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Tear Component Interference in Cytometric Bead Based Assay of IL-8 and IP-10

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Abstract

Purpose: *To validate IL-8 and IP-10 concentrations in tears by comparing CBA and ELISA analyses to see if interference occurs.*
Method: *Three 6.5 μL samples of non-stimulated (NS) tears were collected using 10 μL microcapillary tubes from 20 subjects. Three 20 mL samples of stimulated (Stim) tears were also collected using 20 μL microcapillary tubes from the same subjects. Samples were assayed using CBA and ELISA. Results of analyses were quantified using ANOVA and regression analysis.*
Results: *Positive correlation between CBA and ELISA results for NS and Stim IL-8 indicate uniform interference effects in CBA. ELISA results for IL-8 were 2.7 times higher than CBA results. IP-10 levels were found to be higher in CBA for both NS and Stim tears. The correlation between ELISA and CBA was strong for Stim tears but weak for NS tears, suggesting interference in CBA.*
Conclusion: *The correlation between CBA and ELISA results for tear IL-8 indicates that CBA can predict the more accepted ELISA values. This contradicts the suggestion of other authors that tear IL-8 cannot be reliably measured by multiplexed assay. Correlated IP-10 results for Stim tears by both CBA and ELISA confirm that IP-10 is an important anti-angiogenic factor at the ocular surface. IP-10 CBA of NS tears does not correlate with ELISA data and should be treated with caution.*

Introduction

Cytokines and chemokines are inflammatory mediators that reflect the health of many tissues, including the ocular surface environment[1,2]. As part of an inflammatory response, chemokines attract leukocytes to sites of inflammation[3]. The concentrations of various chemokines can therefore provide useful information about the health of the "immune privileged" ocular surface. By bathing the ocular surface, tears provide a useful source of soluble ocular surface chemokines. However, tears will also contain cytokines secreted by the lacrimal gland.

Interleukin-8 (IL-8) and interferon gamma-inducible protein 10 (IP-10) are two chemokines that may contribute antagonistically to ocular immune privilege[4]. The role of IL-8 in inflammation is to bind (via a conserved glutamic acid-leucine-arginine sequence near the N-terminus) to CXCR1 or CXCR2 receptors, thus attracting neutrophils to the inflammatory site[1,5,6]. Because IL-8 is a pro-inflammatory and pro-angiogenic chemokine, its tear levels would be expected to be higher in disease states than in healthy eyes. Past reports of elevated tear IL-8 levels in dry eye patients confirm that it may be a useful dry eye biomarker[1]. Ocular sources of IL-8 include leukocytes, corneal and conjunctival epithelial cells, and keratocytes[1,7]. We would therefore expect higher levels of IL-8 in non-stimulated (NS) than stimulated (Stim) tears because increased tear flow dilutes the contribution of ocular surface components.

In contrast to IL-8, IP-10 (CXCL10) is an anti-angiogenic chemokine whose properties are consistent with inhibition of corneal neovascularization. For the ocular surface, IP-10 may be a key link between inflammation and angiogenesis. This is based on reports for other tissues that IP-10 promotes inflammation in such a way as to prevent angiogenesis due to shared receptor expression between leukocytes and endothelial cells8. Because IP-10 serves to prevent blood vessel growth, its presence at the ocular surface may relate to immune privilege. Tear concentrations of IP-10 would therefore presumably be relatively high under normal conditions. Previous studies in this laboratory support this assertion, finding IP-10 to be very active at the ocular surface. Using Cytometric Bead-Based Assay (CBA), LaFrance *et al*[2] measured tear IP-10 levels averaging 24,000 pg/mL. By contrast, serum IP-10 levels are typically reported to be in the 50 - 150 pg/mL range[8].

IP-10 is secreted by several cell types in response to interferon-gamma (IFN-). These cell types include monocytes, endothelial cells, fibroblasts and some epithelial cell types, including intestinal epithelium[9]. If IP-10 at the ocular surface is acting as an IL-8 antagonist to counteract its angiogenic effects, the high tear levels of IP-10 may serve to prevent the IL-8 activation of neutrophils[10,11].

