A NEW HERPES-LIKE VIRUS INFECTIOUS FOR THE CANINE

By

RICK KEITH PICKARD

Bachelor of Science

Northwestern State College

Alva, Oklahoma

1968

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1973

A NEW HERPES-LIKE VIRUS INFECTIOUS

FOR THE CANINE

Thesis Approved:

R. E. Corstvet
Thesis Adviser

Elizabeth J. Gandy

Lynn P. Lee

Ralph G. Burkes

N. N. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. Richard E. Corstvet for his direction and encouragement.

Special gratitude is expressed to members of the Advisory Committee for their instructions in the preparation of this thesis.

Particular appreciation is expressed to the various members of the faculty of the College of Veterinary Medicine for their aids and instructions.

Financial support was furnished in part by the American Cancer Society Institutional Grant IN-91A.

Special appreciation is expressed to Dr. Roger Panciera of the College of Veterinary Medicine, Department of Pathology, for the histologic interpretation of infected tissue.

The author also wishes to express sincere gratitude to his wife, Beverly, for her continued encouragement and understanding. Without her support this thesis would not have been completed.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	4
	Herpesvirus Group	4
	New Diseases Attributable to Herpesviruses	5
III.	MATERIALS AND METHODS	7
	Buffer	7
	Trypsin-Versene Digest Solution	7
	Cell Culture Media	7
	Cell Culture Preparation and Maintenance	9
	Bacterial Studies	12
	Source of Viral Isolates	12
	Viral Isolation Technique	13
	Culture Inoculation and Viral Passage	14
	Test Systems	15
	Cell Culture Controls	15
	Cytopathology of Cell Cultures	16
	Viral Characterization Studies	16
	Staining Technique for Cell Cultures	18
	Serum Preparation	19
	Serum Conjugation	20
	Column Preparation	21
	Serum Conjugate Fractionation	21
	Serum Studies	22
	Viral Purification	23
	Viral Titer Determination	24
	Fluorescent Antibody Technique	24
	Beagle Infectivity Studies	25
	Tissue Embedding and Sectioning	28
IV.	RESULTS AND DISCUSSION	29
	In Vitro Viral Propagation	29
	Histopathological Examination of Primary Rabbit Kidney Cell Cultures	30
	In Vitro Characterization Studies	30
	Serum Neutralization Test	47
	Fluorescent Antibody Studies	56
	Beagle Infectivity Studies	57
	Histologic Examination of Biopsied Tissue	61

Chapter Page

V. SUMMARY AND CONCLUSIONS 63

LITERATURE CITED 65

APPENDIX - MATERIALS AND METHODS 67

LIST OF TABLES

Table		Page
I.	Effect of 5-Iodo-2-Deoxyuridine Treatment	36
II.	Effect of Chloroform Treatment on Unpurified Virus	38
III.	Effect of Chloroform Treatment on Purified Virus	39
IV.	Effect of Temperature on Unpurified Virus	40
V.	Effect of Temperature on Purified Virus	42
VI.	Effect of Low Temperature on Stability	44
VII.	Viral Titer Determination	46
VIII.	Viral Size Determination	48
IX.	Serum Neutralization and Titer Determination	50
X.	Cross-Neutralization of Viral Isolates with Heterologous Antisera	55
XI.	Beagle Infectivity Studies	60

LIST OF FIGURES

Figure		Page
1.	Cytopathic Effect of Passage VII Viral Isolates in Rabbit Kidney Primary Cell Monolayers	31
2.	Hematoxylin-eosin Stained Monolayers of Infected Rabbit Kidney Primary Cells	33
3.	Induced Follicular Lesions of the Canine Vagina, Penis, and Conjunctiva on the Tenth Day Post-inoculation	58
4.	Hematoxylin-eosin Stained Sections of Normal and Infected Canine Tissue	62

CHAPTER I

INTRODUCTION

The canine species is host to many infectious agents. However, many clinically distinct disease conditions exist for which there is no identifiable etiologic agent. It was due to such a frequently observed clinical condition in the canine that this research problem was initiated.

An infectious disease frequently observed in the canine, for which the etiology has not been established, is characterized by the presence of lesions on the mucosa of the penis, vagina and the nictitating membrane (third eye lid). Visual examination of these three areas reveals small (1mm to 3mm), raised, clear to erythematous follicles on the surface epithelium. Microscopic examination of tissue sections fixed with Bouin's fluid and stained with Harris' hematoxylin and eosin Y, reveals marked hyperplasia of the subepithelial lymphoid follicles. The centers of these follicles contain large blastic lymphocytes which are in varying stages of mitosis while the non-mitotic lymphocytes are found to be in various stages of maturity (16).

The preputial follicles of the male are coexistent with a pale, yellow, viscous discharge. The vaginal follicles are accompanied by a similar discharge and occasionally Streptococcus faecalis and Streptococcus hemolyticus have been cultured from this discharge (2). The nictitating membrane reveals lymphoid hypertrophy and, when

chronic, will evert and protrude from the free margin of the eye lid. These conditions have been referred to as follicular balanitis, follicular vaginitis and follicular conjunctivitis (13).

Previously the infection was thought to be the adult expression of a visceral hemorrhagic disease of puppies, caused by canine herpesvirus (4, 18, 19). The hypothesis attained credibility when a Lhasa Alpso with a follicular vaginitis whelped five normal-appearing puppies which died on the sixth day post partum with symptoms similar to canine herpesvirus disease. However, this hypothesis was discredited in 1969 when Carmichael reported canine herpesvirus to be specific for puppies under three weeks of age (5). This correlated with the knowledge that the inoculum prepared from the Lhasa Alpso vaginal lesions failed to produce any signs or symptoms in two different litters when injected intraperitoneally and intravaginally (15).

Nevertheless, the problem of characterizing the agent(s) remained, and the hypothesis that the agent(s) for all three follicular conditions were one and the same required confirmation.

An in vitro cell culture system was selected as the best technique for establishing the basic characteristics of the agent(s). Cell cultures of rabbit kidney primary, mouse L cell line,[1] HeLa cell line[2] and canine kidney primary were screened as possible cultures which would promote propagation and cytopathic effect.

---

[1]Mouse fibroblastic cell passed in tissue culture more than 50 times to establish a line of fibroblastic cells.

[2]A cell line that originated from the cervix of Helen Lane, who was a victim of cervical carcinoma.

In vivo studies of infectivity using infected canine tissue were performed. Support of *in vitro* characterization studies was attempted by fluorescent-antibody studies of both cell culture and whole tissue.

CHAPTER II

REVIEW OF LITERATURE

Herpesvirus Group

General Characteristics

The herpesvirus group is composed of those deoxyribonucleic acid (DNA) viruses that have diameters of 180-250 mμ when surrounded by envelopes and 105 mμ when the envelopes were absent. They are lipid solvent sensitive, have cubic or icosahedral symmetry (having twenty faces), possess limiting membranes of lipoprotein with 162 capsomers and are heat labile (6, 10, 23).

Host Range

The herpesvirus group has a wide vertebrate host range. Those members known to infect man are herpes simplex, varicella-zoster, cytomegalovirus, B virus, and Burkitt's lymphoma virus (10). The non-human primates are known to be susceptible to seven herpesviruses (12). Other vertebrate hosts include cattle, sheep, horses, dogs, cats, swine, chickens, frogs, parrots, pigeons, cormorants, and rabbits (12).

Behavior in Cell Culture

Melnick et al. (14) suggested that the herpesvirusus be divided into two groups on the basis of their action *in vitro*. Group A

consists of those herpesviruses which release easily from the cells they infect. Group B consists of those herpesviruses which are cell-associated and release with difficulty.

The type of cellular damage or cytopathic effect (CPE) caused by the herpesvirus group varies with the members of the group (6, 14). In general, the in vitro CPE associated with herpesviruses is the formation of a Cowdry type A intranuclear inclusion which is a basophilic body occupying most of the central area of the nucleus. The inclusion body is surrounded by a clear space or halo (18, 23). Occasional multinucleated giant cells and cellular shrinkage with rounding followed by release of the cells from the surface of the growth container are found in addition to the inclusion bodies.

New Diseases Attributable to Herpesviruses

Burkitt's Lymphoma

A lymphosarcoma or cancer of the lymphatic system in native children of west-central Africa has been recently shown to be virus-associated. Epstein (7), in 1965, showed the presence of a virus resembling herpesvirus in a lymphosarcoma.

Yamaguchi et al. (24), in 1967, confirmed a herpesvirus in Burkitt's lymphoma cell culture P3HR-1 by electron microscopy.

The disease is indigenous to west-central Africa but antibody distribution is widespread in the United States. A strong serologic relationship exists between the Burkitt's lymphoma agent and carcinomas of the post nasal space of patients in China (24).

Renal Carcinoma of Rana Pipiens

Recently a herpesvirus has been associated with renal cancer of the leopard frog, Rana pipiens (9). Rafferty (17) in 1965 isolated a virus from the urine of frogs which were known to have renal neoplasia. The virus seems to be a herpesvirus and identical to the herpesvirus found in intranuclear inclusions of tumor cells taken from frogs with renal neoplasia. The tumor produced by this herpesvirus can comprise up to 13 per cent of the total body weight of the frog with complete involvement of both kidneys (9).

Canine Herpesvirus

Spertzel et al. (18) in 1965, Carmichael et al. (4) in 1965, and Stewart et al. (19) in 1965, investigated a hemorrhagic condition of the thoracic and abdominal organs of fetal and newborn puppies. They established that the condition was caused by a previously unknown member of the herpesvirus group. Carmichael et al. (5) in 1965 showed that equine rhinopneumonitis, bovine rhinotracheitis, pseudorabies and herpes simplex virus were not serologically related to canine herpesvirus. The virus has not been shown to be infective for dogs five to twelve weeks of age when inoculated by intravaginal, intraperitoneal, oral, or nasal routes (5). The virus was later shown to be specific for dogs under two weeks of age (1).

CHAPTER III

MATERIALS AND METHODS

Buffer

Dulbecco's phosphate buffer solutions were autoclaved separately and mixed when completely cool to prevent precipitation (see Appendix).

Trypsin-Versene Digest Solution

This solution was used to digest the canine tissue down to the cellular level and thus help in the isolation of the virus. The solution was also used in preparing rabbit kidney cell cultures. The composition is given in the Appendix.

