Immune corneal rings

II. Diffusion kinetics of equine albumin in the rabbit cornea

Theodore W. Sery, Alexander H. Pinkes, and Rose Marie Nagy*

Intracorneal microinjections of foreign protein, conjugated to I-131 as radioactive tracer, were diminished initially to an average of 71 per cent of the amount injected. After the first day as little as 14 per cent of the intended inoculum remained. However, this did not constitute true diffusion but early leakage of protein through the needle track. The remainder was slowly lost by diffusion at a rate of 10 per cent per day. A preliminary sensitization with 4 mg. of protein intracutaneously 2 weeks prior to the injection of albumin intracorneally did not alter the rate of diffusion. This was true in spite of the occurrence of marked corneal inflammation during measurement of radioactivity. Mechanical trauma to the cornea when the diffusion of protein has achieved a constant rate did not alter this rate. The mechanism of formation of "systemic immune rings" was postulated to occur when the concentration of antigen had dropped to optimum levels for precipitation by specific serum antibody migrating in from the limbus. The precipitation served to attract inflammatory cells in from the limbal circulation, thereby intensifying the ring reaction and explaining the phenomenon of centripetal ring migration.

I n the preceding publication,[1] rabbit corneal response to an injected soluble antigen demonstrated a need for determining the level of this antigen in the cornea at various intervals after sensitization. It was also evident that the visible loss of antigen through the needle track in the cornea, during and immediately following the injection, did not permit an accurate esti-

mation of the initial content of foreign antigen in the cornea. This present study will show that diffusion of equine albumin from the rabbit cornea follows a first order diffusion rate and that it is reproducible for the system used. The data will also demonstrate low values of antigen remaining in the cornea at the time when the Wessely phenomenon or a "systemic immune ring"[1] makes its appearance.

Materials and methods

Preparation and injection of equine albumin paralleled the methods in a previous communication.[1] One exception was the use of a more accurate 100 μl syringe which was a gas-tight model* for gas chromatography. This syringe was filled with sterile antigen solutions by use of a

756

sterile glass capillary pipette to facilitate complete removal of all the trapped air, and was readily refilled thereafter by inserting the needle into the neck of a filled and inverted tuberculin syringe. The equine albumin (EnA)° solution contained 100 mg. per milliliter (assaying at 14.5 mg. N per milliliter)† and contained 0.3 µc per milligram protein. Measurements of radioactivity were made in a shielded well-scintillator in combination with a decade scaler. For counting radioactivity in corneas, animals were sacrificed at appropriate intervals, the corneas removed, placed in Wassermann tubes, and the count obtained. All measurements were corrected for background radiation and natural decay. Cesium-137, as standard, was measured daily to calibrate the equipment. Standard isotope techniques were used to prevent contamination of personnel and equipment.

Results

Experiment No. 1. Radioactive iodine (I-131)-tagged albumin. Intracorneal injections are accompanied by visible loss of material through the needle track and accurate inocula are difficult to obtain or reproduce. The first experiment in this group determined the limits of variability in a series of intracorneal injections.

A micro syringe of 100 µl capacity was first calibrated by measuring 20 µl of EnA–I-131 into each of 17 tubes and then measuring the radioactivity in each. The readings minus background varied between 81,502 and 86,607 c.p.m. with an average of 84,044 ± 540 c.p.m. In the same hour 12 rabbits (23 corneas) were injected intracorneally with 20 µl of EnA–I-131. They were immediately sacrificed, the corneas rinsed with saline, removed, and their radioactivity determined. The variation in content of EnA–I-131 extended between 41,100 and 72,900 c.p.m. in 22 corneas; only one value was as low as 21,300 c.p.m. The range of variation in 22 out of 23 corneas thus represented approximately 49 to 87 per cent of the amount forced from the syringe. The average of all values

was 59,400 c.p.m. and represented 71 per cent of the syringe calibration for 20 µl.

