Isolation of an Agent Producing Ovine Infectious Keratoconjunctivitis (Pink Eye)

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SUMMARY

An agent causing ovine infectious keratoconjunctivitis was isolated in cell cultures. Cytopathic changes were produced. The 5th serial passage of this agent in cell cultures produced keratoconjunctivitis in 1 of 4 sheep. The 15th serial passage in cell cultures produced conjunctivitis in 6 of 7 sheep. Keratitis was observed in 3 of the 6 sheep that had signs of conjunctivitis. The 20th serial passage of this agent was still pathogenic. The virulence of the agent was decreased and the incubation period was increased by serial passage of the agent in cell cultures. This agent did not produce signs of keratoconjunctivitis when inoculated into goats. Gram-negative cocci isolated from infected eyes did not produce signs of the disease and did not enhance the virulence of this agent.

Ovine infectious keratoconjunctivitis (OIK) was reported by Cole [2] as affecting sheep in South Africa. He was successful in transmitting this disease by using material from affected eyes. The infective agent would not pass through siliceous or plaster of Paris filters. On the basis of stained preparations, *Rickettsia* was proposed to be the causative agent. Beveridge [1] confirmed these findings and demonstrated that a carrier state may exist in certain sheep for periods exceeding a year. Immunity was demonstrated which in one instance persisted for over a year. These workers were unsuccessful in isolating the causative agent in chicken embryos. Dickinson and Cooper [3] were unsuccessful in propagating the causative agent of OIK in chicken embryos or tissue cultures. They stated that conjunctival smears prepared from infected eyes contained inclusion bodies which morphologically appeared to resemble those of the psitticosis group more than the rickettsia group. Treatment using chlortetracycline appeared to be effective.

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Materials and Methods

The infective material was obtained from the lacrimal secretions of sheep 1 which had severe signs of OIK. For unrelated experimental purposes, sheep 1 had been isolated from the main flock 2 months prior to the development of signs. The naturally occurring infection appeared during the midwinter of 1963.

Susceptible Sheep.—Infected sheep 1 and susceptible sheep 2 through 19 were obtained from the experimental flock at Substation 14. These were yearling Rambouillets from a flock in which OIK had not been seen for at least 3 years. Other sheep used in these experiments were obtained from flocks that had no signs of a recent infection of OIK. The McCoy synovial cell line was used in the

propagation of this agent and the tissue culture methods that were utilized in this investigation have been described by Livingston and Moore.[5] Antibiotics and serum were not added to the cell culture systems or to the inoculum. Blood agar plates were prepared using blood agar base (Difco) with added 5% defibrinated sheep blood.

Determination of the Infectivity of the Lacrimal Secretions.—Lacrimal secretions from eyes of 4 yearling sheep (2, 3, 4, and 5) were obtained on sterile cotton swabs and streaked on blood agar plates. Then lacrimal secretions from sheep 1, obtained on sterile cotton swabs, were inoculated into the left eyes of these sheep and also streaked on blood agar plates. Lacrimal secretions were collected from the inoculated sheep at 24-hour intervals and streaked on blood agar plates.

Determination of the Sensitivity of this Agent to Penicillin and Streptomycin.—To determine if antibiotics could be utilized in attempts to isolate this agent, lacrimal secretions from sheep 1 were obtained on sterile cotton swabs, and were placed in tubes with 3 ml. of medium 199 * containing 1,000 units of penicillin and 25 mg. of streptomycin per milliliter. The swabs were rotated rapidly several times and removed. Tubes containing 3 ml. of medium 199 but without antibiotics were inoculated similarly and served as controls. The tubes were allowed to remain 10 minutes at room temperature. Material from the tubes containing antibiotics was transferred on sterile swabs to the left eyes of sheep 6 and 7. Sheep 8 and 9 were inoculated similarly but with materials which had not been treated with antibiotics. Ten days postinoculation (PI) sheep 6 and 7 were inoculated with untreated lacrimal secretions obtained from sheep 1. Swabs to be streaked on blood agar plates were obtained from all inoculated eyes at 24-hour intervals. All agar plates were incubated aerobically at 37 C.