Cell Culture Media

Growth Medium for Rabbit Kidney Primaries

A commercially prepared medium of Hank's Basal Medium Eagle (HBME),[1] pH 7.2, containing 10 per cent fetal bovine serum, 200 units/ml. of penicillin and 200 µg/ml. streptomycin was used.

---

[1]Baltimore Biological Laboratory, Division of Bio Quest, Baltimore, Maryland. Basal Medium Eagle 71-0284.

Maintenance Medium for Rabbit Kidney Primaries

This was the same as the growth medium except for the addition of two per cent fetal bovine serum rather than 10 per cent serum.

Mouse L Cell Growth and Maintenance Medium

A. Eyl Growth Medium

100 ml. Earles' 10x concentrate solution (balanced salts)

100 ml. lactalbumin hydrolysate 10x concentrate

10 ml. sodium bicarbonate (7.5% concentration)

690 ml. triple-distilled H_2O

100 ml. horse serum

8 ml. penicillin and streptomycin (concentrations of 200 units/ml. and 200 µg/ml., respectively)

B. Eyl Maintenance Medium

The maintenance medium was the same as the growth medium but contained 50 ml. of horse serum and 740 ml. of triple-distilled water.

HeLa Cell Growth and Maintenance Medium

A. Growth Medium

Medium 199[2] containing 10 per cent horse serum with 200 units/ml. of penicillin and 200 µg/ml. of streptomycin was used.

B. Maintenance Medium

The maintenance medium was medium 199 containing five per cent horse serum.

---

[2]Baltimore Biologic Laboratories, Division of Bio Quest, Cockneyville, Maryland.

Cell Culture Preparation and Maintenance

Rabbit Kidney Primary Cell Preparation

Rabbits between the ages of five and ten days were selected as giving the best yield of viable cells per kidney weight. The rabbits were killed by a sharp blow to the occipital region of the head. The abdomen was immediately opened and the kidneys were removed aseptically.

Using sterile forceps, the kidneys were placed in sterile Petri dishes and covered with Dulbecco's phosphate buffer at 4°C. The kidneys were decapsulated and cut into longitudinal halves. The renal artery and vein and all fatty and connective tissues were removed. The kidney halves were then washed in digest solution and minced in a mincing vessel to achieve 3-5 mm pieces. The tissue was transferred to a trypsinization flask and rinsed with digest solution to remove as many erythrocytes as possible.

Two hundred milliliters of 0.25 per cent trypsin-versene digest solution (see Appendix) were added to the minced tissue at 37°C. The suspension was incubated at 37°C and stirred with a magnetic stirrer for ten minutes. At the end of this period 25 ml. were decanted into a cold flask. The volume decanted was replaced with fresh 37°C trypsin-versene solution and incubated at 37°C with repeated 10-minute pour-offs until the tissue was completely digested.

The pooled cellular suspension was centrifuged at 720 x g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet washed with cold HBME containing 10 per cent fetal bovine serum. The cellular suspension was centrifuged a second time at 720 x g for 10

minutes at $4^{\circ}C$. This step was repeated three more times.

The final pellet was suspended with 50 ml. of HBME containing 10 per cent fetal bovine serum and the cells were counted using a haemocytometer and an objective power of 10x. One ml. portion of suspension containing 2×10^5 to 3×10^5 cells/ml. were planted in 16 x 125 mm. tubes and incubated at $37^{\circ}C$. The cultures were fed with HBME containing 10 per cent fetal bovine serum every other day until 100 per cent confluency was reached.

A. Rabbit Kidney Cell Culture Maintenance

When the kidney cell cultures growing in 16 x 125 mm. tubes reached 100 per cent confluency, they were maintained on HBME with two per cent fetal bovine serum. The maintenance of these cells consisted of daily inspection to evaluate their stability. When cellular metabolism had produced near toxic pH levels, the medium underwent a color change from pink to yellow. This color change was the point at which the medium was removed and fresh medium added.

Canine Kidney Primary Cell Preparation

Three-month old mixed-breed puppies were utilized for kidney primary cell cultures. The preparation procedure followed was the same as the procedure for rabbit kidney primary cell culture.

Mouse L-Cell Preparation and Maintenance

Fresh mouse L-cell monolayers were prepared by scraping the monolayer of cells from the flat surface of a 200 milliliter prescription bottle with a sterile rubber policeman. The cell-medium mixture was

shaken gently to insure a uniform cellular suspension. The suspension was centrifuged at 800 x g for 10 minutes. The cell pellet was added to 48 ml. of Eyl medium containing 10 per cent horse serum. Sixteen milliliters of cell suspension were poured into a sterile 200 ml. prescription bottle and incubated on the flat surface at 37°C until 100 per cent confluency was reached. The monolayers were then maintained on Eyl medium containing five per cent horse serum.

HeLa Cell Preparation and Maintenance

HeLa cells were prepared in a manner similar to that used for mouse L cells; the manner of detachment of the cells from the glass and the growth medium were different.

The spent medium was removed and three ml. of trypsin-versene solution (see Appendix) at 37°C were added and incubated at 37°C until the monolayer detached. The suspension was centrifuged at 80 x g for 10 minutes, washed in cold medium 199 and recentrifuged. The final cell pellet was resuspended in 48 ml. of medium 199 with 10 per cent horse serum at 37°C. The cells were planted in 16 ml. volumes in 200 ml. prescription bottles and incubated at 37°C until 100 per cent confluence was attained. The monolayers were then maintained on medium 199 with five per cent horse serum.

Bacterial Studies

On each series of viral studies performed on tissue culture, bacterial contaminants were ruled out by inoculating blood agar plates, PPLO[3] medium,[4] thioglycollate broth, blood agar with Staphylococcus aureus feeder and PPLO agar overlay[5] (see Appendix).

An inoculating loop was flame sterilized and immersed in the inoculated cell culture fluid. Inoculations were made in the appropriate manner for each medium. All inoculates were incubated at 37°C for 48 hours, except for the PPLO inoculates and they were maintained for eight days.

Source of Viral Isolates

A male English bulldog displaying follicular conjunctivitis and follicular balanitis was anesthetized by intravenous injection of 10 mg. per pound of body weright of a 2.5 per cent sodium Surital solution.[6] Deeper anesthesia was achieved by using a Fluothane[7] -O_2 manual resuscitator.

The penile sheath was retracted exposing the prepuce, and an elliptical 0.5 x 0.25 cm. section of mucosa was surgically removed and placed in 1.0 ml. of HBME containing 10 per cent fetal bovine serum. The incision was sutured with OO chromic gut.

---

[3]Pleuropneumonia-like Organism.

[4,5]Difco Laboratories, Detroit, Michigan.

[6]Sodium Thiamylal, Parke-Davis, Detroit, Michigan.

[7]Ayerst Laboratories, Veterinary Medicine Division, New York, New York.

A female German shepherd displaying follicular vaginitis was anesthetized in the same manner and a 0.5 x 0.25 cm. section of vaginal mucosa was surgically removed and placed in 1.0 ml. of HBME containing 10 per cent fetal bovine serum. The incision was not sutured and healed without complication.

A German shepherd male displaying follicular conjunctivitis was anesthetized in the previously described manner. The lymphoid tissue on the posterior aspect of the membrane nictitans was surgically removed. The tissue was placed in 1.0 ml. of HBME containing 10 per cent fetal bovine serum. The incision was not sutured and healed without complication.

Viral Isolation Technique

All three canine tissue samples were divided into halves to result in two samples for each tissue. One sample of each tissue was placed in a sterile mortar containing 1.0 ml. of fresh HBME in 10 per cent fetal bovine serum. The tissue was ground until a uniform homogenate was obtained. The samples were then removed with sterile 2 cc syringes using 23 gauge needles and placed in 16 x 125 mm. tubes. Five thousand units of penicillin and 5000 µg of streptomycin were added to each tube and allowed to incubate for 30 minutes at room temperature. The samples were designated BPV for the Bulldog penis virus, GVV for German shepherd vaginal virus and GEV for German shepherd eye virus.

The remaining tissue was removed from the HBME and placed in 2 ml. of trypsin-versene digest solution and incubated at 37°C until a cellular suspension was obtained. The suspensions were centrifuged using sterile 40 ml. centrifuge tubes at 720 x g for 10 minutes at 4°C.

The pellets were resuspended and washed in 2 ml. of HBME with 10 per cent fetal bovine serum, centrifuged and washed a second time. The pellets were resuspended following the second washing in 1.0 ml. of HBME containing 10 per cent fetal bovine serum. The same designations assigned to the homogenates were assigned to the digests.

Culture Inoculation and Viral Passage

Rabbit kidney primary cell cultures which had reached 100 per cent confluency were used in the viral propagation studies. The cultures, containing 2 ml. of medium, were inoculated with 0.1 ml. of specific viral isolate.

The inoculated cultures were allowed to incubate at 37°C for 72 hours. This group of virus inoculated monolayers was considered to be a single passage. This passage was divided so that one aliquot could be stored at 2°C and a second aliquot at below -196°C.

One-tenth ml. of the first passage was used in inoculating a fresh cellular monolayer of rabbit kidney primary cells. This second passage was handled in the same manner as the first passage after the 72 hour incubation period.

Test Systems

In Vitro System

Rabbit kidney primary cell cultures grown in Leighton tubes[8] and 16 x 125 mm. tubes, HeLa cell line, mouse L cell line,[9] and canine kidney primary cells were utilized in the initial viral propagation studies.

In Vivo System

Beagles ranging in age from eight months to 24 months were selected for viral infectivity studies.[10]

Cell Culture Controls

Positive Controls

Rabbit kidney primary cell monolayers containing 0.9 ml. of HBME were inoculated with 0.1 ml. of virus suspension under sterile conditions and incubated at 37°C.

---

[8]Baltimore Biologic Laboratories, Division of Bio Quest, Cockneyville, Maryland.

[9]Oklahoma State University, Department of Biochemistry.

[10]Oklahoma State University, College of Veterinary Medicine, Canine Hemophilia Resource Colony. 5 P06 RR 00412-04 Supporting NIH Grant.

Negative Controls

These differed from the positive controls in the addition of 0.1 ml. of "spent" medium (uncontaminated metabolized medium) rather than virus suspension.

Cytopathology of Cell Cultures

The cytopathogenic effect of the virus was determined by comparing infected monolayers with non-inoculated monolayers as negative controls.

