Immune corneal rings

III. Mechanism of local immune corneal ring formation

Theodore W. Sery and Rose Marie Nagy

Immune corneal ring reactions which were earlier defined in two distinct categories under the terms "systemic immune rings" and "local immune rings" have now been shown to be distinguishable by their mechanism of development. Local immune rings formed in properly sensitized corneas, in tissue culture and in whole enucleated eyes, with or without the inclusion of high titer immune serum in the medium or anterior chamber. Weak forms of local rings also developed in vivo by passive transfer of hyperimmune serum followed by optimal amounts of antigen. The local rings have thereby been demonstrated to develop from antibody that is already present in sensitized corneal tissue. Antibody other than precipitin may be responsible for the more severe type of local ring with opaque disc that occurs in vivo. A more dependable method is presented for producing rings in a high percentage of sensitized corneas.

In a previous study immune ring reactions of the cornea were classified in at least two distinct types and given names based upon mechanism of origin and development. The term "local immune ring" was applied to the type of ring reaction that thus far has developed only in corneas that have received at least two (and usually several) injections of soluble antigen. This ring type was first observed by Breebart and James-Witte in 1959. It also had been observed independently in this laboratory the same year. Other investigators studying immune inflammatory processes in the cornea from the time of Wessely in 1911 had failed to obtain or observe this unusual and dramatic response in corneas receiving repeated injections of soluble foreign protein. One possible reason is that the local immune ring occurs with poor regularity or uniformity in identically treated animals and requires proper conditions of hypersensitivity, timing, and antigen dosage. A good comprehensive review on the whole subject of systemic and local immune rings under the title of "anaphylactic keratitis" has been published in a monograph by Breebart. Breebart has proposed that the local immune ring, which he called "super ring," the systemic immune ring, the Wessely phenomenon, and all forms of experimental corneal "anaphylaxis" are variations of one and the same principle. Our own initial study in this subject suggested a different interpretation concerning the nature of this new immune reaction in the cornea. The present study will present evidence that the local immune ring differs from the systemic type in the mechanism of its formation.
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and the chinchilla for producing local and systemic rings. It also demonstrates a better method for obtaining local rings in chinchillas. The injection schedules for different groups are summarized in Table 1, and the incidence of local ring formation is listed as a fraction. In the first six groups all the rings were of weak to moderate intensity except in 2 rabbits, Nos. 682 and 683, which showed unusually strong ring reactions with discs of opacity developing later. With the antigen concentration down to 0.09 per cent (2 to 8 μg per dose) local rings formed only after 5 to 9 injections. These rings were faint and apparently produced no tissue damage. Serum precipitin from these animals was negative by the capillary tube method and mildly positive by the other method. Sera of 3 of the animals sensitized with 0.9 per cent EnA (Nos. 666, 670, and 672) were equally low in precipitin antibody. Precipitates formed only with undiluted sera at an optimum antigen concentration of 10 μg per milliliter.

At 9 per cent EnA the chinchillas produced strong reactions of the Wessely phenomenon on the initial reaction; the corresponding reaction in Dutch rabbits was very weak to negative. A 0.9 per cent EnA injection at 4 week intervals in chinchillas was described in the previous paper and shown to result in local ring response at a rate of 6 out of 20 on the third injection. One of these animals gave a very strong local ring response with an opaque center.

An injection schedule eventually was devised to produce local immune rings in chinchillas with relative ease. It is outlined at the end of Table 1 and in the experiment below on the effect of prednisolone, 21-phosphate on ring formation.

