EPIDEMIC KERATOCONJUNCTIVITIS*‡

I. Isolation and Identification of a Filterable Virus§

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Plates 5 to 7

(Received for publication, September 25, 1942)

The problem of epidemic keratoconjunctivitis has gained prominence in recent years, because of the heavy incidence of this disease in shipyards and other places where industries are concentrated. Aside from the fact that a menace to defense endeavors may be important during time of war, any disease which can temporarily incapacitate thousands of individuals, which is epidemic in character, and may produce some permanent reduction of vision, must be considered an important danger to public health. There is no doubt that this infection, hitherto of unknown etiology, is a disease *sui generis*, and can be differentiated from other conditions affecting the cornea and the conjunctiva. For a complete clinical picture and differential diagnosis, the report of Hogan and Crawford (1) describing the 1941 epidemic on the California coast, and of Rieke (2) describing 600 cases in Oregon, may be consulted. In the Institute of Ophthalmology, the criteria which have been considered characteristic of epidemic keratoconjunctivitis are as follows: an acute follicular conjunctivitis with a scanty exudate; preauricular lymph node enlargement and tenderness; negative bacteriology; mononuclear cellular exudate; and a punctate keratitis, with lesions of varying size. This corneal involvement, occurring 1 week to 10 days after the onset of the disease, is found in 30 to 80 per cent of the cases.

In a previous report (3), preliminary findings were given concerning the recovery of an infectious agent from two patients suffering with epidemic keratoconjunctivitis. Evidence was presented suggesting that the agent is a virus, and its neutralization by serum from patients convalescent from the

* Aided by grants from Knapp Memorial Foundation in Ophthalmology, the John and Mary R. Markle Foundation, and the Warner Institute for Therapeutic Research.

‡ This investigation was carried on in informal collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the United States Army.

§ The authors wish to extend their thanks to Dr. Phillips Thygeson for his kind cooperation in this investigation.

disease indicated a specific relationship between the virus and epidemic keratoconjunctivitis. It was noted also that the virus had been maintained in mice only after preliminary passage through tissue culture, and that the mouse virus had been returned to the eye of a human volunteer with the production of ocular changes characteristic of epidemic keratoconjunctivitis.

The present communication, which contains additional data, as well as the details of data previously reported, may be divided into two phases. The first phase, the study of the virus itself, is concerned with the technique of isolation, the behavior of the virus in laboratory animals, its activity in tissue culture, and its apparent size, based on ultrafiltration experiments. The second phase of the investigation, the identification of the virus, deals with neutralization tests, with the reproduction of the typical clinical picture of epidemic keratoconjunctivitis by inoculation of the mouse agent into the eye of a human volunteer, and with tests for the exclusion of certain viruses.

ISOLATION AND PASSAGE OF VIRUS

Technique of Isolation

While the incidence of keratoconjunctivitis in New York City in no way compared with the epidemic on the West Coast, there appeared to be several hundred cases in the city, most of which were treated by private physicians. In this clinic, eleven cases were studied experimentally, and in nine of these, the diagnosis seemed definite. In the two remaining cases some reasonable doubt remained as to the nature of the conjunctivitis, because of its short duration and the absence of corneal involvement.

The routine procedure which was followed was to inject conjunctival scrapings from the patients into white Swiss mice, intracerebrally as well as intraperitoneally. On two occasions rabbits were injected intracerebrally and intracorneally; twice material was passed in the yolk sac or on the chorioallantois of fertilized eggs. Although pathogenic effects were occasionally observed in a rabbit or in mice injected with egg preparations, the results in general were so confusing and indefinite that both procedures were discontinued in favor of mouse inoculation.

It was early noticed that the conjunctival scrapings, although bacteriologically sterile, produced a distinct but transient effect in mice. In six of the experimentally studied cases, definite but rather vague symptoms appeared in the injected mice in 48 to 72 hours. Most noticeable features of the syndrome were rough coat and lethargy, accompanied by a tendency towards a humped back. The mice recovered in a majority of cases, however, and it was found that the symptoms could be transmitted successfully for only two, three, four, or five mouse passages. Because there was evidence that a nonbacterial agent was being transmitted from patients to mice, by means of the

conjunctival scrapings, but that the transmission was temporary and could not be maintained serially in mice, passage through tissue culture[1] was added to the above procedure, when a case in the early stages of the disease was seen in the clinic. Since this new case was the source of the first virus isolation, a complete history is given.

Case History 1.—Patient E.L., aged 31, a resident physician on an Eye, Ear, Nose, and Throat Service in one of the local hospitals, was first seen on Mar. 18, 1942. He gave the following history.

Mar. 9, 1942. The first symptom noticed was a scratchy sensation in the left eye, as if a foreign body were present. Within 48 hours edema, particularly of the upper lid, and lacrimation were marked. This physician had no known contact with patients suffering with epidemic keratoconjunctivitis, although there were several such cases in his hospital.

Mar. 13. 4 days after the onset of symptoms the preauricular node on the affected side was palpable, and chewing was moderately painful. Previous symptoms became more marked.

Mar. 14. Vision was blurred, and the eye could be opened only by prying the lids apart with the fingers. The preauricular node was by this time more enlarged; chewing was quite painful.

Mar. 15. The patient was hospitalized. The eye was now completely closed, and signs of corneal involvement, *i.e.* photophobia and severe pain, were present. The second eye also became involved, with lacrimation and a scratchy sensation present. No preauricular node was noted, and the symptoms were generally less severe than in the left eye.

Mar. 16. Symptoms in both eyes abated slightly.

Mar. 18. The inoculation tests described below were made. The patient presented the picture usually seen in epidemic keratoconjunctivitis. The mucous membrane of the lower lid was hypertrophied and edematous, and chemosis was marked. In keeping with the photophobia and pain, a slit-lamp examination revealed several corneal opacities, punctate in character, and of varying size. The preauricular node on the side of the eye first affected was tender and enlarged to the size of a small almond. On the same side, the cervical, posterior auricular, and supraclavicular glands could also be palpated. Although these glands were not as large as the preauricular gland, they were, nevertheless, appreciably bigger than the corresponding glands on the opposite side.

Mar. 20. Typical corneal involvement was noted, with superficial punctate lesions that were macular in character. Vision was materially decreased. The patient did not come to the clinic for a visual examination.

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[1] Throughout this study, the term "tissue culture" refers to serum ultrafiltrate, minced embryonic mouse brain preparations, which have been described elsewhere (4, 5). To ensure sterility, the tissue cultures were incubated 48 hours prior to inoculation with virus. The serum ultrafiltrate was prepared by the Warner Institute for Therapeutic Research (associated with the William R. Warner Company, Inc.), and was kindly provided by that organization for use in this investigation.

Later, the patient stated that his vision had improved about two months after the onset of the disease. No further details were available.

Note: The clinical appearance of this patient closely conformed to the criteria of epidemic keratoconjunctivitis considered pathognomonic at the Ophthalmological Institute of Presbyterian Hospital. The actual diagnosis was made by Dr. Phillips Thygeson.

The conjunctival scrapings taken from patient E.L. on Mar. 18 were bacteriologically sterile, and contained large mononuclear cells (Fig. 1). According to the procedure followed in the cases studied previously, the scrapings were immediately put into 1 cc. of Simms buffered salt solution (5), and within half an hour, 0.1 cc. of this material was injected intraperitoneally and 0.03 cc. intracerebrally into mice, each animal being injected by both routes. As can be seen in Diagram 1, two of the three mice which received these scrapings showed symptoms within 2 days. The symptoms were similar to those seen in the mice which had been injected with conjunctival scrapings from other cases of epidemic keratoconjunctivitis, as described earlier in this report. Brain and spleen from one of these two sick animals were injected intracerebrally (0.02 cc.) into six unweaned mice, intracerebrally (0.03 cc.) and intraperitoneally (0.1 cc.) into three adult mice, and intracerebrally into two monkeys and one rabbit. In addition to the intracerebral injections the rabbit and one of the monkeys received an emulsion of brain and spleen tissue rubbed into the scarified surface of their right eyes. None of these animals showed symptoms.

