl of PBS, and analyzed on a flow cytometer (FACSscan [fluorescence-activated cell sorter], Becton Dickinson, Mountain View, CA), equipped with an argon laser emitting at 488 nm. The linear plot showing cellular density (log side scatter) versus cell size (forward-angle light scatter) revealed a single cell population. Analytic gates were set around this population to exclude cellular debris. The number of antigen-positive conjunctival cells was then obtained from a cytogram representing mean fluorescence intensities on a 4-decade logarithmic amplifier. The superior limit of intensity of fluorescence obtained for the control antibody was considered as the threshold of positivity for the two tested antibodies. Percentages of positive cells were thus calculated in each specimen, but because two distinct populations of HLA-DR positive cells were being characterized, with strikingly different levels of fluorescence, separate analyses were done for the two cell groups. For each antibody, at least 1000 cells were analyzed, the threshold for reliable determinations. Poorer specimens were discarded. In most cases, however, analyses were conducted in larger cell populations, between 3000 and 5000 cells for each measurement. All specimens, whatever the procedures used, were examined in a masked manner because the examiners could not know the clinical history of patients.

RESULTS. Of 80 eyes investigated, 56 met the criteria to be reliably analyzed by both methods: 18 eyes from 13 patients with ocular dryness, 22 eyes from 14 patients with chronic conjunctivitis, and 16 eyes from 10 normal subjects. The reason for failure in analyzing paired specimens was poor cell collection, in which 13 of 80 eyes (16%) did not qualify for flow cytometry and 10 of 80 (12%) did not qualify for immunocytochemistry. In those eyes only one method provided the desired information, and the results were not considered in the current data.

Morphologic Analysis. In the 16 specimens we tested from normal eyes, epithelial cells did not express HLA-DR antigens. Only scattered, strongly positive HLA-DR-expressing cells were observed with typical dendritiform antigens, except in dendritic cells; simple increase in density of dendritic cells, without change in epithelial ones; or expression of HLA-DR by a variable proportion of epithelial cells (Fig. 2). Abnormal findings in analysis of cytologic specimens, defined as HLA-DR
FIGURE 1. (top) Dendritic cells positive for HLA-DR in a cytologic specimen collected from a normal subject by impression. The underlying epithelial cells are negative, and can only be seen by the red counterstaining of their nuclei with propidium iodide. Magnification, ×500.

FIGURE 2. (bottom) Expression of HLA-DR by epithelial cells in a patient with chronic iatrogenic conjunctivitis. Magnification, ×800.
Table 1. Immunocytologic and Flow Cytometric Results in Impression Cytology Using Anti-HLA DR and CD23 Monoclonal Antibodies

<table>
<thead>
<tr>
<th></th>
<th>HLA DR Epithelial Expression (CY)</th>
<th>HLA DR Epithelial Expression (FCM)</th>
<th>Cells Highly Positive to HLA DR (FCM)</th>
<th>CD23 (FCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal eyes (n = 16)</td>
<td>0/16</td>
<td>0/16</td>
<td>16/16</td>
<td>2/16</td>
</tr>
<tr>
<td>Number of positive specimens</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Mean % of positive cells</td>
<td>—</td>
<td>—</td>
<td>3.0</td>
<td>65*</td>
</tr>
<tr>
<td>Standard deviation (%)</td>
<td>—</td>
<td>—</td>
<td>1.2</td>
<td>15</td>
</tr>
<tr>
<td>Range (%)</td>
<td>—</td>
<td>—</td>
<td>1–8</td>
<td>56–81</td>
</tr>
<tr>
<td>Dry eyes (n = 18)</td>
<td>11/18</td>
<td>13/18</td>
<td>18/18</td>
<td>13/18</td>
</tr>
<tr>
<td>Number of positive specimens</td>
<td>99</td>
<td>61</td>
<td>5.8†</td>
<td>84</td>
</tr>
<tr>
<td>Mean % of positive cells*</td>
<td>39</td>
<td>61</td>
<td>5.8†</td>
<td>84</td>
</tr>
<tr>
<td>Standard deviation (%)</td>
<td>35</td>
<td>37</td>
<td>4.0</td>
<td>16</td>
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<tr>
<td>Range (%)</td>
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<td>20–98</td>
<td>1–15</td>
<td>50–98</td>
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<tr>
<td>Chronic conjunctivitis (n = 22)</td>
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<td>20/22</td>
<td>22/22</td>
<td>21/22</td>
</tr>
<tr>
<td>Number of positive specimens</td>
<td>52</td>
<td>79</td>
<td>5.9</td>
<td>83</td>
</tr>
<tr>
<td>Mean % of positive cells*</td>
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<td>79</td>
<td>5.9</td>
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<td>Standard deviation (%)</td>
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<tr>
<td>Range (%)</td>
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<td>22–98</td>
<td>1–12</td>
<td>64–95</td>
</tr>
</tbody>
</table>

CY = immunocytology; FCM = flow cytometry.