Viral Characterization Studies

Nucleic Acid Determination

One milliliter of maintenance medium containing 30 µg/ml of 5-iodo-2-deoxyuridine (IUDR),[11] an inhibitor of DNA synthesis in herpesviruses, was added directly to the rabbit kidney primary monolayers and incubated for two hours, at 37°C, followed by .1 ml. inoculation of virus with one hour allowed for absorption. Fresh maintenance medium containing 30 µg/ml of IUDR was then added to the cultures and incubated at 37°C. Positive and negative controls were prepared in a similar manner but without IUDR treatment. All cultures were checked 48-72 hours post inoculation for presence or absence of cytopathic effect.

The viral passage used in this experiment was passage VII. Fifty tubes of primary rabbit kidney cell cultures were used in addition

---

[11] Fisher Scientific Laboratories, Fair Lawn, New Jersey.

to five positive and five negative controls.

Viral Size Determination

Millipore filters[12] having pore diameters of 50, 200, 220 and 330 mμ were selected. Five milliliter samples of passage VII of each viral isolate were withdrawn from the culture tubes by using sterile syringes, which were attached to the swinny filter. The isolates were filtered into sterile 16 x 125 mm. test tubes. A 0.1 ml. volume of each filtrate was used as inoculum. Fifty tubes of rabbit kidney primary cells were inoculated per isolate with five positive and five negative controls.

Chloroform Sensitivity

Chloroform and other lipid solvents inhibit infectivity of enveloped viruses by removing essential lipids. A 0.25 ml. volume of reagent grade chloroform was added to 5.0 ml. of passage VII of each viral isolate and mixed for ten minutes at room temperature. The mixture was then centrifuged at 320 x g for five minutes to layer the chloroform. The viral suspension was removed by pipetting and was centrifuged a second time. Inoculations of 0.1 ml. of treated virus/tube of rabbit kidney primary cell monolayers were made and incubated at 37°C. Five positive and five negative controls were prepared. The inoculates were checked 48-72 hours post-inoculation for presence of cytopathic effect. Fifty tubes of rabbit kidney cells were inoculated for each viral agent.

---

[12]Millipore Filter Corporation, Bedford, Massachusetts.

Temperature Sensitivity

Constant temperature water baths at temperatures of 38°, 39°, 40°, 41°, 42°, 43°, 47°, and 57° were used. The thermometers were certified accurate to within 1°C. Three milliliter samples of each virus were incubated in each water bath for 30 minutes with occasional shaking. One-tenth milliliter of each heat-treated viral isolate was used as inoculum and 30 tubes of rabbit kidney primary cells were inoculated per viral isolate.

The viral passage was passage VII. Ten positive controls and ten negative controls were prepared.

Temperature Stability Determination

One milliliter samples were frozen in glass ampoules and stored at -2°C and at -196°C for two months. The ampoules were removed from storage and 0.1 ml. inoculations of each virus were made on 20 rabbit kidney primary cell monolayers. Five positive controls were prepared using the current passage, passage VII, of each virus by using 0.1 ml. of each as inoculum. Five negative controls were prepared using 0.1 ml. of spent medium. All cultures were incubated at 37°C for four days.

Staining Technique for Cell Cultures

Infected rabbit kidney primary cultures which had been grown on glass coverslips in Leighton tubes were fixed in 1.0 ml. of Bouin's fluid for three minutes. The slides were washed in distilled H_2O and stained for five minutes in eosin Y stain. The cultures were washed

a final time, then air dried and mounted on microscope slides using Permount.[13]

Serum Preparation

Rabbit Serum

Three rabbits, approximately one year of age, were selected for serum preparation. Five milliliters of Freund's complete adjuvant[14] (with Mycobacterium smegmatis) were mixed with five ml. of viral suspension. The mixture was warmed in a water bath at 37°C, then mixed at high speed using a Vortex mixer until the mixture failed to spread when placed on the surface of water.

Rabbit Injection Schedule

Day 1 - 1 cc adjuvant-virus subcutaneously in the neck

0.5 cc adjuvant-virus subcutaneously in right front foot pad

0.5 cc adjuvant-virus subcutaneously in left front foot pad

Day 3 - 1 cc adjuvant-virus subcutaneously in the neck

0.5 cc adjuvant-virus subcutaneously in right hind foot pad

0.5 cc adjuvant-virus subcutaneously in left hind foot pad

Day 7 - Same volume and injection sites as on Day 1

Day 14- Same volume and injection sites as on Day 3

---

[13]Fisher Scientific Company, Fair Lawn, New Jersey.

[14]Difco Laboratories, Detroit, Michigan.

The rabbits were bled on the twenty-eighth day by cardiac puncture. The blood was put in prescription bottles and these were laid in a horizontal position. The bottles were capped and remained at room temperature overnight. The clot formations were cut and the serum poured into sterile 16 x 125 mm test tubes and centrifuged at 2400 x g. The cleared serum was labeled and frozen.

Canine Serum Preparation

Canine serum was acquired by intravenous bleeding of the infected donor dog. The blood was treated in the same manner as that described for the rabbit serum preparation.

Serum Conjugation

Rabbit Serum

Three milliliters of rabbit antisera prepared against each viral isolate were added to 4.4 mg. of fluorescein isothiocyanate[15] in 0.45 ml. of 0.15% carbonate buffer (pH 8.6-8.8). The mixture was allowed to stand for 15 minutes in an ice bath followed by mixing at slow speed on a magnetic stirrer at 4°C for 18 hours.

Canine Serum

The procedure used for rabbit antiserum conjugation was followed.

---

[15]Baltimore Biologic Laboratories, Division of Bio Quest, Cockneyville, Maryland.

Column Preparation

Washing Cellex-D for Column

1. 50 gm. of Cellex-D[16]
2. 1000 ml. of 0.5 N NaOH
3. Mixed, using a magnetic stirrer for five minutes
4. Placed in Buchner funnel, filtered and washed on filter with distilled H_2O until pH of 7.0-7.2 was reached
5. Washed on filter with 0.25M phosphate buffer, pH 7.2
6. Suspended in 0.25M phosphate buffer, pH 7.2, and refrigerated

Column Packing

Standard 100 ml. burettes were used for the columns. They were packed with Cellex-D Cellulose, using phosphate buffer, pH 7.28-7.30. The column height was 10 inches. The columns were pre-run using sufficient phosphate buffer to yield a pH of 7.28-7.30 in the void volume. The columns were stored at $4^{\circ}C$ in pH 7.28-7.30 phosphate buffer until used.

Serum Conjugate Fractionation

The columns were run until the buffer level reached the top of the column. The conjugates were placed on top the Cellex-D and allowed to run down the column until they were level with the column top. Twenty milliliters of phosphate buffer, pH 7.28-7.30, were added to each column. The conjugates were collected in 16 x 125 mm test tubes

---

[16]Anion exchange cellulose, Bio Rad Laboratories, Richmond, California.

between the first and second color changes that occurred on the column. The tubes were labeled and frozen.

Serum Studies

Serum Neutralization

A 1.0 ml. volume of passage VII virus with known titer was mixed with 1.0 ml. of each dilution of ten fold serially diluted donor canine antiserum. The mixtures were allowed to incubate at room temperature for 30 minutes. A 0.2 ml. inoculum was used instead of 0.1 ml. to compensate for the dilution factor of the virus. Five tubes of rabbit kidney primary cells were inoculated per dilution of antiserum. Five positive and five negative controls were prepared.

The neutralization or protection granted was determined by the inhibition of cytopathic change by noting the serum dilution for which the absence of CPE occurred.

Cross Neutralization Reaction

Thirty tubes of rabbit kidney primary cell monolayers, each containing 0.9 ml. of HBME with 10 per cent fetal bovine serum, were inoculated with 0.1 ml. of GVV virus prepared as serial dilutions of 10^{-1} through 10^{-6}. Five tubes of cells were inoculated per dilution and incubated at $37^{\circ}C$.

Thirty tubes of rabbit kidney primary cell monolayers, each containing 0.8 ml. of HBME with 10 per cent fetal bovine serum, were inoculated with 0.2 ml. of GVV virus and donor BPV antiserum. The virus and heterologous sera were mixed in equal volumes (0.1 ml. to

0.1 ml.). The mixture was incubated for 30 minutes at room temperature prior to inoculation.

A homologous serum-virus inoculation was prepared using GVV and GVV donor antiserum. Fifteen tubes of rabbit kidney primary cells were used. Viral dilutions of 10^{-1} through 10^{-6} were prepared and 0.2 ml. inoculations per tube were made. A homologous serum-virus mixture was prepared using BPV and BPV antiserum in the same manner as the GVV-GVV antiserum series.

Five positive and five negative controls were prepared for each viral and antiserum series.

The same procedure and number of monolayers of cells were used for studies on BPV and GEV viruses.

Viral Purification

The frozen viral-cell cultures contained cell fragments, cellular components and whole cells along with the viral particles. Purification was achieved by differential centrifugation. Two 1.0 ml. samples were thawed at room temperature to ensure the maximum release of the virus from whole cells and centrifuged at 1000 x g at 4^{o}C to sediment the cell fragments. The supernatant was then centrifuged at 10,000 x g at 4^{o}C to sediment the major cellular components. The supernatant of this centrifugation was centrifuged at 47,000 x g at 4^{o}C to sediment the virus. The viral pellet was suspended in 0.5 ml. of Hank's Basal Medium Eagle without serum.

Viral Titer Determination

A ten-fold serial dilution of each viral sample was prepared by using 0.6 ml. of virus solution in 5.4 ml. of growth medium. Transfers of 0.5 ml. were made into each of nine test tubes containing 4.5 ml. of growth medium.

A 0.1 ml. volume of each dilution was inoculated per tube of rabbit kidney primary cell monolayer. A total of fifty tubes of cell culture were used with five tubes being inoculated per dilution. The titer of the virus was determined as the reciprocal of the last dilution showing the cytopathic changes. The cytopathic changes were recorded as +1 through +4. The +1 category was the least amount of cytopathic change seen. The +4 category was the greatest amount of cytopathic change seen. The +2 and +3 categories were the intermediate areas of cytopathic changes noted.

Fluorescent Antibody Technique

Tissue Culture

Fifty slides of rabbit kidney primary cell monolayers were used for the fluorescent antibody technique. Ten tubes of monolayers were inoculated with "spent" medium and served as controls. Thirteen monolayers were inoculated using 0.1 ml. of BPV virus, 13 using 0.1 ml. of GVV virus and 14 using 0.1 ml. of GEV virus. All tubes were incubated at 37°C.

