Correlations Between Laser Flare Measurements and Anterior Chamber Protein Concentrations

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The laser flare cell meter quantifies anterior chamber (AC) protein (flare) by measuring light scattering of a helium–neon laser beam in the AC. The relationship between photon count and protein concentration both in vitro and in vivo was assessed. The reproducibility of the in vitro photon count measurements was 7.3%. There was a significant linear relationship between photon count and the concentration of both albumin (r = 1.0, P = 0.0001) and immunoglobulin G (IgG, r = 0.99, P = 0.0001) in vitro, but the linear-regression formulas were different with greater light scattering by IgG than by albumin at the same concentration. Laser flare measurements were done on 22 patients (12 normal eyes, 5 eyes with Fuchs' heterochromic cyclitis, and 5 uveitic eyes) before cataract surgery. Aqueous humor obtained from these patients by paracentesis was analyzed for total protein, albumin, and IgG concentration. There was a significant linear relationship (r = 0.88, P = 0.0001) between the laser flare value (range, 5.8–107.8 photons/msec) and the total aqueous protein concentration (range, 14–388 mg/dl). Use of an in vitro albumin calibration curve to convert photon count into protein concentration was found to overestimate the actual protein concentration. This overestimation was slight in normal eyes and increased with increased blood–aqueous barrier breakdown. The use of such a calibration curve therefore is not appropriate in studies on diseased eyes. The authors recommend that laser flare results be expressed in either photons per milliseconds or converted into an equivalent protein concentration using a calibration curve based on actual AC protein measurements. Invest Ophthalmol Vis Sci 33:2878–2884, 1992

The laser flare cell meter (F.C. 1000; Kowa, Acculas Inc., San Jose, CA) is a recently introduced instrument that quantifies anterior chamber (AC) protein (flare) and particles (cells) by measuring of light scattering of a helium–neon laser beam projected into the AC.[1] It has widespread potential uses in the study of AC physiology and pharmacology and clinical applications in uveitis and anterior segment surgery. In vitro studies have shown that there is a linear relationship between the intensity of light scattering and the concentration of bovine[1,2] and human albumin solution[3] over the range of protein concentration found in the AC in both normal and diseased eyes. These in vitro albumin studies have been used as the basis for calibrating the laser flare cell meter and conversion of photon counts into an equivalent albumin concentration.[1,4] In normal eyes, albumin is the major constituent protein of aqueous humor, but after damage to the

blood–aqueous barrier (BAB), proteins of increasing molecular weight penetrate the AC.[5] The intensity of light scattering by proteins in solution is governed by Rayleigh's law and depends, not only on protein concentrations, but also on molecular size. Therefore, protein solutions of similar concentration but different molecular weights (eg, albumin and immunoglobulin G [IgG]) will cause differing amounts of light scattering.[4] Breakdown of the BAB causes, not only an increase in protein concentration, but also a change in aqueous composition. This will alter the relationship between laser flare values and actual AC protein concentrations. Our study was designed to assess the relationship between aqueous flare measurements and actual protein concentrations in eyes with normal or mildly damaged BABs.

Materials and Methods

The Laser Flare Cell Meter

The laser flare cell meter consists of a helium–neon laser slit lamp, a binocular microscope fitted with a photomultiplier, and a personal computer. The laser beam has a power of 25 μW and a diameter of 20 μm. The beam is projected into the anterior chamber and scattering of the beam in a sampling widow (0.3 × 0.5 mm) is detected by the photomultiplier. Two measure-

ment modes are used: one for protein concentration and the other for cell count. The instrument automatically converts from one mode to the other. Each mode takes 0.5 sec, and thus the total measurement time is 1 sec. Computer analysis distinguishes between photons reflected from cells or protein.

In the protein concentration measurement mode, the laser beam is scanned vertically for a length of 0.6 mm, covering the sampling window. Scattering of this beam is measured when it passes through the window. Measurements also are made when the beam passes above and below the sampling window to assess the background signal (BG1 and BG2). The flare value in photon counts per millisecond is calculated by subtracting the signal value from the mean of the two background counts. In the cell count mode, the beam is scanned two dimensionally (0.6 × 0.25 mm) in the sampling window, and the scanning volume corresponds to 0.075 mm^3. A strong peak of scattered light is obtained when the beam strikes a floating cell, and the number of peaks is counted by the computer.

