EXPERIMENTAL STROMAL HERPES SIMPLEX KERATITIS IN RABBITS*

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THE PATHOGENESIS of the stromal lesions of herpes simplex keratitis remains a mystery. Recent attention has been directed toward two possible mechanisms. One is that stromal damage is caused by the reaction of viral antigen with host antibody, either humoral or cellular, and is thus a hypersensitivity phenomenon.[1-4] The second is based on the belief that the stromal lesions are the direct consequence of viral multiplication, resulting in cell death and the release of toxic products.[5-7] These two mechanisms are not, of course, mutually exclusive.

The first part of this study is designed to test the importance of hypersensitivity by measuring the effect of suppressing it. The second part attempts to assess the nature of endothelial involvement and its possible relation to the stromal lesions. Since we began this investigation, Taktikos and Aurelian[8] have published the results of similar work. The first portion of our study confirms their findings.

A. STUDIES ON THE ROLE OF HYPERSENSITIVITY

Materials and Methods

For the purposes of this study, the hypersensitivity mechanism in rabbits was suppressed by total body X-irradiation. Since direct radiation could also affect the reaction of the corneal cells to herpes virus, and since the only effect desired was suppression of the systemic immune response, it was important to shield the corneas. It has been shown repeatedly that the primary immune response of the rabbit eye is dependent upon the systemic immune mechanism rather than upon local cells and that this primary response can be suppressed by X-irradiation with the eyes shielded.[9-11]

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ANIMALS

The experimental animals were pigmented, outbred rabbits weighing from four and a half to five and a half pounds. The rabbits were paired and from four to 10 pairs were used in each experiment. One rabbit of each pair was irradiated, the other was not.

VIRUS

The inoculum was the RE strain of *Herpesvirus hominis*. This strain was isolated from a human cornea by Dr. Calvin Hanna, who kindly supplied it to our laboratory. It is known to have a strong propensity for producing stromal keratitis in rabbits without incurring a high mortality from encephalitis.

RADIATION PROCEDURE

The rabbits were anesthetized with intravenous pentobarbital, laid on their right sides and curled to fit within a 30-cm. diameter treatment area, and given X-irradiation with a 250-kv., 30-ma. machine fitted with a Thoreus II filter (H.V.L. equals 2.2 mm. Cu). The radiation source was 80 cm. from the table top, and the radiation was delivered at 50.3 r./min. to the skin. In one series of 26, the radiated rabbits received 450 r. and in a second series of 20, the radiated rabbits received 550 r.

A 40 × 80 × 4 mm. lead shield was held immediately over the left eye of each rabbit, and the rabbit was positioned so that the right eye also lay directly below the shield. Probes confirmed that with this arrangement less than 7 per cent of the treatment dose reached either cornea. Increased scatter tended to permit more radiation to reach the lower eye, but this was cancelled approximately by the deeper tissue penetration required. The probes also showed that the fall-off in dose from the center to the periphery of the treatment area was only 5 per cent.

In order to assure that the immunosuppressive effect of the radiation in our experiments was comparable to the effect described in the literature, the experiment of Sellyei and Ellis[11] was repeated. In a small group of rabbits, 4 mg. of bovine serum albumin (BSA) was injected into the corneal stroma to serve as the stimulus for the production of an immune keratitis. This group served as an over-all control series.

INOCULATION PROCEDURE

If the right eyes of the first pair of rabbits in an experiment received virus, the left eyes of the second pair received virus. In this way the

right and left eyes were used alternately for virus inoculation throughout all the pairs of an experiment. In each animal, the opposite eye was inoculated with a sterile virus medium as a control.

The eyes were inoculated 24 hours after radiation. Each cornea to be inoculated was scratched superficially with cross-hatched strokes of a needle. Two drops of virus suspension were then instilled on the cornea, and the lids were held closed for 60 seconds. In all instances the pairs of rabbits were handled sequentially so that any time-dependent artifact affecting the activity of the virus suspension or the rabbit sera would be distributed equally between irradiated and non-irradiated rabbits.

CLINICAL AND HISTOLOGIC EXAMINATION AND GRADING

The rabbits were examined with a slit-lamp and the corneal lesions diagrammed every two or three days. Preliminary studies had shown that by the tenth day after inoculation, the course of the keratitis in any rabbit was usually apparent. By that time (1) the epithelial ulcer had healed without residua, (2) a deep stromal "disciform" haze had appeared, or (3) there was frank stromal necrosis. The rabbits were therefore sacrificed on the thirteenth day after inoculation and their eyes fixed in formalin.

Microscopic sections of the cornea (in the first experiments) and of the whole eye (in later experiments) were prepared and stained with hematoxylin and eosin. The sections were coded so that all histologic grading was done without the examiner knowing the source of the section.

The stromal keratitis was graded according to both its clinical course (Figure 1) and its histologic appearance (Figure 2). Iritis was graded only histologically (Figure 2) since the corneal opacity often precluded an accurate slit-lamp evaluation.

BLOOD STUDIES

On the tenth or thirteenth day after inoculation, blood was obtained from all the rabbits. White cell and differential cell counts were done in several of the experiments as an added check on the effectiveness of the radiation. The sera were stored at −70° C. until neutralizing antibody titers could be determined by the intracerebral inoculation of 15-Gm. Swiss white mice (standard technique and calculations[12]). Since we wished to measure the total early antibody response, including the complement-dependent antibodies,[13] the sera were not heat-inactivated. Each serum sample was tested on 20 mice, five mice for each

