Diffusion of Immunoglobulin G from the Vascular Compartment into the Normal Rabbit Cornea

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In order to study the rate of entry of IgG into the normal cornea, IgG specific for human serum albumin was injected intravenously over a 2-month period into nonimmunized rabbits. The concentrations of total immunoglobulin G (IgG) and IgG specific for human serum albumin in serum, corneal tissue, and aqueous humor were determined with enzyme-linked immunosorbent assays. The experiments showed that the corneal concentration of IgG specific for human serum albumin increased by approximately 1% per day, whereas the total IgG concentration of the corneas used in this study was 70% of the concentration detected in serum. On the basis of these data it was hypothesized that an equilibrium between the serum and corneal IgG concentration was established after approximately 70 days. By injecting IgG preparations with different isoelectric point ranges, the influence of electrostatic interactions on the rate of entry into the cornea was investigated. It was found that charge had no effect on diffusion. From these results it was concluded that, after an antigenic stimulation, newly synthesized antibodies will be confined to the limbal region and will be noted only gradually at points nearer to the center. This indicates that the role of IgG during the immediate inflammatory response of the cornea is limited. Invest Ophthalmol Vis Sci 31:1519-1525, 1990

The distribution of immunoglobulins in human and rabbit ocular tissue, in particular the cornea, is well documented.1-4 The main immunoglobulin present in the cornea is immunoglobulin G (IgG), which is uniformly distributed. Immunoglobulin A (IgA) also is present but at a much lower concentration. Because of its high molecular weight, the presence of immunoglobulin M (IgM) is restricted to the periphery.

Besides the role of molecular weight,5 it is not known whether other factors influence the corneal immunoglobulin distribution. Since aqueous humor and tears contain a small amount of IgG compared to the cornea, and since under noninflamed conditions the limbus and cornea are devoid of antibody-producing cells, it is likely that the IgG molecules present in the corneal stroma are supplied via the limbal vessels.4

In order to allow a better understanding of corneal immune mechanisms, in particular the role of IgG, Allansmith et al8 determined the dynamics of IgG in the cornea. After an intracorneal injection of labeled IgG, the diffusion rate along the stroma and the loss across the endothelium was measured. The current study describes experiments involving nonimmunized rabbits, in which the course of intravenously injected IgG was followed in the cornea. The injected IgG could be detected because of its specificity for human serum albumin. The rate of entry into the cornea was studied by creating a constant serum level of the injected IgG over a period of 2 months. The experimental procedure, which did not involve corneal trauma, is a closer approximation of the in vivo situation. In order to gain a better insight in the function of corneal IgG in immunologic defense mechanisms, the current investigations studied the rate of entry of a circulating antibody into the cornea and the influence of electrostatic interactions on the diffusion of antibodies into the cornea.

Materials and Methods

Experimental Animals

Female Chinchilla rabbits, weighing 1.5-2.5 kg, were purchased from Harlan-Sprague Dawley, Central Institute for the Breeding of Laboratory Animals (HSD/CPB; Zeist, The Netherlands) and housed at our institute. All experiments were carried out in accordance with the ARVO Resolution on the Use of Animals in Research.
IgG Preparations

Rabbits were hyperimmunized with human serum albumin (HSA). Arterial blood was withdrawn from the ears and allowed to clot for 2 hr at room temperature. After separating the serum from the clot by centrifugation (800 g), the serum was heat-inactivated (30 min, 56°C) and stored at −20°C.

Anti-HSA-IgG was purified by affinity chromatography. Collected serum was placed at 4°C and ammonium sulfate was added to a final 50% saturation. This solution was stirred for 30 min and left for another 45 min at 4°C. The solution was then centrifuged for 30 min (5000 g) at 4°C; the precipitate was redissolved in phosphate-buffered saline (PBS; 0.01 M NaH₂PO₄/Na₂HPO₄ + 0.15 M NaCl, pH 7.4) + 0.35 M NaCl to a final volume of half of the original serum volume, and dialyzed against PBS + 0.35 M NaCl.

An immunosorbent column was prepared by coupling HSA to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. The column was equilibrated with PBS + 0.35 M NaCl, and the redissolved ammonium sulfate precipitate was passed over it. The unbound proteins were removed by washing with PBS + 0.35 M NaCl. The bound antibodies were eluted with 0.1 M glycine + 0.9% (weight/volume) NaCl, pH 2.5. The fractions were immediately neutralized by adding a few drops of 1.0 M Tris, and the protein-bound proteins were removed by washing with PBS + 0.1 M NaCl. The IgG preparation then was passed through a sterile 0.45-μm filter (Milllex-HA; Millipore, Mol-}
{sheim, France) to remove large insoluble aggregates and stored at −20°C.