Each set of controls and infected monolayers, following signs of cytopathic change, were fixed in cold acetone for 10 minutes. The infected monolayers were then treated with 1:2.5, 1:5, 1:10, 1:20, and

1:30 dilutions of homologous conjugate and rabbit tissue homogenate. The stained monolayers were incubated at 37°C for 25 minutes in a covered, moist environment.

The excess conjugate-homogenate was removed by washing for five minutes in a 0.01 M phosphate buffered saline (pH 7.2) followed by a 20 minute washing in phosphate buffered saline (pH 7.2). The cultures were quickly rinsed in distilled water and air dried for seven minutes, then mounted on microscope slides using a 1:9 dilution of glycerol in water. All slides were labeled and stored at -2°C.

The slides were viewed using a Seiss fluorescent microscope.

Frozen Tissue Sections

The biopsied tissue of the eye, penis, and vagina from experimentally infected dogs was frozen and mounted on cryostat chucks. A Lipshaw cryostat microtome was used to cut sections, six microns thick, of each of the tissues. The sections were treated with a 1:1 and 1:2.5 dilution of homologous canine conjugate and rabbit tissue homogenate.

The sections were treated in the same manner as that given for tissue culture-fluorescent antibody technique. A Zeiss fluorescent microscope was used to view the slides.

Beagle Infectivity Studies

Beagle Inoculation

Ten Beagle dogs, five males and five females, were used for viral infectivity studies. One male and one female were reserved for controls.

All dogs were anesthetized with sodium Surital, 10 mg/lb of body weight, which was administered intravenously. The dogs were then put on a Fluothane-O_2 manual resuscitator for better control and maintenance.

A German shepherd female, demonstrating a follicular vaginitis, served as the source of infective material for the vaginal inoculations of uninfected female Beagles.

A sterile curette was used to scarify the vaginal follicles of the German shepherd. The area was then swabbed with a sterile, saline-moistened swab.

The same technique was used in making inoculations of the prepuce of the penis and posterior aspect of the third eye lid from a similarly infected German shepherd male having both follicular balanitis and conjunctivitis.

The male beagle, M-18, served as a negative control for both the penis and eye infections. The prepuce was scarified by using a sterile curette and then swabbed with sterile saline. The same procedure was followed for the eye. A female beagle, B-19, served as a negative control in a similar fashion as did the male. The scarification and inoculations were also achieved in the same manner.

All inoculated animals were maintained in individual cages in the same room but individual exercise periods could not be maintained and the dogs were allowed to mix. Daily cleaning of cages was maintained throughout the incubation period but the technique was less than optimal. The temperature and humidity were not controlled but were noted to be variable.

The experiment was conducted for a total of 17 days.

Legend of Beagle Infectivity Studies

Male	Site of Inoculation	
1-5	Eye	
B-20	Eye	
A-3	Penis	
H-15	Penis	
M-18	Eye and penis	Noninoculated control

Female	Site of Inoculation	
A-1	Eye	
A-2	Eye	
H-6	Vagina	
E-24	Vagina	
B-19	Eye and vagina	Non inoculated control

Infected Beagle Biopsy

On the 17th day post-inoculation the Beagles were anesthetized in the manner previously described.

All dogs having received eye inoculations had 1 cm x 1 cm sections removed from the area of inoculation. The dogs which had penile and vaginal inoculations were also biopsied, with 0.5 cm x 0.5 cm sections removed. The control dogs were biopsied in the same manner.

The biopsied tissue was divided into two equal parts. One-half of each section was frozen immediately for virus isolation. The remaining tissue was fixed in Bouin's fluid, which has the composition shown below.

Bouin's Fixative

Picric Acid, saturated aqueous solution	750 ml
40 per cent Formalin	250 ml
Glacial acetic acid	50 ml

The fixation process differed from the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (3rd edition, American Registry of Pathology) in the amount of time the tissue was fixed. The time of fixation for the biopsied Beagle tissue was 24 hours.

Tissue Embedding and Sectioning

The Bouin's-fixed tissue from the Beagle biopsy was embedded in Paraplast[17] and allowed to solidify. The paraplast block was immersed in ice water to achieve the desired firmness, then mounted on a Spencer 820 microtome. All tissues were sectioned at five microns and placed on standard microscope slides. The procedure for staining is given in the Appendix.

[17]Product of W. H. Curtin and Company, Tulsa, Oklahoma.

CHAPTER IV

RESULTS AND DISCUSSION

In Vitro Viral Propagation

Infected canine tissue homogenates and tissue digests were inoculated onto monolayers of rabbit kidney primary cells, mouse L cells, HeLa cells, and canine kidney primary cells. The tissue homogenates failed to demonstrate any CPE after seven passages in any of the cell cultures while the digest failed to cause CPE after seven passages in mouse L cell or HeLa cell cultures. However, the digest samples did demonstrate CPE in rabbit kidney primary cell monolayers during passage VII at 72-90 hours post-inoculation. When these infected cultures were compared with the control cultures, a distinct cytoplasmic granulation, cellular rounding, ballooning, and release from the glass surface was evidenced in the infected monolayers when viewed with a light microscope. No plaque formation was observed in any of the infected cultures. There was, however, an irregular disruption of the monolayer due to cellular death and subsequent release from the glass surface. The canine kidney primary cell inoculations were not conducted beyond the second passage due to an inadequate supply of dogs.

Continued passage of all three viral isolates in rabbit kidney primary cell culture resulted in an increase in CPE, which appeared at

48-56 hours post-inoculation. The cellular changes are demonstrated in Figure 1.

Histopathological Examination of Primary Rabbit Kidney Cell Cultures

Primary rabbit kidney cell monolayers inoculated with passage VII of each viral isolate and incubated for 72 hours revealed multi-nucleated giant cells, basophilic intranuclear inclusion bodies and multi-nucleoli when stained with hematoxylin-eosin stain and viewed with a light microscope.

Both a multi-nucleated cell and a cell containing an intranuclear inclusion body are shown in Figure 2 A. The inclusion body was rather large and filled approximately one-fourth the nuclear mass. The inclusion was bounded by a clear area or halo.

A large number of cells with acidophilic inclusion bodies in the cytoplasm were observed in the control cultures. These bodies were ascertained to be unrelated to the viral isolates because they appeared in both controls and inoculated cultures.

In Vitro Characterization Studies

The study of the basic characteristics of the viral agent(s) was begun once it was established that the viral isolates could be successfully propagated in an in vitro system of rabbit kidney primary cell cultures.

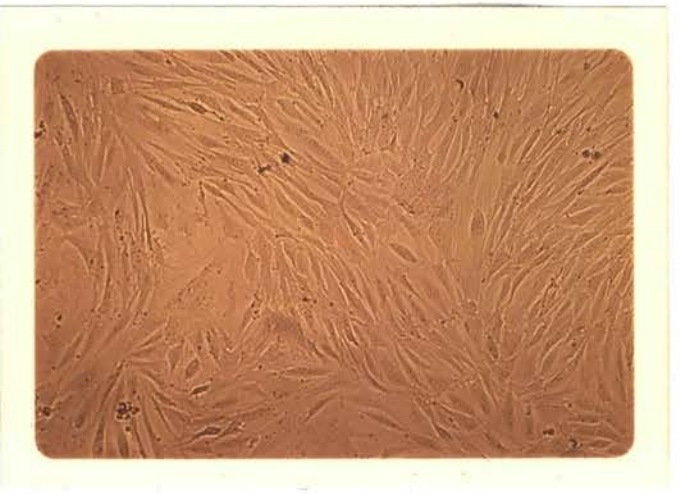
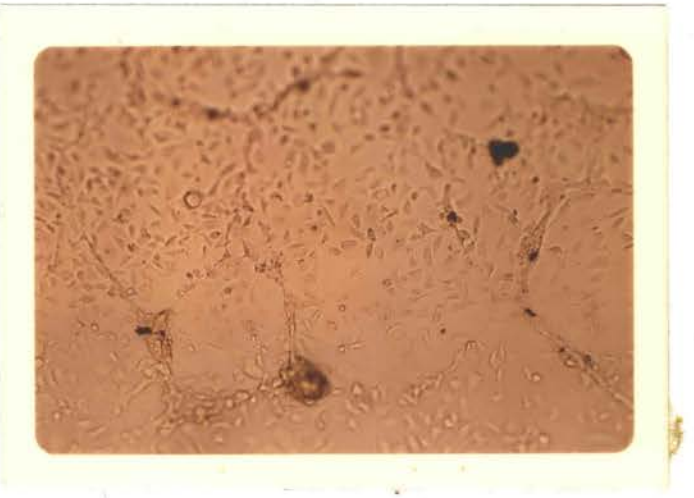


Figure 1. Cytopathic Effect of Passage VII Viral Isolates in Rabbit Kidney Primary Cell Monolayers
A. Control Monolayer at 48 Hours (25x)



B. Monolayers Infected with German Shepherd Eye Virus 48 Hours Post-inoculation (25x)

Figure 1 (Continued)
C. Monolayers Infected with Bulldog Penis Virus 48 Hours Post-inoculation (25x)

D. Monolayers Infected with German Shepherd Vaginal Virus 48 Hours Post-inoculation (25x)

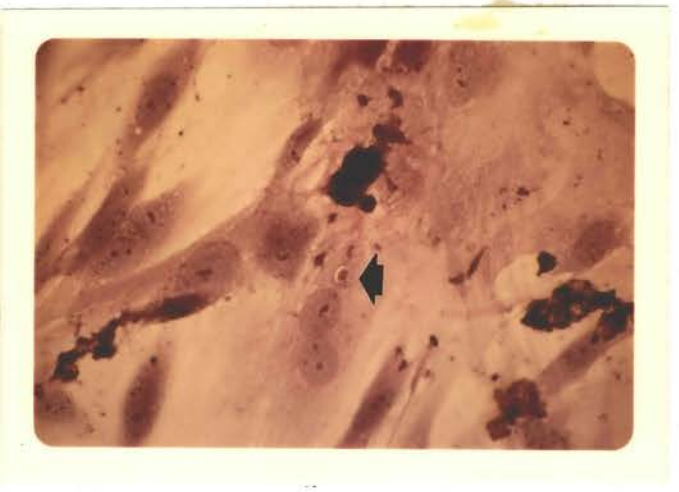


Figure 2. Hematoxylin-eosin Stained Monolayers of Infected Rabbit Kidney Primary Cells
A. Monolayer Infected with Bulldog Penis Virus
Arrow shows Basophilic Intranuclear Inclusion Body and Multinucleated Cell (160x)