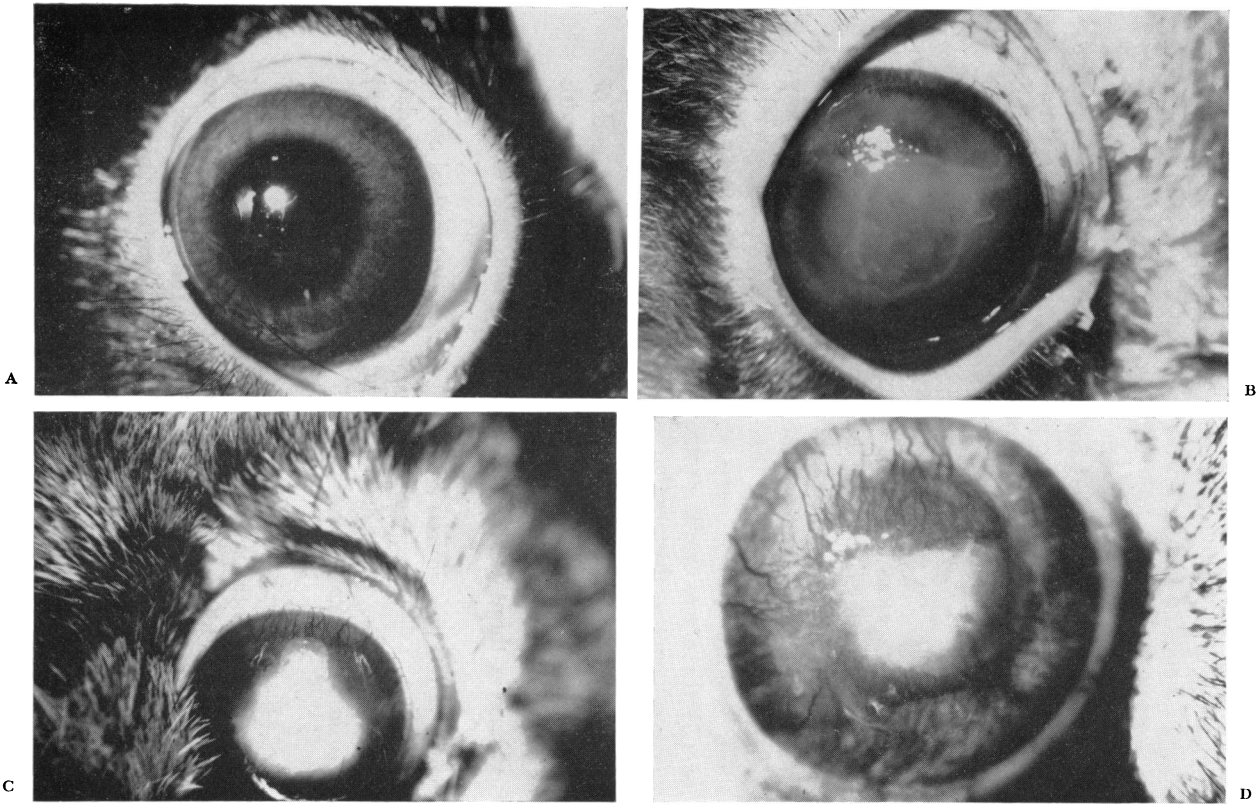
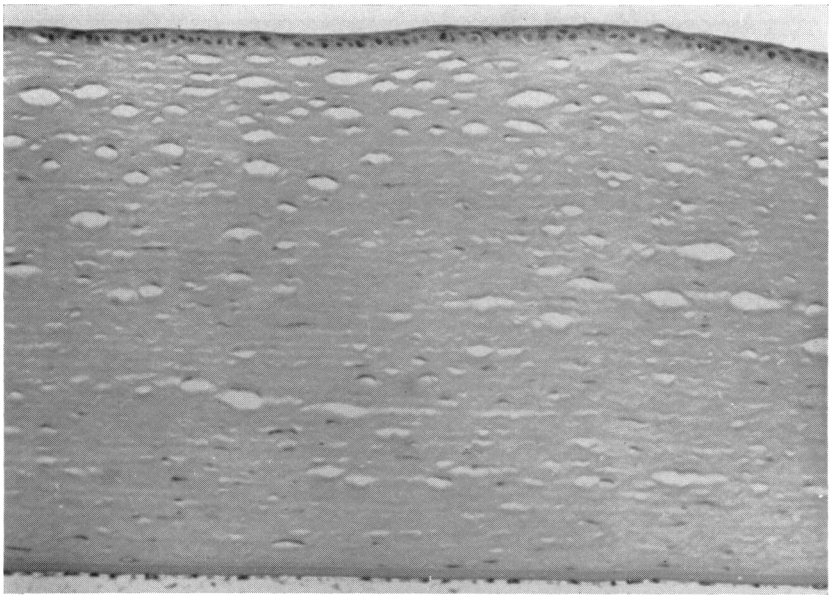


FIGURE 1. CLINICAL GRADING SYSTEM.

A. Grade 1. At day 13: stroma clear; early mild superficial edema, limited strictly to area underlying epithelial ulcer, has disappeared with healing of epithelial lesion. B. Grade 2. At day 13: deep stromal edema, not limited to area of epithelial defect. C. Grade 3. At day 13: dense white stromal necrosis, usually surrounded by a zone of deep stromal edema as in grade 2 lesions. D. At one month: Grade 3 lesion showing persistence and vascularization of central scar.



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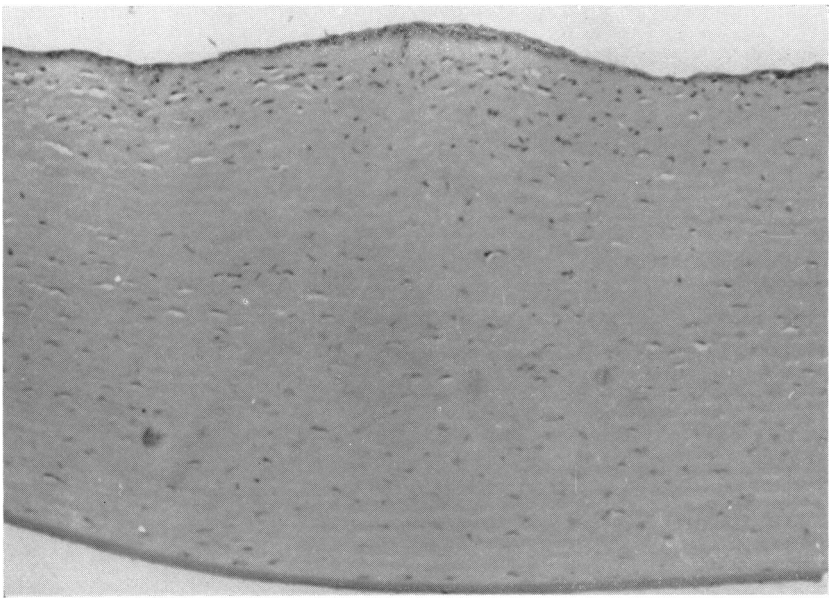