Scans in which the difference between BG1 and BG2 exceed 15% (indicating a nonuniform background) were excluded, and the scan was repeated. To prevent "massaging" of data, after the instrument was aligned, each sequential scan was accepted if the difference between the background counts was less than 15%. The results of five scans then were averaged.

In Vitro Protein Analysis

To calibrate the instrument and assess its accuracy, freshly prepared solutions of human albumin (> 99% purity; Sigma, St. Louis, MO) with a concentration range of 1–1000 mg/dl^3 and human IgG (> 95% purity; Sigma) with a concentration range of 5–600 mg/dl in saline (0.9% NaCl) were placed in plastic cuvettes. These cuvettes were scanned, and an average of five consecutive measurements was calculated. Laser flare measurements were made on 23 in vitro protein solutions (12 albumin and 11 IgG solutions). These were repeated after an interval of several minutes at each protein concentration to assess the reproducibility of the measurements. A cuvette filled with 0.9% saline, but without protein, was used as a control.

In Vivo Anterior Chamber Protein Analysis

We tested 12 patients (mean age, 70.9 yr; range, 52–84 yr) undergoing routine cataract surgery, 5 patients with Fuchs' heterochromic cyclitis (mean age, 52.8 yr; range, 46–63 yr), and 5 patients with chronic uveitis (mean age, 67.8 yr; range, 59–75 yr) who were undergoing surgery for cataract or glaucoma. Laser flare measurements were made before surgery. In-

formed consent was obtained after the nature of the procedure had been explained fully. Before surgery, a paracentesis was made through the limbus with a 25-gauge needle, taking care to avoid blood contamination. Aqueous was withdrawn and frozen at −70°C for later batch processing. Total protein, albumin, and IgG levels were measured using standard techniques in a cerebrospinal fluid laboratory. Total protein was measured by a Bio-Rad protein assay, using an albumin standard and Coomassie brilliant blue G-250. Aqueous albumin and IgG were estimated by radial immunodiffusion on Behring LC Partigen plates with a Behring protein standard.[6] The relative proportion of IgG present in aqueous humor was expressed by a method similar to Zirm.[7] The ratio of aqueous IgG concentration to aqueous albumin concentration (IgG–albumin ratio in percent) was calculated using the following formula.

$$\frac{|x1 - x2|}{(x1 + x2)/2} \qquad (1)$$

Using our calibration curve (based on in vitro albumin analysis in which $y = 1.35x + 0.2$, where y = log of protein concentration in mg/dl and x = log of flare in photons per milliseconds),[3] flare values were converted into their equivalent albumin concentrations. These values then were compared with actual AC protein concentrations to assess the accuracy of such calibration curves and the validity of conversion of photon counts to equivalent albumin concentrations.

Statistical Analysis

Examination of the data showed that both aqueous flare values and AC protein concentrations initially were skewed positively but were approximately normally distributed after logarithmic (log to the base 10) transformation. Therefore, differences in aqueous flare and AC protein concentration between the three patient groups (normal, Fuchs', and uveitic eyes) were analyzed using logarithmic transformed data and a one-way analysis of variance followed by an unpaired Student's two-sided t-test. (The use of untransformed data and equivalent nonparametric tests [Kruskal-Wallis and Mann-Whitney tests] did not alter the statistical significance of the data, thereby confirming the validity of the assumption of normality after logarithmic transformation.) Relationships between aqueous flare and protein concentration (both plotted on a logarithmic scale with flare on the x-axis and concentration on the y-axis) were compared using Pearson's correlation coefficient. P values < 0.05 were considered statistically significant. All flare values were expressed as photons per milliseconds and AC protein concentrations, as milligrams per deciliter.
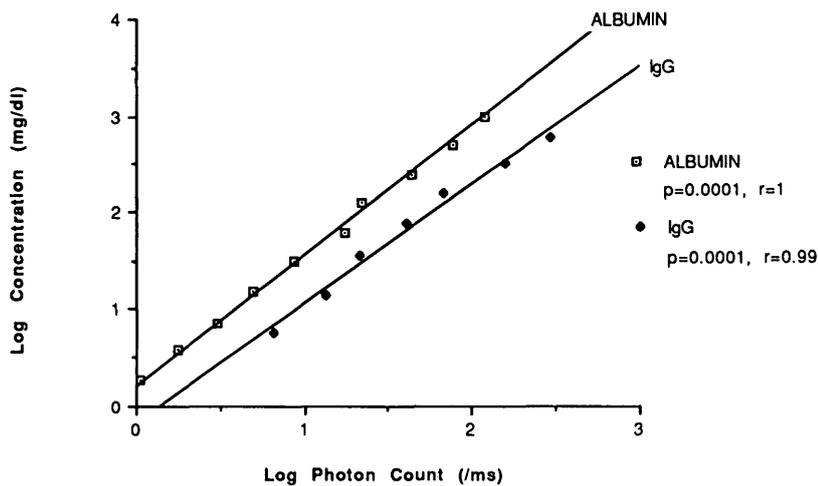
Fig. 1. In vitro photon count and protein concentration. Scatter plot of the log of protein concentration, y (human albumin, and human IgG [mg/dl]) against the log of photon count (per msec), x, with a superimposed line of linear regression (albumin: y = 1.35x + 0.2; IgG: y = 1.23x − 0.17).