However, the picture was entirely different in the case of the six mice which had received emulsion of brain and spleen tissue from the other of the original two sick animals. Two animals in this group became sick in 3 days; two were found dead in 7 days and were discarded. The two mice with symptoms were sacrificed, and their brains were pooled and emulsified. This emulsion was injected intracerebrally (0.02 cc.) into seven unweaned mice, intraperitoneally (0.1 cc.) and intracerebrally (0.03 cc.) into six adult mice, and passed into a sterile tissue culture of embryonic mouse brain and serum ultrafiltrate. As is evident from the diagram, the only animal to show symptoms was one of the adult mice, which became sick in 2 days. The infection could be maintained for only one more passage. The tissue culture, which had also received the brain emulsion from the mouse which was sick on Mar. 23, was incubated for 3 days at 37°C. At the end of that time, ground-up cells plus the supernatant fluid of the culture were injected intraperitoneally (0.1 cc.) and intracerebrally (0.03 cc.) into each of six mice. The symptoms these mice subsequently developed were more severe than the symptoms which had appeared in any of the previously injected animals, which, it will be remembered, had received either human conjunctival scrapings or emulsion of brain and spleen from sick mice. Two of these mice died. The remaining animals, all of which were ill, became the source of *mouse* stock 1, now in its 66th passage.

It should be emphasized that from the point in the scheme of isolation designated as "mouse stock" (Diagram 1), which was reached after passage through tissue culture, the activity of the infectious agent in mice was such as to suggest an increase in potency. Whereas on previous occasions, when passage of human eye material from mouse to mouse resulted in a progressively diminished infectivity, now *all* of the

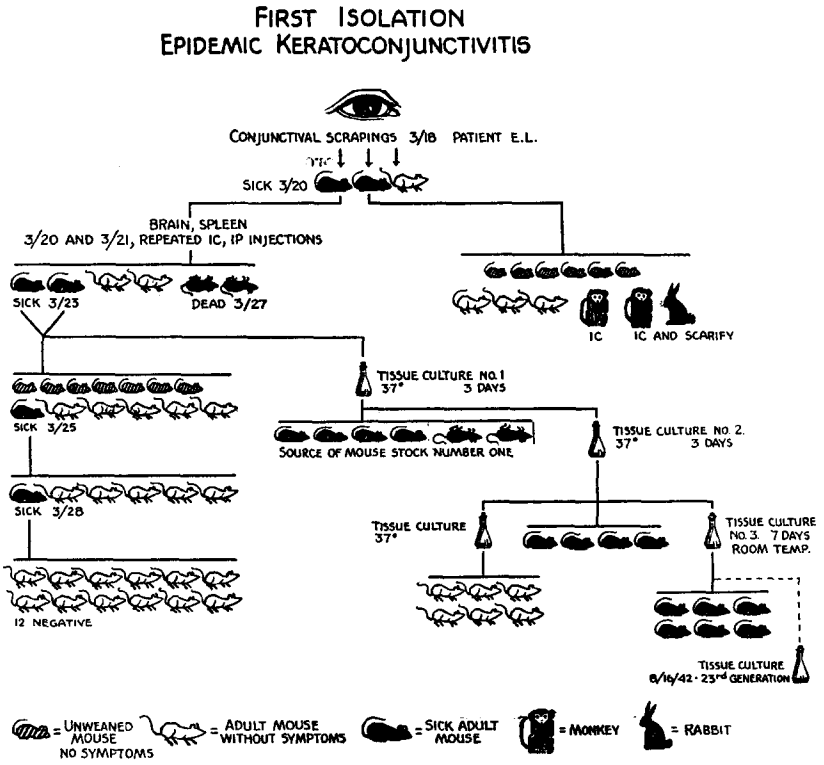

DIAGRAM 1

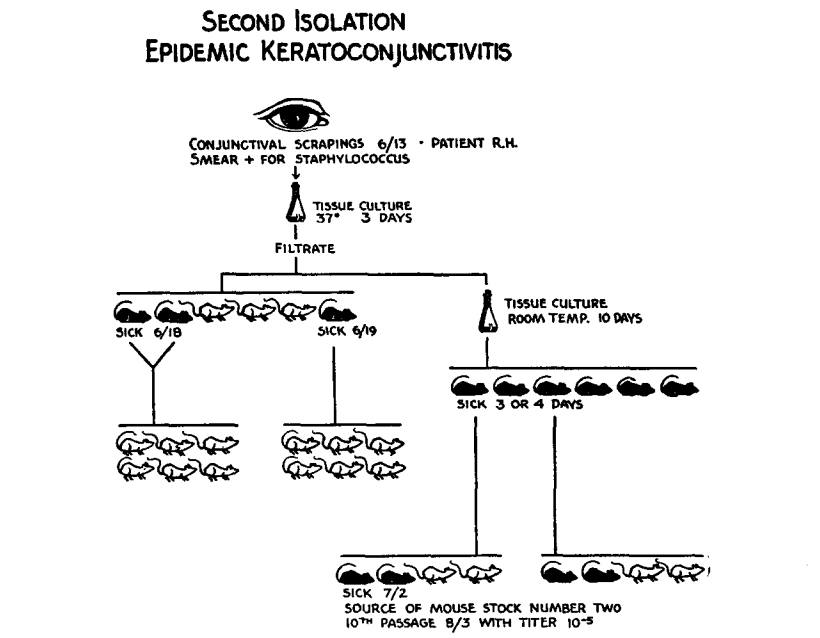

DIAGRAM 2

DIAGRAMS 1 and 2. From the points designated as sources of mouse stock 1 and 2, an agent was obtained which was consistently infectious for mice; after a definite incubation period the mice exhibited a characteristic chain of symptoms which, unlike the transient syndrome seen previously, in all cases led to death. Almost all the injected animals were affected in this manner, and there were no convalescents.

animals injected with the "stock mouse virus" became ill with much more severe symptoms, and, unless sacrificed for subpassage, died. True, the incubation period remained the same (2 to 3 days), but the effect in mice was more definitely a disease entity in that a dependable sequence of *illness* and *death* took place.

½ cc. of the emulsified tissue culture was also transferred to subcultures, which were incubated at 37°C. for 3 days. At the end of this incubation period, 0.03 cc. of the subculture material was injected intracerebrally into each of four mice, causing illness and death in all of them. A further 0.5 cc. transfer was made to two series of tissue cultures. One of these was incubated at 37°C. for 3 days; the other was kept at room temperature for 7 days. The 37°C. cultures were discontinued when the combined intracerebral (0.03 cc.) and intraperitoneal (0.1 cc.) injections failed to infect mice. The series kept at room temperature, however, has been passed to fresh cultures every 7 days. It is consistently infectious for mice, and is the source of *culture* stock 1, now in its 31st generation.

Since both the stock culture and passage viruses will be discussed later, it might be well at this point to deal with the second isolation.

Case History 2.—Patient R.H., aged 58, had been treated at the clinic for about 1 month for an infected chalazion. His general health was not very good, and he appeared to be in a state bordering on malnutrition. He was first seen by us on June 13, 1942. His clinic report is as follows:—

June 5, 1942. Edema of the lids, along with some chemosis of the conjunctiva, was noticeable.

June 6. The conjunctiva, markedly injected and with a follicular reaction, had the appearance characteristic of epidemic keratoconjunctivitis.

June 8. In the morning some secretion was observed, with mucous shreds in the lower cul-de-sac. Culture of secretions taken on this day showed *Staphylococcus aureus*. The preauricular gland was enlarged and tender.

June 11. Marked photophobia and lacrimation were present.

June 13. Additional complications were added to the above picture: anorexia, weakness, pain in the left lower chest, temperature of 101°F. The general symptoms might have been quite independent of the eye affection.

June 15. Multiple punctate corneal opacities were observed.

Aug. 6. During the past month and a half little change was seen. Conjunctivitis with one corneal subepithelial infiltrate near center was present. Preauricular gland was still tender, but questionably palpable.

Aug. 24. Cornea was unchanged.

Sept. 28. Some improvement had occurred and the cornea was fairly clear.

Note: The clinical appearance of this patient closely conformed to the criteria of epidemic keratoconjunctivitis considered pathognomonic at the Ophthalmological Institute of Presbyterian Hospital. The actual diagnosis was made by Dr. Phillips Thygeson.

Conjunctival scrapings taken from R.H.'s eye on June 13 were put into tissue culture and incubated for 3 days at 37°C. (Diagram 2). Although sterility tests showed the presence of staphylococci in the culture, the tissue was ground, diluted

with culture fluid, and filtered through a Berkefeld N filter. When injected intracerebrally (0.03 cc.) and intraperitoneally (0.1 cc.) the filtrate produced symptoms in three of six mice. However, the symptoms could not be transmitted to other mice. The filtrate was also passed to a tissue culture which was kept at room temperature for 10 days. When this tissue culture was emulsified and injected intracerebrally (0.03 cc.) into six mice, they all became ill within 3 or 4 days. Two of these animals were sacrificed, and brain emulsion from each was passed into four mice by intracerebral inoculation. In each case, two of the four injected mice showed symptoms, and the sick animals became the source of *mouse* stock 2. This mouse stock, now in its 15th passage, has a titer of 10^{-5}.

