Isolation of Herpesvirus from Equine Leukocytes: Comparison with Equine Rhinopneumonitis Virus

L. Kemeny and James E. Pearson*

SUMMARY

An agent which possessed the properties of herpesviruses was isolated from the leukocytes of 71 out of 80 (88.7%) apparently normal Iowa horses. It was ether- and heat-sensitive, DNA type, and produced type-A intranuclear inclusion bodies in cell cultures. Electron micrographs revealed a virion of typical herpesvirus structure. Leukocyte isolate virus could be differentiated from equine rhinopneumonitis virus (ERV) by serum neutralization, by growth differences in rabbit kidney cells, and by fluorescent antibody staining. Specific neutralizing antibody against this agent was found in a pooled serum sample from normal horses and in the serums of herpesvirus carrier horses. Serum from a mare inhibited growth of both ERV and leukocyte viral isolates. Normal sheep, calf, and rabbit serum did not neutralize either virus.

RÉSUMÉ

Nous avons isolé un virus herpétique de leucocytes de chevaux (71 sur 80; 80.7%) de l'Iowa apparemment sains. C'est un virus à ADN, sensible à l'éther et à la chaleur, et à inclusions nucléaires de type A en cultures cellulaires. Il a la structure typique des virus herpétiques au microscope électronique. Il peut être différentié de celui de la rhinopneumonie équine (ERV) par les tests de séro-neutralisation, de croissance en cellules rénales de lapin et de l'immuno-fluorescence. Nous avons trouvé un anticorps neutralisant spécifique dans un mélange de sérums de chevaux normaux et dans ceux de chevaux porteurs du virus. Le sérum d'une jument a inhibé la croissance du virus ERV et de ceux isolés des leucocytes. Les sérums normaux de mouton, de veau et de lapin n'ont neutralisé aucun de ces virus.

INTRODUCTION

It long has been recognized that most animal species harbor latent viruses. Little is known about the role of these agents except they appear harmless in the natural host and some are cytopathic for cultured cells. Prominent among the endogenous tissue contaminants are the simian viruses from monkey tissues (5) and herpes-like viruses cultured from human and murine leukemia (4).

In 1963, Plummer and Waterson (12) isolated a herpesvirus which was distinguishable serologically from the classic equine rhinopneumonitis or abortion virus (ERV). They proposed that the ERV be called Equine Herpesvirus type 1, and their isolant, also called "LK" virus, Equine Herpesvirus type 2. Since then, a great number of equine herpesviruses, other than ERV, have been identified in all parts of the world (6, 7, 9, 11).

While attempting to propagate horse leukocytes *in vitro*, we seeded washed leukocytes into rabbit kidney (RK) cell cultures. Small foci of degeneration appeared 14 to 28 days after inoculation.

This report describes the isolation of an agent from leukocytes of normal horses which appears to be an equine member of the herpesvirus group.

*National Animal Disease Laboratory, Animal Disease and Parasite Research Division, ARS, USDA, Ames, Iowa 50010.

MATERIALS AND METHODS

PRIMARY RABBIT KIDNEY CULTURES

Monolayers of RK cells were prepared in plastic tissue culture flasks[1] according to standard methods (5). The growth medium consisted of Eagle's minimum essential medium (MEM) in Earle's balanced salt solution (EBSS), supplemented with 10% fetal calf serum, 0.5% lactalbumin hydrolysate, plus 100 units of penicillin, 100 μg of streptomycin and 100 μg of neomycin/ml. Secondary cultures were prepared in flasks and tubes by subculturing dispersed cells in growth medium. Inoculated cultures were maintained in a medium of similar composition with the addition of 3% inactivated fetal calf serum.

HORSE LEUKOCYTE SUSPENSIONS

White blood cells were separated from freshly collected, citrated or heparinized horse blood by allowing it to stand at room temperature for one hour, then centrifuging the plasma at 1,500 rev/min (500 x g) for seven minutes. The cells were washed three times with phosphate buffered saline (PBS) solution, free from Ca and Mg ions and seeded onto RK cultures.