Contamination with IgA or IgM of this IgG preparation was determined with the use of enzyme-linked immunosorbent assays (ELISA) with heavy-chain specificity. The assays were carried out in a procedure analogous to that described in above for immunoglobulin determination, with the use of peroxidase-labeled antisera to rabbit IgA and IgM (GAR/IgA[Fc]/PO + GAR/IgM[Fc]/PO; Nordic, Tilburg, The Netherlands).

IgG preparations with different isoelectric point (pl) ranges were isolated using anion-exchange chromatography. A diethylaminoethyl (DEAE) column (Sephacel; Pharmacia) was prepared according to the manufacturer’s instructions. The column was equilibrated with 10 mM Tris/HCl, pH 8.0, and the affinity-purified IgG was dialyzed against the same buffer. After this IgG was passed over the column, the column was washed with 10 mM Tris/HCl, pH 8.0. The bound IgG was eluted with a gradient of 0–0.25 M NaCl in 10 mM Tris/HCl, pH 8.0 followed by 0.5 M NaCl in 10 mM Tris/HCl, pH 8.0. The fractions were pooled into four groups, dialyzed against PBS, and stored at −20°C.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Isoelectric Focusing (IEF)

SDS-PAGE and IEF were carried out with PhastSystem (Pharmacia). In brief, for SDS-PAGE, samples (diluted in a nonreducing SDS buffer, pH 8.0) and a combination of reduced low- and high-molecular-weight standards (Bio-Rad Laboratories, Utrecht, The Netherlands) were applied to prepacked PhastGel SDS-PAGE 8–25 gradient media. For IEF, samples and IEF calibration standards, pH range 5–10.5 (Pharmacia) were applied to prepacked PhastGel IEF 3–9 media. Separation and coomassie staining were carried out according to the manufacturer’s instructions.

Immunoglobulin Determinations

IgG and anti-HSA-IgG concentrations were determined with two ELISAs. The tests were performed in flat-bottomed microtiter plates (no. 655101: Greiner, Nürtingen, FRG), and the incubations took place at room temperature on a microtiter ELISA-plate shaking apparatus (Greiner).

Total IgG concentrations were determined with a competition ELISA. Wells were coated for 1 hr with 200 μl coating buffer (0.05 M NaHCO₃/Na₂CO₃, pH 9.6) containing 5 μg purified IgG/ml, after which the microtiter plates were rinsed three times with PBS + 0.1% (volume/volume) Tween 20 (PBS-Tween). Samples were diluted in PBS-Tween and tested in duplicate. In each well, 100 μl sample dilution was added, followed by 100 μl 1:2500-diluted (in PBS-Tween) peroxidase (PO)-labeled antiserum to rabbit IgG (GAR/IgG[H + L]/PO; Nordic). After a 1-hr incubation the plates were rinsed as described above. Bound peroxidase activity was visualized with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Boehringer, Mannheim, FRG) as the chromogenic substrate. To each well 200 μl 0.05 M citric acid, pH 4.0 containing 40 mM ABTS and 0.15% H₂O₂ was added. After a 30-min incubation the reaction product was measured at 405 nm.

Each test plate included a serial dilution of purified IgG (0.25–8.0 μg/ml). Linear regression (with the method of least squares) between the logarithm of the IgG concentration and the extinction yielded a standard curve by which the IgG concentration of the samples was calculated. The coefficients of variation within and between the assays were determined by testing six identical samples in the same plate and testing one such sample on six separate days; they were 6 and 8% respectively.
To determine the anti-HSA-IgG concentrations of the samples, plates were coated for 1 hr with 200 µl coating buffer containing 10 µg HSA/ml and then rinsed. Samples (again in duplicate), diluted in PBS-Tween, were added and incubated for 1 hr. The plates were washed, and each well was filled with 200 µl 1:500 diluted GAR/IgG[H + L]/PO in PBS-Tween and incubated for 1 hr. After another washing procedure, the bound peroxidase was visualized and measured. Each test plate included a duplicate serial dilution anti-HSA-IgG (1.4–44 ng/ml), by which a standard curve was calculated as described above. The coefficients of variation within and between the anti-HSA-IgG ELISAs were 8 and 11% respectively. In control experiments no detectable effect of normal rabbit corneal eluate, tested in several dilutions, was found on the ELISAs used.