The second isolation was not maintained in tissue culture, since the original culture became contaminated in the ice box, and it appeared advisable to concentrate our efforts on the first stock cultures.

Thus, in two instances, it was necessary to pass material from patients through tissue culture and thence into mice, before stable strains of an infectious agent were isolated which could be studied in laboratory animals. If this procedure was not followed, symptoms were not consistently produced in mice. The point of enhanced pathogenicity following the tissue culture passage has been designated as "mouse stock" and from this point death followed in 24 hours the appearance of symptoms. No convalescent animals, therefore, were obtained. Throughout this study titrations have been estimated on the basis of the final dilution of virus capable of killing a majority of the mice injected with it. The titer is essentially a 50 per cent end-point, but it is based on mortality rather than on morbidity. That these two agents belonged to the group of virus infections soon became evident, when it was found that they failed to grow in cell-free media, in synthetic media, or in 10 per cent serum broth; when routine examination of the animal tissue proved it to be bacteriologically sterile; when dark field examination of the various media was negative, and when it was found that the agents could be filtered without difficulty. It now remained to study the activity of the virus in laboratory animals, and to establish a specific relationship between the isolated agent and epidemic keratoconjunctivitis.

Behavior of the Virus in Laboratory Animals

Once the virus had become adapted by way of tissue culture to mice, its activity in laboratory animals was readily defined. The host range investigated included mice, rabbits, monkeys, rats, and guinea pigs. The most susceptible host, and certainly the most convenient one to study, was the white Swiss mouse. Consequently more information is available concerning the action of the virus in this animal than in others. Whereas both strains have been studied in mice, only the virus of the first isolation has been followed in other hosts.

Pathogenicity for Mice.—The adapted virus has been consistently patho-

genic for mice, and within a definite incubation period produces symptoms that lead to death. The dilution activity of the virus has varied from 10^{-5} to 10^{-6}. Although no chemical studies have been done, it was noted that the virus could be preserved for at least 3 months in infected mouse brains kept on carbon dioxide ice.

Mice intracerebrally injected begin to show symptoms in 2 to 3 days for the lower dilutions, and in 5 to 7 days for the higher dilutions. At first the animal is lethargic and has a humped back and a ruffled coat (Fig. 2). Death may follow within a few hours after the onset of lethargy, but in a majority of mice, death is preceded by various types of focal nervous symptoms. The nervous manifestations include tonic and clonic convulsions, sometimes closely resembling those seen in mice which have received intraperitoneal injections of phenolized material. Occasionally, spastic paralyses occur. Another symptom which is sufficiently common to be noteworthy, is a peculiar

TABLE I

Distribution of Keratoconjunctivitis Virus in Mice Following Infection by Intracerebral or Intranasal Routes

Route of infection	Tissue tested					
	Brain	Liver	Spleen	Lungs	Kidney	Blood
Intracerebral	10^{-5} 10^{-6}	−	+*	−	−	−
Intranasal	10^{-3}	−	trace	−	−	−

* Organs other than the brain were tested only for the presence of virus.

sidling gait seen in about 15 per cent of the infected mice. Such animals have a normal gait for 2 or 3 days, and then develop a rotating and sidling gait suggestive of middle ear infection.

In adult mice, infection may be transmitted serially by only the intracerebral and, to a lesser extent, the intranasal routes. Unweaned mice, however, may be infected by the intraperitoneal route. Infection following intranasal injection in adult mice and intraperitoneal injection in unweaned mice, is characterized by an incubation period 1 to 2 days longer than that observed after intracerebral injection. In all cases the symptoms are the same.

An attempt was made to study the distribution of the virus in infected mice. In Table I are the composite data from several such experiments. It is clear that in the case of intracerebrally infected mice, virus could be recovered from brain tissue and, to a slight extent, from the spleen. In mice infected intranasally, there was little demonstrable virus in the spleen. The simplest explanation for this would be that the virus propagates to a greater degree in mice infected intracerebrally. That this is the case is indicated by the differ-

ence in brain titers, the titer in the case of mice infected intracerebrally being 10^{-5} and 10^{-6}, and in the case of mice infected intranasally, 10^{-3}.

As was reported previously, the pathological picture in mice is not striking, and, in fact, shows a relatively mild degree of structural change, considering the capacity of the virus to kill mice. Because little additional information is as yet available concerning the pathology in mice, the findings are essentially those described in the preliminary report (3). The only variations from normal occurred in the central nervous system, in which lesions were scattered diffusely and irregularly through both the gray and white matter. Small inflammatory foci, consisting for the most part of perivascular infiltration by lymphocytes, with occasional polymorphonuclear leukocytes (Figs. 3 and 5), are the principal lesions. A similar infiltration is found to some extent in the perivascular parenchyma (Fig. 4), and occasionally neural elements show degenerative changes (Fig. 6). Early proliferation of microglia cells is found.

Pathogenicity for Rabbits.—As was noted previously, during the period when the virus was being isolated from man, suggestive symptoms were observed in rabbits injected intracerebrally with material from patients' eyes. However, it was not possible to transmit the infection from rabbit to rabbit, or from rabbit to mouse, and the study of this host was temporarily abandoned. When the fixed mouse virus was obtained, its pathogenicity for the rabbit was reexamined with the following results: A 1:50 dilution of 22nd mouse passage brain emulsion injected intracerebrally into four rabbits produced prostration and death in three of the animals within 9 days. In spite of the long incubation period, the infection appeared to be overwhelming, since the animals showed no symptoms for 8 days, and were then prostrated or dead on the 9th day. No focal signs, except for an occasional convulsion, were observed. A 1:50 dilution of emulsion of the pooled brains of two of these rabbits was injected intracerebrally into four guinea pigs, four rats, and two rabbits. Of these animals, only the two rabbits showed symptoms, the incubation period on this occasion being 11 days. The symptoms were similar to those seen previously.

That the agent responsible for the symptoms was similar to the fixed mouse virus was shown by the fact that the rabbit brains were bacteriologically sterile, and that the agent which infected this second group of rabbits was completely neutralized in mice by known convalescent serum. Passage from rabbit to rabbit has produced encephalitic symptoms characterized by the usual type of variable convulsions and focal signs. During the short period the virus has been observed in rabbits, the incubation period has remained 7 to 12 days, and all 17 injected animals have succumbed to the infection. The rabbit series is now in its fifth passage, and it remains to be seen whether a fixed rabbit strain can be obtained. It should also be mentioned that this apparently successful infection of rabbits followed two failures. In one case

where the injected material came from an early mouse passage, no infection was observed in a group of five rabbits injected intracerebrally. In a second case, the 19th mouse passage virus was injected intracerebrally into four guinea pigs, four rats, and two rabbits. Symptoms could be observed in the rabbits within 7 days, but the infection could not be transmitted to other rabbits. Again, the guinea pigs and rats remained free from symptoms.

Pathogenicity for Monkeys.—Portions of the same conjunctival scrapings that had been studied in the first and second isolations of virus from patients E.L. and R.H. were injected intraconjunctivally into four monkeys with no effect. On several occasions the mouse-fixed virus was injected into the conjunctiva and intracerebrally. One monkey developed a brain abscess, three showed no effect whatsoever, and two showed vague nervous symptoms 3 days before death, which occurred in 5 days. Brains from these two animals were sterile, and when injected into mice produced symptoms suggestive of the epidemic keratoconjunctivitis virus. To these may be added two baby *Macacus rhesus* monkeys, which received mouse virus intraconjunctivally. In spite of repeated traumatizing inoculations, the eyes remained entirely free from infection. On one occasion, two adult *Macacus rhesus* monkeys injected intraconjunctivally with mouse virus developed a transient but definite catarrhal conjunctivitis. In the absence of a preauricular node enlargement and because the condition lasted only 2 days, no definite conclusions could be drawn.

Further investigation of monkey susceptibility has been postponed, because of the difficulty in obtaining the animals for experimental purposes.

Pathogenicity for Guinea Pigs and Albino Rats.—Since its isolation the mouse virus has been injected into 36 guinea pigs by intracerebral, subcutaneous, and intraperitoneal routes. None of these animals has at any time shown symptoms, nor have there been any deaths. Similar results were obtained when 12 albino rats were injected with the mouse virus. It is clear that neither guinea pigs nor albino rats can be infected.

Activity of the Virus in Tissue Culture

The serum ultrafiltrate technique has been successfully used for the culture of other viruses (4). This medium, containing a cellular substrate of embryonic mouse brain, was used throughout the present investigation.