VIRAL STRAINS

Two representatives of ERV were: a) the hamster-adapted Kentucky-D strain (Ky-D) which is used in a commercial vaccine[2]; and b) the Army-183 strain[3]. Two viral isolants recovered from the leukocytes of apparently healthy horses were: a) H-15 strain[4]; and b) H-40 strain. The H-40 strain was selected in this study as a representative of 71 viral isolants. All four strains were titrated and passed several times in RK cell cultures before use.

PREPARATION OF VIRUS STOCKS

When 75% of the infected cells manifested CPE, the cell sheet was scraped off the glass; the infectious tissue culture fluid was mixed with 10% sterile skim milk or dimethylsulfoxide (v/v), dispensed in small hard glass vials, and stored at –60°C.

QUANTITATIVE VIRUS ASSAYS

Virus infectivity was measured both by the tube culture and plaque methods. Dulbecco-Vogt (2) phosphate buffered saline (PBS) containing 0.5% bovine albumin[5] was used as diluent in virus assays. Tube cultures of RK cells were inoculated with 0.2 ml of serial ten-fold dilutions of virus, incubated at 37°C for seven to 14 days and the 50% infective dose ($TCID_{50}$) calculated by the method of Reed and Muench (13). Plaque assays were made in RK monolayers growing in 30 ml plastic flasks. The medium was removed, the cell sheets washed with warm PBS and inoculated with 0.1 ml of appropriate virus dilutions. Three flasks were used for each dilution. After three hours' adsorption on a rotary shaker at 37°C, monolayers were covered with 5 ml of methyl cellulose[6] solution. This overlay medium was prepared according to the method described by Zee *et al* (14). Plaques were counted after six days' incubation at 37°C. The viral titer was expressed in terms of plaque-forming units (PFU)/ml of the inoculum.

PREPARATION OF IMMUNE RABBIT SERUMS

Four groups of three rabbits each were immunized with the H-15, H-40, Ky-D, and A-183 reference strains. Antigens were prepared from virus-infected RK cell cultures which were maintained on a serum-free medium. The virus was concentrated 100-fold by centrifugation[7] at 40,000 rev/min (284,000 x g) for two hours. Pre-immunization serum samples were obtained from each animal before inoculation. The first injection of 1 ml of virus intravenously was followed by four intramuscular injections, given at seven day intervals of 1 ml of virus mixed with 1 ml of complete Freund's adjuvant[8]. The rabbits were exsanguinated seven to nine days after the final injection. The serums were stored at –60°C. Control serum also was prepared by inoculating a rabbit with uninfected tissue culture fluid.

---

[1]Falcon Plastic Co., Los Angeles.
[2]Pneumabort-Fort Dodge Labs., Inc., Fort Dodge, Iowa.
[3]Obtained from Dr. J. T. Bryans, University of Kentucky, Lexington, Ky.
[4]Obtained from Dr. J. R. Gorham, Washington State University, Pullman, Washington.
[5]Bovine Albumin Fraction V, Armour Labs., Chicago, Ill.
[6]Methyl cellulose, 4000 centipoises, Matheson Coleman and Bell, East Rutherford, N.J.
[7]Beckman Model L2-65B ultracentrifuge rotor type SW 40Ti.
[8]Difco Labs., Detroit, Mich.

Serum-neutralization test

The constant virus-varying serum technique was applied. Test dose of virus, calculated from titrated stock virus to contain approximately 100 $TCID_{50}$/0.1 ml, was mixed with equal volumes of a series of dilutions of homologous and heterologous inactivated immune serums, incubated at 4°C for two hours, and 0.2 ml of each mixture was inoculated into each of three tubes. The virus control was assayed simultaneously and considered satisfactory when the titer of the test dose of virus was shown to be 100 $TCID_{50}$/0.1 ml. The neutralization titer was calculated and expressed as the reciprocal of the dilution that protected 50% of the inoculated cultures.

Ether sensitivity test

This test was performed by adding 20% by volume of peroxide-free, diethyl ether to undiluted virus suspension, refrigerating the mixture for 18 hours at 4°C, centrifuging at 2,000 rev/min (900 x g) for ten minutes, and titrating the virus infectivity of the aqueous phase. The control virus suspension was held in the same manner without adding ether and tested simultaneously.