Intravenous Antibody Administration

Purified anti-HSA-IgG antibodies were injected into the marginal ear vein. Blood samples were obtained at various time periods from the marginal vein of the opposite ear. At each bleeding 1 ml blood was taken and allowed to clot at room temperature. Serum was obtained by centrifugation (10 min, 800 g).

Tissue Preparations

During the experiments the eyes were regularly examined by slit lamp and showed no signs of trauma or inflammation. The rabbits were anesthetized by means of an intramuscular injection of 10 mg fluanoxone and 0.315 mg phentylcitrate (1.0 ml Hypnorm; Janssen, Goirle, The Netherlands) and sacrificed with 300 mg pentobarbitone sodium (5.0 ml Nembutal; Algin, Maassluis, The Netherlands). After the aqueous humor was collected, the eyes were enucleated and rinsed in distilled water. The dissected corneas (free of all sclera) were rinsed in PBS for 10 sec. and the adherent buffer was removed with a filter paper. The tissue was minced and placed in a preweighed tube containing 600 µl elution buffer (0.01 M Tris/HCl + 0.5 M NaCl + 0.1% Tween 20, pH 8.0). The tubes were reweighed to determine the net weight of the tissue and incubated overnight at 37°C in a shaking water bath. In previous experiments, the amount of IgG extracted from the tissue reached its maximum after a 5-hr incubation (data not shown). The tissue was spun (2500 g), and the supernatant was stored at −20°C for further determinations.

Serum samples were taken immediately before each anti-HSA-IgG injection. The total IgG and anti-HSA-IgG concentration of the samples, taken the day the animals were killed, were set at 100%. The relative amounts present per gram corneal tissue and milli-liter aqueous were calculated according to the equations:

\[
\frac{\text{microgram anti-HSA-IgG/gram corneal tissue}}{\text{microgram anti-HSA-IgG/ml serum}} \times 100 (1)
\]

and

\[
\frac{\text{micrograms anti-HSA-IgG/ml aqueous}}{\text{micrograms anti-HSA-IgG/ml serum}} \times 100 (2)
\]

Statistics

Nonparametric methods were used to test for differences. The Mann-Whitney U test was applied for independent groups. The Wilcoxon matched-pairs signed-ranks test was used when data were measured repeatedly in the same subject (in this case central vs peripheral corneal IgG concentration).

Results

Characteristics of Isolated Anti-HSA-IgG

The diffusion kinetics of IgG from the vascular compartment into the cornea were investigated by intravenously injecting homologous IgG with a known specificity (anti-HSA) into nonimmunized animals. Antibodies specific for HSA were isolated by affinity chromatography. SDS-PAGE of this antibody preparation disclosed only one band, of approximately 150 kD. The antibodies were found to be mainly of the IgG class. Less than 1% was of the IgA class. The pI of the antibody preparation ranged between 5 and 9. The appearance of these antibodies in the cornea was measured with an ELISA with a sensitivity in the nanogram range.

A preliminary study on the appearance of circulating IgG in the cornea was performed by injecting a single dose of anti-HSA-IgG (70 mg/animal). This experiment revealed that the diffusion into the cornea of the injected anti-HSA-IgG extended over several weeks. Elimination of the injected IgG during this period made it difficult to quantify the corneal anti-HSA-IgG concentration. It therefore was decided to inject rabbits at fixed time intervals in order to produce a more constant serum level for a longer period of time.

The biologic half-life of the isolated anti-HSA-IgG was determined by injecting three rabbits intravenously with a single dose. The serum anti-HSA-IgG concentrations were calculated as shown by the disappearance curve (Fig. 1). The elimination from the vascular component followed first-order kinetics, and it was assumed that there were two exponential components. The half-lives were estimated graphically. It was found that the mean half-life of the fast exponen-
Fig. 1. Disappearance of intravenously injected anti-HSA-IgG from the circulation of rabbits (n = 3). Circles indicate the measured anti-HSA-IgG concentrations at different intervals after injection. The concentration of the first sample, taken 15 min after the first injection, was set at 100%. The lines represent the exponential components calculated by linear regression.

Fig. 2. Appearance of intravenously injected anti-HSA-IgG in the cornea. Circles indicate the relative amount present per gram corneal tissue, whereby the serum level is set at 100% (data point 0.0 is omitted). The cross-hatched area represents the relative total corneal IgG concentration (mean ± SD: n = 8).

Fig. 3. Anti-HSA-IgG concentration in the aqueous humor. Circles indicate the relative amount present per milliliter aqueous, whereby the serum level is set at 100% (data point 0.0 is omitted). The cross-hatched area represents the relative total IgG concentration of aqueous (mean ± SD: n = 8).

tial component was 1.6 days. The mean half-life of the slower component was 10 days, and 40% of the injected material was eliminated from the circulation with this half-life.