When the first isolation of the virus had been effected in tissue culture, some difficulty was encountered in maintaining serial subcultures. On three occasions a subculture inoculum consisting of ground-up cells plus culture fluid was compared with one of culture fluid alone. Freshly prepared tissue cultures which received only the culture fluid proved to be non-infectious regardless of the incubation conditions, whereas the flasks receiving ground-up tissue continued to be infectious for mice. Consequently, the routine inoculum

from culture to culture has consisted of both ground-up tissue and culture fluid. Also, because of the instability of the 37°C. series, greater dependence has been placed upon room temperature cultures, and this series is now in its 31st generation, having been subcultured every 6 or 7 days.

TABLE II

Tissue Culture Potencies of Mouse Keratoconjunctivitis Virus Measured by Intracerebral Injection of Mice

Culture generation	Mouse titer
3rd to 10th (no titration done)	Virus present
11th	10^{-2}
12th*	10^{-2}
	10^{-3}
13th	10^{-3}
14th	10^{-4}
15th	10^{-2+}
16th	10^{-3}

* Two tests done.

TABLE III

*Growth of Mouse Keratoconjunctivitis Virus in Tissue Culture at Room Temperature**

Period of incubation	Mouse titer
days	
2	0
3	0
4	10^{-1}
5	$10^{-2.5}$
6	10^{-3}
7	$10^{-2.5}$
8	$10^{-1.5}$

* The 13th subculture was the source of virus for this experiment.

Perhaps the simplest explanation for the necessity of passing both tissue and fluid menstruum from infected to fresh cultures is the relatively low potency of the cultures. As can be seen from Table II, the culture titer in mice has rarely exceeded 10^{-3}. While the tissue cultures seem effective as a link in adapting the virus from human to mouse, and in building up a level of potency which would consistently cause symptoms in these animals, once established in an animal host the virus attains a greater potency than in the artificial medium. It can be seen from Table III, that in subculture no virus is demonstrable until the 4th day of incubation at room temperature, and a

peak is reached on approximately the 6th day. It is for this reason that new culture generations are, in general, started every 6 or 7 days.

It may be noted in passing, that a convenient method of preserving the cultures for fairly long periods of time is to keep the preparations at 4°C., following the completion of their room temperature incubation. Apparently a very slow loss of potency occurs in the refrigerator, as can be seen from the following experiment: The 11th subculture was put into the refrigerator on June 3, 1942, after 8 days' incubation at room temperature. A titration done on a sister culture at that time revealed a potency of 10^{-2}. 7 weeks later (July 24), when the culture in the refrigerator was tested, a potency of 10^{-1} was obtained. The culture was ground up and passed to a

TABLE IV

Filtration of Mouse Epidemic Keratoconjunctivitis Virus through E-K Seitz Filter (Double Pad) Tested by Intracerebral Injection of Mice*

Material tested	Day after injection of virus dilutions	Virus dilutions				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Infected mouse brain before filtration	1st	− − −	− − −	− − −	− − −	− − −
	2nd	+ − −	− − −	− − −	− − −	− − −
	3rd	D + +	+ + +	+ + +	+ − −	− − −
	4th	D D	D D D	D D D	D + +	− − −
	5th				D D	− − −
		1:50	1:100	1:200	1:400	
Filtrate of infected mouse brain	1st	− − − −	− − − −	− − − −	− − − −	
	2nd	+ + + −	+ + − −	− − − −	− − − −	
	3rd	D D D +	D + + +	+ + − −	− − − −	
	4th	D	D D D	D D − −	− − − −	
	5th			− −	− − − −	

Each symbol represents one animal tested: − = no symptoms, + = definite symptoms, D = dead.

* Virus suspension consisted of lightly centrifuged mouse brain emulsion diluted with Simms buffered salt solution.

fresh culture, and the subculture, after 7 days at room temperature, had a potency of 10^{-3}. This method is particularly convenient for providing auxiliary material which may be utilized if stock cultures become contaminated.

The principal object of tissue culture work at this time was to obtain an agent cultivable in series. Preliminary work suggests that once adapted to *in vitro* conditions, the virus may be grown to some extent in embryonic chick tissue, but detailed experiments on this and other culture factors are being carried out and will be reported later.

Filtration Experiments with Keratoconjunctivitis Virus As Maintained in Mice

The mouse keratoconjunctivitis virus passed readily through Berkefeld filters of all sizes, and through an E-K Seitz filter which contained a double

pad. The Seitz filtration was done by comparing the titers of infectious material before and after filtration. The protocol for this experiment given in Table IV shows that there was a shift in the virus potency from 10^{-5} before filtration to 1:200 after filtration. In spite of this loss of potency, which is not unusual in a filter with a large absorption surface, there is no reason to

TABLE V

Ultrafiltration of Mouse Keratoconjunctivitis Virus through Graded Collodion Membranes

Average pore diameter of membranes	Effect on mice receiving intracerebral injection of filtrates
millimicrons	
(Virus of isolation 1)	
175–200	2/4
175–200	2/2
150–175	3/4
150–175	4/4
96	3/4
75–100	2/4
75–100	5/6
75–100	7/10
75–100	8/10
50–75	3/10
50–75	3/10
50–75	3/10
50–75	4/10
25–30	0/6
25–30	0/10
25–30	0/10
(Virus of isolation 2)	
75–100	3/7

Numerator equals the number of mice affected, and denominator equals the number of mice injected.

doubt the ability of the virus to pass through Seitz filters, since this experiment was repeated three times. Ultrafiltration experiments were then begun, initially on graded collodion membranes with an A.P.D. of 175 to 200 millimicrons (6). In all filtration experiments, regardless of the type of filter used, the material filtered consisted of stock mouse brain emulsified and diluted with ground-up tissue culture. The mixture was then centrifuged at about 2000 R.P.M. for 10 minutes, and the supernatant fluid tested.

The filtrates were injected intracerebrally into mice and the symptoms noted. A specimen was considered positive if the mice showed suggestive symptoms in 3 or 4 days. A corroborative test was done on one occasion with material passed through a 75 to 100 millimicron membrane. A mouse which had received this filtrate 4 days previous to the appearance of symptoms was sacrificed, its brain emulsified, and the emulsion tested against a serum known to neutralize the mouse keratoconjunctivitis virus. Evidence of neutralization proved that the filtrate had contained the virus. The filtration experiments were, with one exception, carried out with the virus of the first isolation. In one case the virus of the second isolation was passed through a 75 to 100 millimicron membrane, and three of the seven animals receiving this filtrate showed symptoms.

As is apparent from Table V, the virus consistently passed through membranes with an A.P.D. of 75 to 100 millimicrons, and apparently also passed to some extent through a membrane with an A.P.D. of 50 to 75 millimicrons. Below that range of pore diameter no virus was recovered.

IDENTIFICATION OF THE VIRUS

Neutralization Experiments

From a clinical consideration of epidemic keratoconjunctivitis it can be seen that the lymphadenopathy, which begins within a few days after the appearance of the first symptoms of the disease, and which may involve not only the regional preauricular gland but also the deeper and more distant lymph channels, suggests the possibility of a marked antigenic stimulation. Furthermore, the adenopathy may be apparent for 2 to 6 weeks, and the course of the clinical disease may last from 1 to 3 months. Because of the above immunological considerations, an effort was made shortly after the first isolation to demonstrate neutralizing antibodies in the blood of patients recovered from epidemic keratoconjunctivitis.

The first experiment, in which 0.03 cc. of a serum-virus mixture was injected intracerebrally into mice, failed completely to demonstrate neutralizing antibodies. Since it was known that adult mice are susceptible to epidemic keratoconjunctivitis only by intracerebral and intranasal injections, the second neutralization experiment was carried out on unweaned mice in the hope that an age factor of susceptibility, similar to that seen in Western equine encephalomyelitis, was present. Such an age factor was apparently involved, since the unweaned mice did succumb to infection after introduction of the virus into the peritoneal cavity, and under these experimental conditions, some neutralization was demonstrable. When the technique was further altered to allow for serum-virus binding for 1 hour at 37°C. and for 4 hours at 4°C., the antibody titer was much more clearly defined. Therefore, following this initial period of experimentation, the procedure in neutralization experiments was as follows:—

In each test 0.5 cc. of the undiluted control or convalescent serum was combined with 0.5 cc. of mouse stock virus of either the first or second isolations serially diluted to give the final dilutions indicated in the tables, (Tables VI–VIII). These mixtures were then held for 1 hour at 37°C. and for 4 hours at 4°C., and 0.1 cc. of each mixture was injected intraperitoneally into each of four unweaned mice. Care was taken to have more than one control serum, and the last serum-virus mixture injected was always a control, so that deterioration of virus would not be confused with neutralization.

