The dynamics of IgG in the cornea

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Fluoresceinated IgG was injected centrally into the rabbit's cornea and both its rate of loss from the tissue and its rate of spread in the stroma were estimated by objective fluorometry. A new technique for determining the rate of loss was established which required only the measurement of the maximum fluorescence in the tissue. The rate at which freshly formed IgG in the blood would arrive at the central regions of the cornea was estimated for the human eye. As penetration continues from the limbus and the protein is lost to the aqueous humor, a standing concentration gradient is set up with a minimum at the corneal center. Calculation suggests that this gradient should be small for IgG in the human eye.

Key words: IgG, immunoglobulin dynamics, corneal permeability, fluorometry, connective tissue diffusion

IgG is one of the body's main defenses against foreign substances. At most locations the bloodstream brings it directly to the site where it is required. In the cornea the vascular supply is remote; nevertheless, it has been found that there is a high concentration of IgG present at all times, which is uniformly distributed over its area.1, 2 It would be desirable to know how the immunoglobulin reached this site, whether from the blood or by local production, and how it would change if there were a sudden alteration in the systemic level of any specific antibody. This would allow a better understanding of the cornea's response to infective agents and perhaps of the immune mechanism of graft reaction.

The problem of the distribution and movement in the corneal stroma of another protein, serum albumin, has been treated by Maurice and Watson.3 The concentration of the naturally occurring albumin dropped from the periphery of the cornea toward its center. It was shown that this could be explained if the protein entered from the blood at the limbus and was lost across the corneal surfaces as it diffused toward the center. With the use of radiiodinated albumin, the rate of diffusion in the stroma along the plane of the cornea and the rate of loss across the surfaces were measured. From these data the theoretical steady-state distribution could be calculated, which was found to agree well enough with that determined experimentally.

It was therefore decided to carry out similar experiments with IgG. This is more readily available tagged with fluorescein than with a radioactive tracer, and this required a modification in the techniques previously used. The experiments on the loss across the surfaces and on the diffusion in the stroma will be described separately.

In a few cases fluoresceinated albumin was used as the test substance in order to allow comparisons with previous results.
Loss across corneal surfaces

Theory. If protein is introduced into the center of the cornea, it takes a period measured in weeks for any appreciable quantity to diffuse to its periphery where it can be carried away by the blood. Any loss of material from the tissue must take place across its surfaces, principally the endothelium. In the rabbit, which has a cornea of uniform thickness, the quantity of material remaining should fall off exponentially with time, and the decay constant should be independent of its surface distribution. On a previous occasion this decay was estimated in the case of serum albumin by measuring, with an external counter, the total radioactivity left in the eye after various periods of time.

In the present experiments it would have been possible to measure the total quantity of fluorescent protein in the cornea at any time by using the method of Jones and Maurice. However, it was more convenient to derive this from a measure of the maximum value of fluorescence within the corneal tissue according to the following theory.

An amount, \( M \), of protein is injected at a point in the center of the cornea, and it can be considered as diffusing radially in the stroma as if in a plane. Then after a time, \( t \), the concentration should be given by

\[
F = \frac{M}{\pi D t} \exp(-r^2/4Dt) \exp(-kt)
\]

(1)

where \( k \) is the decay constant, \( D \) is the diffusion coefficient in the plane of the stroma, and \( r \) is the distance from the point of injection. At \( r = 0 \), where the fluorescence is a maximum, this reduces to

\[
F_0 t = \left[ \frac{M}{\pi D} \right] \exp(-kt)
\]

(2)

Since the term in brackets is a constant, the product \( F_0 t \) should fall off exponentially with the time constant \( k \), which it is the purpose of the experiment to determine.

Methods

Fluorometer. A Haag-Streit slit lamp was modified to serve as a fluorometer in a similar manner to that described by Waltman and Kaufman. A fiberoptic probe was cemented into the eyepiece and led to an RCA 931 photomultiplier tube through a Corning 3-69 glass filter. A Baird-Atomic B-4 filter was introduced into the light path of the lamp, and the circular diaphragm was chosen so as to shine a circle of blue light 1.2 mm in diameter on the eye. This image was about three times the size of the fiberoptic probe in the eyepiece. The arms of the slit lamp were brought close together at a known angle, and mea-
Fig. 2. Rate of loss of fluoresceinated IgG from a rabbit cornea. Ordinate: maximum fluorescence in cornea multiplied by experimental time, in arbitrary units. Abscissa: time from injection. Filled circles correspond to actual experimental time. Open circles represent early values modified by the addition of 18 hr to the experimental time to allow for size of injection.

Fig. 3. Early values of fluorescent reading in cornea after injection of fluoresceinated albumin. Crosses represent values of maximum fluorescence; circles, maximum fluorescence multiplied by experimental time. Filled points correspond to actual time; open points, 3 hr added to experimental time. Straight line fits experimental points at long times.
have been insignificant; later on, valid readings could easily be taken on either side of the scar. Background readings were taken from the opposite eye of the animal.