Chemokines can be assayed using single-analyte ELISA or multi-analyte CBA. ELISA has been extensively used to quantify tear cytokines and remains the gold standard for antibody-based tear assay[12]. It is, however, limited to the assay of one cytokine per tear sample. Entire cytokine profiles can be assayed in a single tear sample using a multiplex assay format[2].

Based in part on other reports[13,14] that tear components may produce artifacts in multiplexed (multi-cytokine) assays, the current study was designed to compare tear levels of IL-8 and IP-10 measured by ELISA versus CBA. Comparative results of IP-10 analysis will help determine if the high CBA level is an artifact, and comparison of tear IL-8 levels measured by CBA and ELISA will determine if tear interference is present in CBA of NS and Stim tears as proposed by Sack[14]. If Sack is correct, we would expect IL-8 levels to be higher by CBA than ELISA

of matched tear samples. We would also expect a greater discrepancy in NS tears vs. Stim tears because higher levels of interfering agents have been reported in NS tears[15].

Methods

NS and Stim tears were collected noninvasively from 20 healthy subjects. Subjects collected three 6.5 µL samples of NS tears using 10 µL glass microcapillary tubes placed in the tear meniscus above the lower eyelid margin. Tear flow rate did not exceed 0.33 µL/min during collection. Matching Stim samples (same subjects) were collected using 20 µL microcapillary tubes following collection of an initial 20 µL washout. Inserting a cotton swab along the medial aspect of the nose induced the Stim tears[2]. All tear samples were diluted 1 in 10 with Bioplex Assay Buffer (Bio-Rad, Hercules, CA) and stored at -80°C until use. Tear samples were assayed using a Bio-Rad 27-Plex CBA Assay Kit and read on a Luminex 200 CBA System (Luminex, Austin, TX). Matching samples were assayed using Diaclone WHO-calibrated ELISAs for IL-8 and IP-10 (Cell Sciences, Canton MA). Results of CBA were compared to ELISA to determine the extent and linearity of interference. ANOVA and regression analysis were used to quantify the assay results for all NS and Stim tear comparisons.

Results

While mean NS tear IL-8 levels were 2.7 times higher by ELISA than CBA, there was a strong positive correlation between results for the two assays (Figure 1). The correlation indicates that, if assay interference is present in the NS tear CBA, it appears to be uniform for all measured tear samples. For Stim tear IL-8, a weaker, but still significant correlation between assay techniques was evident (Figure 2). A single data point appeared to skew the Stim tear regression rather than a general non-linear interference effect.

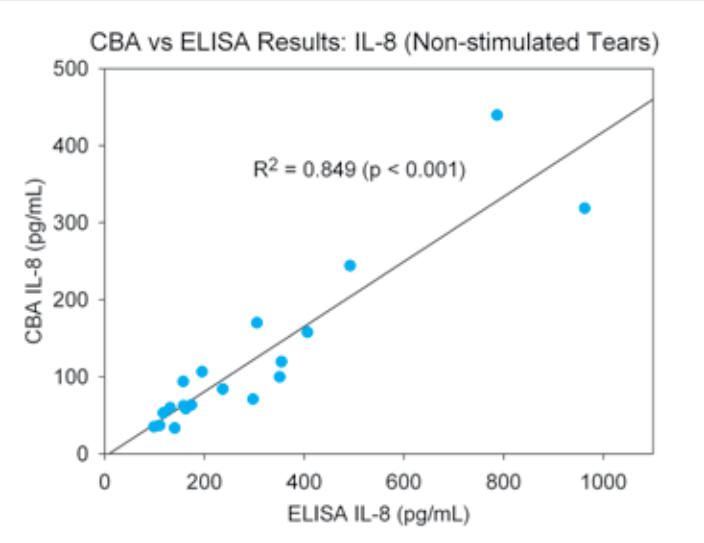

Figure 1. IL-8 in matched NS tear samples measured by CBA (y axis) and ELISA (x axis). ELISA results were 2.7x higher than CBA, but correlated strongly (p<0.001).