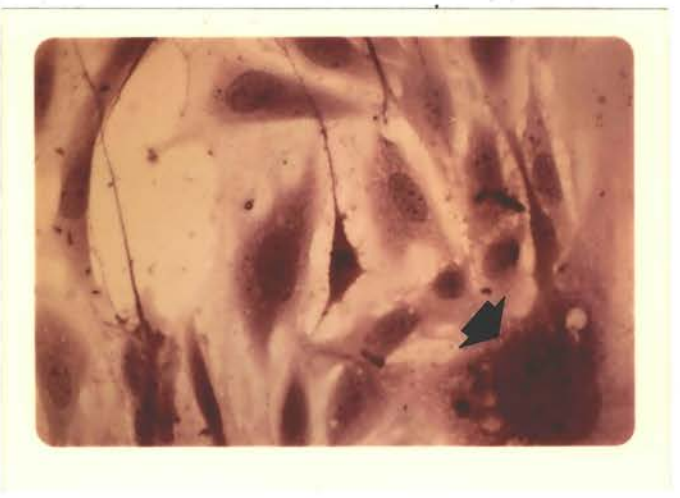


Figure 2 (Continued)
B. Monolayer Infected with German Shepherd Eye Virus (arrow shows Giant Cell) (160x)

Nucleic Acid Determination

Most animal viruses have deoxyribonucleic acid (DNA) as their nucleic acid type. It was believed that by using a DNA inhibitor in the culture medium the presence or absence of DNA in the viral isolate could be determined. The DNA inhibitor selected was 5-iodo-2-deoxyuridine (IUDR) because it inhibits the replication of DNA viruses (22).

All three viral isolates were treated with IUDR (30 µg/ml) and inoculated onto IUDR treated rabbit kidney primary cell cultures. The CPE normally exhibited by all viral isolates was found to be missing 48-72 hours post-inoculation. Table I gives the data for this experiment.

Chloroform Sensitivity Determination

Some animal viruses are encapsulated by a lipoprotein envelope in addition to the protein coat. Stability and infectivity of such viruses can be destroyed by exposure of the virus to certain chemicals such as ether, chloroform, and desoxycholate salts (8, 20). The essential lipids, which have some role in infectivity, are removed from the envelope by this chemical treatment. This characteristic aids greatly in the classification of the different viral groups. Those animal viral groups which possess envelopes are herpesviruses, arboviruses, myxoviruses, and some poxviruses (23).

TABLE I

EFFECT OF 5-IODO-2-DEOXYURIDINE TREATMENT

Inoculum	No. of Untreated Cell Monolayers	Degree of CPE*	No. of Treated Cell Monolayers	Degree of CPE
GVV	5	+4	49	0
			1	+2
GEV	3	+4	48	0
	2	+3	2	+1
BPV	4	+4	50	0
	1	+3		
Spent Medium**	All five tubes of spent medium produced no CPE			

* Positive Control (untreated virus)

** Negative Control

One-tenth milliliter inoculations, using chloroform-treated GEV, GVV, and BPV viral isolates on rabbit kidney primary cell monolayers, failed to produce any CPE (Table II). Both positive and negative controls were included for comparison. When the experiment was repeated, using purified viral isolates, there was no significant differences in the results (Table III).

It was concluded from the data in Tables II and III that GEV, GVV, and BPV viral isolates were sensitive to chloroform and therefore are probably enveloped viruses.

Temperature Sensitivity

Susceptibility to heat is a characteristic property of certain groups of viruses and aids in their classification. However, there are difficulties in determining the specific temperature to which a virus is sensitive. This difficulty is due in part to protection granted by various proteins in the suspension medium and/or the presence of L-cysteine and L-cystine (10).

When unpurified GVV, BPV, and GEV isolates were exposed to temperatures of 43°, 47°, and 57°C for 30 minutes and inoculated on rabbit kidney primary cell monolayers, there was an absence of CPE with viral isolates which had been exposed to temperatures of 47° and 57°. Varied degrees of CPE were noted with all three viral isolates exposed at 43°C. The data for this experiment are found in Table IV.

The experiment was repeated using purified viral isolates and exposing them to temperatures of 38°, 39°, 40°, 41°, 42°, 43°, and 47°C. All viral isolates exposed to 38° produced CPE. There was an absence of CPE in monolayers inoculated with viral isolates exposed to

TABLE II

EFFECT OF CHLOROFORM TREATMENT ON UNPURIFIED VIRUS

Inoculum	No. of Untreated Cell Monolayers	Degree of CPE*	No. of Treated Cell Monolayers	Degree of CPE
GVV	4	+4	50	0
	1	+3		
GEV	5	+4	47	0
			3	+1
BPV	5	+4	50	0
Spent Medium**	All five tubes of spent medium produced no CPE			

* Positive Control (untreated virus)

** Negative Control

TABLE III

EFFECT OF CHLOROFORM TREATMENT ON PURIFIED VIRUS

Inoculum	No. of Untreated Cell Monolayers	Degree of CPE*	No. of Treated Cell Monolayers	Degree of CPE
GVV	5	+4	50	0
GEV	4	+4	48	0
	1	+3	2	+1
BPV	5	+4	50	0
Spent Medium**	All five tubes of spent medium produced no CPE			

* Positive Control (untreated virus)

** Negative Control (spent medium treated with chloroform)

TABLE IV

EFFECT OF TEMPERATURE ON UNPURIFIED VIRUS

	37°C		43°C		47°C		57°C	
Inoculum	No. of Tubes	Degree of CPE*	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE
GVV	8	+4	18	+4	27	0	30	0
	2	+3	12	+3	3	+1		
GEV	8	+4	6	+4	29	0	30	0
	2	+3	20	+3	1	+1		
BPV	10	+4	8	+4	30	0	30	0
			20	+3				
			2	+2				
Spent Medium**	All ten tubes of spent medium produced no CPE							

* Positive Control

** Negative Control

temperatures above 38°C. It was concluded from the data in Table V that GVV, BPV, and GPV were temperature sensitive viruses.

Temperature Stability

Most viruses are stable at -2°C for long periods of time while others require -70°C or less for stability. Such viruses are relatively stable when stored in liquid nitrogen (-196°C). A five per cent solution of dimethyl sulfoxide has been reported to give complete protection to enveloped virus during repeated freezing and thawing (21). Unstable viruses are granted some protection by horse or calf serum; however, this procedure is generally considered to be inadequate (9).

While working with passage VII of the three isolates for propagation of sufficient quantities of virus for characterization studies, it was noted that there was an absence of CPE. Additional ampoules of the viral isolates which had been stored at -2°C were thawed rapidly at 37°C, inoculated onto rabbit kidney primary cell monolayers and incubated at 37°C. There continued to be an absence of CPE. The current passage at that time was passage IX. Passage IX of each viral isolate was placed in 1.0 ml. glass ampoules and stored in liquid nitrogen for two months. At the end of this period the samples were removed and thawed rapidly at 37°C. Inoculations were made onto rabbit kidney primary cell monolayers and incubated at 37°C. CPE occurred between 48 and 60 hours. It was concluded from the data in Table VI that GVV, BPV and GEV were unstable at -2°C and stable at -196°C.

TABLE V

EFFECT OF TEMPERATURE ON PURIFIED VIRUS

	37°C		38°C		39°C		40°C	
Inoculum	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE
GVV	7	+4	30	+4	27	0	30	0
	3	+3			3	+1		
GEV	8	+4	28	+4	26	0	30	0
	2	+3	2	+3	4	+1		
BPV	9	+4	26	+4	28	0	30	0
	1	+3	4	+3	2	+1		

TABLE V (Continued)

	41°C		42°C		43°C		47°C	
Inoculum	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE
GVV	30	0	30	0	30	0	30	0
GEV	30	0	30	0	30	0	30	0
BPV	30	0	30	0	30	0	30	0
Spent Medium*	All ten tubes of spent medium produced no CPE							

* Negative Control

TABLE VI

EFFECT OF LOW TEMPERATURE ON STABILITY

	4°C		-2°C		-77°C	
Inoculum	No. of Tubes	Degree of CPE*	No. of Tubes	Degree of CPE	No. of Tubes	Degree of CPE
GVV	7	+4	27	0	23	+4
	3	+3	3	+1	7	+3
GEV	9	+4	29	0	25	+4
	1	+3	1	+1	5	+3
BPV	8	+4	28	0	26	+4
	2	+3	2	+1	4	+3
Spent Medium**	All ten tubes of spent medium produced no CPE					

* Positive Control (virus which had not been exposed to low temperatures)

** Negative Control

Determination of Virus Titer

The titer for each viral isolate was determined as the reciprocal of the last viral dilution which showed a cytopathic effect. The data for this procedure are compiled in Table VII.

Viral Size

Viral size is characteristic for a given group of viruses. However, apparent size varies with the technique employed and thus single types of measurement produce only a rough estimate of the actual size (10).

Passage VII of the viral isolates was used in the determination of the approximate viral size. The original size determined for passage VII of BPV was 100-200 mμ using Millipore filters, as determined by the presence of CPE in rabbit kidney primary cell monolayers when the filtrate was used as inoculum. Passage VII of BPV was a viral-cell suspension. It was believed that a more accurate determination of size could be achieved by using purified viral samples, since the cells and cellular debris had a tendency to block the filtration of the virus. Purified samples of viral isolates were filtered using Millipore filters having 50, 100, 200, 220, and 300 mμ pore diameters. The filtrates were inoculated onto rabbit kidney primary cell monolayers.

