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Antigenic Relationships Among the Herpesviruses

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ANTIGENIC RELATIONSHIPS AMONG THE HERPESVIRUSES

by

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INTRODUCTION

Herpesviruses are widespread among members of the animal kingdom. More than 50 herpesviruses have been identified from over 30 different animal species, with at least five members of the group infecting man. Other animals which act as a natural host for a herpesvirus or herpesviruses include monkeys, cats, dogs, cattle, horses, pigs, rodents, rabbits, birds, reptiles, frogs, and fish. It seems probable that every species of animal harbors at least one herpesvirus.

All herpesviruses have several characteristics in common. They are ether sensitive, due to the presence of a lipid outer envelope, which is acquired from the nuclear membrane of their host cells (52). Their internal capsid is icosahedral in shape, and is composed of 162 hollow, polygonal capsomeres. The core of the herpesvirion is composed of a molecule of double-stranded DNA. Depending upon the particular herpesvirus, the DNA has a molecular weight ranging from about 3.2×10^7 to 10^9 daltons. Herpes simplex virus of man is composed of 69% protein, 22% lipid, 7% DNA and 2% carbohydrate (47).

Members of the herpesvirus group form inclusion bodies in the nucleus of their host cells. In addition, the so-called cytomegaloviruses can form cytoplasmic inclusion bodies.

Several clinical and pathological similarities exist among members of the herpes group. Many herpesviruses attack the central nervous system, especially in newborns. Clinical manifestations, especially of the central nervous system, are particularly striking with those herpesviruses which can infect an animal other than their natural host. Probably all of the herpesviruses have the ability to remain latent in their hosts, although the exact form in which the viruses exist is unknown. Some herpesviruses, particularly horse herpesvirus type 1 (or, equine abortion virus) and infectious bovine rhinotracheitis virus, invade the fetus and cause abortion (10,33). It seems that most herpesviruses grow better and cause more damage in the very young than in older hosts. Also, several herpesviruses have the ability to persist in the blood leukocytes of their host (6,17,18,25,26,38). Such an ability may be critical to the herpesvirus's survival because they may be protected from circulating antibodies.

The DNA genome of the herpesviruses is rather large in comparison to other DNA viruses, having enough genetic information to code for as many as 100 to 200 proteins (20,42). They are also rather unusual in that they display a wide range of G+C contents, from as low as 33% for the dog herpesvirus up to 72% for pseudorabies virus and infectious bovine rhinotracheitis virus (40). Due to their large amount of genetic information, the members of the group might be expected to be antigenically complex, both in structural and non-structural protein antigens. By polyacrylamide gel electrophoresis, Spear and Roizman (54) were able to separate at least 25 distinct protein fractions synthesized during the course of infection of HEp-2 cells with herpes simplex virus. Using a more stringent purification procedure, they were able to identify 24 viral proteins and glycoproteins by staining and by autoradiography in acrylamide gels (55). These viral proteins ranged in molecular weight from 25,000 to 275,000. The sum of the molecular weights of the proteins was 2,580,000, which represents approximately 47% of the genetic information of the virus. Using a similar technique, Abodeely et al. (1) were able to identify 20 proteins ranging in molecular weight from 13,000

to 115,000 in enveloped virions of horse herpesvirus type 1. De-enveloped virions, prepared by treatment with the non-ionic detergent Nonidet P-40 and sonication, contained at least 14 proteins. Olshevsky and Becker (35), also using the acrylamide gel electrophoresis, identified 9 structural proteins present in mature herpes simplex virus, three of which were located in the lipid envelope. Two proteins were found to be present in empty capsids, while a total of four proteins were contained in unenveloped virions. Robinson and Watson (41), using naked herpes simplex virus which had been highly purified, resolved at least 8 distinct polypeptide peaks by polyacrylamide electrophoresis. Also, at least 10 structural proteins of pseudorabies virus, of which at least four are glycoproteins present in the viral envelope, have been identified (21).

Using antisera prepared against rabbit kidney cells which had been infected with herpes simplex virus, Watson et al. (65) detected 12 virus-specific precipitin lines in immunodiffusion tests against extracts of BHK cells which had been similarly infected. In addition, 10 distinct protein antigens were detected by immunoelectrophoresis. Tokumaru (62) was able to distinguish 7 virus-

specific precipitin lines in immunodiffusion tests using extracts from cells infected with herpes simplex virus and human antisera.

The rate of synthesis of several enzymes has been found to increase after infection of cells with herpes simplex virus and pseudorabies virus. These enzymes include thymidine kinase (28,34), DNA polymerase (23), DNase (23), deoxycytidylate deaminase (9), deoxycytidine kinase (37), and thymidine monophosphate kinase (15). The thymidine kinases from cells infected with either herpes simplex virus or pseudorabies virus have been shown to be antigenically distinct from the thymidine kinase of normal cells (28,15). In addition, the thymidine kinase specified by the two viruses are antigenically unrelated (7), as are the thymidine kinases specified by herpes simplex type 1 virus and herpes simplex type 2 virus (61). The DNA polymerase induced upon infection of BHK 21 or HEp-2 cells with herpes simplex virus has likewise been shown to be immunologically different from cellular DNA polymerase (24). In view of the large genome possessed by the herpesviruses, it is likely that they code for a variety of enzymes which may eventually be shown to be antigenically distinct from cellular en-

zymes. It is also possible that several or all of the herpesviruses code for an enzyme or enzymes which are antigenically identical.