Experiment No. 2. Diffusion rate of EnA–I-131 from the cornea. Groups of animals of 20 to 33 each were injected intracorneally with EnA–I-131 and sacrificed 4 at a time at varying intervals. Corneas were immediately removed and measured for residual radioactivity. Three separate experiments were made in this manner with a total of 73 animals. All measurements were corrected for radioactive decay that occurred from the injection date and are recorded in Fig. 1 as points which are averages of 5 to 8 corneas.

The EnA–I-131 came from two separate shipments of conjugated protein (the two lower curves resulted from the first shipment) and different levels of radioactivity were involved initially. Regardless of the starting level, the results indicate that, after an initial rapid decrease of EnA–I-131, a straight-line relationship is obtained when residual antigen concentration in the cornea is plotted logarithmically against time. The initial rapid loss of radioactivity in the first two determinations (lower curves) was believed to be due to an inflammatory response resulting from trauma during the first day so the third determination (upper curve) was designed to retraumatize 22 out of 66 corneas 5 days after antigen injection and observe if a sudden decrease occurred in the succeeding 24 hours. Measurements of corneas so treated (intracorneal saline injections) are represented by arrows in the graph. It is apparent that no such reaction occurred. The last two points in this trial do not agree with the rest of the graph and may represent either a rapid decrease late in the experiment or an artifact; initial values on day zero are, at best, estimates determined as the fraction, 0.71 of the injected 20 µl volume (Experiment 1). The slopes of the three curves were calculated by the method of least squares and were found to be –0.11, –0.10, and –0.09, respectively, from top to bottom. The intercept on the ordinate was also calculated and the lines

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°From Pentex Corporation, Kankakee, Ill., labeled with a radioactive isotope, iodine-131, by Abbott Laboratories, Oak Ridge, Tenn.

†Analyses prepared by Mrs. Eugenia Wijewski.

plotted from these data. Only the two aberrant points on the upper curve were omitted from the calculations.

Experiment No. 3. Diffusion rate of EnA-I-131 from the cornea in previously sensitized rabbits. Twenty-nine rabbits were given a single intradermal injection of 3 mg. of EnA. Two weeks later each

animal received an intracorneal injection of 20 μl of EnA–I-131 in each eye. The animals were selected at random in groups of 4 at appropriate intervals of time over a period of 2 weeks and sacrificed for measurement of residual corneal radioactivity. Daily observation of all eyes prior to sacrifice demonstrated that 50 per cent of the
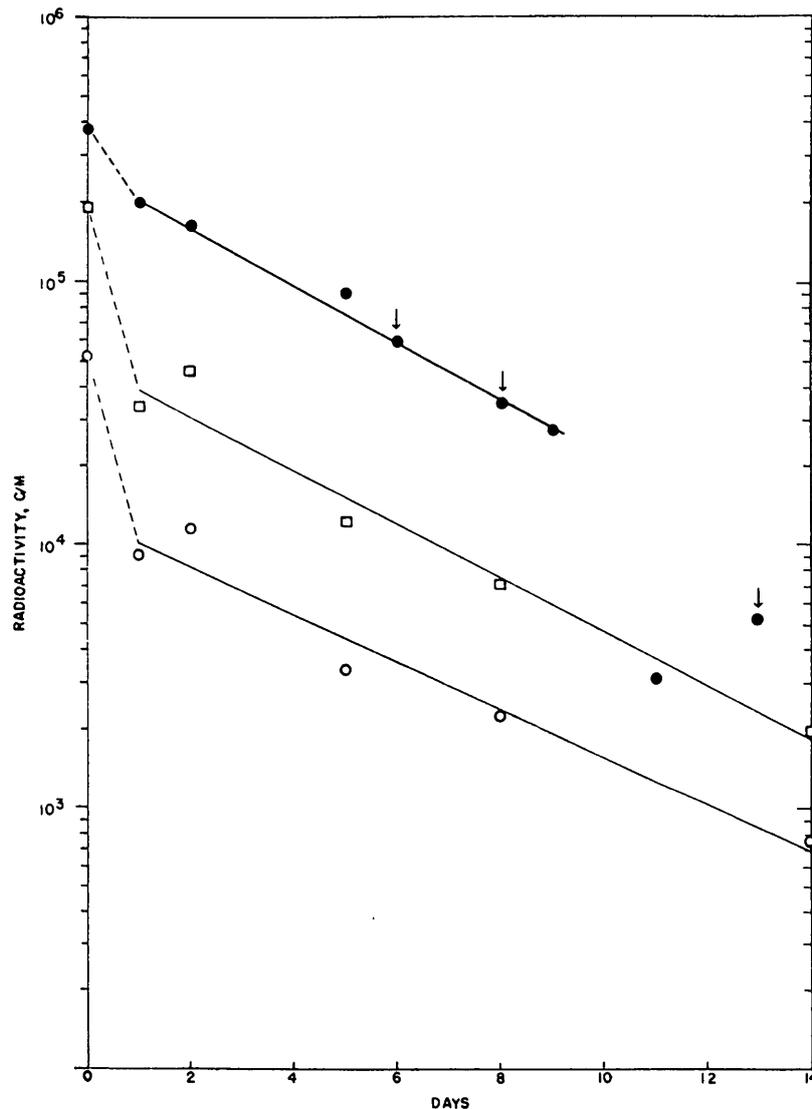