Determination of the Pathogenicity of the Gram-Negative Cocci Isolated from Sheep 2, 3, 4, and 5.—Hemolytic colonies of gram-negative cocci were removed from blood agar plates and suspended in brain-heart infusion broth. Sterile cotton swabs were used to collect the lacrimal secretions from the eyes of yearling sheep 10, 11, 12, and 13. These swabs were streaked on blood agar plates. Then the bacterial suspension was placed in the left eyes of these sheep; the right eyes were not inoculated and served as controls. Eight days PI with the bacterial suspension, the left eyes were inoculated with pooled virulent lacrimal secretions obtained from the OIK-infected eyes of sheep 4 and 5.

Isolation of the Causative Agent in Cell Culture.—Sterile cotton swabs were used to collect the lacrimal secretions from the eyes of sheep 14 and 15. These were streaked on blood agar plates. Infective lacrimal secretions were obtained from the eye of sheep 1 and were placed in the left eyes of these 2 sheep. Eighteen hours after inoculation, cotton swabs were used to obtain lacrimal secretions from sheep 14. The swabs were placed in a tube containing 3 ml. of medium 199, rotated several times, and removed from the tubes. Then 0.2 ml. of this material was added to each of 10 Leighton tubes containing 24-hour-old McCoy synovial cell monolayers. The inoculum was allowed to remain for 1 hour, after which it was removed and replaced with medium 199 without added serum or antibiotics. The cell culture monolayers were incubated at 37 C. and were examined daily for cytopathic effects (CPE). When CPE was marked, the monolayers were harvested, and 0.2 ml. of the medium and cells were inoculated on 24-hour-old monolayers as before.

Determination of the Infectivity of 5th Cell Culture Passage Material.—Swabs containing material

* Difco medium 199, Difco Laboratories, Detroit, Mich.

Legends for Illustrations on Opposite Page

Fig. 1—Keratitis and conjunctivitis produced by sheep-passage ovine keratoconjunctivitis agent (sheep 3).

Fig. 2—Normal, uninoculated left eye of sheep 26. Control for inoculated right eye (see Figure 3).

Fig. 3—Right eye of sheep 26. Keratitis and conjunctivitis produced by the 15th cell culture passage of the ovine keratoconjunctivitis agent.

Fig. 4—Normal McCoy synovial cells. Acridine orange stain; x 1,600.

Fig. 5—McCoy synovial cells in which cytopathic effect was apparent 24 hours after inoculation with 15th cell culture passage of ovine keratoconjunctivitis agent. Acridine orange stain; x 1,600.


