A STUDY OF THE MEMBRANE NICTITANS AND GENITALIUM OF THE CANINE WITH REFERENCE TO LYMPHOFOLLICULAR HYPERPLASIA AND ITS ETIOLOGY

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A STUDY OF THE MEMBRANE NICTITANS AND GENITALIUM OF THE CANINE WITH REFERENCE TO LYMPHOFOLLICULAR HYPERPLASIA AND ITS ETIOLOGY

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PREFACE

This study was concerned with the development of granular appearing nodules on the membrane nictitans and genitalium of dogs. The condition referred to as follicular conjunctivitis, vaginitis and balanitis, is characterized by presence of the nodules on the anterior and posterior surfaces of the third eyelid, the vaginal mucosa, and shaft of the penis respectively. The etiology was presumed to be a new member of the herpesvirus group.

The purpose of this study was to confirm the etiology by demonstrating presence of the virus in the diseased tissues.

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INTRODUCTION AND LITERATURE REVIEW

The Conjunctiva

The conjunctiva is a thin transparent mucous membrane lining the inner surface of the lid (palpebral conjunctiva) and the anterior surface of the eye ball to the cornea (bulbar conjunctiva) (Figure 1). Principle function of the membrane is to serve as a barrier to organisms that may produce disease and to protect the cornea from infectious agents, abrasions and foreign bodies. The conjunctiva is exposed to more irritants than any mucous membrane of the body. The frequency with which pathology is self limiting attests to the ability of the membrane to provide effective defense.

Follicular Conjunctivitis

Small discrete mounds called follicles or nodules are present on the membrane. These follicles are considered to be lymphoid reactions as a part of a chronic inflammation (1). They are reported to be blister-like in appearance and may be distributed over the palpebral and bulbar surfaces of the membrane nictitans as a result of mild persistent irritation (2). Follicular conjunctivitis is a condition defined as an inflammation of the conjunctiva characterized by presence of a large number of these follicles. The follicles are generally considered to be aggregates of lymphocytes. Due to the

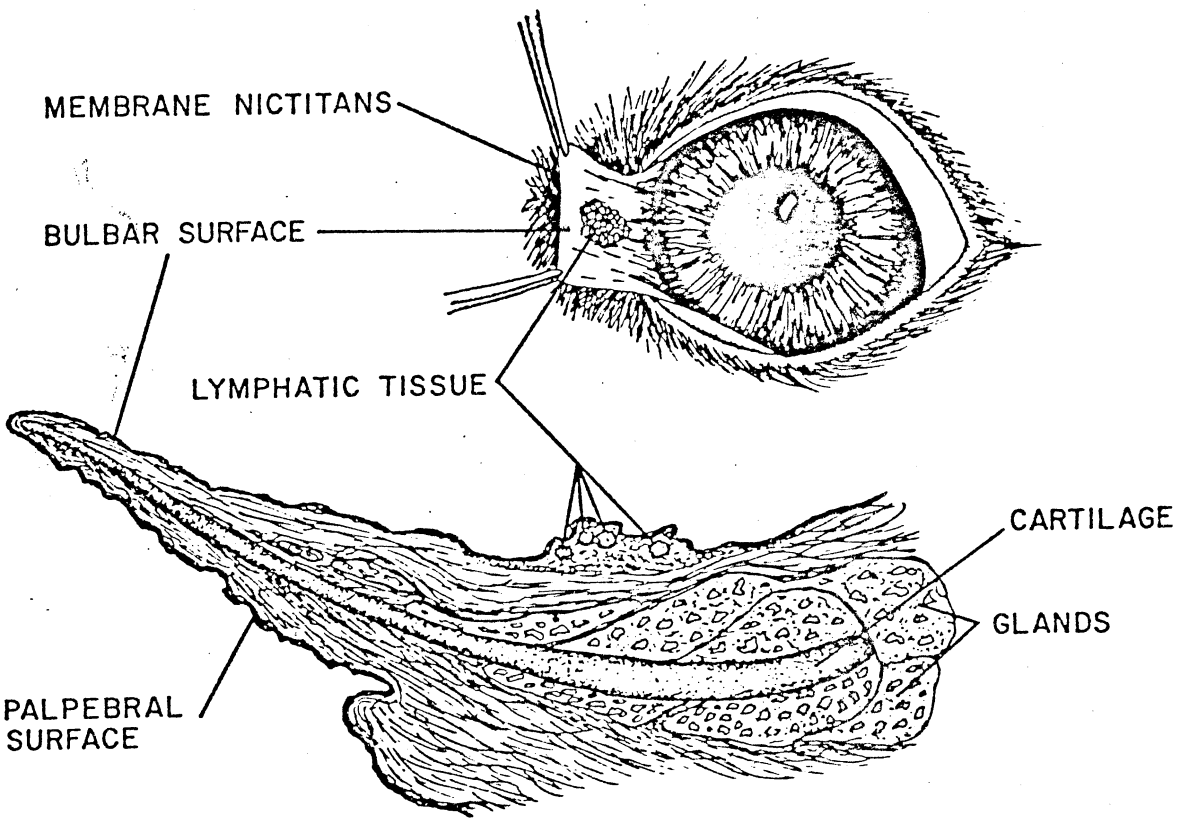

Figure 1. Drawing Depicting the Normal Membrane Nictitans (Drawn from Magrane, reference number 1)

granular appearance imparted to the membrane by the follicles, the condition has been described as a granular conjunctivitis (3). Ocular discharge may or may not accompany the inflammatory changes. The condition is common in humans. It often appears at an early age and persists until age 12 to 15 years when it subsides spontaneously without treatment (4). In domestic animals, follicular conjunctivitis is reportedly associated with tumefaction of the follicles in the palpebral conjunctiva and beneath the membrane nictitans (5).

Eversion of Membrane Nictitans

Entropion, eversion or inversion of the membrane nictitans, occurs congenitally or as an acquired anomaly in certain breeds of dogs; Great Danes, St. Bernards, German Shepherds and Weimaraners. Symptoms are epiphora, or mucoid discharge, and disfigurement (1). The disfigurement results from the protrusion of a tissue mass beyond the membrane producing the condition "cherry eye" (Figure 2). Other dogs affected are the spaniel and Boston terrier breeds. However, epiphora was not reported to be a feature of the condition in these breeds, only hypertrophy of the nictitans gland was reported. Aside from excess tearing, the condition is said to cause little discomfort in most cases. Infiltration of mononuclear cells was seen in some cases but only hyperplasia of secretory tissue in others. Erosion of corneal epithelium with rupture of the anterior chamber and iris prolapse was seen in one case. Removal of the tissue was deemed unnecessary (6). In a study of orbital glands in association with excess tearing, hyperplasia or hypertrophy of secretory tissue was prevalent. Inflammatory elements were observed and cystic ducts and cystic alveoli

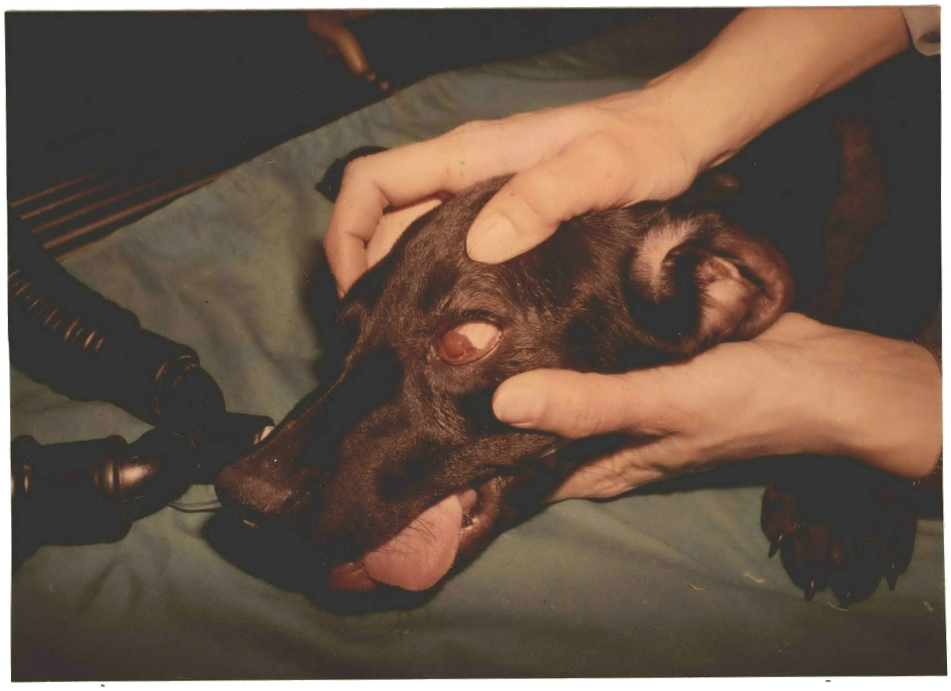

Figure 2. Dog with Condition Called "Cherry Eye" Note Round, Red, Bulging Mass of Tissue on the Third Eyelid.

Figure 3. Normal Eye of the Dog

were enlarged in a manner not characteristic of normal glandular tissue. The cellular infiltrates presented as mononuclear in type. Follicular lymphoid tissue was reported to be minimal and generally not hyperplastic (7).

Vaginitis and Balanitis

Involvement of the genitalium is believed to be a part of the syndrome where follicles are seen on the wall of the vagina and shaft of the penis in the dog. Enlargement of these follicles is reported to occur in cattle of the Grand Island breed (5). A folliculitis is seen on the vaginal mucosal membrane as well as on the membrane nictitans during the summer months while the cattle are on rich pasture. In the dog the genital lesions are seen in areas proximal to the bulb either anterior or posterior, they may be seen in both areas.