TABLE VI

Neutralization of Mouse Keratoconjunctivitis Virus (Isolation 2) by the Serum of Patient E.L. (Source of Isolation 1)

Serum	Day after injection of serum-virus mixture	Virus dilution				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Patient E.L.	1st	----	----	----	----	----
	2nd	----	+---	----	----	----
	3rd	----	D---	----	----	----
	4th	----	---	----	----	----
	5th	+---	+--	----	----	----
	6th	D---	D--	----	----	----
	7th	---	--	----	----	----
	8th	---	--	----	----	----
Control (normal human being)	1st	----	----	----	----	----
	2nd	++--	----	+---	+---	----
	3rd	DD+-	+---	D+--	D---	----
	4th	++	D+++	D++	+++	++++
	5th	DD	DDD	D+	DDD	++++
	6th			D		DDDD

Each symbol represents one animal: − = no symptoms, + = definite symptoms, D = dead.

Technique: 0.5 cc. of undiluted serum was combined with 0.5 cc. of virus dilution to give final dilutions indicated on the table. Mixtures were held for 1 hour at 37°C. and for 4 hours at 4°C. 0.1 cc. of each mixture was injected intraperitoneally into unweaned mice.

Multiple tests carried out on various types of serums have yielded consistent results. The protocol given in Table VI has been selected as a fair example of the course which the neutralization tests follow. This protocol was also selected, because it is one in which the serum of patient E.L., source of the first isolation (case history 1) was tested against the virus of the second isolation, made from patient R.H. (case history 2). Emphasis should be placed on the fact that the neutralization tests throughout this study have been based on mortality rather than morbidity.

It can be seen from Table VI that definite neutralization of at least 10,000 mouse infectious doses was obtained. Whereas the control serum-virus

produced death in all 20 mice (titer of at least 10^{-5}), in the convalescent serum-virus group only three deaths occurred (titer 10^{-1}). The general range in experiments with patients' convalescent serums is between 100 and 10,000 neutralizing doses. Before a summary of the neutralization data is considered, it would perhaps be well to note case histories of two patients who provided convalescent serums for the tests.

Case History 3.[2]—Patient N.S. developed epidemic keratoconjunctivitis on Feb. 10, 1942, while under treatment for staphylococcus blepharitis. The right eye was involved originally, and the left eye became involved on Feb. 15. The picture in both eyes was typical, with edema, epiphora, hyperemia, and enlarged glands on both sides. The right eye developed superficial macular lesions of 1 mm. diameter centrally. No further details on this case are available.

Case History 4.—Patient H.I., aged 46, appeared in the clinic 4 days after he had noticed symptoms of redness and a slight discharge in his right eye. On the day before he came to the clinic, his left eye had also become involved. A diagnosis of epidemic keratoconjunctivitis was made by Dr. Phillips Thygeson. Symptoms in both eyes included edema, chemosis, adenitis, and keratitis. Throughout the course of the disease, the left eye was less markedly affected than the right. Preauricular gland enlargement on both sides was noticed over a period of about 20 days. 3 weeks after onset the patient reported that his eyes had begun to improve. However, there were still a slight bulbar hyperemia and characteristic punctate corneal changes present. The patient failed to return to the clinic and was not seen again.

The summary of neutralization tests in Table VII makes it clear that there is a definite relationship between the two strains of mouse keratoconjunctivitis virus and the serum of patients convalescent from the human disease. Inasmuch as the statistics have been based on mortality rather than on morbidity of tested animals, there has been little possibility for misinterpretation of results.

In addition to the serum from a human volunteer infected with the mouse virus, a total of six convalescent serums have been studied up to this time. Three of the serums have been of particular interest, because they came from patients in California who suffered with the disease during the winter epidemic of 1941. The diagnoses were made by Dr. M. J. Hogan, who sent the serums. The bleedings on these patients were done from 1 to 6 months after the onset of the disease. In two of the three cases, repeated tests revealed 1000 mouse neutralizing doses, and 100 neutralizing doses were demonstrated in the third case. The remaining three convalescent serums came from individuals in the clinic at Presbyterian Hospital, and included the two patients from whose eyes were isolated the two virus strains discussed in this report. The diagno-

[2] We wish to express our thanks to Dr. Michael J. Hogan of the University of California Medical School, for sending serums of patients N.S., J.G., and X (Table VII) from California, so that they might be included in this study.

TABLE VII

Summary of Neutralization Tests Carried Out with Mouse Keratoconjunctivitis Virus and Control or Convalescent Serums

Serum tested		Source of virus (isolation)	Mouse titer*	No. of neutralizing doses
Control	Convalescent			
Normal human being (4 experiments)		1—E.L.	10^{-5}	0
Theiler-hyperimmune		1—E.L.	10^{-5}	0
		1—E.L.	10^{-5}	0
		2—R.H.	10^{-5}	0
Anti-lymphocytic choriomeningitis		1—E.L.	10^{-5}	0
Antiherpes		1—E.L.	10^{-5}	0
Non-specific conjunctivitis or keratitis (7 patients)		1—E.L.	10^{-5}	0
Patient R.H. (isolation 2) at height of disease		1—E.L.	10^{-5}	0
Human volunteer before infection		1—E.L.	10^{-5}	0
	Patient E.L. (isolation 1) 3 mos. after infection	1—E.L.	10^{-1}	10,000
		1—E.L.	10^{-1}	10,000
		1—E.L.	10^{-2}	1,000
		2—R.H.	10^{-1}	10,000
	Patient R.H. (isolation 2) 1 mo. after infection	1—E.L.	10^{-2}	1,000
		1—E.L.	10^{-2}	1,000
		2—R.H.	10^{-2}	1,000
	Patient N.S. (case history 3)‡	1—E.L.	10^{-2}	1,000
		1—E.L.	10^{-2}	1,000
	Patient H.I. (case history 4)	1—E.L.	10^{-3}	100
		2—R.H.	10^{-2}	1,000
	Patient X‡	1—E.L.	10^{-2}	1,000
	Patient J.G.‡	1—E.L.	10^{-3}	100
	Human volunteer (case history 5) 1 mo. after infection	1—E.L.	10^{-2}	1,000

* The final virus dilution producing death in a majority of animals injected.

‡ Patients from the West Coast.

sis was reasonably certain in the Presbyterian Hospital patients, and it should be noted that excellent cross-neutralization of the two strains was obtained with serum from both patient E.L. (first isolation) and patient R.H. (second isolation).

As is also apparent from Table VII, not only were various antiviral serums included in the controls, but also serums from normal individuals, and from seven patients who were suffering with conjunctivitis or keratitis other than epidemic keratoconjunctivitis. The following diagnoses were made for the patients in the last group: superficial punctate keratitis (Fuchs type), chronic conjunctivitis with punctate keratitis, chronic follicular conjunctivitis and keratitis, and marginal ulcer. In no case was neutralization of the mouse virus observed.

An attempt was made to obtain samples of serum both at the beginning of the disease and several weeks later. Unfortunately this procedure was not always practical, but in the case of patient R.H. the development of antibodies was clearly demonstrated. A serum specimen taken at the height of the disease and one taken a month later were tested against the mouse virus in the same experiment. The first specimen acted like a normal serum (serum-virus titer of 10^{-5}), whereas the later specimen of serum neutralized about 1000 mouse doses of virus (serum-virus titer of 10^{-2}). In the case of the human volunteer soon to be reported, a similar procedure was followed with identical results.

It should also be noted that since the present study was concluded, additional serological evidence has been obtained for the specific relationship between the two strains of virus here discussed and epidemic keratoconjunctivitis. Serums from 15 patients suffering from this disease (diagnoses made by Dr. Phillips Thygeson) in another localized epidemic[3] in New York City were tested for neutralizing antibodies by use of the technique described in this report. Results closely paralleling those summarized in Table VII were obtained, *i.e.* 3 to 6 weeks after illness, 100 to 10,000 mouse neutralizing doses were demonstrated in convalescent serum. On six occasions it was possible to obtain two serum specimens from the patients, the first at the height of the disease, the second 3 to 6 weeks later. In these six instances, as in the case of patient R.H., development of neutralizing antibodies was seen. The serum taken at the height of the disease contained no antibodies, whereas a few weeks later neutralization of the magnitude already mentioned was observed.

Production of Epidemic Keratoconjunctivitis in a Human Volunteer

Further evidence of a specific relationship between the isolated virus and the human disease was obtained by the transfer of the mouse keratoconjunc-

[3] Sanders, M., Gulliver, F. D., Forchheimer, L. L., and Alexander, R. C., unpublished data.

tivitis virus to the eye of a human volunteer, with the reproduction of a mild but characteristic human disease. The case history of this individual is as follows:—

Case History 5.—Human volunteer, a white male, 32 years old, in good health, and with no previous history of epidemic keratoconjunctivitis.