Roizman et al. (43) found that the antigenic products specified by herpes simplex virus in HEp-2 cells formed at least five immunofluorescent elements. They also found that each element is segretated in different areas of the cells. The five elements detected were small nuclear granules, large nuclear grandules, an amorphous nuclear mass, cytoplasmic granules, and diffuse cytoplasmic fluorescence. They were also able to show, by absorption experiments, that the nuclear granules were unrelated antigenically to the amorphous nuclear mass, and the cytoplasmic granules differed immunologically from the diffuse cytoplasmic fluorescence. Physical and chemical analysis of the nuclear and cytoplasmic granules indicated that they represented factories and aggregates of viral subunits at different stages of assembly. The nuclear and cytoplasmic amorphous masses could not, however, be characterized. Gerder and Vaczi (13), using similar techniques, obtained essentially the same results as Roizman et al. (43).

The cellular membrane of cells infected with herpes simplex virus acquires a new surface antigen, presumably coded for by the viral genome (19). It has been demonstrated that at least four new glycoproteins are incorporated into HEp-2 cell membranes after infection with herpes simplex virus (53). The same glycoproteins were also found in intact herpes simplex virions; it was, therefore, concluded that these glycoproteins were structural components of the virus and were acquired by the virions during envelopment.

In 1956 Lebrun (31), using the fluorescent antibody test, stated that the earliest viral antigen detectable in herpes simplex virus-infected cells was located in the nucleus. At later times antigens were seen in both the nucleus and cytoplasm, and finally in the cytoplasm only. Koller et al. (29) subsequently suggested that the development of varicella-zoster virus antigens followed the same course. These data were interpreted as indicating that the synthesis of herpes viral antigens takes place in the nucleus, and after viral development, whole virus moves into the cytoplasm. In 1956, however, Sydiskis and Roizman (57) presented evidence indicating that the bulk of proteins specified by herpes simplex virus were syn-

thesized on cytoplasmic polyribosomes. It was later shown, by radioactive labelling, that the viral-specific proteins synthesized in the cytoplasm migrate to the nucleus, where virus maturation then occurs (49). Similar results for herpes simplex virus had been reported earlier by Olshevsky et al. (36). Ross et al. (46) came to the same conclusion using the fluorescent antibody test. Most pseudorabies virus proteins, including the structural proteins, are similarly synthesized in the cytoplasm and migrate to the nucleus. (12,4).

There have been relatively few studies undertaken dealing with the antigenic relationships between the herpesviruses. The best known serological relationship between the members of the group exist among the herpesviruses of primates. Serological relatedness has been demonstrated between herpes simplex virus of man and B virus of rhesus monkeys (8,39,48,66), SA8 of vervet monkeys and B virus (32), herpes simplex virus and SA8 (56) and between the human and vervet monkey cytomegaloviruses (5).

The first studies to determine serological relationships between members of the herpesvirus group were undertaken by Sabin in 1934 (48,49). He determined that

an immunological relationship existed between herpes simplex virus, B virus and pseudorabies virus based on neutralization tests carried out in animals. The relationship between herpes simplex virus and B virus was later confirmed by Burnet (8) and by Plummer (39), although both claimed that only a one-way cross existed by neutralization tests, that being the neutralization of herpes simplex virus by antiserum to B virus. Watson et al. (66), however, later claimed, using kinetic neutralization tests, that significant reciprocal cross-neutralization existed between the two viruses by their respective antisera. In addition, antiserum to herpes simplex virus gave 3 precipitin lines in immunodiffusion tests with extracts from cells infected with B virus. All three lines showed reactions of identity with antigens prepared from cells infected with herpes simplex virus. Both Kaplan and Vatter (22), and Watson et al. (66), reported a lack of cross-neutralization between herpes simplex virus and pseudorabies virus. Plummer (39), however, although noting the lack of cross-neutralization, reported the two to be serologically related based on the complement-fixation test. Watson et al. (66) claimed that antiserum to herpes simplex virus gave a precipitin line in immuno-

diffusion tests with extracts of pseudorabies virus-infected cells. They also established that the precipitin line obtained with pseudorabies virus showed a reaction of identity with an antigen of both herpes simplex virus and B virus. They therefore concluded that there is a group antigen common to at least these three members of the herpesvirus group.

Plummer (39), in 1964, detected serological relationships between several members of the herpes group. Using antiserum to infectious bovine rhinotracheitis virus, complement-fixation was shown with pseudorabies virus, horse herpesvirus type 1, and horse herpesvirus type 2. With antiserum to herpes simplex virus, complement-fixation occurred with pseudorabies virus and horse herpesvirus type 1. Similar serological relationships were demonstrated between horse herpesvirus type 1 and pseudorabies virus, and between horse herpesvirus type 1 and horse herpesvirus type 2.

A relationship between herpes simplex virus and another human herpesvirus, varicella-zoster virus, has been indicated. The relationship is based on the boosting of complement-fixing antibodies to herpes simplex virus in varicella and zoster patients and vice versa (30,44,50).

Although such anamnestic boosting does not constitute undeniable proof of antigenic relatedness, Trilifajova et al. (63) have presented evidence that antigens prepared from cells infected with herpes simplex virus and from cells infected with varicella-zoster show a precipitin line of identity in immunodiffusion tests.