* Calculated by considering only positive specimens.
† Dry eyes, P = 0.05; chronic conjunctivitis, P = 0.02, versus normal specimens.

expression by epithelial cells, were seen in 11 of the 18 eyes with ocular dryness and in 19 of the 22 eyes with chronic conjunctivitis (Table 1). Specimens from eyes of the three patients with Sjögren’s syndrome were positive for HLA-DR (70% to 100% of all cells were positive).

**Flow Cytometry**. Preliminary experiments were done in three normal eyes and in four dry eyes to assess feasibility of the method and to validate the repeatability of results. Repeated measurements were performed in a masked manner in specimens reacted with the two tested antibodies and the control antibody, again by impression cytology a few days or weeks after the first one. Overall variations were less than 10%, either in repeated measurements or in repeated cell collections, and improved because those taking the measurements improved their performance, demonstrating the reliability of our technique.

Results of flow cytometry in specimens from normal eyes showed conjunctival cells at levels of fluorescence that did not differ from those observed in control specimens (Fig. 3), but the results always evidenced a minority of cells that strongly expressed class II antigens (Fig. 3). Flow cytometric count in healthy subjects showed a mean percentage of 3 ± 1.2% of cells highly positive for HLA-DR among the total conjunctival cell population (range, 1% to 8%, Table 1).

According to morphologic analysis, this strongly positive population was likely to be dendritic cells, the only ones to express high levels of class II antigens in cells of clinically normal eyes and the cells that always showed strongest levels of expression in diseased eyes.

In patients suffering from ocular surface disorders, conjunctival cells variably expressed HLA-DR (Fig. 3). Three populations could be observed: negative cells with low levels of fluorescence (similar to those in control and normal specimens), mildly positive cells, and the minority of cells strongly expressing HLA-DR observed in all specimens. These patterns were consistent with those seen with microscopic immunostaining—that is, a variable population of epithelial cells mildly expressing HLA-DR and a minority of dendritic cells positive at the highest levels.

Concerning cells expressing HLA-DR antigens at weaker levels, 13 of the 18 dry eyes (Fig. 3) and 20 of the 22 eyes with chronic conjunctivitis (Fig. 3) showed positive HLA-DR expression in a well-differentiated conjunctival population. Fluorescence levels in those specimens were significantly lower than those in the minority of strongly positive ones (mean fluorescence obtained by FACS scan analysis: 452 ± 109 versus 824 ± 53, respectively, P = 0.001). Again, cells from eyes of the three patients with Sjögren’s syndrome were positive for HLA-DR (80%, 92%, and 95% of positive cells, 6%, 8% and 5% of strongly positive ones respectively).

Percentages of these strongly positive cells from the total cell population also significantly differed in specimens from normal eyes and in specimens from eyes with dryness or chronic conjunctivitis (3 ± 1.2% in normal specimens versus 5.8 ± 4% in dry eyes; P = 0.05 using the Mann–Whitney test; 5.9 ± 5.5% in chronic conjunctivitis, P = 0.02). When considering all diseased specimens in which an abnormal HLA-DR positivity could be found in conjunctival cells, a significant difference could also be found in specimens from normal eyes (6.3 ± 4% versus 3 ± 1.2%, respectively, P = 0.02) but not with HLA-DR-nega-
FIGURE 3. Flow cytometry analyses of HLA-DR and CD23 in impression cytology specimens. (top left) Negative control. (top right) Histogram from a normal conjunctiva showing negativity to HLA-DR, except for a minority (2.5%) of strongly positive conjunctival cells on the right. The vertical line shows the limit of negative control. (middle left) Sjögren's syndrome with two distinct populations of HLA-DR-positive conjunctival cells (weakly positive, 83%; highly positive, 8%). (middle right) A patient with chronic conjunctivitis with a recent episode of allergy to eye drops, showing 12% of negative cells, a first peak (46%) of weakly positive conjunctival cells, a second one at higher levels (37%), and the strongly positive population (5%). (bottom left) No expression of CD23 in a normal conjunctiva. Note the absence of the strongly positive population seen with HLA-DR. (bottom right) Positive staining (91%) to CD23 in a patient with chronic conjunctivitis.

tive specimens, which included those from normal eyes, those from the five dry eyes that were antigen-negative, and the two from eyes with chronic conjunctivitis that were antigen-negative (4.65 ± 2.95% of all conjunctival cells, not significant). This could be explained by the presence of dendritic cells at higher density in eyes with dryness or chronic conjunctivitis, whereas the majority of conjunctival cells remained negative for HLA–DR antigens.

A positive correlation was found between HLA–DR immunolabeling by immunocytochemistry and flow cytometry: Coefficient of correlation was 0.77, }$P =$
0.0001. Although mean percentages of positive cells at mild levels of positivity were slightly higher with flow cytometry than with immunocytochemistry (63% versus 45%), the difference was not significant.

Concerning CD23 expression (Fig. 3) we found positive conjunctival cells in 42 of the 56 tested eyes (2 of 16 normal eyes, 15 of 18 with ocular dryness, 21 of 22 with chronic conjunctivitis). Mean percentages of positive cells calculated taking into account all specimens with positive cells, were 65 ± 15%, 84 ± 16%, and 83 ± 12% in the three groups respectively (Table 1). These percentages did not differ significantly. Using the anti-CD23 antibody, we never found the highly positive cell population observed with HLA-DR (Fig. 3). A low but significant correlation was found between HLA-DR and CD23 expression by flow cytometry (coefficient of correlation: 0.37, P = 0.04).