Fig. 1. Rate of diffusion of I-131–conjugated equine albumin from the cornea of rabbits (chinchilla strain). The rate constants, k, for three separate determinations were –0.11, –0.10, and –0.09 per day, respectively, reading from the top down, and were calculated from the formula for first order kinetics. Different initial levels of radioactivity account for the three different intercepts on the ordinate. The arrows on one line indicate points where corneas were irritated by injection of saline to see if the initial rapid diffusion during the first day was due to nonspecific trauma.

corneas had a strong, accelerated inflammatory response comparable to that in the animals of Experiment 6, Table III, in a previous publication.[1] Half of the remaining corneas had an accelerated but moderate reaction. Data from 4 corneas were rejected because of mechanical difficulty during injection. The results are recorded in Fig. 2.

This graph was calculated and plotted from all the points (omitting the zero time point which is an estimate) as in the preceding figure; the calculated slope was –0.09 per day and was almost identical with the average value obtained from non-sensitized animals in the three separate determinations in Fig. 1. Any irregularity of point averages in Fig. 2 is not due to a variable corneal reaction. Individual corneas undergoing strong or weak immune
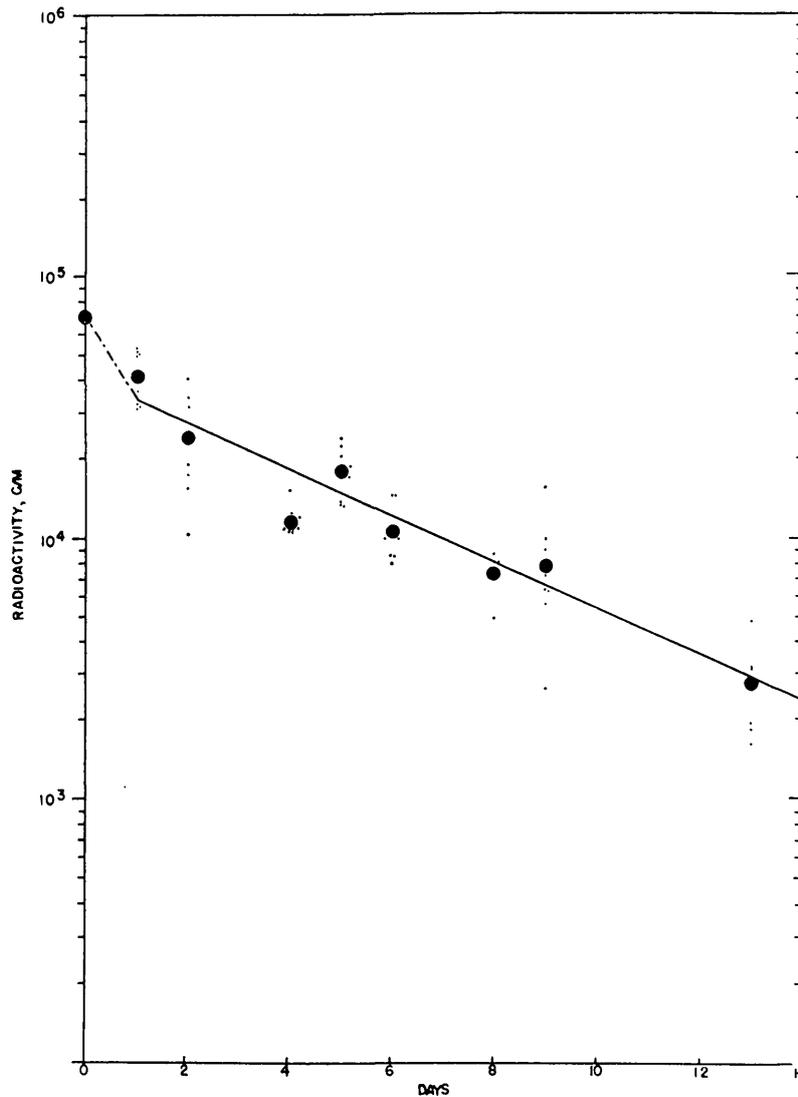

Fig. 2. Rate of diffusion of I-131–conjugated equine albumin as in Fig. 1, but in rabbits that had been sensitized to the protein 2 weeks earlier by an intradermal injection of 3 mg. EnA. The diffusion constant, k, was –0.09 per day. The large solid dots are averages of the radioactivity for individual corneas which are represented as small points.