Aurelian (3) has claimed an immunological relationship between herpes simplex virus and canine herpesvirus. She noted that reciprocal cross-neutralization occurred between the two viruses using their respective antisera, although the homologous virus was neutralized to a much greater extent in each case. Using the fluorescent antibody test, Sharma et al. (51) has tentatively claimed a reciprocal cross-fluorescent staining between Marek's disease virus and pseudorabies virus. A relationship between antigens derived from Burkitt's lymphoma cells, which are known to contain EB virus antigens, and antigens derived from the Lucke' adenocarcinoma of frogs, thought to be caused by Lucke' herpesvirus, has also been claimed (11).

In 1964, Yoshino and Taniguchi (68) reported that rabbits immunized with herpes simplex virus develop complement requiring neutralizing antibodies. Such anti-

bodies developed early in infection, and were followed by non-complement requiring antibodies, although the addition of complement to the latter enhanced its neutralizing ability. Stevens et al. (56) reported that the neutralization of herpes simplex virus and SA8 by their respective antisera were significantly enhanced by the addition of complement, as was the reciprocal cross-neutralization between the two viruses. The most dramatic boosting occurred with early 7S and 19S antibodies, with lower, although significant, boosting occurring with late 7S antibody and whole serum. Late 19S antibody was boosted only in the homologous reaction. Andersen (2), using four strains of human cytomegalovirus, found that two of the strains (Ad 169 and C87) elicited early antibodies which were complement-dependent, while the other two strains (Davis & T27) elicited late antibodies which required complement to neutralize their homologous viruses. He concluded from his data and from the work of others that complement should be added in all herpesvirus cross-neutralization experiments utilizing hyperimmune antisera.

The studies presented herein have been undertaken in order to determine the antigenic relationships among

several herpesviruses. Four viruses were employed: herpes simplex virus type 1, squirrel monkey herpesvirus type 1, infectious bovine rhinotracheitis virus, and horse herpesvirus type 1. Two serological procedures were used, the neutralization test and the indirect fluorescent antibody test. Cross-neutralization tests were carried out by the plaque-reduction method. The effect of complement was also evaluated for its boosting ability in the cross-neutralization experiments. In addition, the localization and sequential development of homologous and cross-reacting antigens in cells infected with the various herpesviruses was studied by the indirect fluorescent-antibody method.

CHAPTER II

MATERIALS AND METHODS

Tissue Culture Media

Medium 199, containing amino acids, nucleic acids, glucose, vitamins and intermediary metabolites in a base of Earle's balanced salt solution, was purchased from Grand Island Biological Co., Grand Island, New York, and was used for the preparation of all tissue cultures. Supplements to the media included 0.3 mg/ml glutamine (Pfanstiehl Laboratories, Inc., Waukegan, Illinois), 1,250 units/ml of buffered potassium penecillin G (E.R. Squibb and Sons, Inc., New York, New York), 1.25 mg/ml of streptomycin sulfate (Chas. Pfizer and Co., Inc., New York, New York), 0.00625 mg/ml funqizone (E.R. Squibb and Sons, Inc., New York, New York), and 375 units/ml of polymixin (Chas. Pfizer and Co., Inc., New York, New York).

Initiator medium, which was used to prepare primary tissue cultures, contained 1.5% sodium bicarbonate (J.T. Baker Chemical Co., Phillipsburg, New Jersey), and 10% lamb serum (Grand Island Biological Co., Grand Island, New York) in addition to the above mentioned supplements.

Maintenance medium, used to maintain tissue cultures after a monolayer was established, contained 2.5% sodium bicarbonate and 5% lamb serum. Overlay medium for plaque titrations and neutralization tests included 3% methyl cellulose (1500 centipoise, Fisher Scientific Co., Fair Lawn, New Jersey) in maintenance medium.

Tissue Cultures

Primary monolayers of rabbit kidney cells were prepared, under sterile conditions, utilizing the kidneys which had been removed from three to four week old baby rabbits (Scientific Small Animals, Arlington Heights, Illinois). After having the surface membrane removed, the kidneys were finely minced with scissors and placed in a trypsinization flask to which 25 ml of 2.5% trypsin (DIFCO, Detroit, Michigan) was added. The suspension was stirred for approximately 30 minutes on a magnetic stirrer, or until the tissue was fully disrupted. The cells were then pelleted by centrifugation at 3,000 RPM for 10 minutes, washed with initiator media and repelleted, and added to 400 ml of initiator media. Cells were then dispended as desired, in 5 ml amounts in small plastic petri dishes (Falcon Plastics, Oxnard, California), in 15 ml amounts in larger petri dishes (Falcon Plastics,

Oxnard, California) which contained fluorescent antibody microscopy slides (Clay Adams, Parsippany, New Jersey), or in 40 ml amounts in 32 oz. glass prescription bottles (Owens-Illinois, Toledo, Ohio). Petri dishes were incubated at 37°C in an atmosphere of 5% CO_2, while bottles were capped tightly and placed in a 37°C normal atmosphere incubator. Medium was changed on growing monolayers after five days.

Viruses

The viruses used in the serological studies were herpes simplex virus type 1, strain Bramson, squirrel monkey herpesvirus type 1, Presbyterian St. Luke's strain, infectious bovine rhinotracheitis virus, strain L.A., and horse herpesvirus type 1 (equine abortion virus, equine rhinopneumonitis virus), Doll strain. In addition, one other strain each of herpes simplex virus type 1 (Watson strain), squirrel monkey herpesvirus type 1 (Baylor strain), infectious bovine rhinotracheitis virus (Colorado strain), and horse herpesvirus type 1 (strain EH 39), which had been passed five times each in either kitten kidney cells or mouse brains, were utilized in fluorescent antibody tests.