July 3, 1942. About 3,000,000 intracerebral doses of mouse keratoconjunctivitis virus were instilled on the conjunctiva of the right eye and rubbed in. A patch was kept over the eye for 30 minutes, and then two more drops of the material added. The infected material consisted of ground-up tissue from the 15th tissue culture generation plus stock mouse brain from the first isolation.

July 4. After a 4 day period in which no symptoms appeared, a slight conjunctivitis with several follicles was noted. The culture of the secretion was negative, and the smear showed a few mononuclear cells, but no bacteria. No edema or enlarged preauricular gland was observed. At this time washings from the right eye were put into the left eye and additional material was rubbed into the lower conjunctiva of the right eye with a swab.

July 10. The diagnosis of epidemic keratoconjunctivitis was made by Dr. Thygeson. There was a mild conjunctivitis with a palpable preauricular node, and apparently typical changes in the lower conjunctiva (Fig. 7).

July 13. General conjunctivitis was more marked, and thickening of the lower conjunctiva of 48 hours' duration was present.

July 18. After 3 days the acute condition of the conjunctiva appeared to subside somewhat, without diminution of mucous membrane hypertrophy. The preauricular gland remained slightly tender, and was still enlarged, although the size was smaller than on July 13. On the evening of the 17th, the patient had the sensation of getting something in his eye, and what appeared to be an acute exacerbation ensued. General acute characteristics noted on the 13th reappeared and were even aggravated. There was some increase in secretion in the morning, and a fairly copious lacrimation. The preauricular node was slightly more tender, and a tender submental node about the size of an almond was palpable.

July 29. There was a general decrease in objective symptoms during the past week. The area of follicular hypertrophy in the lower fornix disappeared. Moderate infiltration and hyperemia persisted.

From Table VIII, a summary of the tests made, it can be seen that there were no neutralizing antibodies present just previous to infection, whereas 1 month later an antibody titer of 1000 mouse doses was obtained.

Tests for the Exclusion of Certain Viruses

A consideration up to this point of the evidence for isolation of the keratoconjunctivitis virus made it clear that additional tests were indicated for excluding the presence of two latent viruses, lymphocytic choriomeningitis and Theiler's spontaneous encephalomyelitis virus.

Lymphocytic Choriomeningitis.—This virus is one of the most common contaminants in laboratory animals, and is easily awakened and maintained

in them. Since the host range includes mice, guinea pigs, and monkeys, there was little reason to expect a relationship between this virus and epidemic

TABLE VIII

Neutralization of Mouse Keratoconjunctivitis Virus (Isolation 1) by Serum from Patient N.S. and from Human Volunteer before and 1 Month after Infection

Serum	Day after injection of serum-virus mixture	Virus dilution				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Human volunteer before infection	1st	----	----	----	----	----
	2nd	----	----	----	----	----
	3rd	+++-	++--	+---	----	----
	4th	DDD-	DD+-	D+--	+---	----
	5th	+	D+	D+-	D---	----
	6th	D	D	D+	+++-	++--
	7th			+	DD+	DD+-
	8th			D	+	D-
	9th				D	-
Human volunteer after infection	1st	----	----	----	----	----
	2nd	----	----	----	----	----
	3rd	----	----	----	----	----
	4th	+---	----	----	----	----
	5th	D+--	----	----	----	----
	6th	D+-	+---	----	----	----
	7th	D-	D+--	+---	----	----
	8th	+	D+-	D---	----	----
	9th	D	D-	---	----	----
Patient N.S. (convalescent from West Coast)	1st	----	----	----	----	----
	2nd	----	----	----	----	----
	3rd	----	----	----	----	----
	4th	----	----	----	----	----
	5th	+---	+---	----	----	----
	6th	D++-	D---	----	----	----
	7th	DD-	+--	----	----	----
	8th	-	D+-	----	----	----
	9th	-	D-	----	----	----

Each symbol represents one animal tested: - = showed no symptoms, + = showed definite symptoms, D = dead.

Technique: 0.5 cc. of undiluted serum was combined with 0.5 cc. of virus dilution to give final dilutions indicated on table. Mixtures were held for 1 hour at 37°C. and then for 4 hours at 4°C. 0.1 cc. of each mixture was injected intraperitoneally into unweaned mice.

keratoconjunctivitis, which is so distinctly non-pathogenic for guinea pigs, and can only produce infection in rabbits and mice. Nevertheless, the variation that is found in lymphocytic strains made it imperative that it be ruled out of the present study. The protocol in Table IX is one of the two experi-

ments carried out with both isolations of the epidemic keratoconjunctivitis virus and anti-lymphocytic choriomeningitis serum.[4] The results in both tests clearly indicated that there was no relationship between lymphocytic choriomeningitis and epidemic keratoconjunctivitis, since the anti-lymphocytic serum contained no neutralizing bodies against the virus of epidemic keratoconjunctivitis.

TABLE IX

Neutralization Test Carried Out with Mouse Keratoconjunctivitis Virus and Anti-Lymphocytic Choriomeningitis Serum

Serum	Day after injection of serum-virus mixtures	Virus dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Anti-lymphocytic choriomeningitis	1st	----	----	----	----	----	----
	2nd	+---	----	+---	----	----	----
	3rd	D---	----	D---	----	----	----
	4th	+++	D+++	+--	----	----	----
	5th	DDD	DDD	D+-	----	----	----
	6th			D-	----	----	----
	7th			+	+++-	+---	----
	8th			D	DDD+	D++-	----
	9th				D	DDD	----
Normal rabbit	1st	----	----	----	----	----	----
	2nd	----	D---	----	----	----	----
	3rd	+---	---	----	----	----	----
	4th	D+--	---	+---	----	----	----
	5th	DD+	++-	D++-	----	----	----
	6th	D	DD+	DD+	+++-	----	----
	7th		D	D	DDD+	----	----
	8th				+	D++-	----
	9th				D	DDD	----
	10th						----

Each symbol represents one animal tested: − = no symptoms, + = definite symptoms, D = dead.

In view of the serological data and the diversity in host range of the two viruses, further tests were considered unnecessary.

Spontaneous Mouse Encephalomyelitis of Theiler.—Because the filtration data on the epidemic keratoconjunctivitis virus suggested that it was an agent of small particle size, and because spontaneous encephalomyelitis of Theiler is present in many mouse colonies, an attempt was made to demonstrate that we were not dealing with the Theiler virus. A serum known to neutralize

[4] Obtained from the laboratory of Dr. Thomas Rivers of The Rockefeller Institute for Medical Research.

large doses of Theiler's virus was tested against the epidemic keratoconjunctivitis virus in mice. Whereas neutralization of Theiler virus with this serum was noted, no neutralization occurred when the same serum was mixed with both isolations of epidemic keratoconjunctivitis. Inasmuch as serological evidence is not entirely significant for the exclusion of Theiler's virus, a protocol of this experiment is not given, but the protocol for the more conclusive test of cross-infection is given in Table X.

A Theiler-immune mouse colony was tested. The colony was immunized as follows: 50 mice were given intraperitoneal injections of 10^{-1} dilution of Theiler virus G.D. VII; the survivors received a second intraperitoneal dose in 4 to 5 days, and this was repeated within a similar period of time. 2

TABLE X

Susceptibility of Theiler-Immune Mice to Intracerebral Injection of 21st Culture Generation of Epidemic Keratoconjunctivitis

Mice tested	Day after injection of tissue culture	Dilutions of culture virus		
		10^{-1}	10^{-2}	10^{-3}
Theiler-immune	1st	D– – – –	– – – – –	– – – – –
	2nd	– – – –	– – – – –	– – – – –
	3rd	D+++	D++++	DD+++
	5th	DDD	DDDD	DDD
Normal control	1st	– – – – –	– – – – –	– – – – –
	2nd	– – – – –	– – – – –	– – – – –
	3rd	D++++	DD+++	DD+++
	5th	DDDD	DDD	DDD

Each symbol represents one animal tested: – = no symptoms, + = definite symptoms, D = dead.

weeks later the remaining mice were tested with a known strain of Theiler virus (0.03 cc. of a 10^{-2} dilution given intracerebrally) and were found to be immune.

This colony then received intracerebral injections of the 21st tissue culture generation (0.03 cc.) of the epidemic keratoconjunctivitis virus in dilutions varying from 10^{-1} to 10^{-3}. As a control, normal mice of the same age group were used. The results, as seen in Table X, indicate that there is no essential difference in the reaction between the Theiler-immune colony of mice and the controls.