Heat inactivation

Stock virus was added to sterile skim milk, prewarmed in a water bath at 56°C. One sample was titrated immediately; further samples were taken at different intervals and assayed for surviving virus.

Staining procedures

Rabbit kidney cell cultures grown on coverslips in Leighton tubes were fixed and stained by Hematoxylin and Eosin, Acridine Orange, and Feulgen's procedures (5).

Fluorescent antibody (FA) staining

Immune rabbit and horse serum-globulin was conjugated with fluorescein isothiocyanate according to standard procedures (1). The direct method was used for detecting viral antigen in infected cells grown on coverslips.

RESULTS

Behavior in cell culture

The two strains of ERV produced rather diffuse CPE and destroyed the cell sheet rapidly in two to three days. The average titer for ERV strains was $10^{6.5}$ to $10^{7.0}$ $TCID_{50}$/ml. In the cultures of the leukocyte viral isolants from 71 horses, the incubation period for CPE development was much longer (seven to 21 days) and sometimes blind passages were necessary to detect virus. The initial CPE consisted of small, round, or oval focal lesions of enlarged, rounded, translucent cells contrasting sharply with the normal cells (Fig. 1). The

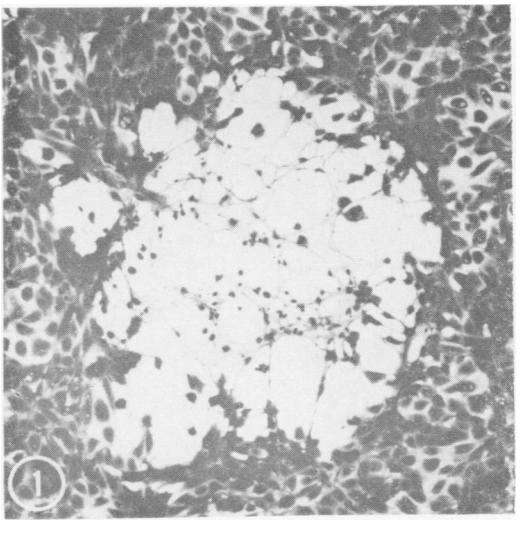

Fig. 1. Cytopathic effect (a plaque), H-40 strain, six days postinoculation. Crystal violet stain; X 125.

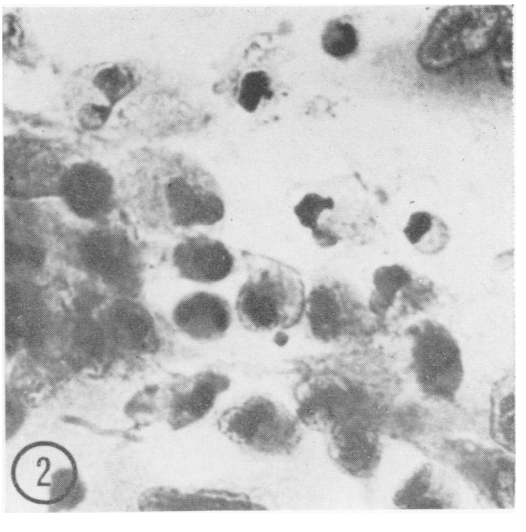

Fig. 2. Type-A inclusion bodies in the nuclei of RK cells, H-40 strain, six days postinoculation; H & E stain; X 320.

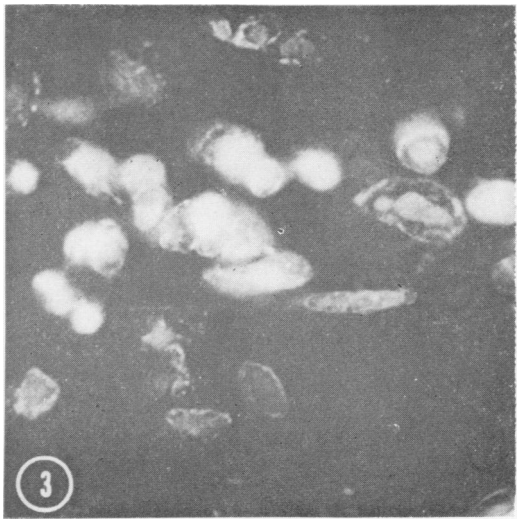

Fig. 3. DNA material in the nuclei of RK cells, H-40 strain, four days postinoculation. Acridine orange stain; X 320.