Virus stocks were grown either in plates or bottles. Monolayers were seeded with virus and virus growth was allowed until nearly 100% cytopathic effect had occurred. The remaining cells were then scraped off into the medium and the virus suspensions were ampuled and stored at -70°C.

Virus Assays

Virus stocks were titered by the plaque assay method. Serial 10-fold dilutions of the viral suspension were made in initiator medium containing 1% lamb serum. From each dilution, 0.2 ml was absorbed onto a tissue culture monolayer. The plates were shaken after 10 minutes, and after a total elapsed time of 15 minutes, the plates were overlayed with methyl cellulose medium. After 48 hours incubation at 37°C in a 5% CO_2 atmosphere, the methyl cellulose medium was poured off, and the monolayer was stained with a solution of 10% methylene blue in distilled water. Plaques were counted microscopically on an inverted microscope.

Preparation of Virus and Viral Antigens for Immunization

Two groups of virus preparations were employed for the immunization of rabbits. The first group of preparations consisted of virus stocks, which had been grown

in monolayers of rabbit kidney cells in medium supplemented with rabbit serum instead of lamb serum. The rabbit serum was obtained from a pre-immunization bleeding of each rabbit. Each virus was grown in the serum from the rabbit which was to ultimately receive that virus.

The second group of preparations consisted of pools of "early" and "late" viral antigens, harvested from cells at various times after infection. These preparations also contained complete, infectious viral particles. Primary monolayers of rabbit kidney cells, which had been grown in medium supplemented with rabbit pre-inoculation serum, were infected with virus. Following a two hour virus adsorption period, the plates were given 3 ml of maintenance medium minus serum. The culture fluid and the cell sheets were harvested together from a series of infected plates at 5, 10, 24, 36 and 48 hours after infection. Each harvest was sonically disrupted (Branson Sonifier, Branson Instruments, Inc., Stamford, Connecticut), and the cell debris was spun down for one hour at 3,000 RPM. The supernatants from each harvest were pooled, ampuled, frozen at -70^{o}C, and used as immunizing antigen.

Preparation of Antisera

Antisera were parpared in two separate groups of rabbits, against four viruses. Two rabbits in each group received the same virus or viral antigen preparation. Antiserum was produced against herpes simplex virus type 1, strain Bramson, squirrel monkey herpesvirus type 1, Presbyterian St. Luke's strain, infectious bovine rhinotracheitis virus, strain L.A., and horse herpesvirus type 1, Doll strain. Two different immunization procedures were followed. One group of rabbits received virus stock preparations, given intramuscularly and intraperitoneally six times at 25 day intervals, with the last inoculation being given in a 50:50 mixture with complete Freund's adjuvant (DIFCO Laboratories, Detroit, Michigan). The rabbits were bled ten days following the second, fourth and final inoculations.

The viral antigen preparations were given four times to the second group of rabbits. Three weekly inoculations were given intramuscularly in complete Freund's adjuvant. Ten days following the third inoculation, the rabbits were boosted with antigen intravenously and intraperitoneally without Freund's. All rabbits were bled out ten days following the final boost. The collected blood was

allowed to clot overnight at 4°C. The clot was then spun out, and the serum was collected, ampuled and stored at -70°C.

Control sera consisted of pre-inoculation serum collected from each rabbit, as well as antiserum prepared in rabbits against uninfected rabbit kidney cells. The cells were from the same monolayer preparations which had been used to grow virus stocks. The same immunization schedule as that used for viral antigens was employed.

Neutralization Tests

Neutralization tests were carried out by the standard doubling dilutions method. Serum samples were serially diluted two-fold, usually from 1/2 to 1/1024. 0.2 ml of each serum dilution was mixed with 0.2 ml of virus suspension (which had been previously titered by the plaque assay method) such that the final mixtures contained 100-200 plaque forming units (PFU)/0.4 ml. The virus-serum mixtures were then incubated in a 37°C water bath for 30 minutes, with shaking after 15 minutes. The number of residual PFU were determined by plating 0.2 ml of each mixture onto a tissue culture. The plates were overlayed with methyl cellulose medium, and after 48 hours incubation, the plaques were counted microscopically.

In neutralization tests utilizing complement, guinea pig serum (Grand Island Biological Co., Grand Island, New York) was diluted and added to the immune serum to give a final complement concentration of 2% (representing approximately 10 hemolytic units of complement). The tests were usually performed in triplicate, one test utilizing untreated antiserum, the second using the same antiserum which had been decomplemented by heating at 56°C for 1/2 hour, and the third utilizing heated antiserum to which was added guinea pig complement to a final concentration of 2%. Control tests were carried out with pre-inoculation sera, anti-normal rabbit kidney cell antisera, and complement alone in maintenance medium. In some of the control tests, the pre-inoculation sera were supplemented with complement to a final concentration of 2%.

Fluorescent Antibody Tests

Fluorescent antibody tests were carried out using the indirect method (67). Before being used in the test, all antisera and fluorescent antibody were absorbed three or more times with an equal packed volume of rabbit kidney cells each time. The fluorescent antibody tests were carried out on monolayers of rabbit kidney cells which had been grown on fluorescent antibody microscope slides.

and which had been infected with virus. Following 24 hours of virus multiplication, the infected slides were treated in cold (4°C) acetone for 30 minutes. They were subsequently washed in phosphate buffered saline (PBS), pH 7.0, three times at two minutes per wash, and air dried. Antiserum, either undiluted or diluted to the desired concentration in PBS, was added and the slides were incubated in a moist chanber at 37°C for 30 minutes. Following incubation, the slides were washed three times for ten minutes per wash in PBS, and again air dried. Fluorescein isothiocyanate conjugated sheep-anti-rabbit-gamma-globulin (Grand Island Biological Co., Grand Island, New York) diluted 1:10 in PBS, was added to the slides and they were again incubated at 37°C for 30 minutes in a moist chamber. They were subsequently washed three times at ten minutes per wash in PBS, and mounted in fluorescent antibody mounting fluid (DIFCO Laboratories, Detroit, Michigan) with glass cover slips. The slides were examined under a Leitz fluorescent microscope, and photographs were taken with Kodak Pan X film.