DISCUSSION

Although specimens taken from the eyes of six of the nine definitely diagnosed cases of epidemic keratoconjunctivitis produced a temporary and transient illness when injected into mice, the filterable agent of the disease

could not be clearly and conclusively demonstrated until material from human beings or from early animal passages was put into tissue culture. Only after this passage into tissue culture did the symptoms in mice become intensified, the incubation period stabilized, and the course of the disease well defined, with death as its inevitable conclusion. Unpublished data on the isolation of three additional strains of the virus from patients suffering with epidemic keratoconjunctivitis indicate that the tissue culture procedure can be repeated successfully. In each of these cases the demonstration of virus closely followed the procedure outlined in the present paper.

Once the virus was established in the mouse, the susceptible animal was a better source of virus than the tissue culture. Tissue culture, however, offers the advantage of giving the investigator control over certain environmental conditions, so that the infectiousness of the inoculum can be temporarily enhanced to the point at which the infection of a new host is possible. Thus, it is easily seen that in the case of an infectious agent naturally occurring on the surface of the ocular mucous membrane, lowering of the incubation temperature might allow a closer approximation to its incubation in nature. It is not possible at present to evaluate what effect the choice of tissue has on the problem.

The mere recovery of a virus from an inoculated laboratory animal did not, of course, signify that actual transmission of a disease from its human host had occurred, and steps were early taken to exclude the more common latent mouse viruses of lymphocytic choriomeningitis and Theiler's spontaneous mouse encephalomyelitis. In this respect, it may be noted that the possibility of wakening a latent virus is appreciably reduced when much of the work is done in tissue culture.

In view of the fact that the epidemic keratoconjunctivitis virus had been recovered from human eyes and was pathogenic for mice and rabbits, it seemed advisable to consider the possibility of confusion between this virus and the virus of herpes. This is particularly true since the herpes virus is capable of a fairly diversified activity. The exclusion of the herpes virus is based on the following considerations: The herpes virus does not pass through collodion membranes with an A.P.D. as small as the minimum A.P.D. range which permits the passage of the epidemic keratoconjunctivitis virus. Further confirmation of the difference in size between the two viruses was seen in the fact that on three occasions epidemic keratoconjunctivitis virus passed without difficulty through an E-K Seitz filter (double pad) which is known to retain the virus of herpes. The absence of inclusion bodies in sections of the brains of mice killed by the epidemic keratoconjunctivitis virus, the inability of this agent to produce keratitis in many tests in rabbits, and its failure to be neutralized by normal human or by anti-herpes serum, together with the above mentioned facts, strongly suggest that the virus of epidemic keratoconjunctivitis is entirely distinct from the virus of herpes.

The specific relationship between the isolated agent and epidemic keratoconjunctivitis was based not only on its apparent transmission from the eyes of patients suffering with the disease to laboratory animals, but also on the consistent neutralization of the passage virus by human serum from patients convalescent in both New York and California. Furthermore, the development of antibodies was demonstrable not only in one of the patients considered in the present study, but also in four other convalescents.[3] These data, together with the fact that a human infection was produced by the mouse virus, and that this infection was accompanied by specific antibody formation, appear to complete the evidence in favor of the specific nature of the agent.

In regard to the human infection just mentioned, it should be noted that even with massive and repeated doses, only a mild infection was induced, and this was limited to the conjunctiva. Since only one volunteer was available, it is still uncertain whether the adapted strain is only mildly virulent for man in consequence of the process of adaptation from one host to another, or whether the healthy condition of the subject rendered him relatively immune against infection.

CONCLUSIONS

1. A virus has been isolated from two patients suffering with epidemic keratoconjunctivitis.

2. At first the virus could be maintained only by the inoculation of tissue cultures (serum ultrafiltrate and embryonic mouse brain) with conjunctival scrapings or with emulsified mouse brains from early passage animals. Later it caused a fatal disease in every mouse into which it was inoculated, and could then be readily maintained in mice.

3. The virus proved pathogenic for unweaned white Swiss mice by the intranasal, intraperitoneal, and intracerebral routes; for adult mice by the intranasal and intracerebral routes, and for rabbits by only the intracerebral route.

4. Although the titer of tissue cultures rarely exceeded 10^{-2} or 10^{-3}, the virus, once established in mice, increased in potency until titers of 10^{-5} and 10^{-6} were attained. Mice injected with either the emulsion of mouse brain tissue or with the tissue culture material in these dilutions developed symptoms within a definite incubation period; once the disease was initiated, it followed a characteristic course for a period of a few to 24 hours, and consistently terminated in death.

5. The pathological changes in mice were limited to the central nervous system, and were not particularly distinctive. The neurotropic character of the virus is further shown by the fact that only the brain tissue was consistently pathogenic for mice.

6. Serial tissue cultures could be maintained only at room temperature, and when the inoculum from virus-infected cultures into fresh tissue cultures contained ground-up cells.

7. The highest level of potency in cultures occurred on about the 6th day at room temperature.

8. The virus passed without difficulty through an E-K Seitz filter (double pads) and through all grades of Berkefeld filters.

9. The virus passed consistently through graded collodion membranes with an A.P.D. of 75 to 100 millimicrons and to a lesser extent through those with an A.P.D. of 50 to 75 millimicrons. Membranes with an A.P.D. of less than 50 millimicrons retained the virus.

10. The mouse virus was not neutralized by anti-lymphocytic choriomeningitis serum, antiherpes serum, normal human serum, or serum from cases of non-specific conjunctivitis or keratitis.

11. Mice hyperimmunized to Theiler's virus were susceptible to the mouse keratoconjunctivitis virus. The latter virus was also not neutralized by hyperimmune Theiler rabbit serum.

12. The mouse virus could be neutralized by serum from the two patients from whom the virus had been isolated, and also by that from the three patients convalescing from epidemic keratoconjunctivitis in California and the serum of a convalescent in New York. The neutralization data were confirmed by tests on 15 additional convalescent serums (unpublished data).

13. A mild but characteristic picture of epidemic keratoconjunctivitis was reproduced in a human volunteer following inoculation with the mouse virus.

14. The serum of the human volunteer, while not neutralizing the mouse virus before infection, contained neutralizing antibodies 1 month after infection.

15. Development of antibodies was demonstrated in one patient (R.H.) in the present series, and in six other patients of another series (unpublished data).

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EXPLANATION OF PLATES

PLATE 5

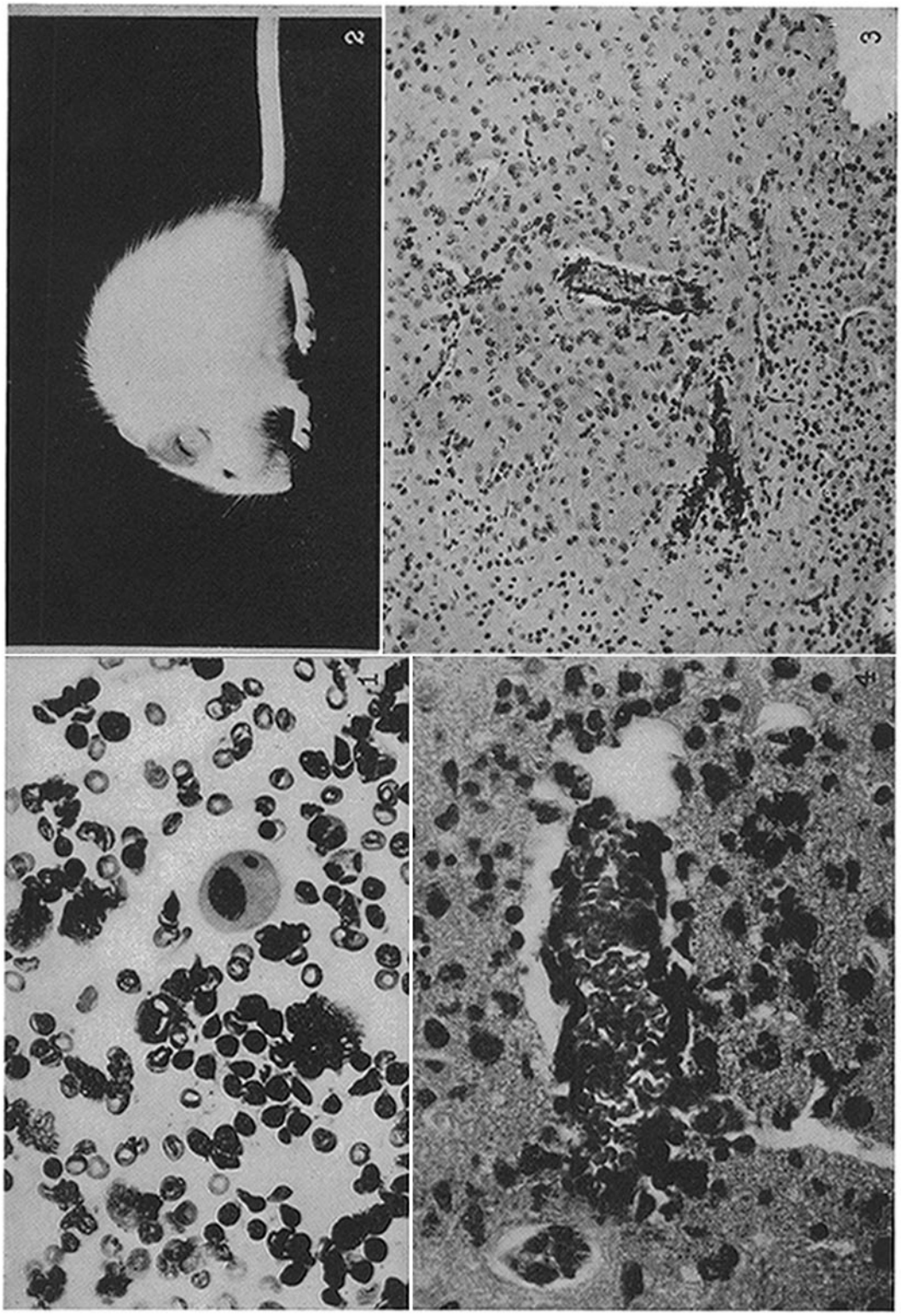
FIG. 1. Conjunctival scraping from patient with epidemic keratoconjunctivitis (Giemsa stain). Note large mononuclear cell and lymphocytes. Oil immersion.