Selective sensitivity for local ring reactions in single animals. Eight chinchilla rabbits (Nos. 526 to 533) were sensitized by an intracorneal injection of 0.02 ml. 9 per cent EnA into the right eye. Strong Wessely reactions with systemic immune rings developed in 3, were weak in 2, and
absent in the other 3. After 26 days when all reactions had subsided, the right cornea was reinjected and the left cornea received its first dose of EnA as above. Within 2 days 7 out of 8 right corneas had developed local rings of varying intensity at the target site of injection. Although strong accelerated Wessely reactions with systemic rings developed in 5 of the left corneas, not one in 8 had even a slight indication of a local ring. Three of the right corneas had quite severe reactions leading to permanent corneal damage. Ten days later skin tests were performed with 0.04 ml. 10 per cent EnA. Strong Arthus reactions were noted in 5 and rated as 4 plus with central blackening of the edematous area. The other 3 rabbits had a weak 1 plus skin edema. Serum precipitin was found in all but was not titrated out. Five sera reacted at least at 1:20 dilution against antigen at 1:1 \times 10^{-4}. This experiment is strong indirect evidence that the local immune ring occurs as a result of a local immune phenomenon. Even though strong circulating precipitins were present in most of these animals, the local immune ring did not or could not develop in the left cornea because it had not been previously sensitized as had the right cornea. Vascularization could not be implicated because the local rings had formed in 4 of the eyes before any vessels appeared.

**Local ring formation in tissue culture.** Nineteen rabbit corneas (chinchilla and Dutch), given local sensitization with 0.9 per cent or 9.0 per cent EnA under varying schedules until they were proved capable of forming local immune rings, were obtained for in vitro studies. Rabbits were locally anesthetized, given their respective concentration of EnA intracorneally at one or more sites, killed, and appropriate sections of the injected antigen site placed at 36° C. in tissue culture medium. Evidence of tissue viability in medium 199 or 50 per cent rabbit serum was noted by acid production and by his-

<table>
<thead>
<tr>
<th>Rabbit type and No.</th>
<th>Sensitization procedure</th>
<th>Rate of local ring formation</th>
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<tr>
<td>Dutch 666-672</td>
<td>a. Two injections 0.02 ml. 9% EnA 49 days apart</td>
<td>14/14</td>
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<tr>
<td>Dutch 682-684, 686, 687</td>
<td>a. Two injections 0.02 ml. 9% EnA 37 days apart</td>
<td>9/10</td>
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<td></td>
<td>b. Third injection after 21 more days</td>
<td>7/10</td>
</tr>
<tr>
<td>Dutch 688-693</td>
<td>a. Two injections 0.02 ml. 0.9% EnA 37 days apart</td>
<td>11/12</td>
</tr>
<tr>
<td></td>
<td>b. Third injection after 21 more days</td>
<td>7/12</td>
</tr>
<tr>
<td>Dutch* 780-781, 783-786</td>
<td>a. Two injections 0.02 ml. 0.9% EnA 2 weeks apart</td>
<td>1/12</td>
</tr>
<tr>
<td></td>
<td>b. Third injection after 14 more days</td>
<td>5/12</td>
</tr>
<tr>
<td>Chinchilla* 649-657 (2 died)</td>
<td>a. Two injections 0.02 ml. 9% EnA 38 days apart</td>
<td>4/14</td>
</tr>
<tr>
<td></td>
<td>b. Third injection after 35 more days</td>
<td>3/8</td>
</tr>
<tr>
<td>Chinchilla 714-716, 718</td>
<td>a. Weekly injections of 0.09% EnA after 5 injections</td>
<td>1/8</td>
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<td>b. After 6 injections</td>
<td>2/8</td>
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<td></td>
<td>c. After 9 injections</td>
<td>5/6</td>
</tr>
<tr>
<td>Chinchilla 213, 217, 219-222, 226, 228, 229</td>
<td>a. One injection of 9% EnA followed 32 days later by one of 0.9% EnA</td>
<td>18/18</td>
</tr>
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</table>

*Very young rabbits, 3 months old, these may have been too young for standard immune response for the 3 injections. 
*1A rather dark type not the same as the gray chinchilla.
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Fig. 1. 682 OD. Enucleated eye injected with antigen just before animal was sacrificed. Twenty hours after incubation at 36° C, a strong local immune ring has developed and one half of it can be seen crossing over a larger, central ring produced 6 days earlier in the living animal.