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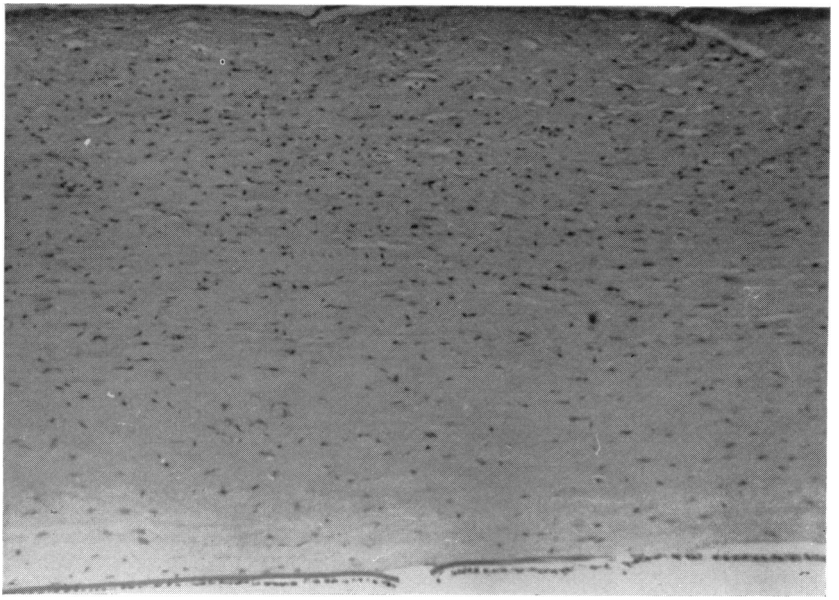
FIGURE 2. HISTOLOGIC GRADING SYSTEM.

The following three pages illustrate the system. Each eye graded according to most severely involved area of central cornea. Central cornea defined as the area greater than one low-power field (× 60) from any point on the limbus.
A. Grade 0 (× 44). At day 13: no inflammatory cells or other differentiating change. (No inflammatory cells or other signs differentiating virus-inoculated cornea from controls.)
B. Grade 1 (× 30). At day 13: inflammatory cells few in number and limited to superficial stroma.



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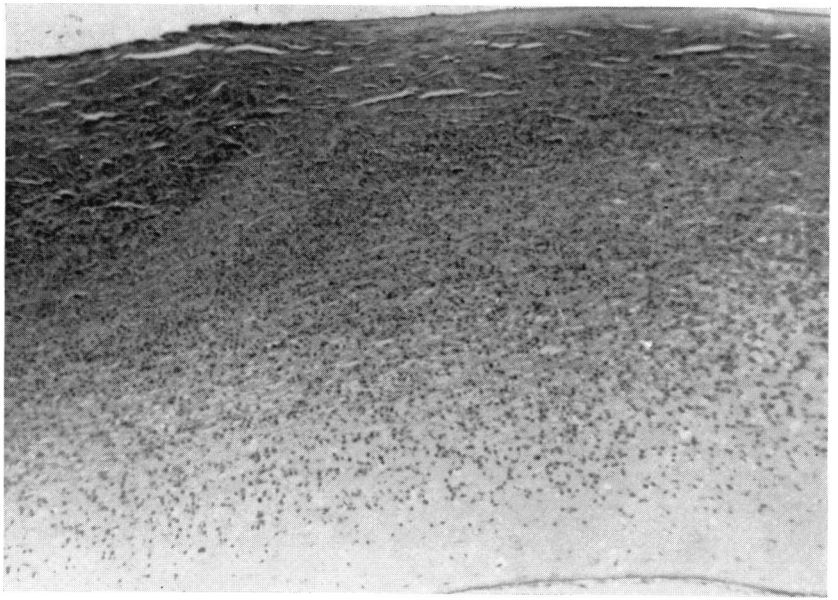

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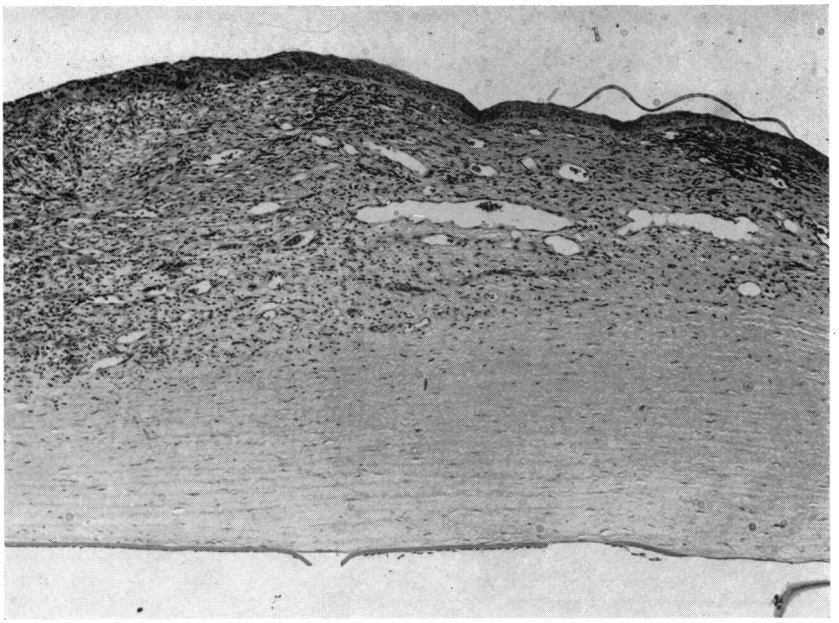
C. Grade 2 ($\times$ 20). At day 13: many inflammatory cells extending throughout width of swollen stroma.

D. Grade 3 ($\times$ 20). At day 13: stromal necrosis involving about the superficial one-third of the cornea.



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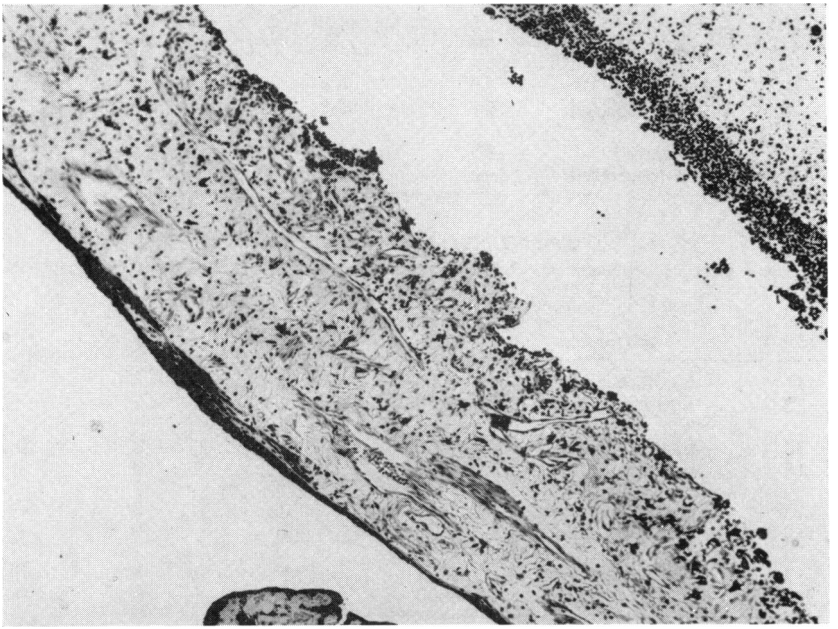

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E. Grade 3 ($\times$ 20). At one month: persistent vascularized scar.
F. Iritis ($\times$ 44). Graded present or absent. Criterion of presence: inflammatory cells in iris and anterior chamber.

of four dilutions. All the sera for any one experiment were run simultaneously.