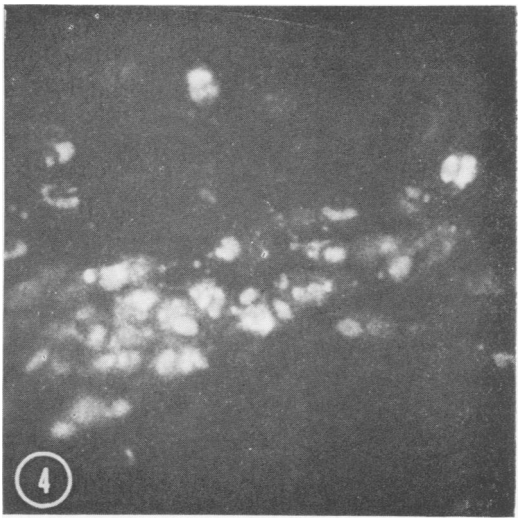

Fig. 4. Viral antigen of the H-40 strain appears brightly fluorescent in the nuclei of RK cells, 24 hours postinoculation. Direct immunofluorescent antibody staining; X 160.

foci progressed centrifugally until the entire culture was destroyed. After six or more passages, the incubation period became shorter (four to seven days), and formation of polykaryocytes was observed. The H-15 and H-40 viral isolants attained a maximum titer of $10^{4.5}$ to $10^{5.5}$ $TCID_{50}$/ml after six passages.

The nuclei of the cells infected either with ERV or with leukocyte isolant viruses showed increased yellow-green staining with acridine orange, and red color with Feulgen's stain as compared to the controls. Type-A intranuclear inclusion bodies surrounded by a clear halo were observed in the nuclei of infected cells after hematoxylin and eosin staining (Fig. 2).

Immunofluorescence was observed in virus infected cells when stained with homologous serum conjugate. Leukocyte

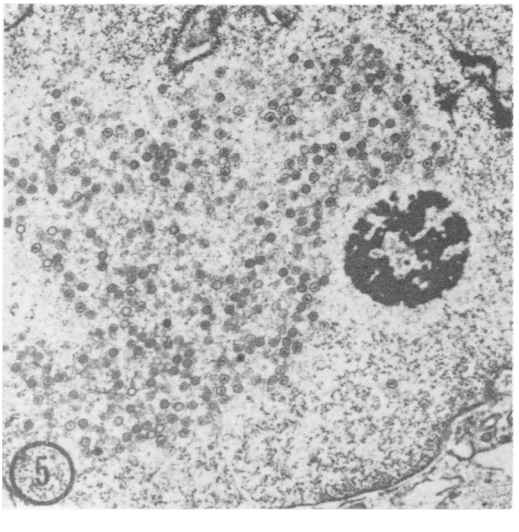

Fig. 5. Electron micrograph of the intranuclear inclusion body, H-40 strain. Virus particles are present in various stages of maturation. Nucleolus is at center right. X 9,800. Preparation, courtesy of N. F. Cheville.

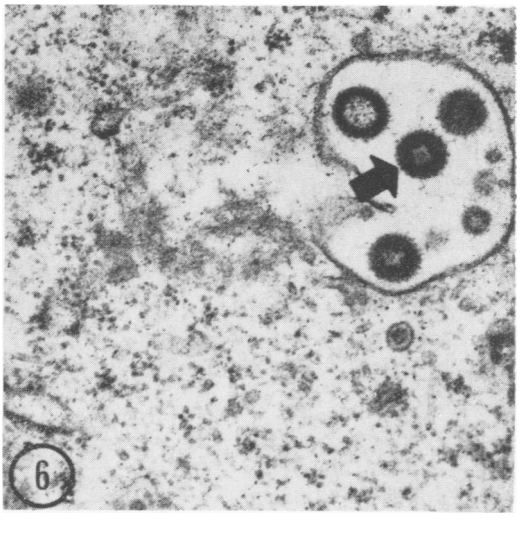

Fig. 6. Electron micrograph of ERV Ky-D strain within a cytoplasmic vacuole, note arrow. X 42,200. Preparation, courtesy of N. F. Cheville.

isolate virus could readily be detected in infected cell cultures by FA staining (Fig. 4). ERV did not give fluorescence with H-40 antiglobulin.