All variables were presented as the mean values (± one standard deviation).

Results

In Vitro Protein Analysis

The results of the relationship between photon count and albumin concentration were published previously; these formed the basis for calibrating our laser flare cell meter.[3] This study was extended to assess the relationship between photon count and IgG concentration. There was a statistically significant correlation between photon count and the concentration of protein in solution for both albumin (P = 0.0001, r = 1.0) and IgG (P = 0.0001, r = 0.99) when plotted on a logarithmic scale (Fig. 1), but the linear-regression formulas were different for the two proteins. Photon counts were higher for IgG (y = 1.23x − 0.17; molecular weight, 150,000) than for albumin (y = 1.35x + 0.2; molecular weight, 69,000) at any given concentration.

Reproducibility

The variation between the first and second laser flare measurements was derived from the within-sample residual mean square and expressed as a coefficient of variation. The average coefficient of variation between the two measurements expressed as a percentage was 7.3%. To compare our results with previous reports on the reproducibility of laser flare measurements in normal eyes,[3,8] the following formula for the coefficient of reproducibility also was used.[8]

$$\frac{\text{Aqueous IgG concentration}}{\text{Aqueous albumin concentration}} \times 100\% \quad (2)$$

where x1 and x2 are the first and second measurements. Using this formula, the coefficient of reproducibility was 8.6%.

In Vivo AC Protein Analysis

Individual clinical patient data, laser flare readings, and protein measurements are summarized in Table 1. Two uveitic aqueous humor samples with high protein concentrations were erroneously not diluted into the reference range for total protein estimation, and therefore, it was possible only to obtain a minimum value for the actual AC total protein concentration for these two patients. These minimum concentrations were used to calculate mean values but were excluded from the subsequent analysis of correlation between photon count and protein concentration.

The mean laser flare values and AC protein concentrations in the three patient groups are shown in Table 2. A one-way analysis of variance of aqueous flare grouped by patient type showed a significant variation between the three patient groups (P = 0.0001). Mean aqueous flare in the normal cataractous eye was 8.8 (± 2.4). Aqueous flare was significantly higher in both the Fuchs' (15.2 ± 4.2, P = 0.002) and uveitic eyes (48.4 ± 35.1, P = 0.0001). Biochemical analysis showed that the mean AC protein concentration in normal cataractous eyes was 22.4 mg/dl, with albumin comprising 54.1% of this. A one-way analysis of variance of aqueous protein concentration showed significant differences in total protein, albumin, and IgG concentration between the three patient groups (P = 0.0001). The mean AC protein concentration was significantly higher in both the Fuchs' (41.2 mg/dl, P = 0.003) and uveitic eyes (> 166.8 mg/dl, P = 0.0002). The mean IgG–albumin ratio was 14.1% in the normal cataractous eyes, 17.4% in the Fuchs' eyes, and 21.6% in the uveitic group, but these differences were not statistically significant (P = 0.25). The mean derived equivalent albumin concentration was higher than the actual AC protein concentration in all three patient groups; the greatest overestimation occurred in the uveitic eyes.

Table 1. Age, pathology, flare values, and aqueous and albumin equivalent protein levels in individual patients