Etiology

Follicular conjunctivitis in human is reported to be accompanied by hypertrophy of lymphoid tissue of upper respiratory passages and considered to be a manifestation of some tendency producing adenoid vegetation of the throat. Attempts to transmit to human and animal have failed but isolation of Adenovirus from the throat of some cases is thought to have some etiological significance (4). Presence of follicles is not specific for virus. Viral conjunctivitis is reported to be characterized by progression from a bilateral serous discharge to a mucopurulent discharge. The etiology may be differentiated from a mycoplasma or chlamydia induced conjunctivitis by conjunctival

cytology and lack of response to chloromycetin (8). Generally, in human, follicular conjunctivitis has been attributed to a variety of causes and agents among which are various allergens, irritants, wind and dust. Ersine and other miotics used to contract the pupils have been incriminated (9). The condition is most often without symptoms and subsides without treatment when it is not accompanied by inclusion bodies, cicatricial changes, or other sequela such as trachoma (5, 8, 9).

In domestic animals the usual causes of follicular conjunctivitis are considered to be the same as for human; allergens, mechanical irritation, wind and dust (5). In one study of follicular conjunctivitis in the canine (2) one intranuclear inclusion was seen in a secretory cell but its significance was not elucidated. In another study (10) intranuclear inclusions were reported in association with the isolation of a Herpes-like virus in primary rabbit kidney cell culture. Transmission of the virus to normal dogs with production of disease was reported. Jackson and Corstvet (11) reported observing an intranuclear inclusion in a germinal epithelial or reticulum type cell. The material examined came from an eye lesion of an experimentally inoculated dog in the investigation. Virus was not isolated in the study, nor was cytopathogenic effect (CPE) noted in rabbit or canine kidney cell cultures. However, presence of the inclusion in conjunction with positive results in transmission studies was considered at that time, circumstantial evidence for presence of a Herpesvirus.

In an attempt to establish the significance of certain bacteria in the eye of the dog and define their role, if any, in the production of disease, a few investigators have studied the bacterial flora

of the normal as well as diseased eye. In the first study Jones (12) reported the predominant organism in the normal conjunctival sac to be a micrococcus sp followed by Staphylococcus aureus, diphtheroids and Streptococcus faecalis in the order named. In the pathological conditions, no reference was made to the kind of pathology, Staphylococcus aureus was the predominant organism followed by Proteus vulgaris and Pseudomonas aeruginosa in that order. A variety of other organisms were reported to be present with equal frequency in the normal and diseased eye. Correa (13) found Neisseria flava II in the conjunctiva of all dogs examined whether there was inflammation or not. The investigator concluded that the organism was a part of the normal flora assuming pathogenicity when the dog's resistance was lowered. Verwer and Gunnick (14) reported Streptococcus canis to be the predominant organism in the eye of the dog in a study of bacteria associated with chronic purulent ocular discharge. The writers did not describe the disease state of the conjunctiva so it is not known whether the condition was accompanied by follicular conjunctivitis. In another study of normal flora of the canine conjunctiva, Urban et. al. (15) reported that the time of year and breed of dog influenced the type of flora.

Mycoplasma agents have been isolated from both the normal and the diseased conjunctiva. Cello (16) reported finding mycoplasma agents in eyes of cats with conjunctivitis. The organisms were isolated from infected eyes only suggesting an etiologic role. Pugh and Hughes (17) in a study of infectious bovine keratoconjunctivitis concluded that mycoplasma agents do not play a major role in the condition. Their presence in the infection was considered to be only coincidental.

Isolation of mycoplasma agents from a litter of piglets involved in an outbreak of conjunctivitis prompted Friis (18) to examine the conjunctiva of swine for presence of the organisms. The membranes were collected from 40 dead piglets and cultured. Only one membrane showed signs of inflammation. The others were normal. Seventeen isolates were obtained. Five were reported to be new serologic variants of Mycoplasma hyorhinix. The significance of the isolations was not elucidated. Mycoplasma has been isolated from the diseased conjunctiva and from the normal membrane of the canine but a pathogenic role was not assigned (19, 20).

Follicular conjunctivitis in bovine of the Grand Island breed was reported to be due to rich carotene content in the diet of these animals (5). The mechanism by which this occurs was not defined.

A chlamydial agent was reported to be responsible for follicular conjunctivitis in the cat (21). The agent was transmitted to a human in which acute disease was produced. The trachoma and inclusion conjunctivitis (TRIC) agents are other chlamydiae affecting human and other primates where acute purulent conjunctivitis is seen in the new-born and follicular conjunctivitis in adults (22).

"Cherry eye" appears to be peculiar to the dog. In the breeds reported to be most commonly affected (1), the etiology was reported to be a defective cartilage causing the membrane to fold forward on itself or backwards, usually in the first year of life, causing the tissue to project beyond the membrane as a red mass, hence the name "cherry eye". Hypertrophy of the nictitans glands was reported to give rise to the condition in the spaniel and Boston terrier breeds (7). The underlying cause of the hypertrophy was not explained.

Recently the condition was seen in a litter of gnotobiotic pups. The "cherry eye" was apparent in three of a litter of five pups at the time the eyes were opened. Attempts to demonstrate an etiology were unsuccessful at this writing.

The etiology of vaginitis and balanitis has been ascribed to a variety of agents. Herpesviruses have long been associated with lesions of the genitalium of man and animals (24, 25, 26, 27).

A mycoplasma agent was isolated from vulvovaginitis in sheep (28) but the disease could not be reproduced in experimental animals, even though the agent multiplied in the vaginal vault.

Edward et al (29) isolated mycoplasma from the bovine genital tract but the pathological significance of the agent was not assessed. Hartman et al (30) inoculated mature heifers intravaginally with a mycoplasma agent known to be pathogenic for the bovine mammary gland. Neutrophilic leukocytosis was reported to be the only detectable clinical manifestation. However, Afsher (31) produced a granular vulvovaginitis in the bovine by inoculating the scarified vaginal mucosa with Mycoplasma bovigenitalium. The lesions were said to consist of granular elevations of the vulvo-vaginal epithelium and a mucopurulent discharge. Nodular collections of lymphocytes, necrosis of epithelium, hyperemia and an infiltration of eosinophils were reported to characterize the microscopic lesion. In another study (32) the same agent was inoculated into the genital organs of bulls. Seminal vesiculitis, epididymitis and ampullitis was seen. There was no report of follicular balanitis.

Certain mycoplasma species were isolated from the genital tract of both diseased and healthy dogs (19). Six types, based on

colonial morphology, were isolated. No significant relationship between the isolation rates and disease was reported. The agents were not considered to be responsible for disease. Rosenthal (33) reported isolation of mycoplasma from the genital tract of dogs, but, again, the agents were not associated with disease.

The follicular vaginitis, accompanied by conjunctivitis, seen in the Grand Island breed of cattle was attributed by Smythe (5) to a purely physiological cause; the rich carotene content of the pastures. There was no mention of balanitis in the bulls and, again, no explanation was given as to specific cause.

The chlamydial agents associated with inclusion conjunctivitis in man are also responsible for disease in human of the genital tract (22), but there appears to be no report of vaginitis or balanitis due to these agents in animals.

CHAPTER II

MATERIALS AND METHODS

Plastic Sections

Seventeen dogs were used in this study. The dogs were injected intramuscularly (IM) with acepromazine[a] followed by an intravenous (IV) injection of pentothal[b] to effect. Following intubation the dogs were maintained by fluothane[c]. Specimens surgically removed from the membrane nictitans were processed in solutions for electron microscopy (Appendix) as follows: They were placed immediately in cold 2% gluteraldehyde solution and minced into approximately 1 sq. mm. fragments. After fixation for two hours in this solution, the tissues were transferred to cold cacodylate buffer for 15 minutes. The 15 minute wash was repeated twice followed by one hour incubation in 2% osmium oxide. The tissues were washed three times in 50% ethyl alcohol then incubated for ten minute periods each in 70, 90, and 95% ethyl alcohol in the order named. The above procedures were carried out at refrigeration temperature. The following procedures were done at room temperature beginning with three ten-minute incubation periods

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[a]Acepromazine Maleate, Barber Lundberg, Oklahoma City, Oklahoma

[b]Sodium theopental, Abbott Laboratories, Chicago, Illinois

[c]Halothane, Ayerst Laboratories, New York, New York

in 100% ethyl alcohol followed by three ten-minute periods in propylene oxide. The tissues were incubated for three one-hour periods in 1:1 propylene oxide and Dow epoxy resin (DER)[d], 1:2 propylene oxide and DER and 1:4 propylene oxide and DER in that order. The tissues were then embedded in 100% DER and placed in a 60°C vacuum oven for 48 hours. The plastic blocks were removed from the oven and trimmed preparatory to sectioning. Thick sections (1.5-2.0u) were cut using a glass knife and a Sorvall MT-2 ultramicrotome[d]. Richardson's stain (34) was then applied to the warmed slide and the section heated for 30 seconds or until desired color intensity was achieved. The slides were rinsed in distilled water, air dried and examined with the light microscope for pathologic changes suggesting viral infectivity. Blocks of sections showing such changes were prepared for electron microscopy by recutting with a diamond knife[d] at a thickness of 60 to 90 nanometers. The silver reflective sections were stained with 5% aqueous uranyl acetate and lead citrate (35). They were then examined and micrographed with the electron microscope[e].

Fluorescent Antibody (Direct)

Smears of Conjunctiva

Twelve pups were used in this study. Five beagle were obtained from the canine hemophilia resource colony maintained at Oklahoma State University College of Veterinary Medicine. They were purchased

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[d]Polysciences, Warrington, Pennsylvania

[e]Phillips 200, Mount Vernon, New York

at six weeks of age, kenneled in isolation quarters until placed on the research program at three months of age. The other seven mongrel pups were whelped in an isolated area where they were kenneled until assigned to the research program at three months of age. The membrane nictitans was examined on several occasions during the interim for presence of follicles. Changes observed during this period were grossly indicative of follicular conjunctivitis. Smears were prepared by rubbing a sterile cotton swab briskly over the membrane and depositing the material on a microscope slide. The slides were air dried and stored at -70°C.