Results

The RE strain of *Herpesvirus hominis* produced a spectrum of disease in rabbits that was comparable to that seen in humans. The dose of 450 r. of X-irradiation reduced the circulating lymphocyte count (Tables 1–3), suppressed the immune keratitis in the control series of BSA-injected corneas (Table 5), and markedly reduced the neutralizing antibody titers to herpes simplex virus in most, though not in all, of the irradiated rabbits (Tables 1–3 and Table 7). The antibody inhibition was sufficient to warrant an attempt to correlate anti-

TABLE 1. EXPERIMENT I—450 R.

Rabbit	Treatment	Neut. index	Lymphocyte count	Keratitis	
				Clinical	Histologic
1	irradiated	9	890	3	3
2	non-irradiated	98	3300	2	2
3	irradiated	7	1100	1	1
4	non-irradiated	96	7100	3	3
5	irradiated	21	670	2	2
6	non-irradiated	98	3700	3	3
7	irradiated	96	1200	2	2
8	non-irradiated	141	6200	1	1

TABLE 2. EXPERIMENT II—450 R.*

Rabbit	Treatment	Lymphocyte count	Keratitis		Iritis
			Clinical	Histologic	
11	irradiated	680	3	3	0
12	non-irradiated	3700	3	3	+
13	irradiated	960	1	2	0
14	non-irradiated	4300	3	3	+
15	irradiated	†	2	3	+
16	non-irradiated	4375	1	0	+
17	irradiated	1450	1	1	0
18	non-irradiated	6600	1	0	0

*The sera for neutralizing antibody titer determinations for this experiment were mishandled and thereby lost.

†This rabbit died on the twelfth day after virus inoculation. Blood was drawn from the other rabbits in this experiment on the thirteenth day, just prior to sacrifice.

TABLE 3. EXPERIMENT III—450 R.

Rabbit	Treatment	Neut. index	Lymphocyte count	Keratitis		Iritis
				Clinical	Histologic	
31	irradiated	46	840	2	3	0
32	non-irradiated	151	4350	1	0	0
33	irradiated	10	*	2	2	+
34	non-irradiated	151	3180	3	2	+
35	irradiated	21	1750	3	3	+
36	non-irradiated	151	3585	1	0	0
37	irradiated	18	1550	2	1	0
38	non-irradiated	100	*	1	1	0
39	irradiated	214	2385	3	3	0
40	non-irradiated	†	5080	1	1	0

*These two rabbits died on the twelfth day after virus inoculation. Anticoagulated blood for white cell and differential cell counts was always drawn on the thirteenth day. In this experiment, serum samples were obtained on the tenth day. Due to the deaths, these earlier sera were used to determine the neutralizing antibody titers for all the rabbits in this experiment.

†Serum lost in processing.

TABLE 4. COMBINED RESULTS WITH 450 R.

	Irradiated rabbits	Non-irradiated rabbits
Clinical grading		
Grade 1	3	7
Grade 2	6	1
Grade 3	4	5
Histologic grading		
Grade 0	0	3
Grade 1	3	3
Grade 2	4	2
Grade 3	6	4
Iritis		
Present	3	4
Absent	6	5

body response with stromal disease (Figure 3). No correlation existed between the antibody titer and the severity of the stromal lesions.

The dose of 550 r. that was given to the second series of rabbits produced approximately a 50 per cent mortality and was thus the maximum dose which could be used. Antibody suppression in the irradiated rabbits was more uniform, but the over-all results were the same as in the 450 r. series. Suppression of the immune response did not suppress either the stromal keratitis or the iritis (Table 6).

TABLE 5. EFFECT OF 450 R. ON THE IMMUNE KERATITIS STIMULATED BY INTRASTROMAL BOVINE SERUM ALBUMIN INJECTION

Rabbit	Treatment	Keratitis (onset of Wessely ring after injection)
41	irradiated	none (observed 23 days after injection)
42	non-irradiated	day 12
43	irradiated	*
44	non-irradiated	day 12
45	irradiated	day 17 (and ring barely visible)
46	non-irradiated	day 12
47	irradiated	day 19
48	non-irradiated	day 12

*This rabbit died on the ninth day after injection.

B. STUDIES ON THE ENDOTHELIUM

Materials and Methods

PREPARATION OF ENDOTHELIAL FLAT MOUNTS

In several of the experiments, the cornea of each eye was halved through the center of the most severe lesion. One half was fixed in formalin for routine sectioning and histologic study; the other half was fixed in a solution of three parts 95 per cent alcohol and one part glacial acetic acid for 24 hours. This second half-cornea was then placed on a slide with the endothelium facing up, stained for 60 seconds with coelestine blue, and mounted with Paragon* and a cover-slip.[14] The coelestine blue stain used in our laboratory has the following composition: coelestine blue, 1 Gm.; ferric ammonium sulfate, 3 Gm.; concentrated sulfuric acid, 2 ml.; glycerine, 10 ml.; water, 100 ml.

Observations on these flat endothelial preparations could be correlated with the clinical and routine histologic observations made on the same corneas. Controls included the endothelium of uninoculated eyes, eyes inoculated with a sterile virus medium, eyes inoculated with heat-inactivated virus, and eyes with keratitis and iritis due to intrastromal injection of BSA or to topical application of 20 per cent sodium hydroxide.

DETERMINING THE TIME COURSE OF ENDOTHELIAL CHANGES

In a separate group of rabbits, the corneal epithelium of both eyes was inoculated with virus. The progress of the endothelial changes was followed for from 2 to 14 days after inoculation by sacrificing the

*Paragon–water-soluble mounting medium, Paragon C. & C. Co., Inc., N.Y.