No.	Age (yr)	Pathology	Pre op. flare	Total protein (mg/dl)	Alb. (mg/dl)	IgG (mg/dl)	Alb. equivalent (mg/dl)
1	84	Normal	8.2	21	10	1	27.1
2	81	Normal	6.9	17	9	0.3	21.5
3	78	Normal	11.8	45	31	3	44.4
4	77	Normal	9.2	22	14	2	31.7
5	77	Normal	11.4	23	14	2	42.3
6	68	Normal	10.4	21	14	4	37.4
7	62	Normal	12.4	16	9	2	47.4
8	55	Normal	6.6	19	12	2	20.2
9	52	Normal	5.8	14	7	1	17
10	67	Normal	6.1	34	6.1	0.4	18.2
11	76	Normal	10.6	23	13	2	38.4
12	74	Normal	6.7	14	6	<2	20.7
13	52	Fuchs'	10.4	51	36	8	37.4
14	57	Fuchs'	13.8	48	34	5	54.8
15	46	Fuchs'	21.7	31	10	2	101
16	63	Fuchs'	16.0	50	18	—	66.9
17	46	Fuchs'	14	26	16	2	55.9
18	59	Uveitis	39.8	>136	90	35	229
19	65	Uveitis	107.8	388	290	50	879.1
20	73	Uveitis	48.2	>179	156	23	296.6
21	68	Uveitis	26.9	69	48	12	134.9
22	74	Uveitis	19.2	62	50	6	85.6

The age, pathology, flare values, and aqueous protein concentrations in 22 patients (12 normal cataractous eyes, five with Fuchs' heterochromic cyclitis and five uveitic eyes). Alb, albumin.

There was a highly statistically significant linear correlation between photon count and AC total protein ($P = 0.0001$, $r = 0.88$, Fig. 2), albumin ($P = 0.0001$, $r = 0.90$), and IgG concentrations ($P = 0.0001$, $r = 0.88$) when plotted on a logarithmic scale. The linear-regression formula for the relationship between photon count and actual total AC protein concentration ($y = 1.01x + 0.4$, where $y = $ log protein concentration and $x = $ log of photon count) was used to produce an in vivo calibration curve for the conversion of photon count into an equivalent AC protein concentration. This calibration curve then was compared with the calibration curve based on in vitro albumin (Fig. 3). There was an increasing divergence between the two linear-regression lines with higher flare values. The discrepancy between the actual measured AC protein concentration and the

Table 2. Mean age, flare, aqueous protein level, and albumin equivalent concentration

	Normal	Fuchs'	Uveitis
No. of patients	12	5	5
Age (yr)	70.9 ± 10.2 (52–84)	52.8 ± 7.3 (46–63)	67.8 ± 6.1 (59–75)
Flare (photons/msec)	8.8 ± 2.4 (5.8–12.4)	15.2 ± 4.2 (10.4–21.7)	48.4 ± 35.1 (19.2–107.8)
Total protein (mg/dl)	22.4 ± 8.9 (14–45)	41.2 ± 11.8 (26–51)	>166.8 ± 132.8 (62–388)
Albumin (mg/dl)	12.1 ± 6.7 (6.1–31)	22.8 ± 11.5 (16–36)	126.8 ± 101.2 (48–290)
IgG (mg/dl)	1.8 ± 1.1 (0.3–4)	4.3 ± 2.9 (2–8)	25.2 ± 17.7 (6–50)
IgG/albumin ratio (%)	14.1 ± 7 (3.3–28.6)	17.4 ± 4.5 (12.5–22.2)	21.6 ± 10.8 (12–38.9)
Equivalent albumin conc. (mg/dl)	30.5 ± 11.1 (17.0–47.4)	63.2 ± 23.6 (37.4–101.0)	325.1 ± 320.4 (85.6–879.2)

Mean (±1 SD, range in parentheses) age, photon count, and aqueous protein concentrations in normal (n = 12), Fuchs' cyclitis (n = 5), and uveitic eyes (n = 5).
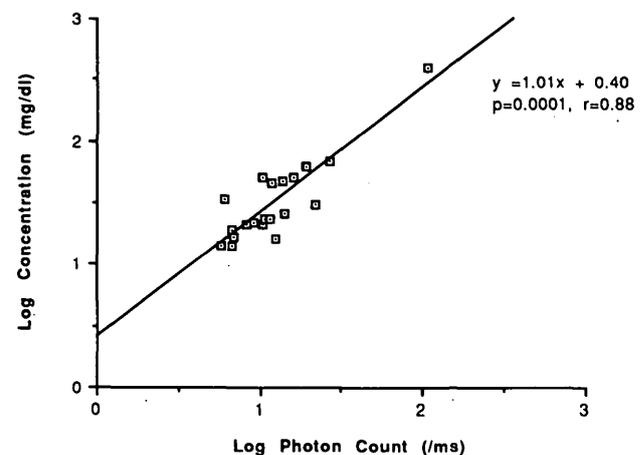