Serum used in this study was canine Herpesvirus - 205 (CHV-205) antiserum[f]. The serum neutralization tissue culture infective dose ($TCID_{50}$) titer was 160 with complement and 40 without complement. The antiserum was prepared in two 18-week old beagles. The pups were inoculated IV with $10^{6.2}$ sucrose gradient purified virus. Three weeks later the pups were again inoculated IM with the virus (untitered) in adjuvant. The pups were bled four weeks from the last inoculation and the serum collected. The diethylaminoethylcellulose (DEAE-cellulose) column was prepared and the serum conjugated with fluorescein according to Corstvet and Sadler (36). Trial runs were made to determine the optimum concentration of the conjugate and the best absorption procedure to minimize nonspecific staining. Best results were obtained when the primary canine kidney cells, used for tissue culture, were mixed, equal parts, with the conjugated serum. The mixture was incubated at 4°C for 30 minutes, centrifuged and the

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[f]Provided by Dr. L. E. Carmichael, Department of Pathology, New York State Veterinary College, Ithaca, New York, 1977.

supernate used in a final concentration of 1:10.

The smears were thawed and placed in 0.01 M phosphate-buffered-saline (PBS) (Appendix) containing 0.1% tween 80 for ten minutes. They were then each treated with approximately 0.1 ml of the stain and incubated for 25 minutes at 37°C in a moist chamber. After incubation the smears were washed in two changes of PBS for 25 minutes followed by a quick rinse in distilled water. The stained smears were mounted with cold fluorescent antibody mounting medium (90% glycerol in PBS) and refrigerated until read. Smears of the dog kidney cells were used as controls. The smears were examined by a Zeiss microscopy with epi-illumination.[g]

Frozen Section of Membrane Nictitans

Twelve eight-week old mongrel pups with the third eyelid showing a mild form of follicular conjunctivitis were scarificed and the membranes used in this study. The pups were given Beuthanasia-D[h] IV to effect. The membranes were surgically removed and representative samples placed immediately on dry ice. The tissue specimens were stored at -20°C until sectioned (4u) with a Lipshaw model 30AB cryostat[i]. The cut sections were mounted on coverslips, stained and examined by the above procedure.

Fluorescent Antibody (Indirect)

Twenty-nine dogs of varying breeds and ages were used in this

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[g]Baltimore Instruments, Dallas, Texas

[h]Chromalloy Pharmaceutal Inc., Oakland, California

[i]Lipshaw Manufacturing Corporation, Detroit, Michigan

study (Table I). The lesions varied from the mild granular follicular conjunctivitis to a more severe proliferative conditions involving both the palpebral and the bulbar surfaces of the membrane nictitans (Figures 4 and 5). Five of the dogs (beagles) were obtained from the canine hemophilia resource colony of Oklahoma State University College of Veterinary Medicine at six weeks of age. An additional five mongrel pups were kenneled since birth in pens isolated from other dogs until they were 12 weeks of age. Another seven dogs (beagles) originating from the hemophiliac resource colony were held in a Stillwater kennel for two months before being returned to the veterinary medicine facility. It should be mentioned that the hemophilia resource colony is maintained in isolation from other dogs. Follicular conjunctivitis has not been seen in the dogs. Seven of the dogs were admitted to the small animal clinic for various complaints and the lesions were detected upon examination. The remaining five dogs were acquired from the Ponca City dog pound. Blood samples were taken from the dogs, the serum collected and stored at -70°C. Paired samples were collected three weeks apart from two of the dogs and used in this study. All other samples were single bleedings.

The virus used in this study was the canine Herpesvirus (CHV-205) provided by Dr. Carmichael. Primary canine kidney cells prepared according to Jackson and Corstvet (11) were used to propagate the virus. The cells were grown in minimum essential medium (MEM) Eagle's[j] with 50 mcg per ml of gentamicin[k] and five mcg per ml of

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[j]Microbiological Associates, Bethesda, Maryland

[k]Gentamicin Reagent Solution, Schering Corporation

TABLE I

SITE OF LESIONS AND RESULTS OF INDIRECT FLUORESCENT ANTIBODY TESTS

Sample No.	Animal	Eye	Genitalium	Results[c]
1	917-58	Positive[a]	Negative[b]	- +
2	916-H1D	Positive	Negative	- +
3	918-Z5	Positive	Negative	+ -
4	904-X3	Positive	Negative	-
5	914-PE3	Positive	Negative	-
6	915-EZ9	Positive	Negative	-
7	913-Y3	Positive	Negative	2 +
8	77385	Positive	Positive	-
9	78465	Positive	Negative	-
10	79132	Positive	Positive	- +
11	F-7998	Positive	Positive	-
12	F-79490	Positive	Negative	- +
13	Springer	Positive	Negative	-
14[d]	915	Positive	Negative	+ -
15	906	Positive	Negative	-
16	X03	Positive	Negative	-
17	216	Positive	Negative	-
18[e]	918	Positive	Negative	+ -
19	939	Positive	Negative	-
20	902	Positive	Negative	- +
21	Baldy	Positive	Negative	-
22	Y01	Positive	Negative	- +
23	C-4	Positive	Negative	- +
24	920	Positive	Negative	+ -
25	A-1	Positive	Negative	+ -
26	A-2	Positive	Negative	+ -
27	A-3	Positive	Negative	-
28	A-4	Positive	Negative	-
29	A-5	Positive	Negative	-
30	Bassett	Positive	Negative	+ -
31	217	Positive	Negative	-

[a]Lesions present at site indicated

[b]Lesions non present at site indicated

[c]Plus-minus (+ -), minus-plus (- +), negative (-)

[d]Second bleeding of animal number 6

[e]Second bleeding of animal number 3

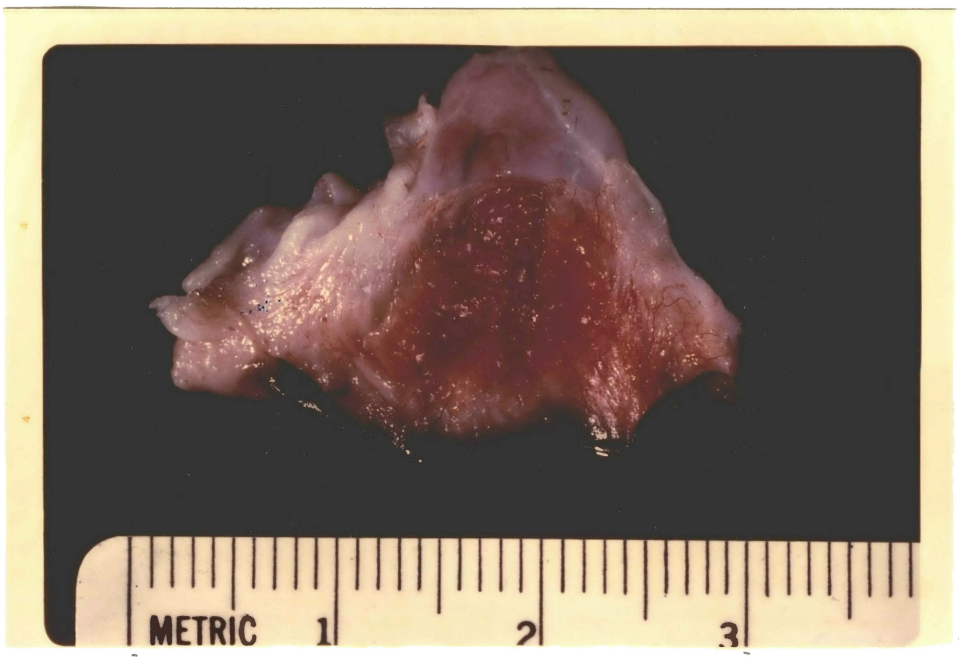

Figure 4. Bulbar Surface of Membrane Nictitans Showing Granularity.

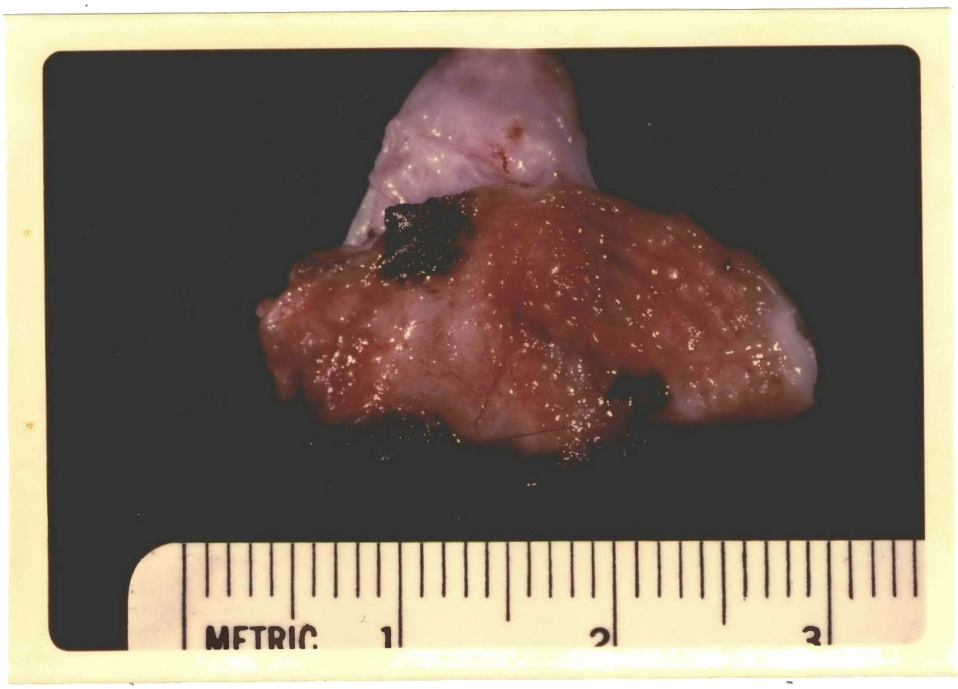

Figure 5. Palpebral Surface of Membrane Nictitans Showing Granularity.