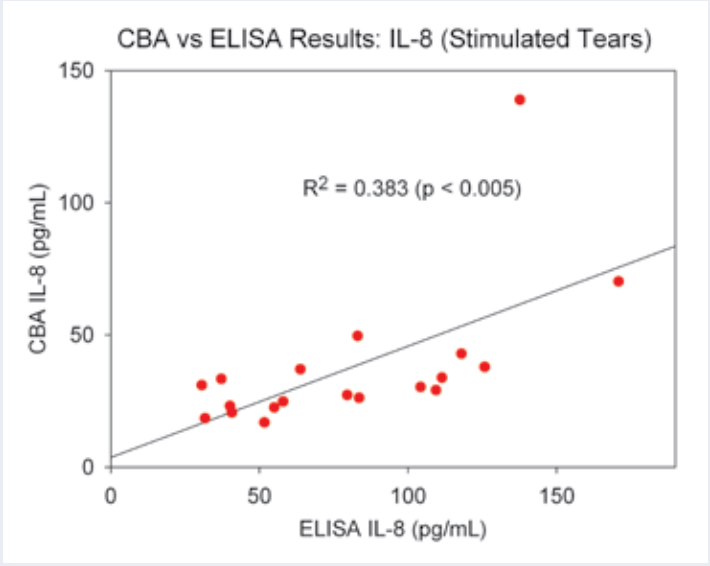

Figure 2. IL-8 in Stim tears measured by CBA (y axis) vs. ELISA (x axis) of matched tear samples. Mean Stim tear IL-8 levels were 2.4x higher by ELISA.

For IP-10, the two assay types show different trends than were evident with IL-8. For both NS (Figure 3) and Stim tears (Figure 4), CBA produced substantially higher IP-10 levels than ELISA. The correlation between assay types was stronger for Stim tears than NS, with a single data point again appearing to skew an otherwise uniform fit. NS tear IP-10 on the other hand showed considerable differences between assay types, suggesting non-linear interference in CBA.

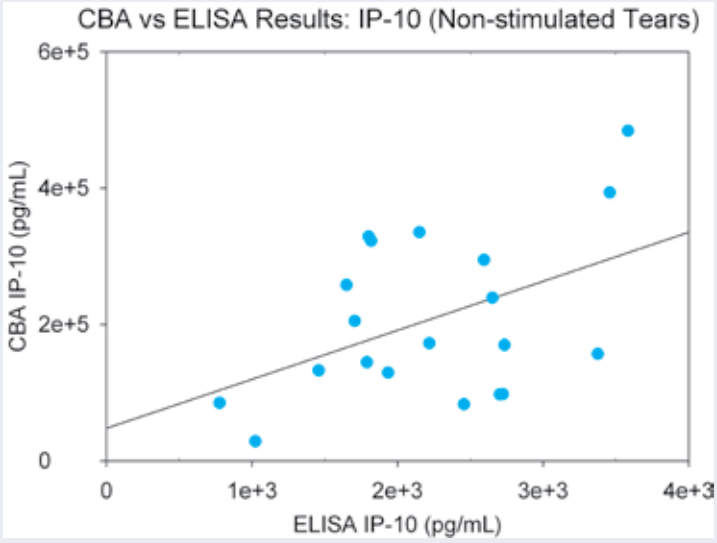

Figure 3. IP-10 in matched NS tear samples measured by CBA (y axis) and ELISA (x axis). CBA results were substantially higher than ELISA, but data is more scattered than for either IL-8 assay comparison.