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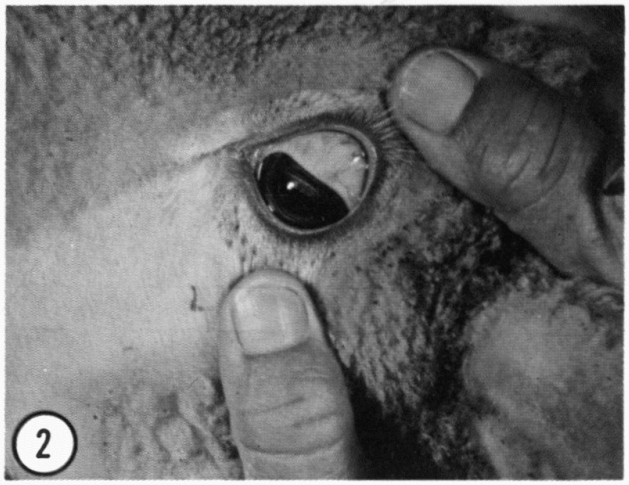
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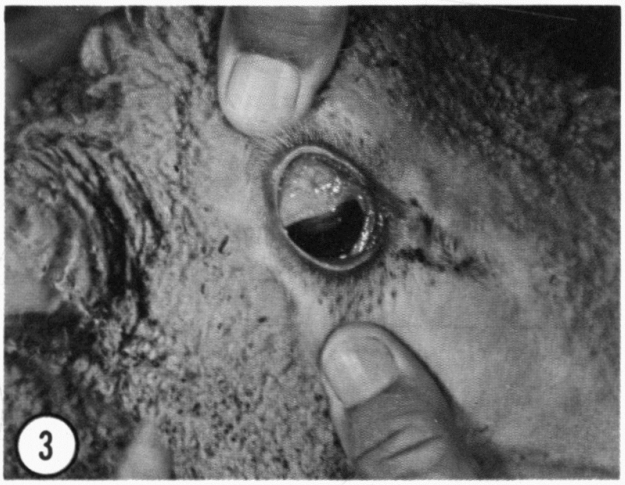
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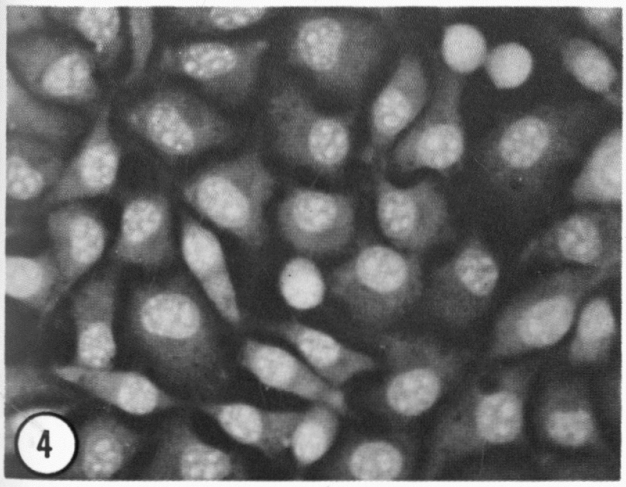
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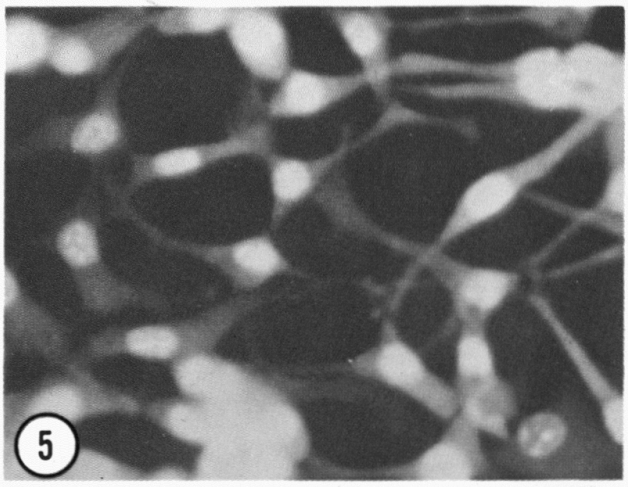
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from the 5th cell culture passage of the agent were used to inoculate the left eyes of sheep 16, 17, 18, and 19. Fourteen days PI, the same eyes were reinoculated with fully virulent material obtained in the lacrimal secretions from sheep 5.

Determination of the Infectivity of the 15th Cell Culture Passage of this Agent to Sheep.—Five aged sheep, 20, 21, 22, 23, and 24, were obtained from the experimental flock of the Texas Agricultural Experiment Station, College Station. These sheep were inoculated in the right eye, using cotton swabs containing material obtained at the 15th cell culture passage. They were observed daily for any signs of infection.

Determination of Effect of Sheep Passage on the Virulence of the Cell Culture-Adapted Agent.—Sheep 25 and 26 were inoculated in the left eye, using swabs containing material from the 15th cell culture passage. Three days after inoculation, lacrimal secretions obtained from the eyes of these sheep were transferred on cotton swabs to the eyes of sheep 27 and 28. Two days after the 1st subinoculation, the lacrimal secretions of sheep 27 and 28 were pooled and were inoculated into the eyes of sheep 29 and 30.

Determination of the Susceptibility of Goats.—Goats 1 and 2 of Angora breeding were inoculated in the eyes with virulent lacrimal secretions obtained from sheep 5. The eyes of Spanish goats 3, 4, 5, 6, and 7 were inoculated with the 16th passage of this agent in cell culture.