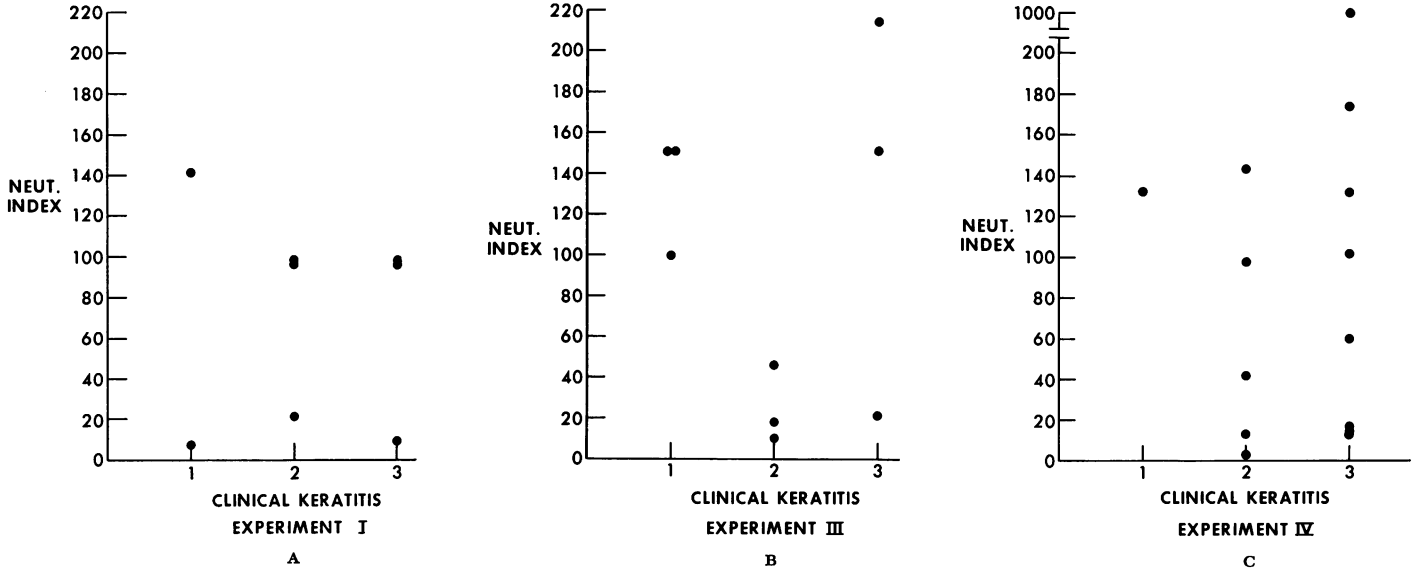


FIGURE 3

Graphs represent attempts to correlate clinical severity of stromal keratitis with magnitude of immune response (measured by serum neutralizing index). Correlation: None.

TABLE 6. EXPERIMENT IV—550 R.

Rabbit	Treatment	Neut. index	Keratitis		Iritis
			Clinical	Histologic	
51	irradiated	*	3	2	0
52	non-irradiated	144	2	2	0
53	irradiated	*	2	2	0
54	non-irradiated	98	2	1	0
55	irradiated	42	2	2	0
56	non-irradiated	132	1	1	0
57	irradiated	14	3	3	0
58	non-irradiated	132	3	2	0
59	irradiated	13	3	2	+
60	non-irradiated	60	3	2	0
61	irradiated	†	3	3	+
62	non-irradiated	174	3	3	0
63	irradiated	13	2	2	0
64	non-irradiated	1000	3	3	+
65	irradiated	‡	3	3	+
66	non-irradiated	102	3	2	0
67	irradiated	3	2	2	0
68	non-irradiated	§	2	2	0
69	irradiated	17	3	3	0

*These two rabbits died on the tenth day after inoculation.
†This rabbit died on the seventh day after virus inoculation.
‡This rabbit died on the eighth day after virus inoculation.
§Serum lost in processing.

rabbits on various days within this period. The right eyes were used for endothelial flat mounts and the left eyes for viral cultures of the endothelium.

CULTURES OF ENDOTHELIUM FOR IDENTIFICATION OF HERPES VIRUS

In order to minimize the possibility of contamination by virus from the epithelium, material for culture was collected as follows: The eye was enucleated, the perilimbal conjunctiva was dissected away, and the underlying sclera and limbus were swabbed with Wescodyne.* An incision was then made through the pars plana parallel to the limbus and was continued around the eye so that the anterior segment with intact lens-iris diaphragm could be lifted off and placed with the

*Wescodyne—organic iodide detergent germicide, West Chemical Products, Inc., Long Island City, N.Y.

TABLE 7. COMBINED RESULTS WITH 550 R.*

	Irradiated rabbits	Non-irradiated rabbits
Clinical grading		
Grade 1	0	1
Grade 2	4	2
Grade 3	6	6†
Histologic grading		
Grade 1	0	2
Grade 2	6	5
Grade 3	4	2
Iritis		
Present	3	1
Absent	7	8

*Neutralizing index: irradiated rabbits—42, 14, 13, 13, 3, 17; non-irradiated rabbits—144, 98, 132, 60, 174, 1000, 102.

†The uneven number of rabbits is due to an anesthetic death.

corneal epithelium down against the table. The iris was then grasped at its root and the iris–lens diaphragm was peeled off the cornea in one sheet. A sterile cotton pledget was next touched to the endothelium near the limbus to drain off excess aqueous. With a platinum spatula or a sterile cotton swab, the central endothelium was then scraped off and placed in minimal essential medium with 2 per cent calf serum. The combined endothelium and medium was transferred to Maben's cell line for viral culture.

Results

Interesting endothelial changes were observed in the herpes-infected corneas (Figure 4); they ranged from apparent cell death to cell

TABLE 8. ANTIBODY SUPPRESSION*

	Neutralizing index	
	<50	<50
450 r. experiment		
irradiated rabbits	2	7
non-irradiated rabbits	8	0
	$p < 0.01$	
550 r. experiment		
irradiated rabbits	0	6
non-irradiated rabbits	8	0
	$p < 0.01$	

*A neutralizing index is considered positive if it is greater than 50.[12]