amphotericin B[1]. Ten percent fetal calf serum[m] was used in the growth medium and 2% in the maintenance medium. Lab Tek slides[n] with four chambers were seeded with the cell suspension and incubated at 37°C in 5% CO_2 in a Bellco incubator[o]. Confluency was achieved in 48 hour. Old medium was poured off and the monolayers inoculated with 0.1 ml of virus suspension containing 10^5 $TCID_{50}$ virus per ml. One hour was allowed for adsorption of the virus after which time maintenance medium was added and the monolayers reincubated. The infected monolayers were incubated for 24 to 48 hours until foci of CPE were noted, but not until cells were detached from the glass. The slides were then prepared for fluorescent antibody study by washing in PBS, fixing for ten minutes in acetone and freezing at -70°C. Upon removal from the freezer, the slides were thawed and placed in PBS containing 0.1% tween 80 for ten minutes. They were then flooded with test serum samples diluted 1:10 and incubated at 37°C for 30 minutes in a moist chamber. Every fifth chamber was used as a positive control receiving the CHV-205 antiserum. Following incubation the slides were again washed in PBS, rinsed in distilled water and stained with rabbit anti-dog IgG fluorescein conjugate[p]. After the slides were incubated a second time, washed and rinsed, they were mounted with cold 90% glycerol in PBS and refrigerated until

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[1]Fungizone, E. R. Squibb & Sons, Inc., Princeton, New Jersey

[m]Pel-Freeze, Biologicals Inc., Roger, Arkansas

[n]Scientific Products, Grand Prairie, Texas

[o]Bellco Glass Inc., Vineland, New Jersey

[p]Miles Laboratories, Elkhart, Indiana

read with the fluorescent antibody microscope. Due to variability in staining, the tests were read as negative (-), minus-plus (- +), plus-minus (+ -), one plus (1 +), two plus (2 +), etc. with the positive control representing a four-plus (4 +) reaction. Serum neutralization tests were done with all samples showing a + - or higher reaction.

Serum Neutralization

Eight of the 31 serum samples used in the indirect fluorescent antibody study were read as a + - or greater reaction. These samples were used for the neutralization tests. The virus (CHV-205) was first titered by preparing ten-fold dilutions using tissue culture medium as diluent. Four confluent monolayers of primary canine kidney cells, in falcon tissue culture tubes[q], were inoculated with 0.2 ml of each virus dilution. Following the one hour adsorption period maintenance medium was added and the tubes sealed and incubated. The infected monolayers were allowed to incubate for six days before the final reading. The $TCID^{50}$ titer of the virus was found to be 5×10^6 per ml. The concentration of virus used for the test was 5×10^4 per ml. The test serum samples were diluted 1:1 with PBS and mixed with equal parts of the diluted virus. After 15 minutes incubation, the serum-virus mixtures were inoculated onto confluent monolayers in Lab Tek slides. The first chamber of each slide was inoculated with 0.1 ml of virus and used as controls. The remaining three chambers were inoculated with 0.2 ml of the virus-serum mixtures. Following

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[q]Curtis Matheson Scientific Inc., Dallas, Texas

the adsorption period, maintenance medium was added and the slides incubated in CO_2 in a Bellco incubator. The monolayers were examined daily for CPE with an inverted microscope[r].

Histopathology

Follicular Conjunctivitis

One membrane nictitans from each of the 12 pups described under direct fluorescent antibody (frozen section) was placed immediately upon removal into Bouin's solution (Appendix). The tissues were fixed in the Bouin's for three hours after which they were placed in isopropyl-sodium-thiosulfite solution (hypo) for five hours. After fixing for eight hours in a second change of hypo, the tissues were placed in 70% ethyl alcohol for a minimum of 24 hours. The specimens were then submitted to the Pathology Department of Oklahoma State University College of Veterinary Medicine for further processing, sectioning and interpretation.

"Cherry Eye"

The tissue mass producing the condition "cherry eye" was observed in one of a litter of five mongrel pups (Figure 2). The mass was first observed when the pup was three months of age. The condition was not always evident during the next month as the tissue mass disappeared and reappeared spontaneously on several occasions. At about four months of age the condition persisted with the mass measuring one centimeter in diameter. The pup was given acepromazine

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[r]Olympus, Actinorex, Springfield, Missouri

.05 mg IM per pound of body weight followed by sodium pentothal given IV at a dose of 6-8 mg/pound of body weight. The tissue mass was excised and placed in Bouin's fixative. Fixation was continued as above and the tissue submitted to the Pathology Department of Oklahoma State University College of Veterinary Medicine.

Bacterial Infections

Mycoplasma Cultures

Twelve dogs were cultured for mycoplasma agents (Table II). Sterile cotton swabs were rubbed over the membrane nictitans, the vaginal mucosa and the shaft of the penis proximal to the bulb. The swabs were inoculated onto an agar selective for the pleuropneumonia-like organisms (PPLO agar)[s]. The medium contained 10% horse serum[t], 1,000 units per ml of penicillin[u] and 0.025% thallium acetate[v]. The inoculated plates were incubated in a candle jar at 37°C for one week. The plates were examined microscopically under low power for presence of mycoplasma colonies.

Examination for Chlamydia

Cultures. Seven dogs, four Irish Setters, one Bassett Hound, one Coonhound, and one Cocker Spaniel were used in this study. The

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[s]Difco Laboratories, Detroit, Michigan

[t]Pel Freeze, Biological Inc., Rogers, Arkansas

[u]Potassium Penicillin G., Pfizer Laboratories, New York

[v]Fisher Scientific Co., Fair Lawn, New Jersey

TABLE II

ANIMALS CULTURED FOR MYCOPLASMA AND SOURCE TISSUE GIVING POSITIVE AND NEGATIVE RESULTS

Animals	Breed	Sex	Age	Source and Results	
				Eye	Genitalium
1	Bassett	Female	1 Yr.	Negative	Positive
2	Coonhound	Female	?	Negative	Positive
3	Irish Setter	Female	9 Mo.	Negative	Positive
4	Irish Setter	Female	9 Mo.	Positive	Positive
5	Irish Setter	Female	9 Mo.	Negative	Positive
6	Irish Setter	Male	9 Mo.	Negative	Positive
7	Mixed	Female	Pup	Positive	Positive
8	Mixed	Female	Pup	Negative	Negative
9	Mixed	Male	Pup	Negative	Negatove
10	Mixed	Female	Pup	Positive	Positive
11	Mixed	Male	Pup	Positive	Positive
12	Cocker	Female	Adult	Positive	Positive

Irish Setters were obtained from a local kennel at four months of age. The Bassett and Cocker Spaniel were patients in the small animal clinic. The Coonhound was donated by an area dog pound. The membrane nictitans of all dogs presented grossly as follicular conjunctivitis, but genital lesions were observed in only two of the dogs (Table III). Sterile cotton swabs were rubbed briskly over the conjunctiva, the vaginal mucosa and shaft of the penis. The swabs were placed in tubes containing 2 ml PBS with 1 mg per ml each of streptomycin[W] and kanamycin[X]. After one hour incubation at room temperature, the swabs were removed from the tubes and 0.2 ml of the suspension inoculated into the yolk sac of five to seven day old chick embryos. The embryos were incubated at 37°C and candled daily. Yolk was blind passaged from the inoculated embryos at weekly intervals to other five to seven day old embryos.

Direct Examination. Yolk sac membranes from the donor embryos were then washed in sterile saline. Impression smears were made, stained by the Gimenez technique (37) and examined microscopically for the intracellular organisms. The remaining PBS suspensions, used as inocula for the chick embryos, were centrifuged and the supernatant decanted. The pellet was resuspended in residual PBS and smeared on a microscope slide. The preparations were air dried, stained by the Gimenez technique, and examined for presence of the organism in the epithelial

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[W]Streptomycin Sulfate, Pfizer Laboratories, New York

[X]Kantrim, Barber-Lundberg, Oklahoma City, Oklahoma

TABLE III

ANIMALS EXAMINED BY CULTURE AND DIRECT SMEAR FOR *CHLAMYDIA SPP*

Animals	Breed	Age	Sex	Lesions		Results	
				Eye	Genitalium	Culture	Smears
1	Bassett	?	Female	Positive[a]	Positive	Negative	Negative
2	Cocker	?	Female	Positive	Positive	Negative	Negative
3	Irish Setter	7 Mo.	Male	Positive	Negative[b]	Negative	Negative
4	Irish Setter	7 Mo.	Female	Positive	Negative	Negative	Negative
5	Irish Setter	7 Mo.	Female	Positive	Negative	Negative	Negative
6	Irish Setter	7 Mo.	Male	Positive	Negative	Negative	Negative
7	Coonhound	?	Female	Positive	Negative	Negative	Negative

[a]Lesions present at site indicated

[b]No lesions present at site indicated. All sites whether positive or negative were cultured and smeared.

cells.

Serology. Five mongrel littermates obtained from a private home at four weeks of age were kenneled in isolation from other dogs. Two months later sufficient proliferation of tissue on the bulbar surface of the conjunctiva was evident to present, grossly, as follicular conjunctivitis. These five pups and one additional beagle pup one year of age were bled. The serum was collected and submitted to a testing laboratory[y] to be assayed for complement fixing (CF) antibodies to chlamydia (Table IV). A second blood sample was collected four weeks later and the serum sent to the above laboratory to be retested for CF antibodies to chlamydia.

Cultures for Other Bacterial Agents

The 12 dogs used in the mycoplasma study (Table II) were cultured for other bacterial agents. Sterile cotton swabs were rubbed over the membrane nictitans, vaginal mucosa and the shaft of the penis proximal to the bulb. The swabs were inoculated onto a heart infusion agar[z] plate containing 5% bovine whole blood with 0.6% sodium citrate[aa] as an anticoagulant. The plates were incubated at 37°C and examined at 24, 48 and 72 hours for bacterial growth.