Fig. 2. 682 OD. Local rings produced in the living animal as they appeared in life. Two concentric rings are evident, a smaller one at the target site and a larger one outside of it. Both developed from the single injection.

tologic observation of epithelial cells growing down upon the cut edge of the stroma. In 8 cultures ring segments were observed to develop in overnight culture; some duplicate cultures of these were negative. Local ring segments formed in the presence of either normal rabbit serum or specific antibody present in autologous serum taken from the animal on the day of sacrifice. When a ring or section of one developed, it was a sharp, very fine white curve of opacity. Control sections of the same cornea injected with saline and incubated for the same period did not form any comparable opacities. Ring segments that became visible at 17 and 24 hours histologically showed no selective cellular accumulation at the ring site. The tissue was edematous and had some randomly scattered round basophilic "nuclei" which resembled atrophied cells.

Ring formation in enucleated eyes. Successful local ring formation in several corneal cultures in vitro suggested that similar results might be obtained in enucleated eyes. This proved to be the case in properly sensitized corneas. Dutch rabbits Nos. 682, 683, 684, 686, and 687 received intracorneal injections of 9 per cent EnA on days: 0, 37, and 58 (No. 683 received one extra on day 50). They were sacrificed, respectively, on days 64, 59, 66, 66, and 66. To control the possibility that specific systemic antibody might be responsible, aqueous humor of the OD was replaced with autologous serum taken at the time of death and aqueous in the OS was replaced with a buffer-gelatin solution at pH 7.2 after two preliminary washings. Weak but definite local rings developed at new 9 per cent EnA injection sites in enucleated eyes Nos. 682 OD and OS, 684 OD, 686 OD and OS, and 687 OD and OS. In rabbit No. 682 two faint rings developed at two separate injection sites each in the OD and OS after only 2 hours' incubation at 36° C. These became progressively stronger after the first 5 hours but still required proper indirect illumination in a dark room for observation. However, after 20 hours the rings in OD were clearly visible to the unaided eye in ordinary daylight and resembled the typical strong local immune rings formed in vivo (Fig. 1). Histo-
Fig. 3. 613 OS. Local immune ring at center of cornea after 25 hours. Polymorphonuclear cells and fibroblasts have accumulated at the ring site. Some of the polymorphonuclear leukocytes may also be seen in the epithelium. (Hematoxylin and eosin. ×450.)

Fig. 4. 213 OD. Local immune ring site in cornea 54 hours after the second injection of 0.9 per cent EnA. The first injection occurred 32 days earlier with 9.0 per cent EnA. The accumulated cells are primarily fibroblasts. Some corneal edema is present. (Hematoxylin and eosin. ×100.)
Fig. 5. High power of previous section. No polymorphonuclear cells are present but a few plasma cells were observed. (Hematoxylin and eosin. ×450.)

Fig. 6. 221 OS. Local immune ring site in cornea 71 hours after challenge. Essentially the same condition prevails as in rabbit eye 213 OD. There is also some degeneration of cells and collagen. (Hematoxylin and eosin. ×100.)
logic examination revealed many fibroblasts in varying states of mobility and degeneration. Fibrocytes were in great disorder. No cell accumulations could be detected as being related to the dense local rings that formed in vitro. Two such accumulations were made up of necrotic cells and were in sites that corresponded to remnants of a previous local ring produced in vivo.

This experiment was also done in rabbits Nos. 688, 689, and 690 which had received sensitization to 0.9 per cent EnA. Their intracorneal injection schedule was similar to the above. Weak rings comparable to the above were observed in Nos. 688 and 689 OD after 20 hours' incubation.