**Results.** Except for a slight swelling around the injection site during the first day and the faint mark visible in the slit lamp, no reaction to the injections was observed. At first, the protein formed a clearly demarcated bleb in the plane of injection, but within an hour it was seen to distribute itself uniformly across the thickness of the stroma and to spread out sideways from its original site. This occurred more quickly with albumin than with IgG.

**Serum albumin.** Fluoresceinated bovine albumin was obtained from ICN Pharmaceuticals, Cleveland, Ohio. Measurements of greater than twice the corneal background could be obtained for more than 2 weeks after the injection. The results of one experiment are plotted on a semilogarithmic scale in Fig. 1, and it will be seen that the values of $F_o$ conform approximately to a straight line. The slope, $f$, of this line in three experiments was 0.08, 0.10, and 0.11/day. These results are compatible with the average values found by Sery et al.\textsuperscript{6} and Maurice and Watson,\textsuperscript{3} 0.09 and 0.22/day, respectively. It will be noted that the latter workers preferred the value of Sery et al.\textsuperscript{6} to their own.

**IgG.** Fluoresceinated rabbit antibovine albumin was obtained from Cappel Laboratories, Downingtown, Pa. In these experiments readings could be carried on for a month, and again the results can be fitted with a straight line (Fig. 2). Readings were taken from five eyes and gave values of the decay constant lying in the range 0.019 to 0.036/day, with a median of 0.025/day.

**Sources of error.**

**Finite size of origin.** The protein is not concentrated at a true point immediately after injection, as is required by the theory, but is distributed over about 2 mm of the surface. This has a serious effect on the short-term measurements, although the values after many days are little affected. A simple correction consisting of adding a constant time to each of the experimental periods has been introduced which fits the earliest readings to the theoretical straight line.

This is shown in Fig. 3, which plots both points corresponding to the uncorrected times and others where 3 hr have been added to every experimental time. The latter fit much better to the straight line that was drawn through the experimental points obtained at longer periods.

The assumption underlying the correction is that the lenticular volume injected into the cornea approximates the Gaussian distribution of the protein that would be found if it had started diffusing from a point source some 3 hr before the moment of injection. The size of the bleb and the diffusion constant of albumin in the cornea are compatible with this assumption.

Similar corrections are applied in Figs. 1 and 2 with time increments that are found by successive approximations to give the best linearity over the entire time range. It will be seen from the figures that the use of the correction makes a small but definite difference in the estimate of the slope from that which would have been made merely by ignoring some of the earliest points.

**Loss at the limbus.** When the injected material diffuses to the edge of the cornea, a distance of 8 mm, it will be removed by the perilimbal blood vessels, thus giving rise to a rate of loss greater than transfer across the surfaces alone. Calculation shows that this effect should be small under the experimental conditions. From the published\textsuperscript{3} rate of diffusion of serum albumin in the stroma, $1 \times 10^{-2}$ cm\textsuperscript{2}/day, and that given later in this paper for IgG, $2 \times 10^{-3}$ cm\textsuperscript{2}/day, the time taken for 5% of the total material to pass across the limbus and perhaps be carried away would be 10 and 50 days, respectively. These times would have to be considerably increased before the concentration at the center reflected the loss taking place at the edge. Since the method employed depends on the measurement of the central concentration, $F_o$, it seems that edge effects can be neglected over the experimental periods employed.
Irregular corneal geometry. Other assumptions underlying equation 1 are that the corneal stroma is a flat sheet of constant thickness whose surfaces have a constant permeability from center to edge. The human cornea thickens about 1 1/2 times toward its periphery, but the rabbit cornea shows no significant variation over its entire surface. The effects of the spherical rather than plane shape were considered by Maurice and Watson, and it was found that an iterative numerical computation of albumin distribution based on the true shape of the cornea gave results that were insignificantly different from a theoretical calculation based on the planar assumption. This is unlikely to give rise to serious errors under any circumstances, therefore. Changes in permeability over the cell layers on either surface cannot be ruled out, but their uniform morphology renders this unlikely.

Loss of label. The experimental value of \( k \) might be high because of the breakdown of the fluoresceinated IgG molecules into smaller fragments which could find an easier passage across the endothelium. For example, either the fluorescein tag could detach entirely or the IgG molecule could be slightly unstable and break down into its components during the weeks that are necessary for the determination of \( k \). In some separate experiments it was found that prolonged incubation of the labeled IgG with corneas in vitro produced no fluorescent material not precipitable with trichloroacetic acid, which casts some doubt on the first alternative.