Fig. 2. Photon count and A.C total protein concentration. Scatter plot of the log of total aqueous protein concentration (mg/dl), y, against the log of laser flare value (photon/msec), x, with a superimposed line of linear regression (total protein: $y = 1.01x + 0.4$)
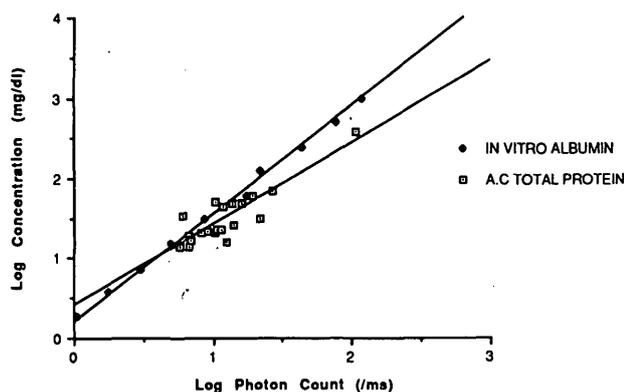
Fig. 3. A comparison of calibration curves based on in vitro albumin and in vivo aqueous protein. Scatter plots of the log of protein concentration (in vitro albumin and in vivo total aqueous protein (mg/dl)), y, against the log of photon count (per msec), x, with superimposed lines of linear regression (in vitro albumin: y = 1.35x + 0.2; in vivo aqueous total protein: y = 1.01x + 0.4). There is increasing divergence between the two lines of linear regression with increasing protein concentration.

predicted concentration (using both the in vivo and the in vitro albumin calibration curve) was calculated for each patient using the following formula.

$$\frac{\text{Actual A.C concentration} - \text{predicted concentration}}{\text{Actual A.C concentration}} \times 100\% \quad (3)$$

The mean discrepancy between actual measured AC protein concentration and the predicted AC protein concentration using the in vivo calibration curve was +11.1 ± 37.9% in the normal eyes, +7.5 ± 53.7% in the Fuchs' eyes, and −13.8 ± 14.8% in the uveitic eyes. The difference between actual and predicted protein concentration was much greater when the in vitro albumin calibration curve was used to convert photon count into a protein concentration. The extent of the overestimation increased with increasing flare value (+46.1 ± 59.7% in the normal eyes, +72.4 ± 100.0% in the Fuchs' eyes, and 86.7 ± 44.9% in the uveitic eyes).

Discussion

The reproducibility of laser flare measurements of in vitro protein solutions was 7.3%. This value agreed with our previously reported coefficient of variation of measurements in normal eyes of 8.6%[3] and with the coefficient of reproducibility of 12.5% reported by others.[8] The significant correlation between photon count and protein concentration for a single molecular weight protein in solution was consistent with Rayleigh's law (Fig. 1). The effect of molecular size on light scattering was shown by comparing the linear-re-

gression formulas for albumin and IgG. Using these calibration curves, a flare value of 100 photons/ms was equivalent to 794.3 mg/dl of albumin and 195 mg/dl of IgG (greater light scattering with larger molecules).

Previously, AC paracentesis was used to obtain samples of aqueous humor for protein analysis and studies showed that paracentesis did not alter the protein concentration or composition significantly.[7,9] Measurements of low concentrations of protein in small volumes of fluid (such as aqueous) require careful handling of the spe   and depend to some extent on the technique i   n our study, the mean aqueous protein concentration in normal cataractous eyes was 22.4 mg/dl (range, 14–45 mg/dl). This was consistent with the results of previous studies.[5,9,10] In these studies, aqueous protein concentrations were between 5–30 mg/dl in normal eyes with slightly higher values in cataractous eyes.[5,9] The increase in total aqueous protein in both the Fuchs' (41.2 mg/dl; range, 26–51 mg/dl) and uveitic eyes (166.8 mg/dl; range, 62–388 mg/dl) also agreed with previous reports. Using a trichloroacetic acid precipitation method, aqueous protein was measured in 90 eyes with active uveitis.[10] It was found that 48 of these eyes had protein concentrations between 8–118 mg/dl; 34 eyes, between 127–861 mg/dl; and only 8, > 1000 mg/dl. In this earlier study, all eyes with Fuchs' cyclitis had protein concentrations < 80 mg/dl. The relative increase in the proportion of IgG in the aqueous of the uveitic eyes we studied also was reported earlier[8] using a radial immunodiffusion technique. A relative excess of IgG in 28 of 35 cases of Fuchs' cyclitis was found;[8] more recently (in a report using an enzyme-linked immunosorbent assay), 65% of patients with Fuchs' and 70% of patients with uveitis had evidence of local synthesis of IgG compared with 44% of the control group.[11]