reactions did not demonstrate any significant differences in residual radioactivity at time of sacrifice. In Fig. 2 the radioactivity in individual corneas is represented as small dots to demonstrate the range of variation that occurred in this type of study.

Experiment No. 4. The stability of EnA–I-131 in corneal tissue. Four corneas were injected with EnA–I-131 in the usual manner, the animals sacrificed within minutes, and the whole corneas transferred to separate 250 ml. Erlenmeyer flasks. Ten milliliters of Parker's tissue culture medium 199 with 10 per cent bovine serum was added to each vessel. The flasks were sealed and incubated at 35° C. for 48 hours on a rotary shaker. The fluids were then pooled and dialyzed against 40 ml. of distilled water (8° C., overnight, with shaking). Comparison of radioactivity in the incubated corneas with that found in the fluids before and after dialysis showed that 92 per cent of the radioactivity diffused into the culture fluids but that only 6 per cent of this activity was able to pass into the dialysate. Up to 3 per cent radioactivity of the original radioactive albumin was considered by the manufacturer to be unbound iodine. It is evident, therefore, that the living tissue does not appreciably degrade equine albumin and liberate diffusible I-131 products.

Discussion

Intracorneal injection of soluble proteins by the methods currently available entails a high degree of unexpected quantitative error. The above data show that statistically only about 70 per cent of injected foreign protein is retained initially. In a matter of 24 hours (less than 3 hours by more recent determinations) this has further decreased to as low as 14 per cent, and at best to 37 per cent, of the intended inoculum. Thus one must expect that more than 50 per cent of a given injection into the cornea will be lost by both rapid and delayed leakage through the needle track made by a 30 gauge needle.