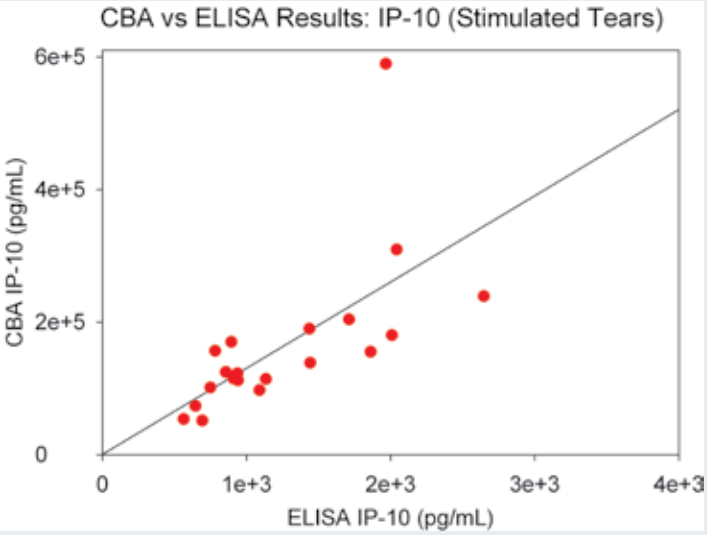

Figure 4. IP-10 in Stim tears measured by CBA (y axis) vs. ELISA (x axis) of matched tear samples. Mean Stim levels were substantially higher by CBA than ELISA. Assay techniques for tear IP-10 measurement correlate more strongly for Stim tears than NS.

Discussion

IL-8

Using ELISA as the standard, CBA results indicated moderate systematic interference effects. It is possible that partial IL-8 epitope blocking in the CBA[16] was responsible for the lower measured tear values. However, NS tear IL-8 levels correlated well between the two assay types. Ranking tests demonstrated that both assays produced essentially the same order of cytokine concentration among tear samples. These findings contradict those of Sack *et al*[14], who reported that multiplexed assay values were artificially high for tear IL-8.

Stimulated tears showed a less robust correlation between CBA and ELISA, suggesting that non-systematic interference may be a factor. With the multi-antibody/multi-antigen format of CBA, the chance for tear component-induced interference increases greatly[16]. Sample components have been reported to form primary to secondary antibody bridges, especially in multiplexed assays[16]. In tears, this would produce false positive artifacts. However, interfering agents may alternatively bind to the primary antibody and produce epitope shielding for the tear antigen, thus reducing measured tear levels. This may explain the lower levels of tear IL-8 measured by CBA, although epitope shielding would be expected to produce a non-linear dose-response curve for tear IL-8. A simpler explanation is that the differences were due to non-equivalent IL-8 standards for the two assays.

As expected, IL-8 levels were lower in Stim than NS tears. This confirms the prediction that ocular surface cells are the primary source of IL-8, and that reflex tears act simply as a diluting agent to decrease IL-8 concentration in the collected tear samples.

IP-10

Much higher levels of IP-10 by CBA than ELISA for NS and Stim tears indicate that CBA produces artificially elevated IP-10 values. This suggests assay interference and may explain the very high tear values found by LaFrance *et al*[2]. The poor correlation between CBA and ELISA values for NS tears indicates non-linear interference in CBA. Therefore CBA levels would not be able to reliably predict ELISA values through a simple calibration factor. Interference in Stim tear CBA appears more linear based on the significant correlation between assays. CBA levels of IP-10 in Stim tears could therefore be used to obtain a reasonable estimate of ELISA levels. Non-linear interference in NS tear IP-10 CBA is further supported by the lack of correlation between levels of tear IP-10 and its inducer, IFN-γ (also measured by CBA; data not shown). A strong correlation between Stim tear IP-10 and IFN-γ (an expected result) further supports the greater reliability of CBA Stim than NS tear IP-10 values. This is consistent with the finding of Shiozawa *et al*[11] report that IFN-γ is a potent inducer of IP-10 from many cell types. One possible explanation for the interference in NS tear IP-10 CBA may be binding of IP-10 to α_2-macroglobulin or another cytokine transport protein in NS tears. This would be less likely in freshly secreted Stim tears. Similar levels of tear IP-10 in NS and Stim tears (measured by ELISA) indicate that the lacrimal gland may be an important source of ocular surface IP-10.