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[y]Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma

[z]Difco Laboratories, Detroit, Michigan

[aa]Fisher Scientific Company, Fair Lawn, New Jersey

TABLE IV

ANIMALS TESTED FOR COMPLEMENT FIXING ANTIBODIES TO *CHLAMYDIA SPP*

Animal	Breed	Age	Sex	Eye	Genitalium	Results	
						Sample 1	Sample 2
1	Mixed	4 Mos.	Female	Positive[a]	Negative[b]	Negative	Negative
2	Mixed	4 Mos.	Female	Positive	Negative	Negative	Negative
3	Mixed	4 Mos.	Male	Positive	Negative	Negative	Negative
4	Mixed	4 Mos.	Male	Positive	Negative	Negative	Negative
5	Mixed	4 Mos.	Male	Positive	Negative	Negative	Negative
6	Beagle	1 Yr.	Female	Positive	Negative	Negative	Negative

[a]Lesions present at site indicated

[b]Lesions not present at site indicated

Viral Isolation Attempts

Forty-two dogs of varying ages and breeds were cultured for virus. The third eyelid and genitalium of each animal were examined. Proliferation of lymphoid or glandular tissue was evident on the bulbar surface of the membrane nictitans of all the dogs, but follicles were present on the genitalium of only four of the animals. Conjunctival and genital scrapings were obtained by currettage. The tissue fragments were removed with sterile cotton swabs. The swabs were placed in a tube containing 1 ml of a cleaning solution comprised of MEM with 200 mcg/ml of gentamicin[bb]. After one hour incubation at room temperature, the swabs were removed and 0.2 ml of each tissue suspension inoculated onto two monolayers of primary dog kidney cells in falcon tissue culture tubes. The inoculated monolayers were incubated at 37°C for one hour to allow for attachment of the virus, after which, the inoculum was poured off and maintenance medium added. The tubes were sealed and the inoculated monolayers incubated at 37°C and examined daily for CPE. One of the two monolayers was blind passaged at 96 hour intervals by trypsinization as follows; the old medium was decanted and saved and the monolayers washed with saline A (Appendix). Trypsin solution was added and allowed to stand on the cells for five to ten minutes at 37°C. The spent medium was added back to the tube along with sufficient growth medium to permit dividing the cell suspension into two parts. The divided cells suspensions were again incubated at 37°C.

[bb]Gentamicin reagent solution, Schering Corporation, Kenilworth, New Jersey

Control monolayers were passaged in the same manner. After the monolayers had been passaged and observed for two to three weeks, they were discarded as negative. All material for culture was inoculated onto primary dog kidney cells prepared as in the previous investigation (11). However, on a few occasions, an established line dog kidney cell, Martin-Darby dog kidney (MDDK)[cc], was also inoculated and processed as above.

Transmission Studies

Animal to Animal

Four Irish Setter pups 12 weeks of age were used in this study. The donor animals were a female Bassett hound and female Cocker Spaniel. Both had marked follicular conjunctivitis and vaginitis. Material from the donor animals was obtained by curettage and the specimen taken with swabs. The swabs were rubbed over the third eyelid and genitalium of the experimental, recipient, pups. The experimentally inoculated pups were examined twice weekly. The inoculated areas were swabbed and cultured for mycoplasma, chlamydia and virus as described above. Two Irish Setter pups from the same litter were used as uninoculated controls.

Culture to Animal

Viral. The CHV-205 virus used in the fluorescent antibody study was inoculated onto the membranes nictitans and genitalia of two

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[cc]Supplied by Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma

mongrel pups to determine whether or not lesions comparable to those seen in natural infections would develop. Swabs dipped into infected primary dog kidney cell suspensions were rubbed over the third eyelid and genitalium of the experimental pups in the same manner as above. The inoculated pups were examined twice weekly, swabbed and cultured for presence of the virus as described under viral isolation attempts. One mongrel littermate was maintained in isolation for a control.

Bacterial. Five mongrel pups, 14 weeks of age, and one Beagle pup, one year of age, were anesthetized and the genitalia examined. All were found to be void of lesions suggesting vaginitis or balanitis. The agent used in this study was Streptococcus canis. The agent had been isolated in pure culture from the vagina of two dogs with follicular vaginitis. The agent was subcultured once on heartinfusion agar slants washed off with sterile milk, lyophilized[dd] and stored. The day before use, the organism was inoculated into heart infusion broth and incubated at 37°C. A sterile swab was dipped into the 24 hour broth culture and rubbed over the vaginal mucosa of one of the females and the shaft of the penis proximal to the bulb of two male pups. The remaining three pups, one female and two males, were sham inoculated with a sterile swab dipped into sterile heart infusion broth and used as controls. All animals were cultured just prior to inoculation for presence of Streptococcus canis. The inoculated animals were examined for lesions twice weekly for two weeks and again at the end of the third week.

[dd]Atmo-vac Refrigeration for Science, Island Park, New York

CHAPTER III

RESULTS

Plastic Sections

Only five of the sections presented as abnormal. Microscopic examination of these sections revealed cytoplasmic vacuolation of mucosal epithelial cells, lymphoid follicles composed primarily of reticuloendothelial cells with a scattering of lymphocytes and some necrosis as attested by presence of karyorrhexis and hemorrhage[a] (Figure 6 and 7). Electronmicrographs of these sections were void of particles resembling virus.

Fluorescent Antibody (Direct)

Smears of Conjunctiva

Results of the direct fluorescent antibody study of the smears were negative. Non-specific staining was a problem. However, staining in the test samples was not found to be of any greater intensity than was observed in normal controls. Hence, it was concluded that no *Herpesvirus* antigens were present in the tissues of the membrane nictitans of these animals.

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[a]Read by Dr. E. L. Stair, Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma

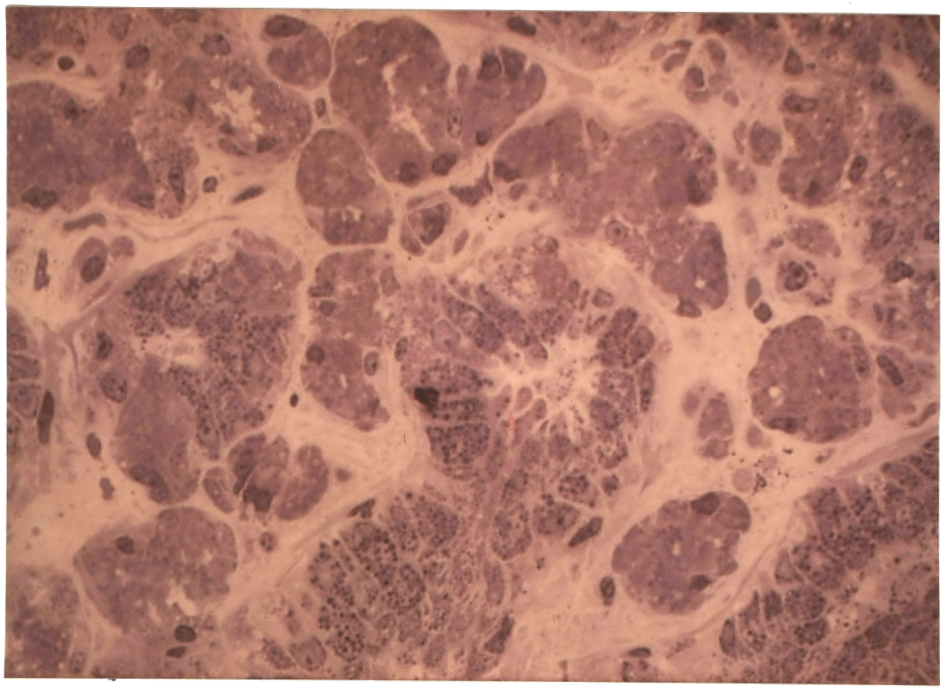

Figure 6. Plastic Section of Normal Membrane.
Stained with Richardson's.

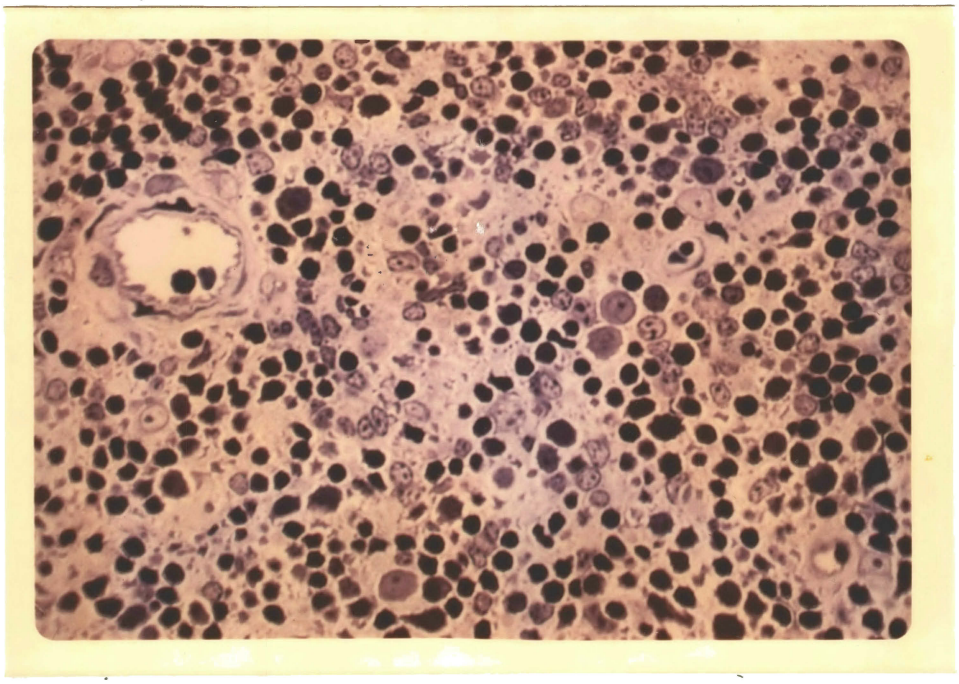

Figure 7. Plastic Section of Membrane Showing Inflammatory Changes. Stained with Richardson's.

Frozen Sections of Membrane Nictitans

Results of this study were also negative. Controls were not used in this study as no clear eyelids were available for sectioning. However, the staining intensity was such that all sections were considered normal and without Herpesvirus antigens.

Fluorescent Antibody (Indirect)

Results of the indirect fluorescent antibody studies were varied. The degree of staining intensity of the specimen varied from no staining at all (negative) to an intensity approaching that of the positive controls (Figure 8 and 9). Sixteen of the test specimen were read as negative, seven as minus-plus (- +), seven as plus-minus (+ -) and one as two-plus (2 +) to express the difference in degree of staining (Table I). At this point in the investigation it was assumed that the dogs whose serum gave a positive test possessed antibodies to the virus.