Attempts to Increase the Virulence of the 20th Cell Culture Passage of the Agent by Adding a Suspension of the Gram-Negative Cocci Obtained from Infected Eyes.—Two 4-month-old sheep (31 and 32) were inoculated in the right eyes, using swabs containing material obtained from the 20th passage of this agent in cell culture. The left eyes were inoculated with a mixture of the suspension of a freshly isolated gram-negative cocci and material obtained from the 20th cell culture passage. In addition, 2 similar sheep (33 and 34) were inoculated in the right eye with only material from the 20th cell culture passage; these sheep served as controls for sheep 31 and 32. Two weeks after inoculation, the immunity of sheep 31, 32, 33, and 34 was challenged with lacrimal secretions containing the fully virulent agent.

Results

Determination of the Infectivity of the Lacrimal Secretion from Sheep 1.—Swabs containing lacrimal secretions obtained from sheep 1 were negative when streaked on blood agar plates. Swabs containing lacrimal secretions from both eyes collected before inoculation, at 24 hours PI, and at 48 hours PI from sheep 2, 3, 4, and 5 were negative when streaked on blood agar plates. Only a few colonies which were contaminates were found on some of the plates. At 72 hours PI, gram-negative cocci were isolated from the lacrimal secretions of the inoculated eyes of these sheep. These colonies were hemolytic and formed almost pure cultures.

Sheep 2, 3, and 5 had an excess of lacrimal fluids in the inoculated eyes 48 hours PI. These secretions moistened the wool around the eyes which made the condition more obvious. Seventy-two hours PI, conjunctivitis and congestion of the blood vessels of the sclera were observed in all sheep. Keratitis developed in all 4 sheep within 7 days PI (Fig. 1). A corneal ulcer developed in the inoculated eye of sheep 2. The results are summarized (Table 1).

Determination of the Sensitivity of This Agent to Penicillin and Streptomycin.—Lacrimal material treated with antibiotics did not produce signs of OIK in sheep 6 and 7. When immunity was challenged with untreated infective material, these 2 sheep developed a conjunctivitis within 18 hours. The conjunctivitis was evident in sheep 6 for 3 days. Forty-eight hours after inoculation, keratitis was observed in sheep 7. Material obtained from the eyes and streaked on blood agar plates was essentially negative for 48 hours. At 72 hours, gram-negative cocci were easily isolated on blood agar plates.

Untreated lacrimal secretions produced conjunctivitis within 18 hours and keratitis within 72 hours in sheep 8 and 9.

Determination of the Pathogenicity of the Gram-Negative Cocci Isolated from Sheep 2, 3, 4, and 5.—Changes were not observed in the eyes of sheep 10, 11, 12, and 13 after inoculation with this bacterial suspension. When the immunity of these 4 sheep was challenged with the pooled lacrimal secretions from sheep having signs of the disease, excessive lacrimation was observed in all 4 sheep at 18 hours PI. Opaque areas at the upper and lower edges of the cornea were observed at 48 hours PI in sheep 11, 12, and 13. Keratitis developed in sheep 11, 12, and 13 at 72 hours. Severe conjunctivitis and congestion of the blood vessels of the sclera occurred in sheep 10 at 72 hours. Gram-negative cocci were isolated

from preinoculation specimens obtained from sheep 10, 11, 12, and 13. These organisms were not abundant, but hemolytic colonies were isolated occasionally on the plates.

obtained at 48 hours from the eyes of lamb 14. The lacrimal secretions from sheep 15 appeared to be sterile when inoculated on blood agar plates. All inoculated monolayers had CPE 48 hours after inoculation.

TABLE 1—Summary of Eye Lesions and Immunity Status of Sheep Used in This Experiment