In 5 more rabbits (Nos. 800 to 804) similarly prepared as the above, 3 separate injections were made intracorneally in each eye after enucleation with: (a) 9 per cent EnA, (b) 9 per cent rabbit serum albumin, and (c) physiologic saline. Local rings developed in 6 out of 10 corneas but only at the site of the 9 per cent EnA. Wherever a large volume of EnA was injected, i.e., over 0.02 ml., a broad arc rather than a ring traversed the cornea. A very small dose of 0.002 ml. produced a small ring 1.5 mm. in diameter.

**Attempts at passive transfer.** Six normal chinchilla rabbits were given multiple intracorneal injections in both eyes with serum from rabbit No. 682. This serum had a precipitin titer of 1:80 at an optimum antigen concentration of 10 μg per milliliter. This rabbit had produced exceptional local immune rings and the serum date corresponded to the time when these rings were forming (Figs. 1 and 2). The injections per cornea (from 5 to 10 of approximately 0.15 ml. total) were distributed over the whole cornea so that very little uninjected space remained. A central area of about 4 to 5 mm. diameter was deliberately left clear in all corneas. The injections per cornea (from 5 to 10 of approximately 0.15 ml. total) were distributed over the whole cornea so that very little uninjected space remained. A central area of about 4 to 5 mm. diameter was deliberately left clear in all corneas. After varying intervals of 6, 17½, 24, and 136 hours for different corneas, each was challenged with an intracorneal injection of 0.02 ml. 9 per cent EnA both in the center of the cornea and at a peripheral location. Observations of all eyes over several days with optimum lighting conditions showed only diffuse haziness or云ing at the antigen injection sites. No local immune rings were observed.

In another series 6 chinchilla rabbits were sensitized passively with 0.03 ml. undiluted antiserum in the center of the cornea. After 24 hours the same site received 0.02 ml. EnA at concentrations varying in twofold dilutions from 90 mg. to 0.04 mg. per milliliter. Very faint, delicate local-type rings formed in only 2 corneas—those receiving approximately 0.16 per cent and 0.02 per cent EnA, or an estimated 10 and 1.25 μg of protein. Extreme care and proper lighting were necessary to visualize these rings. Between these concentrations the cornea receiving 5 μg EnA produced a barely visible and transient ring. These rings resembled the local rings in location and timing of development. The cornea that received 1.25 μg of EnA also formed a faint disc of central haziness within the ring as seen in some in vivo local immune rings. With concentrations of EnA at 0.16, 0.08, 0.04, and 0.02 per cent, a series of 3 eyes each (4 for 0.02 per cent) were given the same treatment with an interval of 3 days between sensitization and challenge. Only the 4 corneas at 0.02 per cent EnA responded with the same extremely faint local-type immune ring.

Additional passive transfer studies with varying antiserum concentrations, between undiluted and 1:512, versus constant antigen at 9.0 per cent failed to produce local immune rings.

**Effect of an anti-inflammatory corticosteroid on the formation of local immune rings.** Thirty chinchilla rabbits were given a preliminary injection of 9 per cent EnA into each cornea. The first 10 animals (Nos. 201 to 210) were given topical treatment for 21 consecutive days with prednisolone, 21-phosphate, 0.5 per cent. The schedule consisted of 1 drop every half hour between 9 A.M. and 5 P.M. The remaining 20 control rabbits (Nos. 211 to 230) were given saline topically following the same
schedule. The outcome of this initial system was that the steroid-treated eyes and corneas remained totally clear and uninfamed while the control animals developed severe Wessely reactions with systemic immune rings in 50 per cent of the eyes and mild or weak reactions in the remainder.