TABLE VII

VIRUS TITER DETERMINATION

		Viral Dilutions										
		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Inoculum	Tube No.	Degree of CPE*	Degree of CPE									
Passage VII GVV	1	+4	+4	+4	+3	+3	+2	0	0	0	0	0
	2	+4	+4	+3	+3	+4	0	0	0	0	0	0
	3	+4	+4	+4	+4	+3	0	0	0	0	0	0
	4	+4	+3	+4	+4	+4	+1	0	0	0	0	0
	5	+3	+3	+4	+3	+4	0	0	0	0	0	0
Passage VII GEV	1	+4	+4	+4	+3	+2	+3	0	0	0	0	0
	2	+4	+4	+4	+3	+3	+3	0	0	0	0	0
	3	+4	+3	+4	+4	+2	+2	0	0	0	0	0
	4	+4	+4	+4	+3	+2	+1	0	0	0	0	0
	5	+4	+4	+4	+4	+4	+3	0	0	0	0	0
Passage VII BPV	1	+4	+4	+4	+3	+4	+3	+4	+2	0	0	0
	2	+4	+4	+4	+3	+4	+4	+3	0	0	0	0
	3	+4	+4	+4	+4	+4	+4	+3	0	0	0	0
	4	+4	+3	+4	+4	+4	+3	+2	0	0	0	0
	5	+4	+3	+4	+4	+3	+3	+3	0	0	0	0
Spent Medium**	All five tubes of spent medium produced no CPE											

* Positive Control

** Negative Control

As Table VIII indicates there was an absence of CPE with all isolates when filtered with 50 mμ filters but a presence of CPE when filters of 100 mμ pore diameters and larger were used. The data indicate that the viral particles are larger than 50 mμ and smaller than 100 mμ. The size differential between the three viral isolates and the herpesvirus group cannot be readily explained.

Serum Neutralization Test

Canine Antisera

When fixed concentrations of each virus isolate were mixed in equal volumes with ten-fold serially diluted homologous canine antiserum and inoculated onto rabbit kidney primary cell monolayers, neutralization of each viral isolate was determined.

Canine BPV antiserum, when mixed with BPV virus, granted protection when diluted to 10^{-8}. Both GVV and GEV viruses were neutralized by their homologous antisera when diluted to 10^{-7}. These data are shown in Table IX.

Rabbit Antisera

Rabbit antisera prepared to the viral isolates and mixed in a similar manner as described for the canine antisera studies resulted in a viral neutralization. The data from both experiments are compiled in Table IX.

TABLE VIII

VIRAL SIZE DETERMINATION

Unfiltered Virus			Filtered Virus					
			Pore Diameter of Filter					
				50 mμ	100 mμ	200 mμ	220 mμ	300 mμ
Inoculum	Tube No.	Degree of CPE	Tube No.	Degree of CPE				
GVV	1	+4	1	0	+4	+4	+4	+4
	2	+4	2	0	+4	+4	+4	+4
	3	+4	3	0	+4	+4	+3	+4
	4	+3	4	0	+4	+4	+4	+4
	5	+4	5	0	+3	+4	+4	+4
			6	0	+4	+4	+4	+4
			7	0	+4	+4	+4	+4
			8	0	+4	+4	+4	+4
			9	0	+4	+3	+4	+4
			10	0	+4	+4	+4	+4
GEV	1	+4	1	0	+3	+4	+4	+4
	2	+4	2	0	+4	+4	+4	+4
	3	+4	3	0	+4	+4	+4	+4
	4	+4	4	0	+3	+4	+4	+4
	5	+4	5	0	+3	+4	+4	+4
			6	0	+3	+4	+2	+4
			7	0	+4	+4	+4	+4
			8	0	+4	+3	+4	+3
			9	0	+4	+4	+4	+4
			10	0	+4	+4	+4	+4

TABLE VIII (Continued)

Unfiltered Virus			Filtered Virus					
			Pore Diameter of Filter					
				50 mμ	100 mμ	200 mμ	220 mμ	300 mμ
Inoculum	Tube No.	Degree of CPE	Tube No.	Degree of CPE				
BPV	1	+4	1	0	+4	+4	+4	+4
	2	+4	2	0	+4	+4	+4	+4
	3	+4	3	0	+4	+4	+3	+4
	4	+4	4	0	+4	+4	+3	+4
	5	+4	5	0	+4	+4	+3	+4
			6	0	+3	+4	+4	+4
			7	0	+4	+4	+4	+4
			8	0	+4	+4	+4	+4
			9	0	+4	+4	+4	+4
			10	0	+4	+3	+4	+4
Spent Medium*	All five tubes of spent medium produced no CPE							

* Negative Control

TABLE IX

SERUM NEUTRALIZATION AND TITER DETERMINATION

Inoculum	Tube No.	Degree of CPE*	Tube No.	Homologous Canine Anti-Serum Dilution									
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
				Degree of CPE									
GVV	1	+4	1	0	0	0	0	0	0	+2	+3	+4	+4
	2	+4	2	0	0	0	0	0	0	0	+4	+4	+4
	3	+4	3	0	0	0	0	0	0	0	+3	+4	+4
	4	+4	4	0	0	0	0	0	0	0	+3	+3	+4
	5	+4	5	0	0	0	0	0	0	0	+4	+4	+4
GEV	1	+4	1	0	0	0	0	0	0	+2	+3	+4	+4
	2	+4	2	0	0	0	0	0	0	0	+3	+4	+4
	3	+4	3	0	0	0	0	0	0	0	+4	+4	+4
	4	+3	4	0	0	0	0	0	0	0	+4	+4	+4
	5	+4	5	0	0	0	0	0	0	+1	+4	+4	+4
BPV	1	+4	1	0	0	0	0	0	0	0	0	+3	+4
	2	+4	2	0	0	0	0	0	0	0	0	+3	+4
	3	+4	3	0	0	0	0	0	0	0	0	+4	+4
	4	+4	4	0	0	0	0	0	0	0	+2	+4	+4
	5	+4	5	0	0	0	0	0	0	0	0	+3	+4

TABLE IX (Continued)

Inoculum	Tube No.	Degree of CPE*	Tube No.	Homologous Rabbit Anti-Serum Dilutions									
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
				Degree of CPE									
GVV	1	+4	1	0	0	0	+4	+4	+4	+4	+4	+4	+4
	2	+4	2	0	0	0	+3	+4	+4	+4	+4	+4	+4
	3	+4	3	0	0	+2	+3	+4	+4	+3	+4	+4	+4
	4	+4	4	0	0	0	+4	+4	+4	+4	+4	+4	+4
	5	+4	5	0	0	0	+3	+4	+4	+4	+4	+4	+4
GEV	1	+4	1	0	0	0	+3	+4	+4	+4	+4	+4	+3
	2	+4	2	0	0	0	+3	+4	+4	+4	+4	+4	+4
	3	+3	3	0	0	0	+4	+4	+4	+4	+4	+4	+4
	4	+4	4	0	0	0	+4	+3	+4	+4	+3	+3	+4
	5	+4	5	0	0	0	+4	+4	+4	+4	+3	+4	+4
BPV	1	+4	1	0	0	0	0	+3	+4	+4	+4	+4	+3
	2	+4	2	0	0	0	0	+3	+4	+4	+4	+4	+3
	3	+4	3	0	0	0	0	+4	+4	+4	+3	+4	+4
	4	+4	4	0	0	0	+2	+4	+4	+4	+4	+4	+4
	5	+5	5	0	0	0	+1	+4	+4	+4	+4	+4	+4
Spent Medium**		All five tubes of spent medium produced no CPE											

* Positive Control
** Negative Control

Cross-Neutralization Test

If a serologic relationship, i.e., cross-neutralization, could be demonstrated between the viral isolates and heterologous donor dog antisera, it would further aid in determining whether the isolates were similar. Studies showed that cross-neutralization occurred when BPV virus was incubated with GVV antiserum and inoculated onto rabbit kidney primary cell monolayers. Positive controls for BPV showed good cytopathic effect at a dilution of 10^{-5}. Neutralization of BPV virus occurred when incubated with GVV antisera. When GVV virus and GEV virus were mixed with heterologous antisera, cross-neutralization was again demonstrated. Data in Table X show the serologic similarity existing between GEV, GVV, and BPV viral isolates.

Canine Herpesvirus Neutralization Test

Aliquots of rabbit and donor dog antisera to GEV, GVV, and BPV were sent to the College of Veterinary Medicine, Cornell University, Ithaca, New York, for neutralization test with canine herpesvirus.

Dilutions of 1:5 of each antiserum were prepared and mixed with canine herpesvirus. All sera at 1:5 dilution failed to neutralize or cause plaque reduction of canine herpesvirus (5). This is sufficient evidence to conclude that canine herpesvirus and the three viruses under investigation were not related serologically.

TABLE X

CROSS-NEUTRALIZATION OF VIRAL ISOLATES
WITH HETEROLOGOUS ANTISERA

		Viral Dilutions					
Inoculum	Tube	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
BPV-BPV Serum*	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
BPV-GVV Serum	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
BPV No Serum*	1	+4	+4	+4	+4	+3	0
	2	+4	+4	+4	+4	+3	0
	3	+4	+3	+3	+4	+4	0
	4	+4	+4	+3	+4	+2	0
	5	+4	+4	+4	+4	+3	0

TABLE X (Continued)

Inoculum	Tube	Viral Dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
GVV-GVV Serum*	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
GVV-GEV Serum	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
GVV No Serum*	1	+4	+4	+3	+4	+3	0
	2	+4	+4	+4	+4	+3	0
	3	+4	+4	+4	+3	+2	0
	4	+4	+4	+4	+4	+3	0
	5	+4	+4	+3	+2	+3	0

TABLE X (Continued)

Inoculum	Tube	Viral Dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
GEV-GEV Serum*	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
GEV-BPV Serum	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	0	0	0	0	0
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
GEV No Serum*	1	+4	+4	+4	+3	0	0
	2	+4	+3	+4	+4	+2	0
	3	+4	+3	+4	+4	+2	0
	4	+4	+4	+4	+2	0	0
	5	+4	+4	+3	+2	0	0

* Controls

Fluorescent Antibody Studies

Tissue Culture

Three groups of slides representing fifty tubes of rabbit kidney primary cell monolayers which had been inoculated 72 hours previously with GVV, GEV, and BPV were fixed with cold acetone and stained with homologous conjugate at dilutions of 1:2.5, 1:5, 1:10, 1:20, and 1.30. The slides were also covered with rabbit tissue homogenate to absorb the excess fluorescein dye. These preparations were examined using a Zeiss fluorescent microscope. Although faint fluorescence of a few nuclei was noted, the results were inconclusive.

The procedure was repeated using monolayers inoculated 12 hours previously, in hopes that the first attempt was made too late in the viral replicative cycle to show the areas of fluorescence. Dilutions of 1:1 and 1:2.5 of the homologous conjugate-tissue homogenates were prepared and the slides stained. The examination revealed faint fluorescence with good control of non-specific fluorescence. In the control slides there were only a few cells in the 1:1 dilution which showed faint fluorescence of the nuclei. The results were again considered inconclusive.