Sheep No.	Type of inoculum	Results	Immunity test (inoculation with virulent OIK agent)
1	Natural infection	Severe keratitis	Not done
2	Lacrimal secretions from sheep 1	Severe keratitis and corneal ulcer	Not done
3		Severe keratitis	Not done
4		Severe keratitis	Not done
5		Severe keratitis	Not done
6	Lacrimal secretions from sheep 1 with antibiotics added	Negative	Keratitis
7		Negative	Keratitis
8	Lacrimal secretions from sheep 1 without added antibiotics	Keratitis	Not done
9		Keratitis	Not done
10	Suspension of gram-negative cocci isolated from sheep 2, 3, 4, and 5	Negative	Conjunctivitis
11		Negative	Keratitis
12		Negative	Keratitis
13		Negative	Keratitis
14	Lacrimal secretions from sheep 1	Keratitis	Not done
15		Keratitis	Not done
16	5th cell culture passage of OIK agent	Negative	Keratitis
17		Keratitis	Mild keratitis of short duration
18		Negative	Keratitis
19		Negative	Keratitis
20	15th cell culture passage of OIK agent	Keratitis	Not done
21		Conjunctivitis	Not done
22		Conjunctivitis	Not done
23		Conjunctivitis	Not done
24		Negative	Not done
25	15th cell culture passage of OIK agent	Excessive lacrimation	Not done
26		Keratitis 7th day	Not done
27	Pooled lacrimal secretions from sheep 25 and 26, 3 days PI	Excessive lacrimation	Not done
28		Keratitis 5th day	Not done
29	Pooled lacrimal secretions from sheep 27 and 28, 2 days PI	Keratitis 5th day	Not done
30		Keratitis 5th day	Not done
31	20th cell culture passage of OIK agent with a suspension of gram-negative cocci.	Slight keratitis	Immune
32		Slight keratitis	Immune
33	20th cell culture passage of OIK agent	Keratitis	Immune
34		Normal	Immune

Isolation of the Causative Agent in Cell Culture.—Forty-eight hours PI, conjunctivitis with congestion of the blood vessels in the sclera was observed in lambs 14 and 15. A few gram-negative cocci colonies were

The fibroblastic-type cells increased in length, forming long, narrow cells. Very few cells detached from the surface of the tubes. A group of those monolayers which were harvested at 72 hours and inoculated

onto other monolayers produced a CPE more rapidly and more cells were observed to be abnormal. At the 5th cell culture passage of this agent, CPE was well advanced in 24 hours and nearly all of the cells appeared to be affected (Fig. 5). The pH change was more acid in the inoculated tubes than in the uninoculated controls.

Determination of the Infectivity of the 5th Cell Culture Passage Material.—The eyes of sheep 16, 18, and 19 remained normal. Severe conjunctivitis with congestion of the scleral blood vessels developed in the eye of sheep 17 within 24 hours PI and remained for 6 days. The cornea of sheep 17 became opaque at 48 hours PI and remained so for 5 days.

After the eyes were inoculated with known virulent lacrimal secretions, conjunctivitis developed in all 4 sheep within 18 hours PI. At 24 hours PI, the corneas were slightly opaque at the upper margins. At this time, keratitis was evident in sheep 17; however, at 48 hours PI, the sign of infection had almost disappeared with only barely perceptible keratitis remaining. In sheep 16, 18, and 19, OIK followed the usual course.

Determination of the Infectivity of the 15th Cell Culture Passage.—Twenty-four hours PI, excessive lacrimation with a slight conjunctivitis were observed in the eyes of sheep 20, 21, and 22. Seventy-two hours PI, conjunctivitis and congestion of the blood vessels of the sclera were observed in sheep 20. At 96 hours PI, a slight excess of lacrimal secretions and conjunctivitis were observed in the eyes of sheep 23. At this time, keratitis had developed in sheep 20. Sheep 24 remained normal.

Determination of Effects of Sheep Passage on the Virulence of the Cell Culture-Adapted Agent.—Twenty-four hours after inoculation, excessive lacrimation was observed in the left eyes of both sheep. This condition remained unchanged until the 7th day PI, at which time slight keratitis, severe conjunctivitis, and congestion of the scleral blood vessels were observed in the left eye of sheep 26 (Fig. 3). The uninoculated right eye remained normal (Fig. 2). The eyes of sheep 25 had returned to normal at this time.

Five days after the 1st subinoculation, conjunctivitis, congestion of the scleral blood vessels, and keratitis were observed in the eyes of sheep 28. On the 7th day after the 1st subinoculation, excessive lacrimal secretions were observed in sheep 27.