Photon counts increase with age in normal eyes.[3,12] They were in the range of 3.68 ± 0.59 in subjects younger than 30 yr of age and increased to 6.52 ± 1.24 in those older than 70 yr of age.[3] In our study, the mean photon count was 8.8 ± 2.4 in the normal cataractous eyes, 15.8 ± 4.2 in the Fuchs' eyes, and 48.4 ± 35.1 in the uveitic eyes. In our experience, flare counts after cataract surgery in normal eyes range from 30–250 immediately after surgery (mean, 96 ± 65.3 on the first postoperative day). Flare counts in acutely inflamed uveitic eyes usually are in the range of 50–400; the highest count we found was 800–1000 in eyes with fibrinous exudates and hypopyon. The highest flare count in the current study was 107.8, and therefore, this study was done in eyes with mild damage to the BAB. Eyes with greater damage are, of course, much less likely to be operated on electively, and the other main source of aqueous samples,

diagnostic paracentesis, rarely is required or done in our practice.

There was a highly statistically significant linear correlation between photon count and total aqueous protein concentration in the aqueous flare range of 5.8–107.8 with a protein concentration range of 14–388 mg/dl that demonstrated the validity of laser flare measurements as an indicator of BAB breakdown. However, the linear-regression formula obtained from this in vivo study differed from our calibration formula derived previously from in vitro albumin solutions.[3] Using a scanning ocular spectrofluorophotometer to measure light scattering in the rabbit AC, others also found such a discrepancy between a calibration curve derived from rabbit albumin and a curve based on actual aqueous humor protein measurements.[13] Light scatter was twice as great from aqueous humor from commercial purified rabbit albumin at the same protein concentration. Use of an albumin calibration curve leads to an overestimation of AC protein concentration because it neglects the effect of larger molecular weight proteins. This overestimation is slight in the normal eye but increases with breakdown of the BAB. Flare values of 100 and 1000 photons/ms gave a protein concentration of 267.9 and 2754.2 mg/dl, respectively, using our in vivo calibration curve, an equivalent albumin concentration of 794.3 and 17,782 mg/dl using our in vitro derived albumin calibration formula,[3] and a concentration of 1380 and 23,758 mg/dl using the calibration formula derived from bovine serum albumin.[1] These higher values obviously were unrealistic; they were greater than the total plasma protein concentration. This marked overestimation of protein concentration in diseased eyes showed that the conversion of photon counts into equivalent albumin concentration was inappropriate and misleading in studies on inflamed eyes. The reason for this overestimation can be understood easily by an analysis of the effects of BAB breakdown on aqueous proteins. Albumin is the major constituent of normal aqueous in the uninflamed eye, and this explains the relatively small difference between actual and derived protein concentration. Breakdown of the BAB causes both an increase in protein concentration and a change in composition.[5] Up to 130 mg/dl, there is no qualitative change in the composition of the aqueous. At protein concentrations between 130–670 mg/dl, new serum macromolecular fractions begin to appear in aqueous. Above 700 mg/dl, all serum fractions are present in the aqueous, but macromolecules are still proportionately underrepresented. At concentrations above 1000–1200 mg/dl, the aqueous becomes qualitatively similar to serum. Therefore, as the eye becomes progressively more inflamed, there is an increase in both

the concentration and proportion of higher molecular weight proteins, both of which increase light scattering.

These findings have two main consequences. First, it is difficult to convert a flare value into an equivalent AC protein concentration over a large range of values using a single linear calibration curve. Second, a calibration curve based on albumin alone always will overestimate the protein concentration. Therefore, even though use of a calibration curve derived from in vivo studies is more accurate than the use of an albumin calibration curve, extrapolation of such a single linear in vivo curve over a larger range of concentrations also may cause a discrepancy between the derived and actual concentration. We think that it is more appropriate for results obtained using the laser flare cell meter in diseased eyes to be expressed in photons per millisecond rather than as an albumin-equivalent protein concentration (as has been the practice in many publications on this subject).[4,14–16] However, comparisons of results using photons per milliseconds between eyes also may not be valid completely because of the effects of both protein concentration and composition on light scattering (ie, the same photon count in two eyes does not necessarily imply the same aqueous protein concentration because the protein composition may be different).

Key words: aqueous protein, blood–aqueous barrier, laser flare cell meter, uveitis

Acknowledgments

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