FIG. 2. Mouse 3 days after intracerebral injection with fourth generation mouse keratoconjunctivitis virus. Note the humped back and ruffled fur. The animal was prostrate and died a few hours after photograph was taken.

FIGS. 3 and 4. Sections from brain of mice infected with keratoconjunctivitis virus.

Fig. 3. Cerebral cortex near frontal pole; note perivascular infiltration by lymphocytes in lower cortical layer. Hematoxylin-eosin. × 110.

Fig. 4. Cerebral cortex; perivascular infiltration by lymphocytes and occasional large mononuclear cells. Similar infiltration in perivascular parenchyma with early microglial activation. Hematoxylin-eosin. × 230.



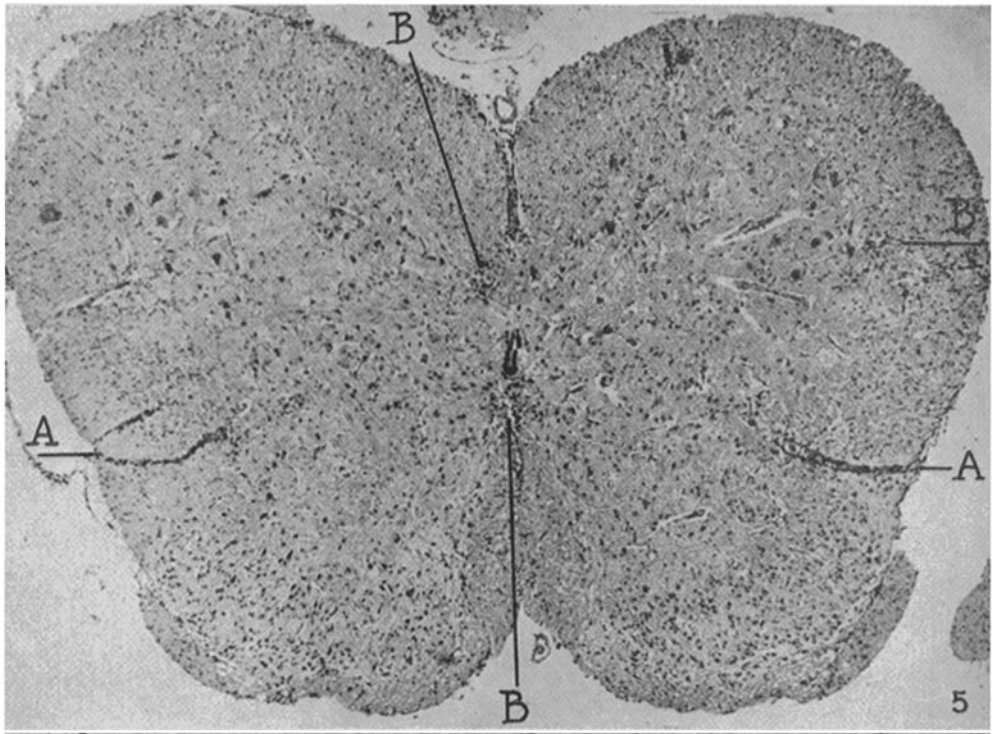
(Sanders and Alexander: Epidemic keratoconjunctivitis. I)

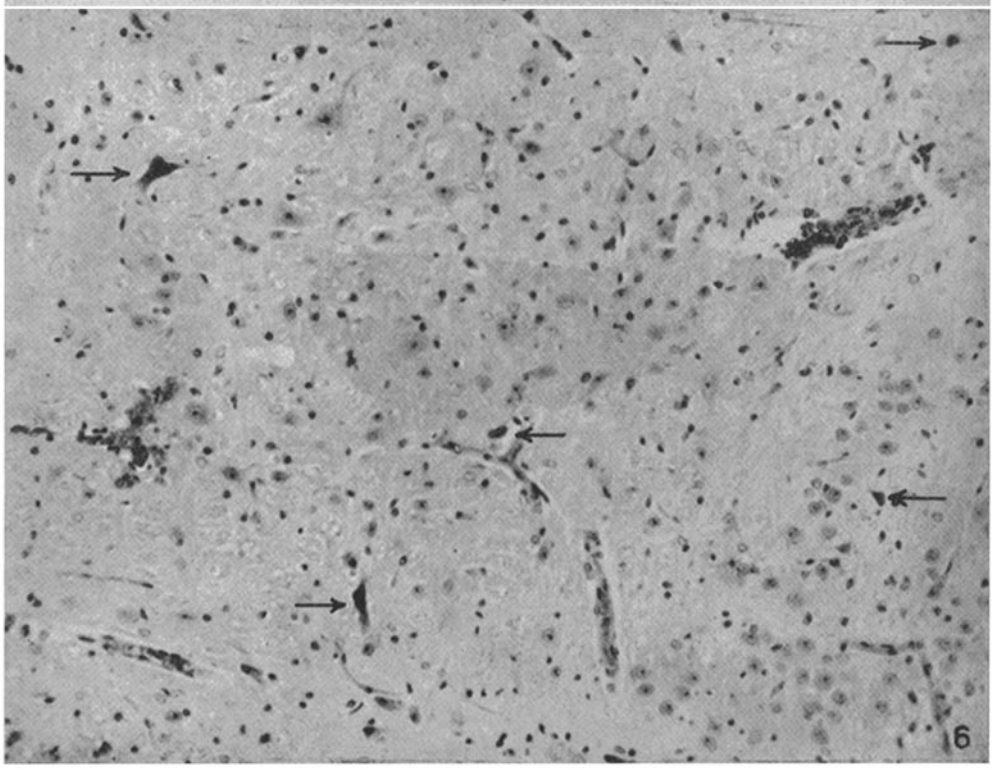
PLATE 6

FIGS. 5 and 6. Sections from cord and brain of mice infected with keratoconjunctivitis virus.

Fig. 5. Lumbar cord; note mild leptomeningeal and perivascular infiltration in lateral columns (A) and small foci of diffuse infiltration (B). Hematoxylin-eosin. × 95.

Fig. 6. Pons; note mild perivascular infiltration and occasional shrunken, degenerating nerve cells (arrows). Hematoxylin-eosin. × 195.





(Sanders and Alexander: Epidemic keratoconjunctivitis. I)

PLATE 7

FIG. 7. Right eye of human volunteer 1 week after infection with mouse keratoconjunctivitis virus. Note follicles and exudate on edematous and hyperemic palpebral conjunctiva. × 2.

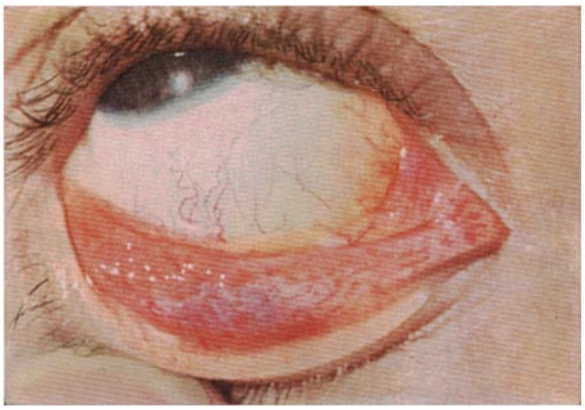


FIG. 7

(Sanders and Alexander: Epidemic keratoconjunctivitis. I)