To follow the sequential development and localization of homologous and heterologous viral antigens, monolayer slide cultures were inoculated with virus, and fol-

lowing a two hour viral adsorption period, slides were removed and fixed in acetone after 3, 4, 5, 7, 12 and 26 hours of infection. The infected slides were stored frozen until they could be stained.

To determine the fluorescent antibody titers of the antisera, serum samples were diluted two-fold in PBS and each dilution was tested against a virus-infected monolayer.

Controls consisted of fluorescent antibody tests carried out with each antiserum on uninoculated monolayers of rabbit kidney cells, virus-infected cells treated with fluorescent antibody alone, fluorescent antibody tests carried out on virus-infected cells utilizing pre-inoculation sera and anti-rabbit kidney cell antisera, and tests on cells infected with vaccinia virus, a member of the poxvirus group. In addition, the fluorescent antibody titrations were repeated, using squirrel monkey herpesvirus antiserum, with each virus which had been passed several times in either mouse brains or cat kidney tissue cultures, and also with a different strain of each of the four viruses which had also passed several times. This procedure was carried out in order to insure that the

four viruses being tested did not each have a common contaminant which was responsible for the cross-fluorescence that was observed.

CHAPTER III

RESULTS - NEUTRALIZATION TESTS

The neutralization test was the first serological procedure chosen to determine if antigenic relationships exist between several members of the herpesvirus group. Cross-neutralization tests were performed between herpes simplex virus type 1 (HSV), squirrel monkey herpesvirus type 1 (SMHV), infectious bovine rhinotracheitis virus (IBR), and equine herpesvirus type 1 (EHV), using antisera prepared in rabbits against HSV, SMHV, IBR virus and EHV. Neutralization tests were carried out by the doubling-dilutions method (refer to Materials and Methods section for procedure).

Cross-Neutralization Tests Utilizing Primary and Hyperimmune Antisera

Table 2 represents a comparison of the cross-neutralization titers obtained with primary (first bleeding) and hyperimmune (final bleeding) rabbit anti herpesvirus antisera. These antisera were prepared by injection of rabbits with suspensions of virus stock which had been grown in monolayers of rabbit kidney cells. Neutralization tests which utilized primary antisera revealed weak

but definite reciprocal cross-neutralization between HSV and SMHV. Slight neutralization of both herpes simplex virus and squirrel monkey herpesvirus by primary antiserum to infectious bovine rhinotracheitis virus and equine herpesvirus type 1 also occurred. There was no cross-neutralization between IBR virus and EHV by their respective primary antisera. Hyperimmune anti-herpesvirus antisera were found to have titers boosted from between two-fold to eight-fold over that of their respective primary antisera. The crossings obtained with hyperimmune antisera were the same as those obtained with primary antisera, i.e., definite reciprocal cross-neutralization between herpes simplex virus and squirrel monkey herpesvirus, with both IBR virus and EHV antisera slightly neutralizing HSV and SMHV. In addition, hyperimmune SMHV antiserum slightly neutralized IBR virus, making the weak crossing between these two viruses a reciprocal one. Controls, consisting of neutralization tests carried out with pre-inoculation serum corresponding to each antiserum, revealed little or no neutralization of any of the four herpesviruses (Table 1).

TABLE 1. PRE-INOCULATION SERA AND ANTI-RABBIT KIDNEY CELL SERA vs. HERPES SIMPLEX VIRUS, SQUIRREL MONKEY HERPESVIRUS, INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS, AND EQUINE HERPESVIRUS TYPE 1.

SERA	HERPES SIMPLEX VIRUS 50% ENDPOINT	SQUIRREL MONKEY HERPESVIRUS 50% ENDPOINT	INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS 50% ENDPOINT	EQUINE HERPESVIRUS TYPE 1 50% ENDPOINT
Pre-Inoc #301 Untreated	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #302 Untreated	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #303 Untreated	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #304 Heated, No C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #305 Untreated	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #306 Heated, No C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #307 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #309 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #310 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #311 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #313 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Pre-Inoc #315 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Anti-RK Cell #317 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Anti-RK Cell #318 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Anti-RK Cell #319 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4
Anti-RK Cell #320 Heated, +2% C'	< 1/4	< 1/4	< 1/4	< 1/4

TABLE 2. TITERS OF CROSS-NEUTRALIZATION TESTS OBTAINED WITH PRIMARY AND HYPERIMMUNE ANTI-HERPES VIRUS ANTISERA.