Thirty-two days after the initial injection all eyes were reinjected but with 0.9 per cent EnA. The same animals were given steroid or saline as before but only on 5 consecutive days for each of 3 weeks. Another exception was that only the 9 most responsive animals from the untreated group, Nos. 213, 217, 219 to 222, 226, 228, and 229 were selected for the second antigen challenge. The results of 3 more weeks of observation again demonstrated the inability of steroid-treated eyes to produce corneal reactions of any type. All of the remaining corneas, Nos. "213 to 229," developed distinct, easily visible, well-defined local rings. Some of these included an opaque area within the ring. Thus a system for the formation of local immune rings was developed (see Table I) in which a high percentage of success could be expected. Finally, 6 of the latter rabbits, Nos. 217, 219, 220, 222, 226, and 229, were again injected with the lower concentration of 0.9 per cent EnA (28 days after the previous injection) but these eyes were now treated with prednisolone, 21-phosphate for 3 days. Having shown the capacity to form local rings these eyes would be expected to retain the property for subsequent injections. All 12 corneas did respond, beginning at 6 hours, with local immune rings of comparable intensity to their previous reaction.

Histologically the local immune ring has been shown to acquire an accumulation of inflammatory and other type cells at the ring site.\(^1\)\(^2\)\(^3\)\(^4\) This, however, is only true about 24 or more hours after injection of antigen. Figs. 3 to 6 demonstrate the region of the ring site at 25, 54, and 71 hours after the challenge injection. Cell accumulations were not observed in corneas showing local rings at 2½, 8¼, and 16 hours. In No. 613 OS (Fig. 3) at 25 hours the predominant cell seen is an eosinophil granule-bearing cell and is presumably a polymorphonuclear cell, which in rabbits may resemble an eosinophil. In sections made on later specimens (54 and 71 hours) the predominating cell appears to be the fibroblast \((Figs. 4 to 6)\) with few or no leukocytic elements present.

Discussion

It can now be stated with greater certainty that the mechanism for local immune ring formation is primarily a local tissue phenomenon. Indirect evidence supporting this hypothesis was obtained in the experiment on selective sensitivity of one cornea over another in the same animal. In these animals the presence of a high systemic immunity did not lead to local immune ring formation in the unsensitized cornea. Further evidence that an active limbal circulation was unimportant was obtained \((a)\) in those instances of local ring development in tissue culture where nonimmune serum was the major component of the medium and \((b)\) where local rings developed in enucleated eyes in the absence of specific circulating antibody. This evidence, however, did not rule out the possibility that previous injections had attracted serum antibody into the cornea. If systemically derived antibody was present when the cornea was rechallenged with antigen it could conceivably be responsible for local ring formation. The very faint rings observed in passive transfer experiments with a high titer precipitin-type antiserum certainly suggest that systemic antibody could be involved. However, there are two major differences between passively and actively induced local rings. One is the extreme faintness of passively induced rings and the other is the need to limit the concentration of antigen somewhere in the range of 0.02 per cent EnA. For passively induced local rings, concentrations above 0.16 per cent were ineffective. Local rings induced by active
Fig. 7. 118 OS. Albino rabbit with a strong local immune ring and disc after receiving 4 separate injections of EnA over a 2 month period. The reaction appears as shown 2 days after a final challenge of 0.9 per cent EnA near the limbus.

Sensitization were produced with 9.0 and 0.9 per cent EnA and were seldom so faint as to be inapparent in ordinary light. Besides this, these rings were often very prominent, producing permanent discs of tissue damage1 (Figs. 7 to 10).

The composition of the local ring may be considered to be initially a true precipitation between antigen and local antibody similar to that described for the systemic immune ring.1 This would account for the faint appearance of the ring in the first few hours during which no cellular component can be found. The intensified reaction after 24 hours is a reflection of the accumulation of leukocytes and fibroblasts at the ring site. The failure to see such cell organization in cultured tissues may have been due to a nutritional failure.