Recovery of virus from horse leukocytes

Herpesvirus was isolated from 71 horses, or 88.7% of those tested. The horses were in four separate groups that were never in contact. Virus was readily recovered from leukocytes but not from plasma or serum. One isolation was made following inoculation of material from 24 nasal swabs onto RK cells.

Ether and heat sensitivity

Ether sensitivity studies revealed that the ERV, Ky-D, A-183 strains, and the H-15, H-40 isolants lost about 75% of their infectivity in 20% ether after 18 hours' incubation at 4°C. The same four strains of virus were partially inactivated by heat at 56°C for 15 minutes and completely inactivated after 60 minutes.

Plaque formation

All four reference strains formed small (1-2 mm in diameter) clear plaques in RK cells under methyl cellulose. Plaque development was complete after three to five days in cultures infected with ERV, and in six to seven days in those which received leukocyte virus isolants (Fig. 1,7). The average PFU was 1.2 x 10^6/ml for H-40 strain and 8.2 x 10^6/ml for the ERV, Ky-D strain.

Neutralization test

Immune rabbit serums were tested in reciprocal cross neutralization tests (Table I). The anti-H-15 and anti-H-40 serums neutralized both isolants, but not the two ERV strains. Conversely, there was no cross neutralization between the anti-ERV, Ky-D and A-183 serums and the two viral isolants from leukocytes.

Table II shows 50% neutralizing endpoint titers from different animal species. Serum samples of 16 horses from which virus had previously been isolated had antibody titers greater than 1:4 against the H-40 isolant. Also, serum of one mare neutralized the ERV, Ky-D strain. The serum obtained from a newborn foal, separated from its mother and reared in isolation on artificial milk replacer, failed to neutralize either virus. Hyperimmune rabbit serums neutralized the homologous virus in a high titer but not the heterologous virus. Pooled serums from normal horses also neutralized the H-40 strain of virus, while serums from other species failed to prevent CPE.

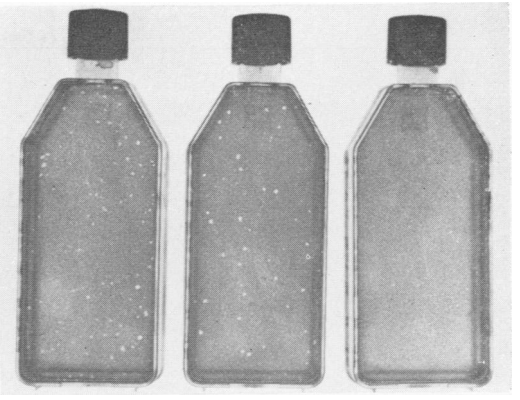

Fig. 7. Plaques produced in rabbit kidney cell cultures by $10^{-3.6}$, $10^{-3.9}$, $10^{-4.2}$ dilutions of H-40 virus, six days postinoculation. Methyl cellulose overlay, crystal violet staining.

Preliminary results, using the plaque-reduction neutralization test, also indicated that the leukocyte isolate virus is serologically distinct from ERV. Homologous immune serum reduced the number of plaques more than 90%, whereas heterologous immune serum had no effect.

DISCUSSION

In many generalized virus infections, leukocytes serve to carry viable virus within the body. The relationships between viruses and leukocytes have been extensively re-

TABLE I. Reciprocal Cross Neutralization Among Two Viral Isolants and ERV, Ky-D, A-183 Strains

	Antiserums to				
Virus	H-15	H-40	Ky-D	A-183	TC-C
H-15	+	+	Neg.	Neg.	Neg.
H-40	+	+	Neg.	Neg.	Neg.
Ky-D	Neg.	Neg.	+	+	Neg.
A-183	Neg.	Neg.	+	+	Neg.

+ = Neutralization.