Serum Neutralization

Serum neutralization tests using the serum samples giving a plus-minus (+ -) or stronger reaction failed to support the notion that the samples possessed antibody to the virus. Even the serum giving the strongest reaction (2 +) failed to neutralized infectivity of the virus (Table V).

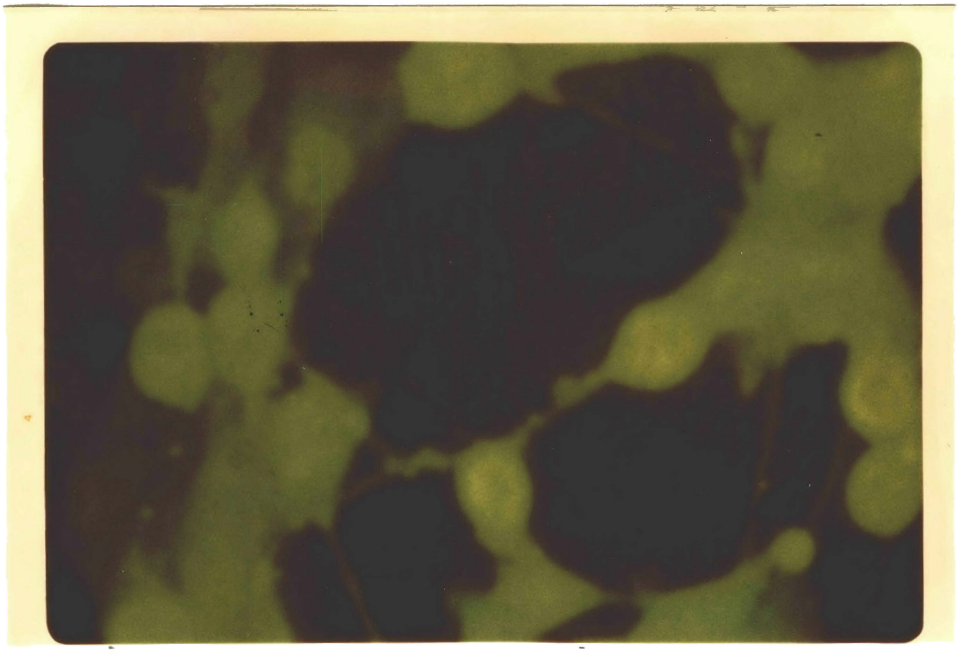

Figure 8. Positive Indirect Fluorescent Antibody Test.

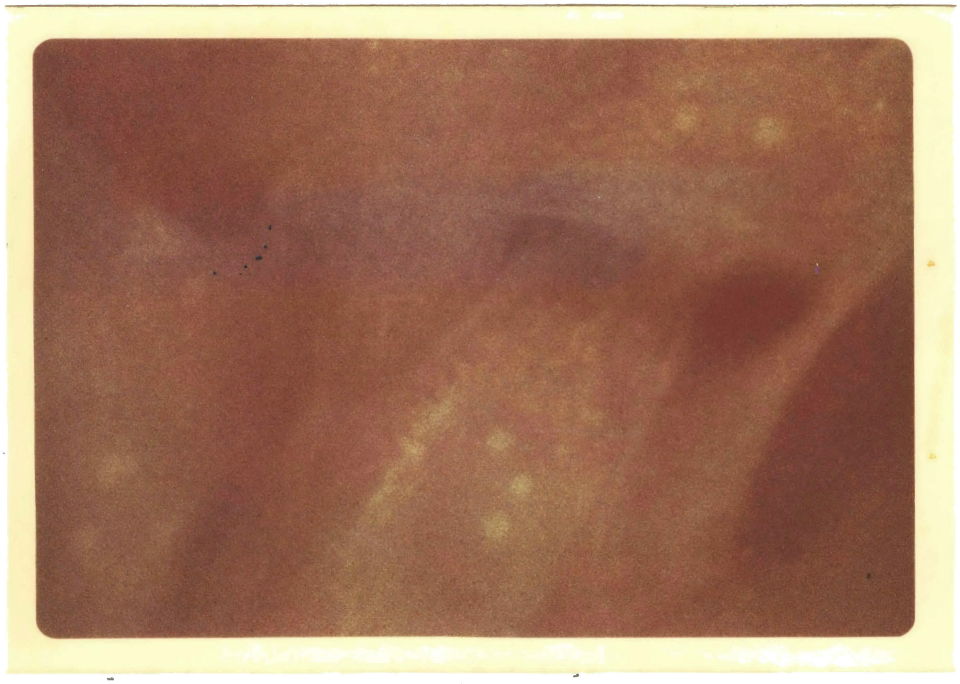

Figure 9. Negative Indirect Fluorescent Antibody Test.

TABLE V

RESULTS OF SERUM NEUTRALIZATION TESTS FOR ANTIBODIES TO HERPESVIRUS (CHV-205)

Sample No.	Animal	Eye	Genitalium	Results
3	918-25	Positive[a]	Negative[b]	Negative
7	913-Y3	Positive	Negative	Negative
14	915	Positive	Negative	Negative
18	216D	Positive	Negative	Negative
24	920	Positive	Negative	Negative
25	901	Positive	Negative	Negative
26	A-1	Positive	Negative	Negative
30	A-5	Positive	Negative	Negative

[a]Lesions present at site indicated

[b]No lesions present at site indicated

Histopathology

Follicular Conjunctivitis

Study of the histopathologic sections revealed the predominant tissue to be glandular in type. Mounds, blebs on the membrane nictitans appearing grossly, and presenting clinically as lymphofollicular proliferation (Figure 4 and 5) were found, upon microscopic examinations, to be glandular tissue, for the most part, with occasional foci of lymphoid tissue (Figures 10 and 11). Inflammatory changes were minimal and not considered to be of pathological significance. Generally, the sections were interpretated as being within normal limits of a canine third eyelid.[b]

"Cherry Eye"

The condition could hardly be considered normal. The mass of tissue was found to be glandular in type rather than lymphoid as was suspected. Part of the section was comprised of cartilage in the shape of a horseshoe (Figure 12).

Bacterial Infections

Mycoplasma Cultures

Based on colonial morphological, four different *Mycoplasma spp* were isolated from the dogs. The agents were isolated from the genitalium only of five dogs, and from the eye and genitalium of five dogs. Two of the dogs were negative (Table II).

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[b]The sections were read by Dr. B. Osburn, Department of Pathology, College of Veterinary Medicine, Davis, California

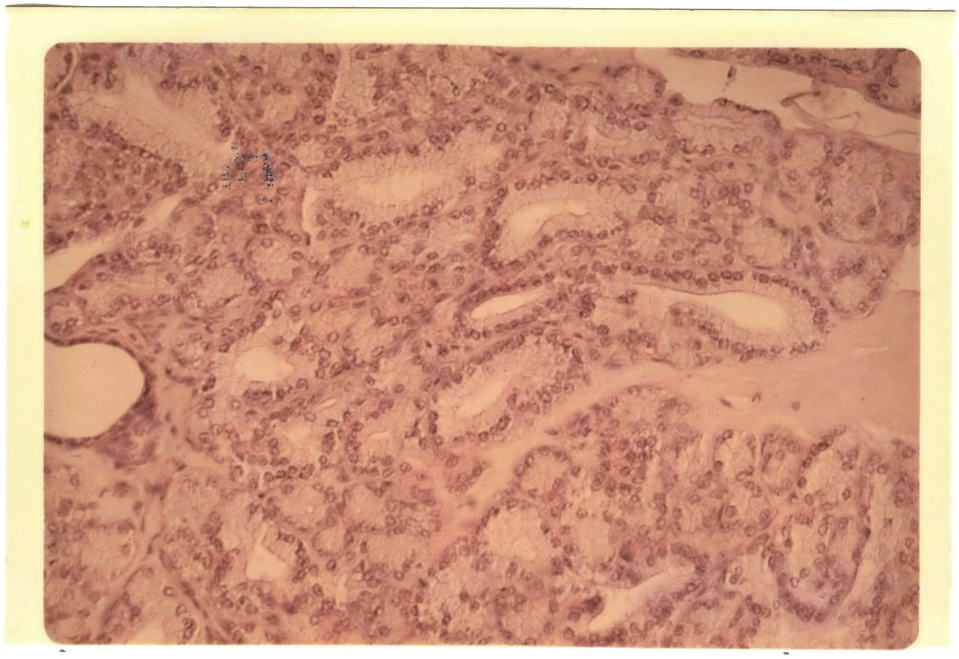

Figure 10. Microscopic Section of Membrane (Figure 7) Showing Glandular Tissue. H&E Stain.

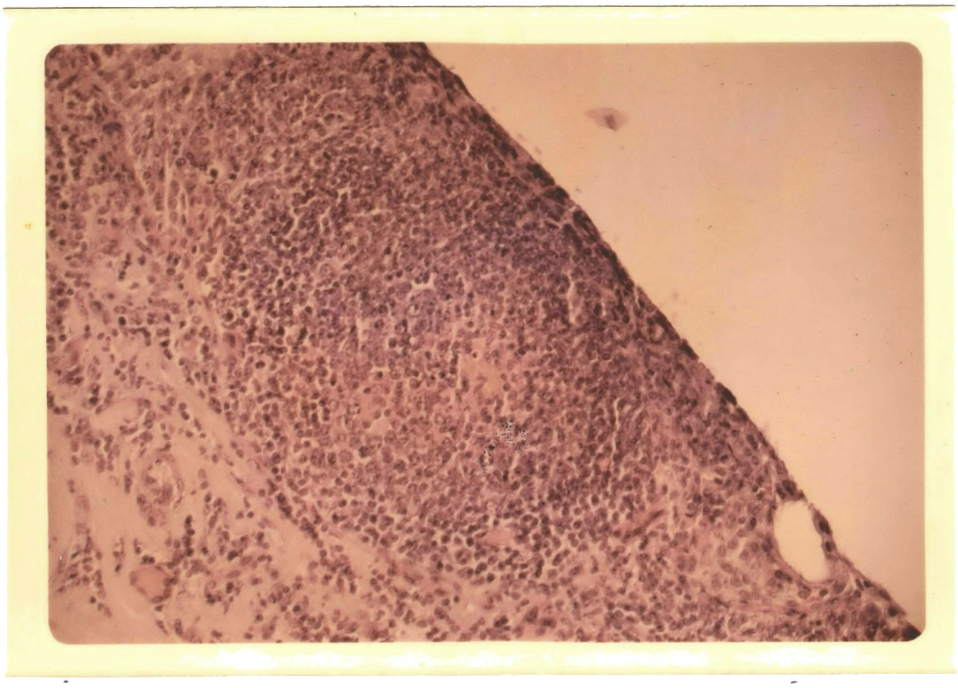

Figure 11. Microscopic Section of Membrane (Figure 7) Lymphoid Follicle. H&E Stain.