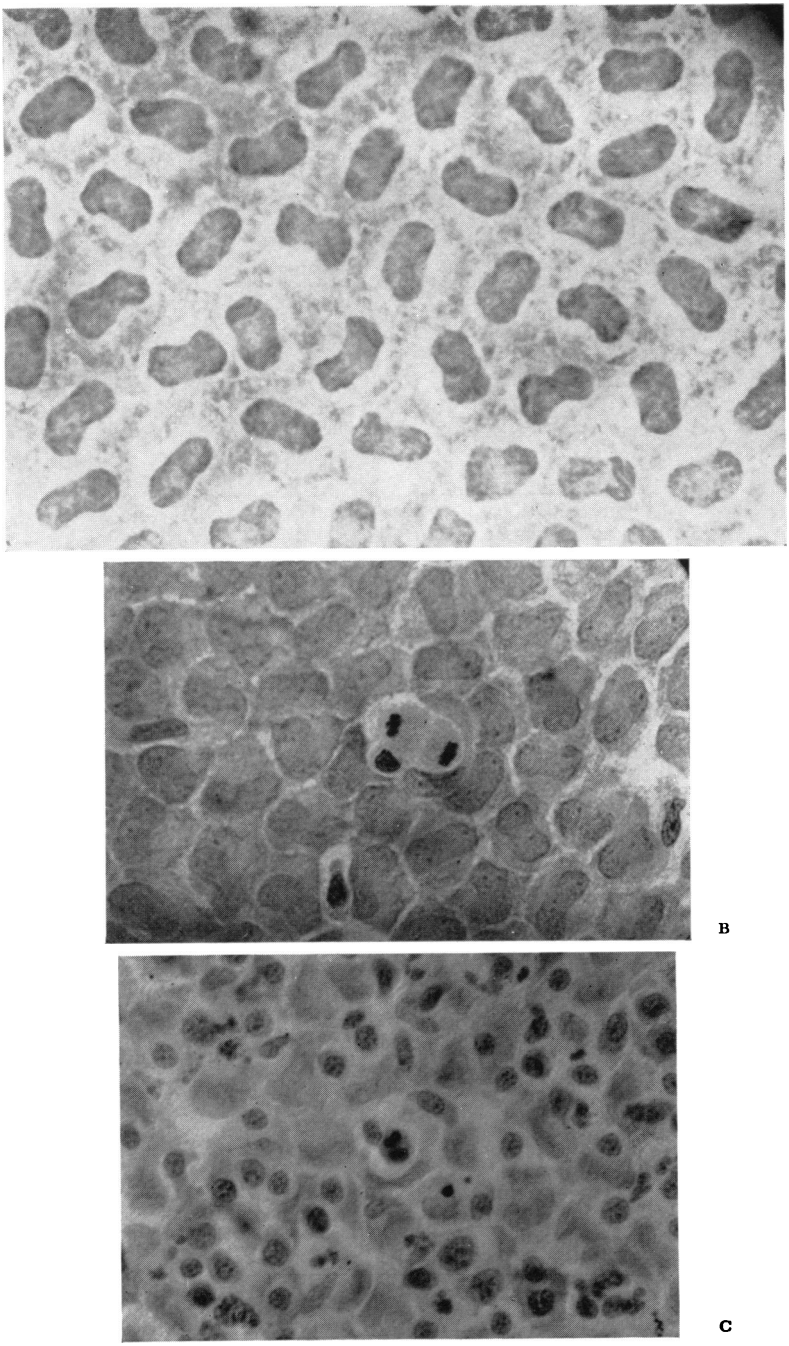
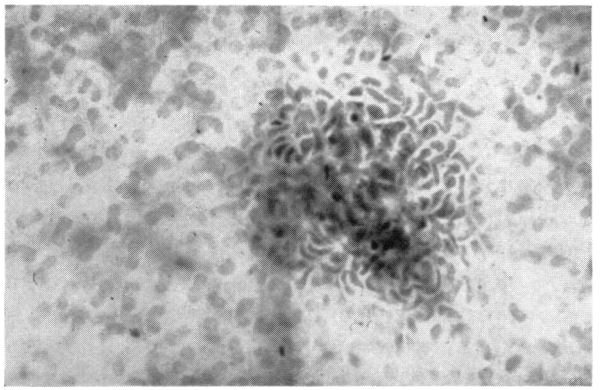


FIGURE 4. ENDOTHELIAL CHANGES.

A. Control eye inoculated with sterile virus medium, shows uniform appearance of endothelial cells. ($\times$ 250) B. Virus-inoculated cornea; note shrunken pyknotic cells in vicinity of



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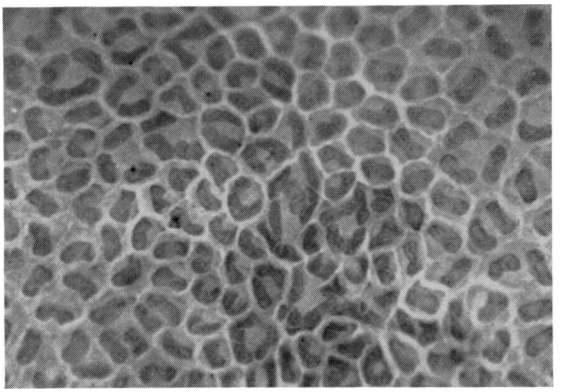

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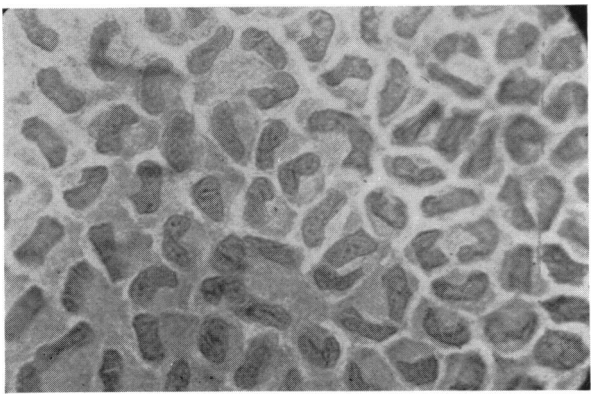

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cell undergoing mitosis. (× 1000) C. Virus-inoculated cornea; note area of severe endothelial damage showing marked nuclear pleomorphism. (× 400) D. Virus-inoculated cornea; note proliferation of endothelial cells forming mound of cells projecting into aqueous; pyknotic cells are scattered around base of mound. (× 250) E. Narrow ridge of three-dimensional proliferation containing multinucleated giant cells; note apparently normal endothelial cells on both sides of focal area of abnormal proliferation. (× 250) F. Higher magnification shows giant cells in area of focal three-dimensional proliferation. (× 400)

multiplication with mitoses, multinucleated giant cells, and focal proliferation away from the normal flat plane so as to produce localized mounds of endothelial cells projecting into the aqueous. This focal proliferation resembled that described as occurring in tissue culture monolayers subjected to chronic herpes simplex infection.[15] Such changes were not found in the controls which were uninoculated, inoculated with a sterile virus medium, or inoculated with heat-inactivated virus. However, they could be produced in both the BSA-injected and the sodium-hydroxide–treated corneas. They thus appear to be non-specific signs of endothelial damage and regeneration, differing from the endothelial changes noted by previous authors in various types of anterior chamber inflammation.[16]

Endothelial changes were noted as early as four days after epithelial virus inoculation, but at this early time the affected cells were few and widely scattered. By the eighth or tenth day after inoculation, many rabbits showed areas of severe endothelial damage in which more than 50 per cent of the cells were affected. The presence of such severe damage correlated positively with the occurrence of deep stromal edema. Although difficult to quantitate, this correlation was manifest as follows: (1) So far as the individual rabbit was concerned, it was apparent in the experiments in which all the rabbits were sacrificed 13 days after inoculation. (2) So far as the time of onset of the endothelial changes was concerned, it was apparent in the experiments in which the rabbits were sacrificed at two-day intervals following inoculation.