The remainder of the inoculum makes its exit from the cornea by diffusion into the aqueous and the limbal circulation. The rate of diffusion of equine albumin from the rabbit cornea follows a logarithmic decrease in concentration with respect to time. The rate therefore depends upon the concentration of albumin which is present in the cornea at any given moment. This result is in agreement with those shown by Korngold and associates[2] on the rate of diffusion of labeled foreign protein from rabbit skin. Korngold also demonstrated a greater retention of antigen in the skin sites where nonspecific as well as specific Arthus reactions were induced. This was interpreted as evidence of increased retention at the site where any active inflammatory reaction was taking place. We were not able to find this variation in diffusion rates from the cornea in specifically immune rabbits. In Experiment 3 where rabbits were given preliminary sensitization via the skin, the diffusion rate of equine albumin from inflamed corneas was comparable with that in normal corneas. In experiments by Jarowslow and Smith[3] where the labeled antigen was administered intravenously, a more rapid elimination of antigen appeared to occur during the immune phase than during the nonimmune period. Differences in retention of antigen in these tissues are apparently a reflection of the wide difference in tissue structure of the types involved, i.e., nonvascularized, vascularized, and fluid. Gitlin and co-workers[4] have shown the half-lives of intravenously injected human and bovine serum albumin to be 5 days. This was not altered whether iodine-conjugated or native protein was used. Our own half-life of equine albumin in the cornea is approximately 3 days. There is a discrepancy between our results and those of Negoro[5] who used I-131–labeled egg albumin in the rabbit cornea. He found the antigen to disappear more rapidly in corneas of sensitized rabbits. We are unable to account for this difference.

The initial rapid decrease in injected

antigen during the first 24 hours in our study was unrelated to inflammation arising from mechanical trauma. Part of this rapid drop probably results from elimination of the 2 or 3 per cent of free iodine that is present in the labeled antigen and in labeled smaller molecules. The rest of the initial decrease is due to early leakage through the needle track. This is readily apparent in a new method we have used in another study to be published on equine gamma globulin. The rapid initial decrease actually occurs within 2 to 3 hours.

The possibility of antigen diffusion rates being affected by immune factors could not be expected until the end of the second week when the Wessely phenomenon would become an active process. There was only a hint of this possibility in one out of three trials in Fig. 1. This may have been an artifact and is certainly not supported by the data of Fig. 2 where an accelerated corneal inflammatory reaction was evident from the second through the fourteenth day without any evidence of greater retention or release of antigen.

Thompson and Olson[6] measured the retention of hen ovalbumin injected into the rabbit cornea and found that approximately 2 per cent remained in corneal fluids after 2, 5, and 7 days. Estimates of our own residual equine albumin after 7 days vary from 5 to 14 per cent of the estimated injected amount and these percentages were proportionally higher on preceding days. The discrepancy between their results and our own can be attributed to the sensitivity of the methods used. They used a serologic test which is probably not as sensitive as that involving the use of a tagged antigen.

In regard to the amount of protein needed to lead to the formation of systemic immune corneal rings, it would appear from various publications (reviewed in Part I of this series) that approximately 1 mg. protein (*intended* injection) is necessary. This is essentially our own experience with equine albumin in 2 to 3 kilogram chinchilla

rabbits when the amount intended for intracorneal acceptance was 1.8 mg. (0.02 ml. of 9.0 per cent EnA). When this was diluted 1:10 and tried in 20 eyes (cf. animals Nos. 607 to 616, Part I) no corneal or uveal reaction occurred in a single case after the first injection, or even after one reinjection one month later. Therefore, the limiting dose of equine albumin lies at, or not very much less than, 0.25 mg., i.e., 1.8 mg. × 14 per cent, but definitely more than 0.07 mg., i.e., 0.18 mg. × 37 per cent. At the end of 14 days (at the beginning of systemic immune ring formation), 0.25 to 0.67 mg. of retained albumin (14 to 37 per cent of 1.8 mg.) would have decreased to 9 to 23 μg by diffusion. Thus the residual amount needed to form a ring reaction with antibody migrating in at the limbus is approximately 10 to 20 μg.

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