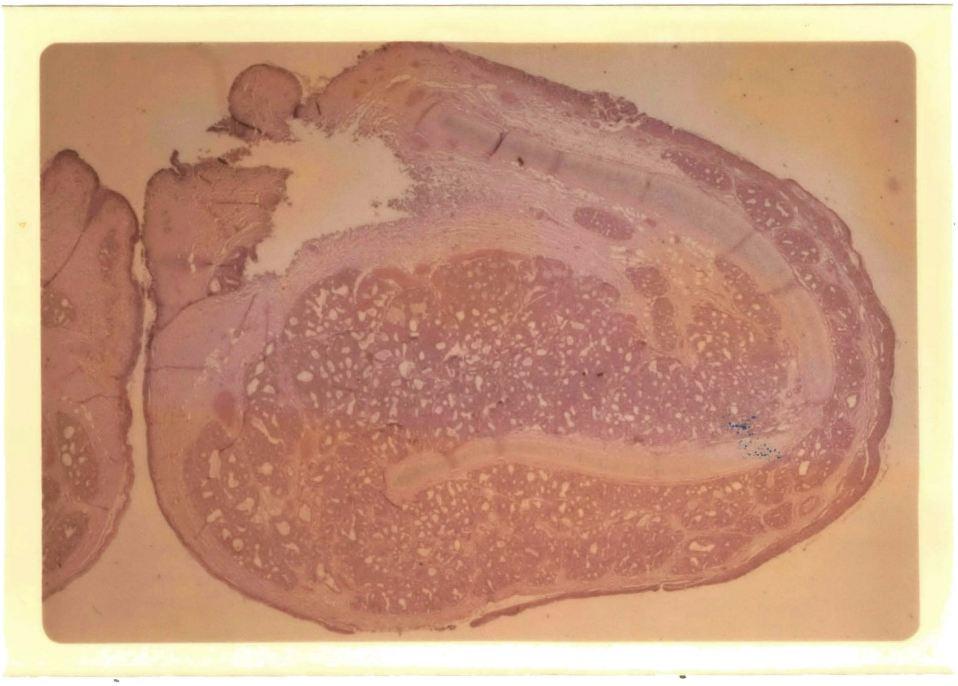

Figure 12. Microscopic Section of Tissue ("Cherry Eye" Figure 2) Showing Glandular Tissue and Bent Cartilage.

Examination for Chlamydia

Cultures. No chlamydial agents were isolated from any of the seven dogs cultured.

Direct Examination. No intracellular organisms were found in the smears prepared of the yolk sac membranes. The conjunctival scrapings were void of organisms resembling chlamydia (Table III).

Serology. Serum samples collected from the six pups were all

found to be void of complement fixing antibodies to chlamydia (Table IV).

Culture for Other Bacterial Agents

A variety of organisms were isolated from these animals as would be expected since all membranes cultured possess normal bacterial flora. A beta hemolytic streptococcus identified as Streptococcus canis was isolated from dogs number 1 and 2 (Table II). The organism was isolated from lesions of the vaginal mucosa of these dogs.

Viral Isolation Attempts

No evidence of viral infection was observed in any of the cultures. Cellular degeneration of the MDDK monolayers was encountered but was found to be due to mycoplasma in the cell cultures as the uninoculated controls were also involved.

Transmission Studies

Animal to Animal

Animals inoculated with material from other animals did not develop lesions. When compared to uninoculated controls, the membranes appeared to be without change.

Culture to Animal

Viral. Animals receiving the virus CHV-205 developed a mild conjunctivitis characterized by erythema and edema which was quite

obvious when compared to the uninoculated control. Follicular or vesicular lesions were not evident. The reddened, thickened membrane was the only change noted. When cultured by swab, the membrane yielded virus for 16 days post-inoculation.

Bacterial. In the bacterial transmission study no inflammatory changes were observed for the three week post-inoculation period. When cultured at the end of the three week period, Streptococcus canis was recovered suggesting that it did multiply on the membranes producing no pathological lesions.

CHAPTER IV

DISCUSSION

The plastic sections were of good quality for study of cellular detail (Figure 6), but primary fixation by formalin or Bouin's fixative might have enhanced visualization of inclusion bodies. However, since the sections showing inflammatory changes were negative for viral particles by electron microscopy, it is not considered likely that inclusion bodies were present in the samples. The inflammatory changes; karyohexis, hemorrhage, vacuolation and necrosis (Figure 7), while compatible with viral infectivity, cannot be considered due exclusively to a virus infection.

Limitations were imposed by the small size of the samples examined by electron microscopy and cells containing viral inclusions and viral particles could have been missed. Tissue samples must be minced into minute (1 mm) fragments for proper fixation and impregnation. All of the fragments were embedded in plastic, sectioned and examined with the light microscope to locate the areas showing pathologic changes. It is possible that cells containing virus were missed in, at least, some of the plastic blocks. In considering the total number of tissue fragments sectioned and examined, the requirements for adequate sampling were met and there is justification for stating that virus was not present in the tissues.

Richardson's stain did not provide the best differential stain

but was superior in quality to the hemotoxylin and eosin (H&E) stained plastic sections because eosin was absorbed by the plastic rendering the specimen unsuitable for interpretation.

The virus and its antiserum used in the fluorescent antibody and serum neutralization studies were not under investigation. They were used in an attempt to demonstrate cross-reactivity with the suspect agent(s). A Herpes-like virus was reported to be associated with the condition under study by Pickard (10) and Jackson (11). Certain Herpesviruses, even from different species of animals, have been shown to possess antigens in common (25, 38, 39, 40) so it was considered desirable that these studies be done. Results obtained fail to lend support to the Herpesvirus etiology.

In the early stages of the investigation membranes with mounds of tissue over the surface had a granular appearance that was classified as follicular conjunctivitis. It was found subsequently that all dogs examined had such membranes to some degree. Even pups maintained in isolation since whelping to be used in transmission studies developed the condition and were no longer considered negative. Histopathologic studies were prompted by the over all prevalence of the condition and by the fact that clinicians were not in agreement as to what constituted the abnormal membrane. Ocular discharge reported in other studies (1) was not a prominent feature of the condition in dogs seen by this investigator. In the previous investigation (11) and in the early part of this study, a granular appearance was considered to be indicative of the presence of lymphoid follicles. The membranes nictitans from the 12 pups described under the section histopathology were removed and embedded in paraffin so the palpebral surface with

the granular appearance would be sampled. The mounds were comprised of predominately glandular tissue. Lymphoid follicles were present but so sparse they could not have accounted for the irregular, granular appearance of the membrane. The canine membranes nictitans classified in this study as follicular conjunctivitis, based on the gross granular appearance, were within the limits of normal for a third eyelid. Particularly, since the lymphoid follicles observed were not considered to by hyperplastic. Some eyelids had more glandular and lymphoid tissues than others. In the absence of established limits of normal it is difficult to attach clinical significance to the changes seen especially, since normal limits may be affected by age, breed and environmental factors (4).

Histopathologic sections were not prepared by this investigator in the previous study (11). The membranes were scraped and the material used as inocula for tissue culture, transmission studies and cytology. Hence, no comparison can be made between the two studies relative to histopathologic appearance.

Biopsy specimens studied by Pickard (10) of both the membrane nictitans and genitalium were reported to be abnormal based on lymphoid hyperplasia characterized by large hyperplastic areas with a high percentage of mitotic cells. Since the membrane nictitans was biopsied and not sectioned in its entirety, as in the present study, no comparison can be made between the two studies regarding the relative amounts of glandular and lymphoid tissues present on the membrane. "Massive proliferation of lymphoid tissue" was reported (10) indicating that the lesion was comprised predominately, if not entirely, of lymphoid tissue. This is in contrast to findings of the

present study.

As to "cherry eye", it is not clear what the underlying pathology is, but presence of the bent cartilage in the section (Figure 12) is of considerable interest in that defective cartilage was reported by Magrane (1) to contribute to the anomaly. In the previous study by Jackson (11), the "cherry eye" mass was excised, processed for use as inoculum for tissue culture and transmission studies. No histopathologic sections were prepared so, again, no comparisons can be made with findings of the present study. The mass was considered grossly by some clinicians to be lymphoid tissue. Microscopic sections prepared from two cases of "cherry eye" were both comprised of hyperplastic glandular tissue. Cartilage was observed in only one of the sections. Lymphoid follicles were not entirely absent, but were too few in number to be considered significant.

In the study by Pickard (10), no mention of the "cherry eye" condition was made. Sources of the three viral isolates were reported to be the third eyelid presenting clinically as follicular conjunctivitis, the penis, and the vagina, not "cherry eye". But had more dogs with "cherry eye" been available in the present study, a better evaluation could have been made of the condition. The writer submits that inversion or eversion, whatever the cause, could lead to hyperplasia of the glandular tissue and the presence of mononuclear inflammatory cells (lymphoid follicles) because of membrane nictitans irritation.

Isolation of the mycoplasma agents from the membrane nictitans should probably not be considered of significance since these agents have been isolated from normal membranes (18, 19, 20, 21, 22, 30).

Their role has not been defined as pathogenic particularly in the condition under study.

Failure to demonstrate the presence of chlamydia or its antibody in the dogs examined suggest that these agents are not associated with the condition in dogs as they are in man and certain animals (8, 22).