Appearance of Anti-HSA-IgG in the Cornea and Aqueous Humor

An injection schedule was calculated with the data described above and the equation for first-order kinetics:

\[ C_t = C_0 e^{-K_e t} \]

where \( t \) = time in days, \( C_t \) = concentration at \( t \), \( C_0 \) = concentration at \( t = 0 \), and \( K_e \) = elimination constant of anti-HSA-IgG. Since the half-life of the purified IgG was found to be 10 days, it was not necessary to administer anti-HSA-IgG with small time intervals to establish an approximative constant serum level. It was decided to give the animals a priming dose of 12.0 mg anti-HSA-IgG followed by 2.1 mg on every 3rd day or 3.0 mg on every 4th day. Before starting the experiments, no anti-HSA-IgG was detected in the circulation in any of the rabbits. They were killed at different time intervals (between 0 and 63 days after the priming dose), and the corneal tissue was processed as described above.

The mean IgG and anti-HSA-IgG concentrations of the two corneas or two aqueous humor samples from each individual rabbit were used. During the experiment, the serum IgG concentration was 6.0 ± 1.4 mg/ml (mean ± SD, n = 8). Fluctuations noted for each individual rabbit were only minor. The concentration of the injected anti-HSA-IgG fluctuated between 26 and 36 \( \mu \)g/ml serum at the time points measured. The concentration of total IgG per gram corneal tissue was 70 ± 20% (mean ± SD, n = 8) of that detected in serum. These circumstances enabled us to calculate a rate of entry of IgG into the cornea. The injected anti-HSA-IgG accumulated slowly in the cornea (Fig. 2). Per day the corneal anti-HSA-IgG concentration increased by approximately 1% of the serum concentration, as determined by nonlinear regression. Since the experiment lasted only 63 days, the equilibrium with the vascular compartment was not reached. In contrast, the equilibrium between circulating IgG and IgG present in aqueous humor was established after 2 days and remained stable (Fig. 3).
The Effect of Charge on the Diffusion of IgG into the Cornea

Charge was considered a possible factor influencing the diffusion of IgG into the cornea. For this reason, two groups of four rabbits were injected with anti-HSA-IgG preparations of different pI range (5.8-6.9, 6.7-8.2) (Fig. 4). A constant serum level of anti-HSA-IgG was achieved as described above and maintained for 15 days. The excised corneas were separated into a central and peripheral part with a 6.75-mm trephine. The weight of the central corneal tissue pieces was 16.2 ± 0.7 mg (mean ± SD, n = 16), whereas the weight of the peripheral pieces was 41 ± 2.1 mg (mean ± SD, n = 16).

The mean total IgG level of all central corneal tissue pieces was not significantly different from the average IgG level of the peripheral tissue pieces (center, 3.8 ± 1.2 mg/g vs periphery, 3.6 ± 1.5 mg/g; mean ± SD, n = 8). Moreover, no significant differences were observed between the relative anti-HSA-IgG concentration of the central parts of the two groups and the peripheral parts: center high-pI group, 7.6 ± 0.9% vs center low-pI group, 7.1 ± 2.6%; periphery high-pI group, 16.9 ± 3.3% vs periphery low-pI group, 13.4 ± 4.4% (mean ± SD, n = 4) (Fig. 5). By contrast, in both groups, the anti-HSA-IgG concentration detected in the corneal periphery was significantly higher (P < 0.05) than the concentration in the center: low-pI group, 7.6 ± 0.9% vs 16.9 ± 3.3%; high-pI group 7.1 ± 2.6% vs 13.4 ± 4.4% (mean ± SD, n = 4).

Fig. 4. IEF of isolated IgG. Scale at left is pI. Lane A represents the markers by which the pI of the samples was estimated. The pI range of the IgG preparation present in lane B is 6.7-8.2; the pI range of the preparation in lane C is 5.8-6.9.