Three days after the 2nd subinoculation both sheep 29 and 30 developed severe conjunctivitis with congestion of the blood vessels of the sclera. By the 5th day, the corneas were clouded at the margins and blood vessels were observed invading the corneas. The severity of the keratitis progressed until the 9th day after the 2nd subinoculation, at which time blood vessels had involved areas on upper and lower margins of the corneas of sheep 29 and 30.

Determination of the Susceptibility of Goats.—The eyes of all goats remained normal.

Attempts to Increase the Virulence of the 20th Cell Culture Passage of the Agent by Adding the Gram-Negative Cocci Obtained from Infected Eyes.—Twenty-four hours PI, excessive lacrimal secretions containing purulent flakes, conjunctivitis, and slight congestion of the blood vessels of the sclera were observed in sheep 31 and 32. The condition of the eyes remained unchanged for 6 days. On the 7th day PI, blood vessels which were barely perceptible had invaded the upper and lower margins of the corneas of both sheep. The eyes appeared normal 9 days PI. There was no difference in the severity of the infection of eyes inoculated in this manner and of eyes inoculated with only the cell-culture-adapted agent. When the immunity of sheep 31 and 32 was challenged with virulent lacrimal secretions, signs of infection were not observed.

Sheep 33 developed signs of OIK; sheep 34 remained normal. Both sheep were immune when inoculated with virulent lacrimal secretions.

Discussion

Since this agent was susceptible to penicillin and streptomycin in amounts commonly employed for virus isolation in chicken embryo, it appeared best to attempt to isolate the OIK agent without employing antibiotics. At the onset of conjunctivitis, usually from 18 to 48 hours PI, bacteria

were either absent or present in negligible numbers in the lacrimal fluids. It was during this period that the lacrimal fluids were collected for tissue culture inoculation. Bacterial or fungal contamination did not materially interfere with these isolations. After 48 hours PI it was impossible to isolate this agent because of contamination by bacteria. In isolating this agent, it is essential to obtain material from sheep in which the infection has just begun.

In the cell culture monolayers, CPE could be observed within 24 hours. The fibroblastic-type cells became greatly increased in length and formed long, needle-shaped cells. As a result, irregular spaces were formed between the cells. Several cells coalesced and formed clumps of cells. These changes are similar to those produced by the pleuropneumonia groups of organisms in tissue cultures.[4] A rapid increase in pH was observed in the cell culture fluid containing the OIK agent. The titer of the infective agent was low and never exceeded 10^3 log dilutions.

The susceptibility of the OIK agent to antibiotics and the requirements of cell cultures for the propagation of this agent indicates that this agent may be either a *Rickettsia* resembling Coles organism or a bacteria with strict growth requirements. Although numerous enriched media preparations have been used in attempts to propagate this organism in a cell-free medium, the possibilities of obtaining such a medium have not been exhausted. Attempts to propagate this agent in a cell-free medium are continuing. The relationship of this agent with the cell cultures is being determined using differential staining methods. The classification of this organism remains upon the completion of these studies.

The 5th cell culture passage of the OIK agent was of low virulence. It is possible that the titer of this agent in this passage was insufficient to produce signs of infection in all sheep. The virulence of the OIK agent increased in later passages although the signs were not as severe as are usually seen in natural infections. Subinoculation of the OIK agent into susceptible sheep increased the virulence and decreased the incubation period.

Gram-negative cocci did not produce any signs of OIK when inoculated alone, nor did they potentiate the virulence of the OIK agent when inoculated simultaneously with the OIK agent. Gram-negative cocci appear to be normal inhabitants or opportunists of the eye which cause no apparent damage.

Goats inoculated with the lacrimal secretions containing the virulent OIK agent or with the 15th cell culture passage did not have signs of OIK. This agent appears to be species specific.

In the Edwards Plateau region of Texas it is a common practice the place sheep and goats in the same pasture. Epizootics of infectious keratoconjunctivitis affecting sheep in contact with goats usually remains confined to sheep. Also, the converse appears to be true: epizootics of infectious keratoconjunctivitis affecting goats in contact with sheep seldom involve sheep.

The classification of this agent has not been determined precisely and investigation must continue before this can be accomplished.

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