Streptococcus canis was isolated in this study from the vagina of two dogs presenting grossly as follicular vaginitis. Its role as a primary pathogen was assessed by inoculation of the three experimental pups. In one study (16) the organism was reported to be the predominant organism of the canine eye where chronic purulent discharge was a feature but its role in this condition was not defined. Since no lesions were produced in the experimental animals, the writer assumes that the organism was present as in the original dogs as normal flora or as a secondary invader.

As regards the viral cultures, the absence of CPE in tissue culture does not necessarily rule out the presence of virus. But viral particles were not demonstrated in the tissues by direct fluorescent antibody tests nor by electron microscopy of pathological specimens and antibody to the virus was not detected by indirect fluorescent antibody. Histopathologic sections revealed foci of mononuclear infiltration and other changes compatible with virus infectivity, but no vesicles or inclusion bodies, the hallmark of Herpesvirus infection, were observed in any of the sections. Hence the writer concludes that virus was not present in the tissues cultures. The foregoing conclusion is not arrived at without reservation. The evidence presented does not necessarily offer

conclusive proof of the absence of virus in the condition studied. It would be naive on the part of the investigator to imply that all resources for the detection and isolation of virus were exhausted and all possibilities explored. Virus may possibly be present in low numbers at certain times in the tissues escaping detection by methods used. Tissues may have to be ground and the fluid concentrated by ultracentrifugation, ultrafiltration or other more sophisticated techniques before virus is demonstrated. However, the condition, glandular hyperplasia of the membrane nictitans, does not appear to be serious enough, where epiphora is not a feature, to warrant such attention. The time and money would be better spent on research with the known canine Herpesvirus where there is debilitating disease and death.

Transmission studies, with regard to the eye condition, were impeded by a lack of negative, clear eyelids to inoculate. Pups held in isolation since birth all developed changes considered, upon gross examination, to be consistent with follicular conjunctivitis and therefore could not be used for experimental inoculation. In addition, the 12 pups acquired at eight weeks of age were found to possess the granular appearing eyelids and could not be used either.

In view of the foregoing observations, it is now difficult to think of the follicular conjunctivitis as reported in the early study (10, 11) as well as in this investigation as being a pathological condition initiated and perpetuated by an infectious, transmissible, agent. The evidence presented in one study (10) leaves some doubt as to its validity and as to whether or not a Herpes-like virus was actually isolated from the dogs. In absence of positive fluores-

cent antibody results and electron microscopy, conclusions drawn were based solely on CPE in tissue culture. In fact, all evidence presented for the presence of virus was based on the presence or absence of CPE. Since antibody titers, again, based on CPE in tissue culture, are unrealistic and without precedent for a Herpesvirus, even in the hyperimmunized animal, the results indicate that degeneration of monolayers might have been misinterpreted as viral CPE or that a contaminating virus or bacterium was present in the inoculum. The foregoing could apply to the characterization studies as well since the findings were based on the presence or absence of CPE. The CPE shown was not necessarily peculiar to nor consistent with a canine Herpesvirus. Degeneration of cells in tissue culture is common place in the virology laboratory. It was observed by this writer in both the previous study (11) and the present one. Cytoplasmic inclusions reported by Pickard (10), however, were not seen.

The intranuclear inclusions reported by Pickard (10) and the one intranuclear inclusion reported by the present investigator (11) were considered to be evidence of the possible presence of a Herpesvirus. However, in the science of Virology, intranuclear inclusions are not considered definitive of a particular virus unless supported by virus isolation or by demonstration of viral particles by the electron microscope. In the one study (10) virus was not isolated. In the other study (11) evidence of virus isolation is subject to challenge. Finally, virus particles were not demonstrated by electron microscopy, hence, this investigator is not convinced that a canine Herpesvirus was present.

The strongest evidence remaining in the previous study (11)

for presence of virus is the positive results of the transmission studies. These findings were based on the difference in appearance of the inoculated membranes when compared with uninoculated membranes. In the present study, it became apparent that the differences were not sufficient in past studies to constitute a positive finding. The changes might have occurred in the absence of the inoculations as they did in the control animals of the present study. It is theoretically possible, but highly unlikely, that an infectious, transmissible agent was present as the causative agent. It is now obvious that changes seen on the membrane nictitans were misinterpreted as positive evidence in those transmission studies.

In the transmission studies of the present investigation, the membranes were not scarified to the extent that they were in the previous study (11). The swabs were simply rubbed briskly over the membranes. The reason for this inoculation technique was two-fold. First; scarified membranes of the previous investigation responded with inflammatory changes to the scarification, as expected, which rendered interpretation more difficult, in spite of controls. Second; in a preliminary study, Herpesvirus (CHV-205) was inoculated onto the membrane nictitans by the above procedure. The virus was replicated and changes produced which were readily distinguishable from conditions of the sham inoculated control membranes. The investigator was concerned that when a membrane is damaged sufficiently by scarification, disease may be produced by its normal flora. Since the preliminary study proved that virus could be introduced with subsequent disease production without intense scarification, and since the problems associated with non-specific inflammation due to trauma were

avoided, deviation from the previously used inoculation procedure was considered justified if not warranted. Since the inoculation procedures of this study differed from those of the previous studies (10, 11) and since the inoculated membranes nictitans of the present study were not clear as in the previous studies, a comparison of findings is not possible.

Lesions of the genitalium, believed to be a manisfestation of the follicular conjunctivitis syndrome, were seen in only four of the dogs studied. The association was reported in the previous studies (10, 11) but evidence of the present study suggests that the genital lesions do not necessarily accompany the eye lesions. Too many dogs were seen in this study with eye lesions in the absence of genital lesions. The association might be a valid one where a true follicular conjunctivitis exist but when the eye lesion is comprised of glandular tissue the genital lesions do not appear to be uniformly present. Two dogs having the eye condition when first seen were held for six months. The genitalia of these animals remained clear and unchanged.

Finally, evidence presented in the two previous studies appears tenuous and circumstantial at best. In the present study, it is felt that a sufficient number of tissues, representing both acute and chronic changes and, perhaps, stages in between, were examined to rule out the kind of pathology associated with virus infection, particularly, Herpesvirus.

The writer thus concludes that the condition under study presenting, grossly, as follicular conjunctivitis is a glandular proliferation of unknown etiology. Glandular hyperplasia was the

prominent feature of the few cases of "cherry eye". Histopathologic sections were not prepared of genital lesions so no assessment was made as to tissue type.

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APPENDIX A

SOLUTIONS FOR ELECTRON MICROSCOPY FIXATION

1. 8% gluteraldehyde

   1 vial 70% gluteraldehyde
   15.5 ml distilled H_2O

   Rinse vial out with distilled water in order to get all of the gluteraldehyde. Stir well.

2. .27 M Cacodylate buffer
   57.7854 gm/1000 ml H_2O
   (or) 5.779 gm/100 ml H_2O
   (or) 28.895 gm/500 ml H_2O

3. Buffered wash

   60 ml of .27 M cacodylate buffer
   140 ml distilled H_2O
   12.3 gm sucrose
   200.0 ml

4. Osmium

   25 ml distilled H_2O
   1/2 gm vial osmic acid (crystalline)
   (takes a few days to dissolve; freeze extra in small vials)

Gluteraldehyde Fixation

10 ml 8% gluteraldehyde
30 ml .27 M cacodylate buffer
.5 ml acrolein (1 squirt with pasteur pipette)
.02 gm $CaCl_2$ (2 $CaCl_2$ crystals)
10 ml H_2O

Adjust pH to 7.4 with 1 N HCl

APPENDIX B

PHOSPHATE BUFFERED-SALINE

8.5 gm Nacl
100 ml 0.1 Molar phosphate buffer
qs to 1 liter with distilled H_2O

Solution A:
27.6 gm Na H_2PO_4 H_2O
1000 ml distilled H_2O

Solution B:
28.4 gm Na_2 HPO_4
1000 ml distilled H_2O

0.1 M phosphate buffer
28 ml solution A
72 ml solution B
qs to 200 ml with distilled H_2O

APPENDIX C

BOUIN'S FIXATIVE

picric acid - - - - - - - - - - 750.0 ml

37-40% formalin - - - - - - - - 250.0 ml

Glacial Acetic acid - - - - - - 50.0 ml

Hypo Solution

Saturated sodium thiosulfate in 70% isopropyl alcohol.

APPENDIX D

TRYPSINIZING SOLUTIONS

Saline A

	1X	10X
NaCl	8.00 g	30 g
KCl	0.40 g	4 g
Glucose	1.00 g	10 g
$NaHCO_3$	0.35 g	3.5 g
(Double) distilled water	1000 ml	1000 ml

Reference: Journal of Experimental Medicine 104 (4): 615-629 (Oct. 1956).

This may be prepared in 10X concentration, dispensed in 100 ml amounts, autoclaved and frozen. Dilute with 900 ml of sterile distilled water before use. Add 250 units penicillin and 250 mcg streptomycin.

This is a calcium and magnesium free balanced salt solution used to wash cell sheet to remove media containing calcium and magnesium salts which interfere with the trypsin.

Saline A with Versene* and Trypsin

1. Make up Saline A.
2. Prepare 1% trypsin solution.
3. Prepare 1% Versene solution.
4. Filter to sterilize--Seitz or Selas.

Add 50 ml of 1% trypsin solution to 1 liter of Saline A.
Add 20 ml of 1% Versene solution to above mixture.

Note: To prepare large amount of Saline A with Trypsin and Versene

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*Versenes, Incorporated, Framingham, Massachusetts

This gives a total of 1700 ml. Filter combined solutions through Seitz filter. Freeze in 170 ml amount. To reconstitute, sterilize 830 ml of double distilled water in autoclave; cool. Then add bottle containing 170 ml of Saline A + T + V to make up to 1000 ml.

VITA

James Andrew Jackson

Candidate for the Degree of

Doctor of Philisophy

Thesis: A STUDY OF THE MEMBRANE NICTITANS AND GENITALIUM OF THE CANINE WITH REFERENCE TO LYMPHOFOLLICULAR HYPERPLASIA AND ITS ETIOLOGY

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