Conclusion

Tear IL-8 levels measured by CBA are lower than ELISA, presumably due to interference effects in the former assay. Interference appears sufficiently linear to allow inter-conversion between ELISA and CBA results with a simple calibration factor. Results of the current study suggest that the membrane microarray assay artifact reported by Sack[14] to produce falsely elevated tear IL-8 levels does not affect CBA. CBA should therefore be a more reliable multiplex assay when studying tear cytokine profiles that include IL-8.

Tear IP-10 levels for both CBA and ELISA exceed reported serum values[10] supporting the theory that IP-10 is an important ocular surface anti-angiogenic factor. Non-linear interference in NS tear IP-10 CBA suggests that multiplex assay data for this chemokine in NS tears should be interpreted with caution. Stim tears show greater potential for providing reliable relative measures of IP-10 at the ocular surface. Studies of tear IP-10 in patients with corneal neovascularization may shed further light on the potential anti-angiogenic role of tear and ocular surface IP-10. Other studies could explore the nature of CBA interference by determining which blocking agents are most effective in reducing interference. Similarly, dissociation

studies could be used to determine if the IP-10 interference effect is due to protein binding *in vivo*, for example by α_2-macroglobulin. Finally, non-ELISA based assays (e.g. MALDI-TOF-MS or gene expression studies) could be used to confirm or refute the high ocular surface levels of IP-10 found in the current study.

References

1. Fodor, M. *et al*. (2006). Enhanced release of IL-6 and IL-8 into tears in various anterior segment eye diseases. Ophthalmic Research 38:182-188.

2. LaFrance, M., Kehinde, L. and Fullard, R. (2008). Multiple cytokine analysis in human tears: an optimized procedure for cytometric bead-based assay. Current Eye Research 33:525-544.

3. Luster, A. (1998). Chemokines - chemotactic cytokines that mediate inflammation. The New England Journal of Medicine 338: 436-445.

4. Taylor, A.W. (2009). Ocular immune privilege. Eye 23:1885–1889.

5. Stern, M., *et al* (2005). Evaluation of ocular surface inflammation in the presence of dry eye and allergic conjunctival disease. The Ocular Surface 3:161-164.

6. Nour, M. and Chodosh, J. (2005). Chemokine signaling pathways in corneal fibroblasts. The Ocular Surface 3:149-151.

7. McDermott, A., *et al*. (2005). Pathways of corneal and ocular surface inflammation: a perspective from the Cullen Symposium. The Ocular Surface 3:131-138.

8. Gotsch, F., *et al*. (2007). CXCL10/IP-10: A missing link between inflammation and anti-angiogenesis in preeclampsia? Journal of Maternal-Fetal Neonatal Medicine 20:777–792.

9. Dwinell, M.B., *et al*. (2001). Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells. Gastroenterology 120:49–59.

10. Streiter, R.M., *et al*. (1995). Role of C-X-C chemokines as regulators of angiogenesis in lung cancer Journal of Leukocyte Biology 57: 752-762.

11. Shiozawa, F., *et al*. (2004). Enhanced Expression of Interferon-Inducible Protein 10. Arthritis Research & Therapy 6:R78-R86.

12. Pflugfelder, S., *et al*. (1999). Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca. Current Eye Research 19:201-211.

13. Sack, R., *et al*. (2007). Antibody array characterization of inflammatory mediators in allergic and normal tears in the open and closed eye environments. Experimental Eye Research 85:528-538.

14. Sack, R., *et al*. (2005). Membrane array characterization of 80 chemokines, cytokines, and growth factors in open- and closed-eye tears: angiogenin and other defense constituents. Investigative Ophthalmology and Vision Science 45:1228-1238.

15. Kuizenga, A., *et al*. (1996). Analysis of human tear fluid components inhibiting protein adhesion to plastic surfaces. Experimental Eye Research 63:319-328.

16. Phillips, D.J., *et al*. (2006). Interference in microsphere flow cytometric multiplexed immunoassays for human cytokine estimation. Cytokine 36:180–188.