ANTISERA	HERPES SIMPLEX VIRUS		SQUIRREL MONKEY HERPESVIRUS		INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS		EQUINE HERPESVIRUS TYPE 1	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
HSV ANTISERUM #301 PRIMARY	▷1/512[a]	1/128[b]	1/8	◁ 1/4	◁ 1/4	◁ 1/4	◁ 1/4	◁ 1/4
SMHV ANTISERUM #303 PRIMARY	1/4	◁ 1/4	▷1/1024	1/256	◁ 1/4	◁ 1/4	◁ 1/4	◁ 1/4
IBR ANTISERUM #305 PRIMARY	1/8	◁ 1/4	1/4	◁ 1/4	1/512	1/64	◁ 1/4	◁ 1/4
EHV ANTISERUM #307 PRIMARY	1/4	◁ 1/4	1/16	◁ 1/4	◁ 1/4	◁ 1/4	1/512	1/16
HSV ANTISERUM #301 HYPERIMMUNE	▷1/1024	1/256	1/64	◁ 1/4	◁ 1/4	◁ 1/4	◁ 1/4	◁ 1/4
SMHV ANTISERUM #303 HYPERIMMUNE	1/64	1/16	1/2048	1/512	1/8	◁ 1/4	◁ 1/4	◁ 1/4
IBR ANTISERUM #305 HYPERIMMUNE	1/4	◁ 1/4	1/8	◁ 1/4	1/256	1/64	◁ 1/4	◁ 1/4
EHV ANTISERUM #307 HYPERIMMUNE	1/8	◁ 1/4	1/16	◁ 1/4	◁ 1/4	◁ 1/4	▷ 1/1024	1/128

a. Highest serum dilution resulting in a 50% plaque reduction
b. Highest serum dilution resulting in a 100% plaque reduction

The Effect of Complement

Reports by several investigators (2,14,16,56,58,59,60,64,68) have indicated that the addition of guinea pig complement to both early and hyperimmune anti-herpesvirus antisera significantly enhances their neutralizing ability. The neutralization tests were therefore repeated with the addition of guinea pig complement to hyperimmune antisera to a final concentration of 2% in order to see if the heterologous neutralizing titers could be boosted, of if cross-reactions not previously detected would be revealed.

Table 3 contains the results of neutralization tests carried out with two hyperimmune anti-HSV antisera. Tests with both sera were run in triplicate, in order to evaluate the enhancing effect of complement, if any, on the homologous and heterologous neutralizing titers. Serum samples were thus tested either untreated, heated at 56°C for 30 minutes to destroy their natural complement, or heated followed by the addition of guinea pig complement to a final concentration of 2%.

Antisera samples which had been heated to destroy complement showed a slight drop in neutralizing titer from that of normal, untreated antisera. The addition of

TABLE 3. HYPERIMMUNE HERPES SIMPLEX VIRUS ANTISERA (ANTI-VIRUS STOCK), WITH AND WITHOUT COMPLEMENT, vs. HERPES SIMPLEX VIRUS, SQUIRREL MONKEY HERPESVIRUS, INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS AND EQUINE HERPESVIRUS TYPE 1.

HYPERIMMUNE ANTISERUM	HERPES SIMPLEX VIRUS		SQUIRREL MONKEY HERPESVIRUS		INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS		EQUINE HERPESVIRUS TYPE 1	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
Anti-HSV #301 Untreated	> 1/1024	1/256	1/64	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4
Anti-HSV #301 Heated, No C'	1/2048	1/256	1/16	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4
Anti-HSV #301 Heated, +2% C'	NT	NT	1/64	< 1/4	1/4	< 1/4	< 1/4	< 1/4
Anti-HSV #302 Untreated	1/2048	1/256	1/32	< 1/4	1/32	< 1/4	< 1/4	< 1/4
Anti-HSV #302 Heated, No C'	1/1024	1/256	1/32	< 1/4	1/32	1/4	< 1/4	< 1/4
Anti-HSV #302 Heated, +2% C'	> 1/4096	1/512	1/64	1/8	1/8	< 1/4	< 1/4	< 1/4
2% C' in Maintenance Medium	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4

NT = Not Tested

complement to decomplemented antisera resulted in either a restoration to normal titer or a slight boost in neutralizing power. Significant cross-neutralization of SMHV by both anti-HSV antisera was again evident in these tests. The highest neutralization titers occurred with antiserum samples which had been supplemented with complement. Significant neutralization of IBR virus was also seen with one of the anti-HSV antisera, although the highest neutralizing titer unexplainably occurred with serum which had been heat-inactivated. EHV remained unaffected by HSV antiserum, even when the serum was supplemented with complement. Controls consisted of each virus mixed with a dilution of 2% complement in maintenance medium. Complement alone had no neutralizing effect on the herpesviruses.

Similar neutralization tests which employed anti-SMHV antisera and anti-IBR virus antisera yielded comparable results, i.e., the addition of complement had a boosting effect on these antisera (Tables 4 and 5). Hyperimmune anti-SMHV antiserum again showed strong neutralization of HSV and weak neutralization of IBR virus, although a second antiserum preparation (#304) failed to neutralize either HSV or IBR virus (Table 4). EHV was

TABLE 4. HYPERIMMUNE SQUIRREL MONKEY HERPESVIRUS ANTISERA (ANTI-VIRUS STOCK), WITH AND WITHOUT COMPLEMENT, vs. SQUIRREL MONKEY HERPESVIRUS, HERPES SIMPLEX VIRUS, INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS AND EQUINE HERPESVIRUS TYPE 1.