Diffusion along stroma

Theory. A quantity of tracer is injected into the center of the cornea as before, and its distribution after time \( t \) is derived from equation 1:

\[
F_r = K \exp(-r^2/4Dt)
\]

where \( K \) is a constant independent of \( r \).

Making a change to rectangular coordinates,

\[
F_{x,y} = K \exp -[(x^2 + y^2)/4Dt]
\]

and so the distribution along a linear element \( y = a \) is given by

\[
F_x = [K \exp(-a^2/4Dt)]\exp(-x^2/4Dt)
\]

where the term in brackets is a parameter independent of \( x \). It is therefore necessary only to determine the distribution of fluorescence along any straight line on the corneal surface after time \( t \) in order to derive a value of \( D \).

Methods. The experiments with IgG were carried out very much as described in the previous section. However, the animal was killed after a determined period of time, and the cornea was excised and the level of fluorescence in the stroma measured at every millimeter in a straight line from limbus to limbus passing near the center. The opposite, un.injected, cornea was treated similarly to give background levels.

Initially the scanning was carried out on the stage micrometer of a Zeiss immunofluorescence...
microscope. The cornea was mounted flat on a slide by means of radial cuts. The condenser was reduced so as to illuminate the tissue with a 1 mm spot of light passed through both a BG 12 and 465 excitation filter. After passage through a 53 barrier filter, the fluorescent light was collected by a fiberoptics probe in the eyepiece and carried to a photomultiplier. In later experiments a strip of cornea about 4 mm wide was cut, and the scanning was carried out with the slit-lamp fluorometer previously described.

Results. It is possible to arrive at a value for D by fitting a Gaussian curve to the experimental values as required by equation 3. The fitting is made easier by approximating the integral \( \int F(x) \, dx \), which should give a linear relationship when plotted against x on probability paper, where the slope of this line is proportional to D. The stages of one such calculation are laid out in Fig. 4 and the detailed result of an experiment in Fig. 5.

In three experiments lasting 11, 30, and 49 days, the values of D were 1.9, 2.05, and \( 1.8 \times 10^{-3} \) cm\(^2\)/day, respectively. Four experiments carried out for less than 6 days gave a mean value for D of \( 2.3 \times 10^{-3} \) cm\(^2\)/day after 15 hr were added onto the experimental times to allow for the area of the initial injection, this being the average increase required to best fit the points on the decay curves. The results taken as a whole indicate that the proper value of D is close to \( 2.0 \times 10^{-3} \) cm\(^2\)/day for IgG.

It may be noted that small molecules present in the initial injection will leave the stroma faster than IgG; for example, fluorescein would effectively disappear from the eye in 12 hr. Thus the longer the experiment lasts the more likely it is that IgG is being measured. Furthermore, any smaller components will spread more quickly and be over-represented at the tail of the curve. If this occurred to any significant extent, it would make the cumulative fluorescent plot nonlinear. This was not seen, and the plots were quite linear, especially in the central region where the heavy molecules are most concentrated. Other sources of error have been discussed in the previous section, and it is probable that the value of D is quite accurate for the fluoresceinated protein.

Discussion

The methods described in this paper allow both D and k to be determined accurately from a single experiment. Mathematical reduction of the data is simple, very little experimental material is required, and few assumptions are necessary. Thus it would not be difficult to obtain values for many other substances. If a method of measuring the distribution around the cornea in the intact eye were developed, nonacute experiments could be carried out, and its application to man is not inconceivable.

It would of course be possible to perform similar experiments in which the penetration of the tagged tracer from the blood to the center of the cornea was measured. This would have several disadvantages:

1. Labeled fragments of the injected protein would move preferentially into the cornea because of their greater rate of diffusion and smaller exclusion volume. The label would have to be extracted from the tissue and identified.

2. The mathematical reduction of the data would be extremely complex, all the more so if the blood level of the tracer changed with
time. The derivation of k would be particularly difficult, since it would scarcely affect the distribution except after several weeks, when equilibrium was being approached.

3. This analysis would require separate determinations of (a) the physiological radius of the cornea—the point beyond the limbus where equilibrium is established between tissue and blood and (b) the value of this equilibrium ratio.

4. Only a single point could be obtained from each rabbit. It might need a very large number of animals, depending on the reproducibility of the experiments, in order to obtain values of D and k of a useful order of accuracy.

5. A relatively large quantity of material would be required for each animal.

**Diffusion in stroma.** The IgG molecule has a molecular weight of 140,000 and is known to be a Y-shaped molecule, 12 nm long, whose three arms are rods about 3.5 nm in diameter. It is thus intermediate in size between serum albumin and mammalian hemoglobin, which are roughly spheres of 6 nm diameter and diffuse about 9 times more slowly than in free solution, and Planorbis hemoglobin, roughly 15 nm in diameter, which is too large to diffuse in the stroma.8 The free diffusion constant of IgG corresponds to $5.5 \times 10^{-2}$ cm$^2$/day at 37°C, and the present results show that it is slowed 27 times in the stroma, which appears to be compatible with the behavior of other proteins.