A question still remains unanswered and that is the failure to obtain a local ring by passive transfer comparable to the more intense types seen in vivo. It may be that the number of trials by passive transfer have thus far been insufficient or that the antigen-antibody concentrations chosen were not appropriate to stimulate this type of response. The more likely answer is that a precipitation of antigen is only a subsidiary component and quite unimportant to the over-all local ring reaction and that another different type of antibody is involved in the actively sensitized cornea. We suspect antibody of the type that is fixed to tissues or cells (quite possibly produced locally) and which is involved in a reaction related to that of a delayed hypersensitivity. That antibody is already present in the cornea at the time of reinjection is further amplified by the ability of local rings to develop despite topical treatment of corneas with an anti-inflammatory steroid. In the case of the Wessely phenomenon and systemic immune rings, such treatment totally inhibited all immune response. This could mean either that systemic antibody manufacture was greatly inhibited or that the rate of diffusion of antigen from the cornea was greatly accelerated. Quite possibly when a faint type of local ring forms on the target site of

Fig. 8. 610 OD. Seven separate injections were given over 6 months in each cornea with 0.9 per cent EnA. The separate intervals were 28, 28, 34, 60, 3, and 28 days. No reaction occurred in either eye until the fifth injection but the cornea was totally unresponsive until the seventh. In this instance permanent damage is noticeable 11 days after the last dose.
injection and is weak enough to be absorbed in several days, this ring is of a pure precipitate type. The more prominent local rings depend upon an additional component(s) and further study is continuing with this view in mind.

REFERENCES

Discussion
Dr. S. P. Halbert, New York, N. Y. In recent years, the development of techniques for the analysis of antigen-antibody reactions in gels have proved extremely valuable for many immunologic, as well as biochemical investigations. The gels employed have included such diverse materials as agar, gelatin, synthetic polymers, and cellulose acetate films. The studies reported here, and by previous workers, have made it clear that the ground substance of the clear cornea also constitutes a medium in which two-directional immunodiffusion precipitates can occur. The present authors have confirmed the observation.
that systemic circulating antibody diffuses into the cornea from the limbus. Antigen then injected into the central portion of the cornea forms a ring of precipitate peripherally as it diffuses outward and reacts in suitable proportions with the intracorneal antibody. Other studies have shown that a line of precipitate can develop if antigen is injected into one side of the cornea, and antibody into the opposite portion. This, of course, is exactly the same type of reaction that occurs in agar gels with the use of the Ouchterlony technique.

The present studies have shown that another type of immunologic ring of precipitate may apparently form, if repeated doses of antigen are given intracorneally. Dr. Sery and Miss Nagy have termed this the "local immune ring," and it appears from their data that this more restricted reaction also is immunologic in nature. One of the difficulties in analyzing such observations is the lack of quantitative information regarding residual antigen at the injection site, or of antibody concentrations within the corneal substance itself. One might expect that data of this sort would be most helpful in understanding the processes involved.

The amount of residual antigen at various times could be rather easily determined if the albumin were tagged with a radioisotope, such as I-131. On the other hand, the extremely sensitive hemagglutination test of Boyden might have been employed more extensively to estimate the local concentrations of antibody in the cornea, as well as in the serum or aqueous. An even more highly sensitive in vivo technique for antibody determination is available in the "passive cutaneous anaphylaxis" method.

Last, it may be worth pointing out that Thompson has shown that local antibody formation can apparently occur within the cornea. This locally formed antibody may also be involved in the reactions described by the authors.

**Dr. Sery (closing).** Data on I-131-conjugated antigen are now available in Part II of this published series. Dr. Halbert's remarks about quantitation of corneal, serum, and aqueous antibody by hemagglutination and by passive cutaneous anaphylaxis are well taken and we hope to have information of this type at a later date. We have measured hemagglutinating antibody titers in sera and are continuing the study in corneal extracts. There has been a considerable amount of work on local production of antibody in the cornea appearing in recent years in the German literature (primarily by Schwab and associates), and we are inclined to believe that one or more antibody types from this source are responsible for that special form of local immune ring reaction which produces an opaque disc within the local ring in the stroma. Although this reaction closely resembles clinical cases of corneal homograft rejection, we are unable to offer evidence at this time showing a relationship between the two responses. However, it is hoped a detailed elucidation of the mechanism involved in producing permanent damage in the stroma, merely by the use of a bland foreign protein, soluble in water, will provide helpful information for a better understanding of corneal homograft rejection.