HYPERIMMUNE ANTISERUM	SQUIRREL MONKEY HERPESVIRUS		HERPES SIMPLEX VIRUS		INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS		EQUINE HERPESVIRUS TYPE 1	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
Anti-Sq. Mo. #303 Untreated	1/2048	1/512	1/64	1/16	1/8	< 1/4	< 1/4	< 1/4
Anti-Sq. Mo. #303 Heated, No C'	1/1024	1/256	1/8	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4
Anti-Sq. Mo. #303 Heated, +2% C'	> 1/4096	> 1/512	1/64	1/4	NT	NT	NT	NT
Anti-Sq. Mo. #304 Untreated	> 1/2048	1/256	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4
Anti-Sq. Mo. #304 Heated, No C'	1/2048	1/128	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4
2% C' in Maintenance Medium	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4

NT = Not Tested

TABLE 5. HYPERIMMUNE INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS ANTISERUM (ANTI-VIRUS STOCK), WITH AND WITHOUT COMPLEMENT vs. INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS, HERPES SIMPLEX VIRUS, SQUIRREL MONKEY HERPESVIRUS, AND EQUINE HERPESVIRUS TYPE 1.

HYPERIMMUNE ANTISERA	INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS		HERPES SIMPLEX VIRUS		SQUIRREL MONKEY HERPESVIRUS		EQUINE HERPESVIRUS TYPE 1	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
IBR Antiserum #305 Untreated	1/256	1/64	1/4	< 1/4	1/8	< 1/4	< 1/4	< 1/4
IBR Antiserum #305 Heated, No C'	1/1024	1/32	1/8	< 1/4	1/8	< 1/4	< 1/4	< 1/4
IBR Antiserum #305 Heated, +2% C'	1/256	1/8	1/16	< 1/4	1/16	< 1/4	< 1/4	< 1/4
IBR Antiserum #306 Heated, No C'	1/1024	1/128	1/4	< 1/4	1/4	< 1/4	< 1/4	< 1/4
2% C' in Maintenance Medium	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4

not neutralized by either of the SMHV antisera preparations. Hyperimmune anti-IBR virus antiserum showed weak cross-neutralization of HSV and SMHV, with the highest neutralization titers being attained with serum supplemented with complement (Table 5).

Hyperimmune equine herpesvirus type 1 antiserum was tested only with the addition of complement to a final concentration of 2%. Both HSV and SMHV were neutralized to some extent (Table 6). IBR virus was again unaffected by EHV antiserum.

Table 7 better summarizes the effect of complement in boosting the neutralizing power of herpesvirus antisera. Both herpes simplex virus and squirrel monkey herpesvirus antisera, when brought to a final concentration of 2% complement, showed from between a two-fold to eight-fold increase in neutralization titer in both the homologous and heterologous reactions. It must be stressed, however, that the enhancing effect of complement was not a uniform effect, i.e., not all sera were boosted equally, nor was the boosting of a single serum equal against each of the viruses. It can be concluded, however, that the addition of complement is of some value in performing cross-neutralization tests with herpesviruses.

TABLE 6. HYPERIMMUNE EQUINE HERPESVIRUS TYPE 1 (ANTI-VIRUS STOCK), WITH COMPLEMENT, vs. EQUINE HERPESVIRUS TYPE 1, HERPES SIMPLEX VIRUS, SQUIRREL MONKEY HERPESVIRUS, AND INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS.

HYPERIMMUNE ANTISERA	EQUINE HERPESVIRUS TYPE 1		HERPES SIMPLEX VIRUS		SQUIRREL MONKEY HERPESVIRUS		INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
EHV Antiserum #307 Heated, +2% C'	> 1/1024	1/64	1/8	< 1/4	1/16	< 1/4	< 1/4	< 1/4
2% C' in Maintenance Medium	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4

TABLE 7. THE EFFECT OF COMPLEMENT ON HOMOLOGOUS AND HETEROLOGOUS NEUTRALIZING ANTIBODY TITERS.

HYPERIMMUNE ANTISERUM	HSV				SMHV			
	-C'		+C'		-C'		+C'	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
HSV	1/2048	1/256	1/4096	1/512	1/32	< 1/4	1/64	1/8
SMHV	1/8	< 1/4	1/64	1/4	1/1024	1/256	> 1/4096	> 1/512

Neutralization Tests Utilizing Antiserum Produced Against Viral Antigen Preparations

Cross-neutralization tests between the four herpesviruses were again repeated with antisera which had been prepared in rabbits against viral antigen preparations instead of virus stocks. The viral antigens were prepared by sonic disruption of virus-infected tissue cultures. All antisera were heat-inactivated and supplemented with complement to a final concentration of 2% before use. The results are presented in Table 8. Although the homologous neutralization titers obtained with viral-antigen antisera were generally higher than those obtained with the corresponding virus stock antisera, the cross neutralization titers were slightly lower. However, the same pattern of cross-neutralization was seen. The strongest crossing occurred reciprocally between HSV and SMHV by their respective antisera, with weaker reciprocal crossing between SMHV and IBR virus. Weak neutralization of IBR virus by anti-HSV antiserum and of SMHV by EHV antiserum was also seen again. No cross neutralization occurred between infectious bovine rhinotracheitis virus and equine herpesvirus type 1. Controls consisted of tests using pre-inoculation sera corresponding to each

TABLE 8. HYPERIMMUNE VIRAL-ANTIGEN ANTISERA, WITH COMPLEMENT, vs. HERPES SIMPLEX VIRUS, SQUIRREL MONKEY HERPESVIRUS, INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS, AND EQUINE HERPESVIRUS TYPE 1.