Cultures of the endothelium were positive for herpes virus at 2, 4, and 6 days after epithelial inoculation, but were uniformly negative thereafter.

DISCUSSION

Three types of stromal lesions are seen in herpetic keratitis in rabbits. The first is a mild, superficial haze strictly limited to the area underlying the epithelial ulcer and disappearing when the ulcer heals. A similar superficial haze is seen after merely wiping off an area of epithelium with a cotton swab. The second type of lesion is a deep stromal edema, not limited spatially or temporally by the epithelial ulcer. In the present study, this edema was seen to correlate with the severity of endothelial damage. The third type of lesion is dense white stromal necrosis, which begins superficially and extends for varying depths into the stroma.

When an immune disciform keratitis has been produced experimentally by topical application of antigen, the severity of the keratitis

has correlated very well with the serum antibody response.[3] In our experiments with herpes virus, however, neither the deep stromal edema nor the stromal necrosis was ameliorated by suppression of the rabbit's antibody response. It would thus seem that in herpes-infected corneas these two lesions are probably not dependent upon a specific immune response to viral antigen but rather are caused by the presence of live virus within the endothelial and stromal cells, respectively, with resultant cell damage and non-specific inflammatory response. The same reasoning appears to hold true for the iritis observed in these experiments.

Whereas herpes simplex was previously believed to be strictly epitheliotropic, this concept has more recently proven false. Herpes virus has been demonstrated in corneal stromal cells by fluorescent antibody in rabbits[17] and by electron microscopy in both rabbits and humans.[18] It has been cultured from the endothelium and from the ciliary body.[18,19]

In the present study it was noted that, in corneas with a well-developed central "disciform" area of stromal edema, the central endothelium seemed most severely damaged. However, when rabbits were sacrificed early, the endothelial changes first appeared near the limbus in many cases. This difference was difficult to measure, but it might indicate that the virus penetrates to the endothelium more rapidly at the limbus than centrally. This would be in keeping with the fluorescent antibody studies which have shown that in the central cornea the virus is usually restricted to the superficial stroma.

SUMMARY AND CONCLUSIONS

1. An attempt was made in rabbits to assess the role of systemic hypersensitivity in the production of stromal lesions in herpes simplex keratitis. Suppression of the immune response by total body X-irradiation, with the eyes shielded, did not diminish the stromal lesions. It would thus seem improbable that hypersensitivity plays the major role in their production.

2. Endothelial changes were observed that seemed to correlate with the time of appearance and the severity of the deep stromal edema. Although the endothelial damage was non-specific morphologically, the fact that it was not affected by suppression of the immune response and that virus could be cultivated regularly from the endothelium suggested that the damage is probably due to the presence of virus within the endothelial cells.

3. These findings support the concept, previously proposed by

others,[19] that, at least with some strains of *Herpesvirus hominus*, direct endothelial damage by living virus is more important than immune mechanisms in producing the stromal edema of disciform herpes simplex keratitis.

ACKNOWLEDGMENTS

Mr. Gerald Collins refined the endothelial staining techniques as presently used in this laboratory and guided the authors in their use.

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DISCUSSION

DR. WILLIAM F. HUGHES, JR. It is a pleasure to open the discussion of a paper whose senior author is a junior member of the distinguished Irvine clan. Even more, it is good to see them continuing their academic leanings, and under the direction of an honest scientist who is the junior author. The authors were good enough to send me not one but two copies of the manuscript with illustrations, and I took the liberty of having Dr. A. W. Holmes, virologist at our hospital, read it over for his comment.

Stromal herpes keratitis has been theoretically attributed by some to the stroma acting as an immunologic blotter which reacts with herpes antigen. However, the virus has been demonstrated (mostly in rabbits) by electron microscopy, fluorescent antibody methods, and culture in all layers of the cornea and ciliary body. The present paper certainly indicates that the virus damages the endothelium in acute herpetic keratitis in rabbits.

In spite of the elaborate precautions which the authors took to prevent contamination by virus from other ocular tissues in culturing the endothelium, it is theoretically possible. Have they done or do they know of other methods such as the fluorescein antibody technique or electron microscopy which have also demonstrated the virus in the endothelium? It would be reasonable to assume that the virus spreads directly through the cornea to the endothelium. The deleterious spreading action of local steroids in dendritic ulcer would support this idea. However, the authors' observation that the peripheral endothelium is affected first might indicate that the virus arrives *via* the limbal vessels or aqueous. Have blood cultures been performed to rule out an early viremia, so common in most virus infections?

The present studies, using the method of suppressing both humoral and cellular antibodies by total body irradiation excluding the eye, indicate that the severity of the keratitis in acute herpetic keratitis in non-immune animals is not affected. Holmes made a technical comment that failure to inactivate sera used in neutralization tests might allow non-specific inhibitors to react. He thought it would be better to add exogenous (i.e., guinea pig) complement to the reaction mixture for neutralization.

In a study more comparable to the stromal disease in adult humans who have circulating antibodies, Taktikos and Aurelian recently showed that the level of circulating antibodies in immune rabbits was not related to the severity of the stromal disease as one might expect if this were an antigen-antibody reaction. The only possibility would be that the virus changes the antigenicity of the cornea itself, but such an autohypersensitivity with anti-corneal antibodies has not been shown in herpetic infections. Also, the pathology of stromal herpes does not suggest an autohypersensitivity.

In conclusion, one must say that acute herpes infections in non-immune rabbits do not have all of the characteristics of either a simple dendritic

ulcer or a chronic stromal keratitis in humans. Outside of fewer takes and less severe infection, the clinical picture in immune rabbits is also somewhat different from humans. However, this paper's nice demonstration of endothelial damage by the virus can easily explain the most common form of stromal herpes in humans, viz., an acute and then chronic edema of the stroma, modified by secondary factors such as the use of steroids, secondary infection, ulceration, vascularization, and iritis. Like eternal youth, the theory of hypersensitivity as a cause of stromal herpes is fast fading.