This rate of diffusion may also be considered appropriate to the human cornea, since its structure is little different from that of the animal cornea. Confirmation by direct measurements on excised human corneas such as were attempted by Stock and Aronson are not easy, since the diffusion of large molecules in the stroma will be very dependent upon its state of hydration and this is difficult to control over long experimental periods. Stock and Aronson used autopsy eyes, which probably had swollen corneas; accordingly, the rate of diffusion of IgG which they found seems to be very fast in comparison to that reported here.
mated by using published curves. It is assumed that the human cornea is a cylindrical disc of uniform thickness and of 6 mm radius; the physiological limbus was found to be slightly outside of the scleral margin in the rabbit (Fig. 6).

Under these conditions the antibody diffusing in from the limbus should start to cause a concentration rise at a point 3 mm off-center in 4 days and at the center in 9 days, and within a few weeks it should produce a concentration halfway to equilibrium with the blood (Fig. 7). This contrasts with the amount entering from the aqueous humor where the IgG concentration is 7 mg/100 ml, compared to 900 mg in the plasma. Allowing that the exchange coefficient, 0.025/day, holds for entry into the cornea, the concentration of IgG from this source would be only 0.2% of the plasma equilibrium value and would reach a maximum of 0.8%. On the other hand, in the inflamed eye much higher aqueous concentrations can occur, and it is possible for these figures to be increased manifold.

Loss across endothelium. As regards the movement of proteins across the endothelium, the value of k for albumin found here, 0.10/day, converts to a permeability of about $7.2 \times 10^{-4}$ cm/day by the use of the formula $P = kqr$, where q is the stromal thickness and r is the partition coefficient between stroma and saline, 0.2 in the case of albumin. This permeability is very low, about 2000 times less than that of the sodium ion, for example. Since the value of k for IgG appears to be at least four times less than that for albumin, its permeability should be lower by an equal proportion.

These very small protein permeabilities suggest a transfer across the cell layer in vesicles as proposed in the capillary endothelium. Some question arises as to the correct value of r to be used in the case of IgG, however. Since steric hindrance decides its value, it should be smaller for the larger IgG molecule than for albumin. However, Allansmith and McClellan found its in vivo value of r to be 0.5, and Stock and Aronson, 0.04. This discrepancy requires clarification.

Protein distribution. Maurice and Watson showed that the steady-state distribution of serum albumin within the rabbit cornea could be explained on the basis that it entered from the blood vessels at the limbus and was lost across the endothelium to the aqueous humor as it diffused centrally within the stroma. The equation used to describe the theoretical concentration distribution under these circumstances was

$$C_p = C_P \frac{I_0(p \sqrt{k/\lambda D})}{I_0(p \sqrt{k/\lambda D})}$$

where $C_p$ denotes the concentration at any radius $p$, $C_P$ the concentration at the limbus P, and $I_0$ indicates a Bessel function. The factor that determines the concentration distribution is the ratio $\sqrt{k/\lambda D}$. With the value of k for albumin found in the present paper, this assumes a value of 3.2 cm$^{-1}$ and leads to the calculated concentration of protein in the center of the cornea of the rabbit being one third of that at the periphery, in accordance with experimental observation.

For IgG the factor $\sqrt{k/\lambda D}$ found for rabbits in the present experiments is 3.5 cm$^{-1}$. The correctness of this value should be checked against the experimentally determined standing distribution of the protein in the normal cornea. The only determinations that appear to have been published are those of Allansmith and McClellan, which were carried out on autopsy eyes. They could not distinguish any difference between its concentration in a 5 mm central button and in the remainder of the cornea up to the scleral boundary. The human cornea is thicker than the rabbit's, and assuming that their permeability and diffusional properties are the same, this would change $\sqrt{k/\lambda D}$ to about 2.8 cm$^{-1}$. When the tissue is divided as described by Allansmith and McClellan, it can be calculated by equation 5 that the outer segment should have an average concentration about 35% greater than that in the center. The variability of the determinations was sufficiently large that such a difference might not have been picked up, and they can be considered not inconsistent with this calculation.

The findings of Allansmith and McClellan
present a serious problem, however, for although the IgG concentration was uniform, that of serum albumin showed a large dip at the center of the cornea. The data are sufficiently consistent to make it improbable that the difference between the proteins is a result of a statistical variation. On the basis of similar \( \sqrt{k/D} \) values found in the rabbit, on the other hand, they should have almost identical distributions.

It is not fruitful at present to speculate as to the cause of this discrepancy; it might result from several causes, including a species difference in the properties of the cornea. Until it is resolved by further experimentation, the present values of \( D \) and \( k \) can be considered as only approximations to those of the human cornea.

REFERENCES