**DISCUSSION.** Impression cytology is a simple, useful, noninvasive tool for exploring superficial conjunctival layers.1-5 It can be done repeatedly to assess evolution of conjunctival disorders or efficacy of treatment; and, using immunocytochemistry, the method may be of real interest for use in a wide variety of studies. Through impression cytology, the three cell populations of the conjunctival epithelium: epithelial cells; goblet cells; and, with appropriate markers, Langerhans’ cells, may be examined and numbered.4,5

In a previous report, we described immunocytochemistry by standard impression cytology to analyze inflammatory changes in the epithelium. We found,4,6 in chronic inflammatory disorders from various origins, a strong reliable expression of HLA-DR and CD23 by epithelial cells, as well as an increased density of Langerhans’ cells compared with those seen in healthy specimens. An abnormal epithelial expression of class II antigens has also been found in trachoma7 and in Sjögren’s syndrome,8 together with a strong upregulation of ICAM-1, another inflammatory marker that may also be abnormally expressed by conjunctival epithelial cells in allergic conditions. However, immunocytochemistry has only been used in a limited way in combination with impression cytology, although there has been major interest in this method.3,5,7,8

Nevertheless, as in any morphometric investigation, this procedure remains subject to intrinsic technical limits that may impair quality and reliability of results. Transfer to glass slides may cause significant loss or damage of cells; transparent membranes, Biopore (Millipore, St. Quentin, France), for example, could avoid this problem8 but are thin and are difficult to use in a routine procedure, both for cell collection and for immunocytochemistry. Moreover, whatever material is used, readings are done in a limited number of cells and appreciation of both percentage of positive cells and intensity of immunostaining remain subjective, and susceptible to inter- and even intraobserver variations. This does not favor a wide use of such methods in multicentric protocols.

In contrast, flow cytometry is an objective technique, commonly used for cell analyses in many domains. It allows a rapid, computerized analysis of fluorescence in large and varied populations of cells. As a standardized method, flow cytometry may thus be done by different laboratories. Another advantage of flow cytometry is that cells may be extracted by gentle agitation of membranes in PBS, thus avoiding possible trauma or loss in transferring cells to glass slides. A good correlation can be found between the two methods, morphologic and cytofluorometric, and our current results are quite consistent with those previously described using immunocytologic procedures.4-7 However, although promising, flow cytometry still raises technical problems, especially in obtaining enough cells. Thus in 13 of 80 specimens we collected, the cell population was not large enough to obtain reliable results by flow cytometry. Future research should be directed to improving the specimen yield, either by more suitable membranes or by other techniques of cell extraction. Brush cytology has been proposed for conjunctival cell collection,9 and served for flow cytometry to demonstrate HLA-DR expression by conjunctival epithelium in Sjögren’s syndrome.10 Although brush cytology is also a promising technique, impression cytology allows a homogenous collection of a single epithelial cell layer and does not depend on the strength and duration of application on the conjunctiva. Brush cytology may therefore collect deeper cells, which could be of interest in some ocular diseases.

Flow cytometry also may allow an objective count of a cell population strongly positive for class II antigens, probably belonging to the dendritic cell family, which is known to express high levels of class II antigens. Although the presence of an atypical group of highly positive epithelial cells could not be totally eliminated, this would not be consistent with immunocytologic patterns of typical epithelial cells mildly expressing HLA-DR and of a minority of dendritic cells positive at the highest levels. Therefore the cytofluorometric characteristics (very high levels of fluorescence in a distinct population) were strongly suggestive of Langerhans’ cells. The possibility of an artifactual increase of fluorescence by the aggregation of some epithelial cells could be eliminated because this pattern was found in all eyes (healthy or diseased) with HLA-DR immunostaining, but never with CD23 or the control antibody. We are currently looking for a double immunostaining procedure with more specific markers for Langerhans’ cells. Dendritic cells constitutively express immune markers at high levels11,12 and play a major role in immune defense of the ocular surface. We found a sig-
significant increase of this cell population in dry eyes, eyes with chronic conjunctivitis, and, globally, in inflammatory specimens, compared with our findings in normal conjunctivas. In the future a specific analysis of Langerhans’ cells could thus become an interesting way for exploring conjunctival inflammation.

In conclusion, flow cytometry in impression cytology combines the comfort of a nontraumatic technique for collecting superficial conjunctival cells and the objectivity and reliability of a computerized cell analysis. Our preliminary results showed this method to be feasible for routine use and suggested some of its numerous potential applications. Flow cytometry presents many major advantages toward morphologic techniques, but standard immunostaining procedures remain the only way to understand cell morphology, to look for eventual metaplastic changes, and to investigate the presence of goblet cells, pathogens, or inflammatory infiltrates.

Key Words
conjunctiva, dry eye, flow cytometry, HLA-DR, impression cytology

References