TABLE II. Neutralization Titers of Serums of Different Animal Species Against Equine Herpesviruses

Animal species	No. of specimens	Neutralization			
		H-40		ERV — Ky-D	
		No. positive	Titer[a]	No. positive	Titer[a]
Horse	16	16	>1:4	1	1:10
Foal	1	0	Neg.	0	Neg.
Rabbit/A	3	3	1:870	0	Neg.
Rabbit/B	3	3	1:870	0	Neg.
Rabbit/C	3	0	Neg.	3	1:670
Sheep[b]	1	0	Neg.	0	Neg.
Calf[b]	1	0	Neg.	0	Neg.
Rabbit[b]	1	0	Neg.	0	Neg.
Horse[b]	1	1	>1:4	0	Neg.

[a]50% neutralization endpoint.
[b]Pooled serum from normal animals.
A = Anti-H-40; B = anti-H-15; C = Anti-ERV-Ky-D.

viewed (3). Inapparent, latent, or recurrent infection with herpesviruses is common in man and in animals (8). The ability of these agents to emerge repeatedly in the presence of antibody also has long been recognized. Virus apparently persists intracellularly where it is protected from humoral antibodies. The removal of antibody by repeated washings and the use of primary RK cell cultures enabled us to detect herpesvirus infection in the leukocytes of healthy horses, but oftentimes two or more blind passages were necessary before evidence of virus was observed.

Asymptomatic infection of rabbits with herpesvirus is known to occur (10). For this reason, it was essential to verify that isolants did not originate from the rabbit tissue culture as endogenous viral contaminants. The equine origin of the isolants could be supported by the following evidence: (a) pooled rabbit serum as well as 12 preimmunization rabbit serum samples did not neutralize virus isolants, (b) neutralizing antibody was present in the serums of virus carrier horses, (c) conjugated horse serum-globulin gave specific fluorescence, (d) uninoculated cultures of primary or subpassaged RK cells did not show spontaneous viral CPE during three

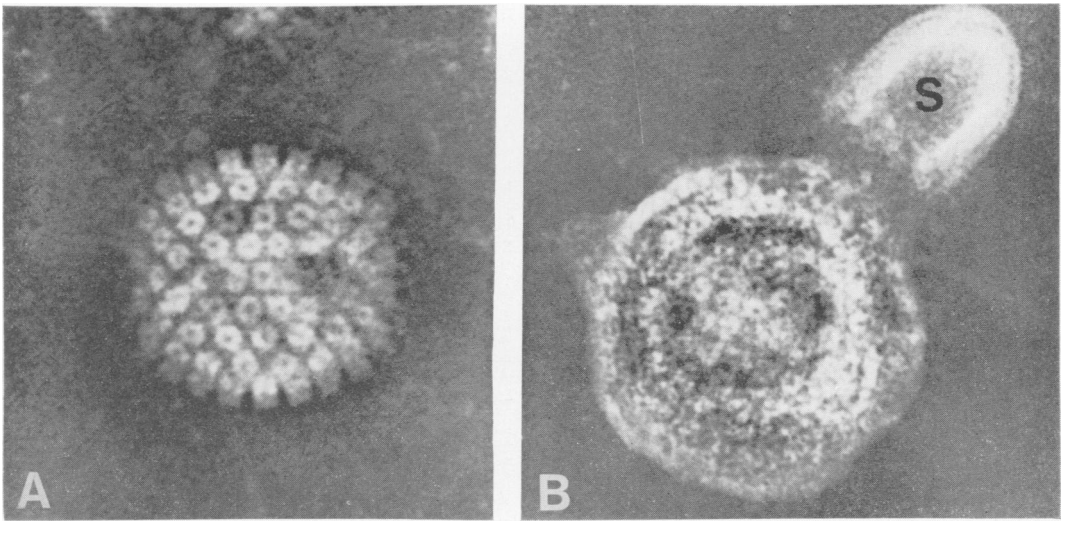

Fig. 8. Equine herpesvirus, H-40 strain, negatively stained with potassium phosphotungstate; X 330,000. A. Unenveloped capsid with typical herpesvirus capsomeres and geometry. B. Enveloped (mature) virion with membranous "stalk" (s). Note that some capsomeres are discernible within the enveloping membrane(s). Preparation, courtesy of A. E. Ritchie.

to four weeks' observation.