Frozen Sections

Sections of nictitating membrane, vagina, and penis tissues, cut six microns thick, were placed on cover slips and fixed in acetone. The tissues were stained with 1:1 and 1:2.5 solutions of conjugate. The examination of the slides revealed only faint fluorescence of a

few nuclei.

The failure of fluorescence in both frozen section and tissue section and tissue culture was attributed to the poor quality of Cellex-D cellulose. The manufacturer stated that the problem was due to the complete binding of all globulin fractions to the cellulose. Lack of sufficient canine serum negated the possibility of concentrating the serum by ammonium sulfate precipitation and removal of excess fluorescein isothiocyanate by dialysis.

Beagle Infectivity Studies

Following the studies of the basic characteristics of the three isolates, the study of the transmissibility of the agent(s) was undertaken. Ten Beagles from the hemophilia resource colony were selected for this study because of their history of being free of any of the follicular lesions. The male dogs were assayed as normal and though the females were transmitters, their factor VIII levels ranged from 25 per cent to 50 per cent.

Five animals, L-5, A-3, H-15, B-19 and E-24 were examined on the seventh day post-inoculation and showed no evidence of follicular lesion. On the ninth day post-inoculation dogs M-18, A-2, B-20, A-1 and A-3 were examined. This examination revealed B-20, A-1, and A-2 to have light to moderate follicular lesions in the inoculation sites. A-3 and M-18 were found to have no follicular lesions. All dogs were examined on the tenth day with eight test animals demonstrating follicular lesions (see Figure 3). The control animals showed no lesions. The results of the experiment are compiled in Table XI.

On the 17th day post inoculation the dogs were anesthetized and the

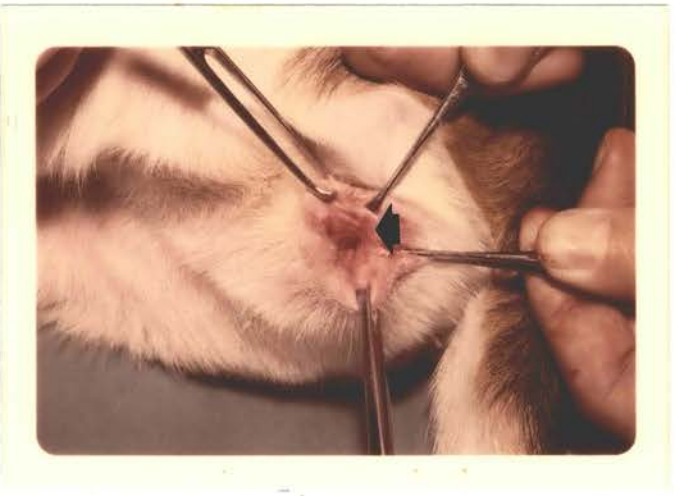
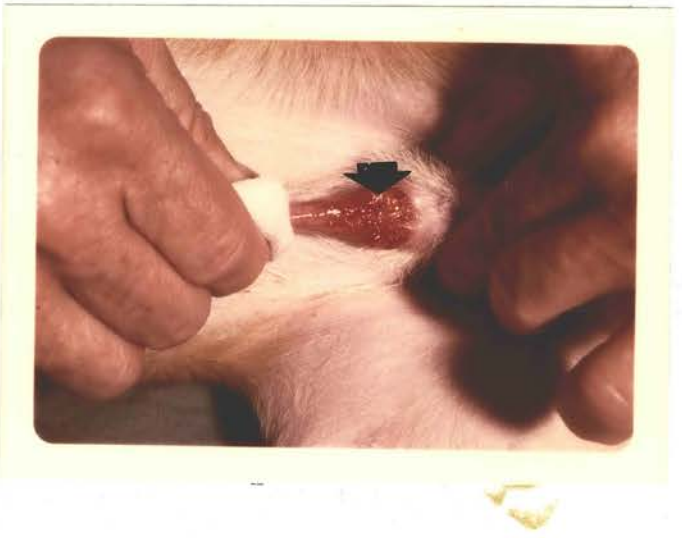


Figure 3. Induced Follicular Lesions of the Canine Vagina, Penis, and Conjunctiva on the Tenth Day Post-inoculation
A. Vaginal Follicles in E-24 (arrows)



B. Penile Follicles in H-15

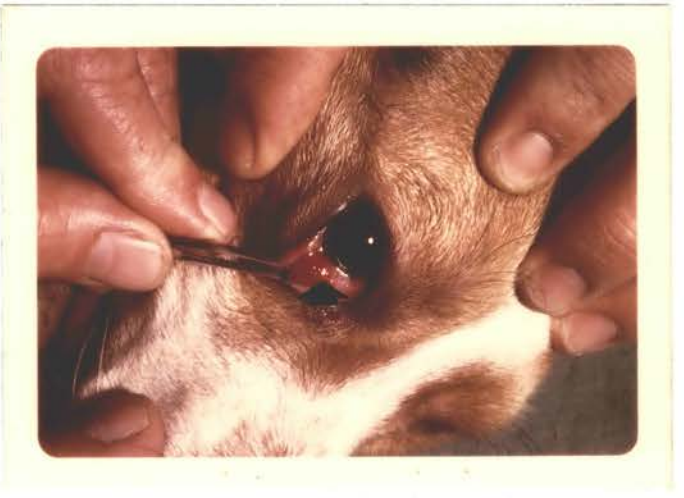


Figure 3 (Continued)
C. Follicles of the
Membrane Nictitans
L-5

TABLE XI

BEAGLE INFECTIVITY STUDIES

Examination Results on 10th Day

Dog	Treatment Site	Lesions
L-5	Eye, bilateral	Present
B-20	Eye, bilateral	Present
A-3	Penis	Present
H-15	Penis	Present
M-18*	Penis	Absent
	Eye, bilateral	Absent
A-1	Eye, bilateral	Present
A-2	Eye, bilateral	Present
H-6	Vagina	Present
E-24	Vagina	Present
B-19*	Vagina	Absent
	Eye, bilateral	Absent

Examination Results on 17th Day

Dog	Treatment Site	Lesion	Site of Secondary Lesions
L-5	Eye, bilateral	Present	Penis
B-20	Eye, bilateral	Present	
A-3	Penis	Present	Eye, right
H-15	Penis	Present	Eye, right
M-18*	Penis	Present	Penis
	Eye, bilateral	Present	Eye, left
A-1	Eye, bilateral	Present	Vagina
A-2	Eye, bilateral	Present	Vagina
H-6	Vagina	Present	Eyes
E-24	Vagina	Present	Eye, right
B-19*	Vagina	Present	Vagina
	Eye, bilateral	Present	Eyes

* Controls

inoculation sites were biopsied. The controls were found to be infected; M-18 had both penis and right nictitating membrane follicles while B-19 had vaginal and bilateral membrane follicles. It was also noted that other susceptible areas of the inoculated animals were demonstrating the follicular lesion. Data for this experiment are compiled in Table XI.

An interesting note and one that prompts further investigation was the lessening of the degree of follicular vaginitis of E-24 during estrus. This was observed on the 17th day post-inoculation.

Histologic Examination of Biopsied Tissue

When sections of infected Beagle tissue were stained with Harris' hematoxylin-eosin Y stain and examined with a light microscope, a massive proliferation of lymphoid tissue, or more apecifically, a lymphoid hyperplasia was observed (16).

This hyperplastic condition was exhibited in tissue biopsied from all three sites of infection. Normal, non-infected tissue of these areas characteristically has lymphoid nodules as evidenced in Figure 4A but not in a state of hyperplasia, as noted in the inoculated culture in Figure 4B. Lymphoid hyperplasia is the expected pathological response to the presence of a pathogen (16).

Figure 4B shows a large hyperplastic area in which the central portion contains a high percentage of mitotic cells in varying degrees of maturation. The peripheral cells are stained much darker and have a more uniform size which is smaller than the mitotic cell. The centers of hyperplasia are responsible for the irregularities or follicles of the surface epithelium.

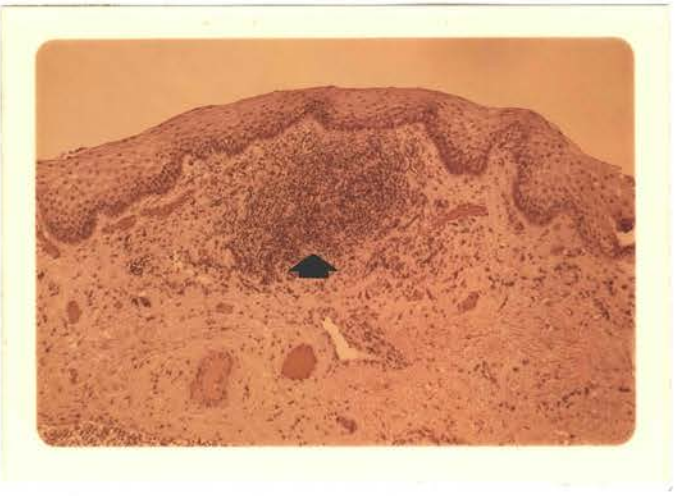
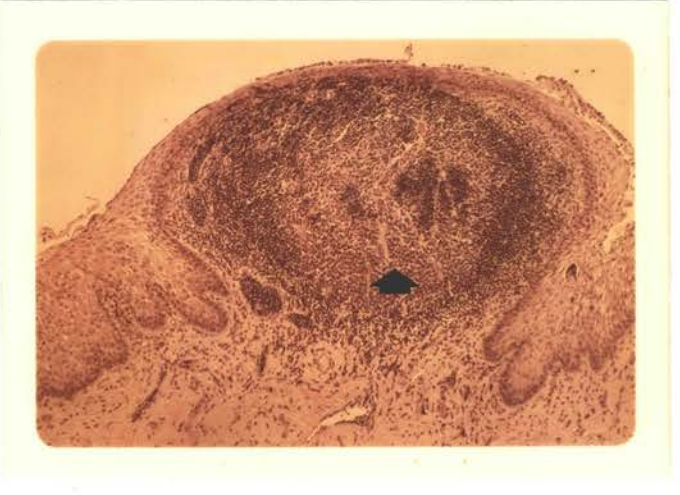


Figure 4. Hematoxylin-eosin Stained Sections of Normal and Infected Canine Tissue
A. Normal Mucosa of the Penis. Non-hyperplastic Lymphoid Tissue (arrow)(25x)



B. Infected Mucosa of Penis Lymphoid Hyperplasia (arrow) (25x)

CHAPTER V

SUMMARY AND CONCLUSIONS

Follicular hyperplasia of the vagina, penis, and nictitating membrane in the canine were not caused by a bacterial, fungal, or mycoplasmal agent or by three different viruses as first suspected, but by a single virus which appears to belong to the herpesvirus group.