Fig. 5. Influence of charge on the diffusion of anti-HSA-IgG into the cornea. Circles indicate the relative amount present per gram corneal tissue, whereby the serum level is set at 100%. The left side of the graph represents data of rabbits injected with IgG with a pI range of 6.7-8.2 (mean ± SD, n = 4), data on the right side represent animals injected with IgG with a pI range of 5.8-6.9 (mean ± SD, n = 4).
Discussion

The quantity of IgG present in tissue is believed to be correlated with the amount of vascularization. This does not, however, apply to the eye. The avascular cornea contains the highest IgG concentration of all ocular tissues.\(^3\)

The function of antibodies in corneal inflammation has always been a subject of controversy. Some experimental evidence for the action of antibodies in corneal inflammation is provided by Wessely's phenomenon.\(^9\) When sensitized by an intracorneal injection of antigen, rabbits develop an inflammatory response after a quiescent period of 10 days. This reaction is triggered by the formation of immune complexes and the subsequent activation of the complement system. However, attempts to induce this Arthus-type reaction passively in the avascular cornea, by means of an intravenous injection of anti-serum followed by an intracorneal injection of the corresponding antigen, have not succeeded.\(^10\)-\(^13\) The slow rate entry of circulating antibodies into the cornea, as shown in this study, may provide an explanation for these negative findings.

By creating a constant serum level for 63 days we were able to determine the rate of entry of circulating IgG into the cornea. It was calculated that approximately 1% of the injected IgG concentration present in the vascular component diffuses daily into the normal rabbit cornea. Since the total IgG concentration in the rabbit corneas used in this study was 70 ± 20% of that detected in serum, an equilibrium had not been reached after 63 days. Therefore, it can only be hypothesized that an equilibrium is reached after approximately 70 days. The interpretation of the data is further complicated by the fact that, to our knowledge, it is not known whether the half-life of corneal IgG is the same as that of serum IgG. If it is assumed that corneal IgG has a longer half-life than that of serum IgG, the time lapse between IgG injections and collection of the tissues might result in relatively high corneal IgG levels.

Allansmith et al\(^8\) determined the dynamics of intracorneal-injected IgG. A comparison between those results and the results of this study revealed only minor differences. The increase in the IgG levels in the corneal tissue noted in the current study is slower than the increase given by the hypothetical curve postulated by the aforementioned study. This could be due to the fact that the Allansmith and co-workers' results were calculated for the human cornea, which is smaller and therefore reaches equilibrium faster. Moreover, our results concern the whole cornea, whereas those of Allansmith et al were predicted for the center of the cornea and a point 3 mm off-center. Furthermore, our experiments did not involve corneal trauma.

It is clear that the diffusion of IgG from the circulation into the normal cornea is a very slow process. As we have stated, this probably accounts for the inability of previous investigators to generate a passive Arthus reaction in the cornea. The induction of a passive corneal Arthus reaction, under conditions in which sufficient antibody levels have reached the cornea, will be the subject of further study.

In the current experiment, the total IgG concentration of rabbit aqueous humor was 1 ± 0.3% of the concentration detected in serum. In contrast to the slow accumulation of anti-HSA-IgG into the corneal tissue, an equilibrium between the serum and aqueous anti-HSA-IgG concentration was established within 2 days.

So far, only molecular weight has been identified as a factor influencing the diffusion of IgG in the cornea.\(^5\) Waldrep\(^14\) investigated the influence of electrostatic interactions on the distribution of IgG in ocular tissues by intravenously injecting IgG with various pl ranges. However, even after 5 days, none of the injected IgG was detectable in the cornea. The method described in the current paper is more sensitive: injected IgG was detected in the cornea after 2 days. However, no evidence was found that charge affects the diffusion rate of IgG into the cornea. A comparison between the steady-state ratios of stromal to serum IgG with the use of IEF showed a restricted pl profile for the normal cornea as compared to serum.\(^15\) The cornea seems to lack IgG species with extreme high and low pl values. These results indicate that the role of charge, if any, is limited and can probably only be revealed by the use of IgG species with extreme pl ranges. However, it can be concluded that the proteoglycans of the corneal stroma do not affect IgG diffusion by means of their negative charge; if they did, a shift to the cationic IgG species would have been noted.

The fact that 15 days after the initial injection the anti-HSA-IgG concentration was found to be higher in the corneal periphery than in the center indicates that IgG reaches the cornea via the limbal vessels, rather than through the aqueous or tears, as has been suggested by Allansmith and McClellan.\(^4\)

From the current investigation it can be concluded that after a sudden rise of IgG in the circulation (due to antigenic stimulation) antibodies will be confined to the limbal region, and will be noted only gradually at points nearer to the center. On the other hand, during inflammation, vasodilation in the limbus, changes in the corneal structure due to edema, and local production of IgG may increase the rate of entry of IgG into the cornea. Since IgG is one of the few
immunologic components present in the normal cornea, studies dealing with the kinetics and localization of these antibodies under normal or inflammatory conditions can provide valuable information concerning the immunologic defense mechanisms of the cornea.

**Key words:** cornea, rabbit, IgG diffusion, corneal physiology, electrostatic interactions

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**References**