Of much interest was the high incidence of this agent in the Iowa horse population. The observation that a foal, separated from its mother and reared in isolation, did not develop antibody suggests that this virus is spread by contact. The finding that the serum from a mare inhibited replication of both ERV and leukocyte viral isolates suggests the possibility that a fresh virus isolant may not be homogeneous in composition. The importance of this agent as a pathogen is not known. This study has shown that the agent is not related, other than morphologically, to ERV and may exist merely as a latent passenger virus. The antigenic relationships of the 71 isolated virus strains to the other previously described types of equine herpesviruses (7, 12) must yet be established.

ACKNOWLEDGMENTS

The authors thank Dr. J. R. Gorham, USDA, ARS, ADPR Division, Washington State University, Pullman, Wash.; Dr. J. T. Bryans, Professor, Department of Veterinary Science, University of Kentucky, Lexington, Ky., for supplying virus strains; Dr. N. F. Cheville, Mr. A. E. Ritchie for providing the electron micrographs, Dr. M. Van Der Maaten for preparation of labeled globulin; Mr. R. M. Glazier for the illustrations, and Mr. C. S. Becvar for technical assistance.

REFERENCES

1. **CHERRY, W. B., M. GOLDMAN and T. R. CARSKI.** Fluorescent antibody techniques in the diagnosis of communicable diseases. Public Health Service Pub. No. 729, Washington, D.C.: U.S. Government Printing Office. 1960.
2. **DULBECCO, R. and M. VOGT.** Plaque formation and isolation of pure lines of poliomyelitis viruses. J. exp. Med. **99**: 167-182. 1954.
3. **GRESSER, I. and D. J. LANG.** Relationships between viruses and leukocytes. Progr. Med. Virol. Volume 8. pp. 62-130. Basel/New York: Karger. 1966.
4. **HENLE, G., W. HENLE and V. DIEHL.** Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc. Nat. Acad. Sci. U.S. **59**: 94-101. 1968.
5. **HSIUNG, G. D.** Diagnostic Virology. New Haven, Conn: Yale University Press. 1964.
6. **HSIUNG, G. D., H. R. FISCHMAN, C. K. Y. FONG and H. R. GREEN.** Characterization of a cytomegalo-like virus isolated from spontaneously degenerated equine kidney cell culture. Proc. Soc. exp. Biol. Med. **130**: 80-84. 1969.
7. **KARPAS, A.** Characterisation of a new herpes-like virus from foal kidney. Annls. Inst. Pasteur 110: 688-696. 1966.
8. **KIBRICK, S. and G. W. GOODING.** Pathogenesis of infection with herpes simplex virus with special reference to nervous tissue. In: NINDB Monograph No. 2, Slow, Latent, and Temperate Virus Infections, pp. 143-154. U.S. Public Health Service Publication No. 1378. Washington, D.C.: U.S. Government Printing Office. 1965.
9. **KONO, Y. and K. KOBAYASHI.** Cytopathogenic equine orphan (CEO) virus in horse kidney cell culture. I. Isolation and properties. Natn. Inst. Anim. Hlth Qt., Tokyo, Jap. **4**: 10-20. 1964.
10. **NESBURN, A. B.** Isolation and characterization of a herpes-like virus from New Zealand albino rabbit kidney cell cultures: a probable reisolation of virus III of Rivers. J. Virol. **3**: 59-69. 1969.
11. **PETZOLDT, K.** Die Virusausscheidung beim Stutenabort (Rhinopneumonitis). Arch. Exp. Veterinaermed. **21**: 115-119. 1967.
12. **PLUMMER, G. and A. P. WATERSON.** Equine herpes viruses. Virology **19**: 412-415. 1963.
13. **REED, L. J. and H. MUENCH.** A simple method of estimating 50 percent end points. Am. J. Hyg. **27**: 493-497. 1938.
14. **ZEE, YUAN CHUNG, S. H. MADIN and A. J. HACKETT.** Development of plaque assay for vesicular exanthema of swine virus (VESV) under methyl cellulose overlay. Arch. ges. Virusforsch. **21**: 53-60. 1967.