DR. LUDWIG VON SALLMANN. I greatly enjoyed this paper. I understand the authors do not consider the endothelial changes as virus-induced or specific. Several years ago, when I worked with prolonged perfusion of the anterior chamber with a balanced salt solution, cytopathologic changes of the endothelium were produced which were almost identical with those shown by the authors—giant nuclei, mutlinucleated cells, and various forms of cell degeneration as the response to the non-specific but possibly irritating stimulus. This observation strengthens the impression of the authors.

DR. LORENZ E. ZIMMERMAN. A few years ago before this Society, Drs. Hogan, Kimura, and Thygeson,[1] in reporting on the pathology of stromal herpes, described a granulomatous reaction around Descemet's membrane. We have seen this reaction in a number of cases of chronic ulcerative and deep keratitis,[2] and we have been particularly impressed with the frequency with which this reaction is seen in cases that appear to be stromal herpes.[3] I am particularly anxious to learn whether, in these experimental studies, this same deep granulomatous reaction centered on Descemet's membrane has been observed.

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DR. ALSON E. BRALEY. Before we bury hypersensitivity completely, I feel compelled to say at least a few words. This study is in the rabbit, and to me there is no doubt that the virus does invade deeply in the rabbit. I think it has been demonstrated in several tissues in the stroma. I do not know how many of you remember the Gifford Lecture that I gave on herpes some time ago in which I theorized on the subject of delayed hypersensitivity. In this purely theoretical discussion, I included the type of hypersensitivity in which antibodies reacted with the herpes virus. Since the virus does invade the stroma, and since there is a form of hypersensitivity that does occur in herpes, particularly in the human, I feel we cannot bury the theory completely, although I will prepare a casket.

DR. ALEXANDER IRVINE. I want to thank Dr. Hughes and the other discussers. Dr. Hughes asked some very good questions; some of them are the questions we have been asking ourselves.

First, he asked if we have been able to demonstrate the virus in the endothelium by any method other than cultures. We have tried immunofluorescence and to date have not been able to get good flat preparations of the endothelium that were free of the terrific non-specific fluorescence of Descemet's membrane, so we have not had any luck yet with a technique by which we could study the endothelial cell by the immunofluorescent method. Nor have we turned the electron microscope on the endothelium in these rabbits yet, but I think it is important to mention, as Dr. Hughes stated, that the electron microscope now has shown herpes virus in human corneal stroma. Dr. Chandler Dawson, at the University of California in San Francisco, is studying the corneal buttons removed from herpes cases at keratoplasty, and he has found herpes deep in the stroma in five human cases. He has not found virus in the endothelium, but then the cases have been ones of late corneal scarring, not of reversible disciform stromal edema. He has often found residua of severe damage in the endothelium and Descemet's membrane. We thus are left with cultures as the only evidence that the endothelial damage we found was caused by a viral presence; and, admittedly, the possibility of contamination exists. In view of the way the cultures were taken, however, the only likely source of contamination was the aqueous or the anterior surface of the iris. Since attempts to culture virus directly from aqueous were futile, it does not seem likely that this could be a source of contamination.

The second question was whether the virus might spread to the endothelium by a hematogenous route, rather than through the cornea or limbus. There certainly is a viremia in rabbits about two to three days after epithelial virus inoculation. However, I think the fact that the opposite eye, which served as a control, never showed any endothelial involvement would tend to rule out simple viremia as the method of spread from epithelium to endothelium.

The question of heat inactivation of the serum is a good one. Yoshino has shown that in order to measure the total early antibody response to herpes in the rabbit he had to use serum that was not heat-inactivated, since the early response was largely complement-dependent. As Dr. Hughes has suggested, it would have been preferable had we heated the sera and then added a standard amount of guinea pig complement, rather than merely relying on the rabbit complement in the unheated sera. I do not believe, however, that this would have made any significant difference in the relative size of the antibody titers within our experiment.

Our emphasis upon endothelial damage and upon the presence of culturable virus in the endothelium may remind one of the long-known entity of "herpes corneae posterior." In Dr. Stocker's monograph I believe he indicated this condition is probably misnamed and not related to the actual endothelial infection with herpes virus we are discussing today. Since I see

Dr. Stocker is here this morning, I hope he will comment on his present feelings regarding this.

Dr. von Sallmann questioned the specificity of the endothelial damage. We are forced to agree with him. At first we thought the giant cells and other changes that we found were virus-specific, but more recently we have found similar changes in control eyes which had a keratitis due to sodium hydroxide or bovine serum albumin. So, we are really only saying that in herpetic keratitis there is endothelial damage which correlates in timing and severity with the deep stromal edema. We believe that this endothelial damage, rather than any immune reaction in the stroma, plays the main role in production of "disciform stromal edema." In proposing that this endothelial damage is caused by live virus, we are basing our claim on our ability to culture virus from the endothelium and on the fact that the process is not inhibited by immune suppression.

Dr. Zimmerman asked whether we had found a granulomatous reaction around Descemet's membrane similar to what Dr. Hogan has described. In the routine histologic sections of the corneas we did not find any such granulomatous reactions in the stroma or on Descemet's membrane. The closest thing we have seen to it are these endothelial cells that look like Langhans' giant cells. Perhaps this is the difference between a primary subacute keratitis in the rabbit and the recurrent or quiescent late keratitis that Dr. Hogan was observing in humans.

Dr. Braley, I do not think we can totally bury the hypersensitivity mechanism, but I think we can say that this is not the major factor in the rabbit. If it were playing a major role, we could not vary the hypersensitivity response so greatly without varying the keratitis at all.

Some prominent investigators have stated that, since herpes is an intracellular microorganism, it can do no more than kill cells, and that, therefore, it could not possibly account for the collagen necrosis and other extracellular damage seen in stromal herpes. They state that cell death alone does not lead to such extracellular damage, since one can freeze-thaw corneas, killing the stromal cells, and yet maintain stromal transparency. In order to account for this extracellular damage, they feel forced to invoke a hypersensitivity mechanism. I believe our study has shown that such reasoning is false. Herpes virus is capable of bringing about extracellular damage without relying on a hypersensitivity mechanism. Although the means by which it does so are not yet fully understood, many can be postulated.

If our work proves to have any value, I think it will be this. We have confirmed the finding that *Herpesvirus hominis* can produce stromal disease without relying on an antigen-specific hypersensitivity mechanism, and we have pointed to the endothelial damage and its role in the production of disciform herpetic keratitis. Neither of these points is original, but in the present state of controversy regarding herpetic keratitis, they warrant our affirmation.