This conclusion is based on *in vitro* studies using rabbit kidney primary cell culture monolayers, beagle infectivity studies, serologic, and histopathologic studies. The formation of Cowdry type A intranuclear inclusion bodies, lipid solvent sensitivity, presence of DNA, temperature instability at -2°C, and the irregular cellular release from the growth surface when infected, tend to indicate the German shepherd and Bulldog viral isolates are one and the same virus. The characteristics of this virus tentatively place it in the herpesvirus group.

Antibodies, from the donor dogs, to this virus caused neutralization of the virus *in vitro*. The fact that there was cross-neutralization of the viral isolates with heterologous donor dog antisera further indicates a serologic relationship. The virus is not serologically related to the canine herpesvirus. This does not dispute the conclusion drawn but rather affirms the variability that exists among the members of the herpesvirus group. For example, in 1965

Carmichael et al. reported that canine herpesvirus was not serologically related to other herpesviruses on the basis of cross-neutralization studies (3).

In dogs free of the follicular hyperplasia there is a high degree of infection when the virus is introduced both directly and indirectly. Secondary sites of infection appear which indicates hematogenous or contact spread or a combination of these factors.

Histological examination of infected canine tissue reveals lymphoid hyperplasia which is characterized by a number of rapidly dividing cells in varying stages of mitosis. This mitotic area is surrounded by a more mature, non-replicating area of lymphoid cells.

There is no credible explanation for the size differential between this virus and the herpesvirus group. However, as stated previously, single types of measurement produce only a rough estimate of the actual size. The electron microscope will ultimately determine its true size.

LITERATURE CITED

1. Appel, M. J. G., M. Menegus, I. M. Parsonson, and L. E. Carmichael. Pathogenesis of Canine Herpesvirus in Specific Pathogen Free (SPF) Dogs. Unpublished.

2. Buckner, Ralph G. (1969). Department of Pathology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma. Personal Communication.

3. Carmichael, L. E., J. D. Strandberg, and F. D. Barnes (1965). Identification of a Cytopathogenic Agent Infectious for Puppies as a Canine Herpesvirus. Proc. Soc. Expt. Biol. and Med. 120:644-650.

4. Carmichael, L. E., R. A. Squire, and L. Krook (1965). Clinical and Pathological Features of a Fatal Disease in Newborn Pups. Amer. J. Vet. Res. 26:803-814.

5. Carmichael, L. E. (1969). College of Veterinary Medicine, Cornell University, Ithaca, New York. Personal Communication.

6. Crandall, R. A. (1966). The Herpesvirus Group. Amer. J. Vet. Res. 28:577-588.

7. Epstein, M. A. (1965). Herpes-like Virus in a Lymphosarcoma. J. Expt. Med. 121:761-763.

8. Feldman, H. A. and S. A. Wang (1961). Sensitivity of Various Viruses to Chloroform. Proc. Soc. Expt. Biol. and Med. 106:736-740.

9. Gravell, M., A. Granoff, and R. W. Daerlington (1968). Viruses and Renal Carcinoma in Rana pipiens. Virology 36: 467-475.

10. Hamparian, V. V., M. R. Hillerman, and A. Ketler (1963). Contributions to Characterization and Classification of Animal Viruses. Proc. Soc. Expt. Biol. and Med. 112:466-471.

11. Humason, G. L. (1967). Animal Tissue Technique, 2nd ed. W. H. Freeman and Co.

12. Hunt, R. D. and L. V. Melendez (1969). Herpesvirus Infection of Non-Human Primates. Lab. Ani. Care 19:221-232.

13. Jones, E. M. (1969). Department of Medicine and Surgery, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma. Personal Communication.

14. Melnick, J. L., M. Midulla, I. Wimberly, and J. G. Barrera-Oro (1963). A New Member of the Herpesvirus Group Isolated from South American Marmosets. J. Immuno. 92:595-601.

15. Osborn, B. I. (1969). College of Veterinary Medicine, University of California, Davis, California. Personal Communication.

16. Panciera, R. J. (1969). Department of Pathology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma. Personal Communication.

17. Rafferty, K. A., Jr. (1965). The Cultivation of Inclusion Associated Viruses from Lucké Tumor Frogs. Ann. N. Y. Acad. Sci. 126:127-145.

18. Spertzel, R. O., D. L. Huxsoll, and J. J. McConnell (1965). Recovery and Characterization of a Herpes-like Virus from Dog Kidney Cell Cultures. Proc. Soc. Expt. Biol. and Med. 120:1250-1255.

19. Stewart, S., J. David-Ferriera, and E. Lovelace (1965). Herpes-like Virus Isolated from Neonatal and Fetal Dogs. Science 148:1341-1343.

20. Theiler, M. (1957). Action of Desoxycholate on Arthropod-born Viruses. Proc. Soc. Expt. Biol. and Med. 96:380-382.

21. Wallis, C. and J. L. Melnick (1968). Stabilization of Enveloped Viruses by Dimethyl Sulfoxide. J. Virol. 2:953-954.

22. Watson, J. D. (1965). Molecular Biology of the Gene. W. A. Benjamin, Inc., 290-291.

23. Wilner, B. I. (1965). A Classification of the Major Virus Groups of Human and Animal Viruses. Burgess Publishing Co. 3rd ed.

24. Yamaguchi, J., Y. Hinuma, and J. T. Grace, Jr. (1967). Structure of Virus Particles Extracted from Burkitt Lymphoma Cells. J. Virol. 1:640-642.

APPENDIX

MATERIALS AND METHODS

Dulbecco's Phosphate Buffer

1. Solution I

NaCl	8.0 gm
KCl	0.2 gm
Na_2HPO_4	1.15 gm
KH_2PO_4	0.2 gm
Deionized H_2O	300 ml

Solution II

$CaCl_2$	0.1 gm
Deionized H_2O	100 ml

Solution III

$MgCl_2 \cdot 6 H_2O$	0.1 gm
Deionized H_2O	100 ml

Trypsin - Versene Digest Solution

NaCl	8.0 gm
KCl	0.4 gm
Dextrose	1.0 gm
Versene[1]	0.2 gm (.02%)
Trypsin	2.5 gm (.25%)
$NaHCO_3$	0.35 gm
1% solution phenol red	0.5 ml
Deionized H_2O	1000 ml

The solution was filter sterilized by using a Seitz filter.

---

[1] Disodium dihydrogen ethylenediaminetetraacetate dihydrate, Frederick Smith Chemical Company, Columbus, Ohio.

Bacterial Media

Blood Agar

1. 25 gm heart infusion agar (Difco)
2. 5% bovine citrated blood
3. 20 gm agar
4. 1000 ml distilled H_2O

PPLO Inhibited

1. 35 gm PPLO agar
2. 10 gm yeast extract
3. 900 ml distilled H_2O
4. 1 cc of thallium acetate/100 ml
5. 5000 units of penicillin G
6. 100 ml horse serum

pH was 7.6

Stains and Staining Technique

Harris Hematoxylin Stain

Hematoxylin crystals	5 gm
Ethanol 100%	50 ml
Aluminum ammonium sulfate	100 ml
Distilled H_2O	1000 ml
Mercuric oxide (red)	2.5 gm

Two milliliters of galcial acetic acid were added per 100 ml. of stain solution. The stain solution was then filtered and kept at room temperature.

Eosin Y Stain-stock Solution

Eosin Y, water soluble	1.0 gm
Distilled H_2O	20 ml
Ethanol, 95%	80 ml

A. Eosin Y Working Solution

One part Eosin Y stock solution and three parts 80% ethanol comprised the working solution. Prior to use, 0.5 ml. of glacial acetic acid was added per 100 ml. of stain.

Hematoxylin-eosin Staining Technique

Tissues treated with Bouin's fixative was run through the following procedure and mounted on standard microscope slides using Permount.

1. 2 1/2 minutes in xylol
2. 2 1/2 minutes in xylol
3. 1 1/2 minutes in 100% isopropyl alcohol
4. 1 1/2 minutes in 100% isopropyl alcohol
5. 1 1/2 minutes in 80% isopropyl alcohol
6. 2 minutes in distilled water
7. 3 minutes in Harris hematoxylin solution
8. 1 quick rinse in distilled water
9. 2 quick rinses in 1% acid-alcohol
10. 10 dips in 1% ammonium hydroxide
11. 2 minutes in 1% ammonium hydroxide
12. 2 minutes in distilled water
13. 1 quick dip in 1% phosphotungstic acid

14. 2 minutes in distilled water
15. 2 minutes in 1% ammonia hydroxide
16. 2 minutes in distilled water
17. 2 minutes in 70% isopropyl alcohol
18. 2 minutes in eosin Y solution
19. 5 dips in 95% isopropyl alcohol
20. 20 dips in 100% isopropyl alcohol
21. 1 minute in 100% isopropyl alcohol
22. 2 minutes in 50% isopropyl alcohol-xylol solution
23. 2 minutes in xylol
24. 2 minutes in xylol
24. air dry

VITA

Rick Keith Pickard

Candidate for the Degree of

Master of Science

Thesis: A NEW HERPES-LIKE VIRUS INFECTIOUS FOR THE CANINE

Major Field: Microbiology

Biographical:

Personal Data: Born in Woodward, Oklahoma, April 20, 1943, the son of Walter E. and Helen V. Pickard; married to Beverly Ann Saltmarsh on May 28, 1966; one daughter, Stephanie Christina, born December 5, 1968.

Education: Graduated from Woodward High School, Woodward, Oklahom, in 1961. Served with the United States Air Force and was honorably discharged in 1965. Received the Bachelor of Science degree in Biology and Chemistry from Northwestern State College at Alva, Oklahoma, in 1968. Completed requirements for the degree of Master of Science at Oklahoma State University, Decmeber, 1973.

Professional Experience: Production Chemist for Allergy Laboratories, Oklahoma City, Oklahoma, 1968. Teaching Assistant, Department of Microbiology, Oklahoma State University, 1968-1969.