HYPERIMMUNE ANTISERA	HERPES SIMPLEX VIRUS		SQUIRREL MONKEY HERPESVIRUS		INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS		EQUINE HERPESVIRUS TYPE 1	
	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT	50% ENDPOINT	100% ENDPOINT
HSV Antiserum #309 Heated, +2% C'	> 1/2048	1/512	1/16	< 1/4	1/8	< 1/4	< 1/4	< 1/4
HSV Antiserum #310 Heated, +2% C'	> 1/2048	1/512	1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4
EHV Antiserum #311 Heated, +2% C'	< 1/4	< 1/4	1/4	< 1/4	< 1/4	< 1/4	> 1/2048	1/256
SMHV Antiserum #313 Heated, +2% C'	1/64	< 1/4	> 1/2048	1/1024	1/8	< 1/4	< 1/4	< 1/4
IBR Antiserum #315 Heated, +2% C'	< 1/4	< 1/4	1/4	< 1/4	> 1/0248	> 1/2048	< 1/4	< 1/4
2% C' in Maintenance Medium	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4	< 1/4

antiserum (Table 1). Pre-inoculation sera were also supplemented with complement to a final concentration of 2%. Little or no neutralisation of the four herpesviruses was seen. In addition, antisera were produced in rabbits to normal rabbit kidney cells. Cells from the same monolayer cultures which had been used for the growth of the four viruses for immunization were used for the control rabbits. The serum collected from these control rabbits had no neutralising effect on the four herpesviruses (Table 1).

CHAPTER IV

RESULTS - FLUORESCENT ANTIBODY TESTS

Cross-fluorescent antibody tests were performed utilizing four herpesviruses -- HSV, SMHV, IBR virus and EHV -- and antisera to HSV, SMHV, IBR virus and EHV. The indirect test was used on virus infected monolayers of rabbit kidney cells. Before use, both the anti-herpesvirus antisera and the fluorescent antiserum had to be repeatedly absorbed with rabbit kidney cells in order to remove non-specific staining components. The effectiveness of the absorptions seemed to depend upon the trypsin preparation which was used to disrupt the kidneys into single cells, since different trypsin preparations left cells with different capabilities of absorbing out non-specific staining components. Antisera and fluorescent antibody were not used until they completely lacked the ability to non-specifically stain uninoculated monolayers of rabbit kidney cells.

Cross-Fluorescent Staining Between HSV, SMHV, IBR Virus and EHV

The initial tests were carried out with undiluted anti-viral antisera. The same antisera which had been

used in the neutralization tests were utilized in the fluorescent antibody tests. The results are presented in Table 9. As can be seen, all four viruses showed serological relatedness by this test, although to different degrees. The strongest relationship existed between HSV and SMHV, as had been previously found by the neutralization test (Plate 1). IBR virus and EHV, the two viruses which were unrelated by the neutralization test, showed the weakest cross fluorescence (Plates 2 and 3). Cross-fluorescence of intermediate intensity occurred reciprocally between HSV and IBR virus, HSV and EHV, SMHV and IBR virus, and SMHV and EHV. All controls showed either very faint or a complete absence of fluorescent staining (Tables 9 and 10, Plates 4, 5, and 6).

The strength of the fluorescent-antibody cross-reactions was further evaluated by performing endpoint titrations with each antiserum against the four herpesviruses. The results of these titrations are presented in Table 11. The highest cross-fluorescent endpoint titers occurred reciprocally between HSV and SMHV, and the weakest between IBR virus and EHV. Although the endpoint titers were not high, they did confirm the observations made with the neutralization tests and led to the conclu-

TABLE 9. CROSS-REACTIONS BETWEEN THE HERPESVIRUSES BY THE FLUORESCENT ANTIBODY TEST.

Undiluted Antiserum	STAINING INTENSITY VS: HSV	Sq.Mo.	IBR	ERPV	CONTROLS Vaccinia	Normal Cells
Anti-HSV #302	5+[a]	4+	2-3+	2+	0	0
Anti-HSV #309	5+	4+	2+	1-2+	0	0
Anti-SMHV #303	4+	5+	1+	1+	0	0
Anti-SMHV #313	4+	5+	2+	2+	0	0
Anti-IBR #305	3+	3+	5+	1-2+	0	0
Anti-IBR #315	2+	3+	5+	1+	0	0
Anti-EHV #307	2+	2+	1-2+	5+	0	0
Anti-EHV #311	3+	2+	1+	5+	0	0

[a] Staining intensity rated in degrees from 0 to 5+, with 5+ being maximum intensity.

TABLE 10. FLUORESCENT ANTIBODY TESTS CARRIED OUT WITH PRE-INOCULATION SERA, ANTI-RABBIT KIDNEY CELL SERA AND FLUORESCENT ANTISERUM ALONE.

	STAINING INTENSITY VS:				CONTROLS	
Serum	HSV	Sq.Mo.	IBR	EHV	Vaccinia	Normal Cells
Pre-Inoc. #302	0	0	0	0	0	0
Pre-Inoc. #309	0	0	0	0	0	0
Pre-Inoc. #303	0	0	0	0	0	0
Pre-Inoc. #313	0	0	0	0	0	0
Pre-Inoc. #305	0	0	0	0	0	0
Pre-Inoc. #315	0	0	0	0	0	0
Pre-Inoc. #307	0	0	0	0	0	0
Pre-Inoc. #311	0	0	0	0	0	0
Anti-RK Cell #317	0	0	0	0	0	0
Anti-RK Cell #318	0	0	0	0	0	0
Anti-RK Cell #319	0	0	0	0	0	0
Anti-RK Cell #320	0	0	0	0	0	0
Fluorescent Antiserum Alone	0	0	0	0	0	0

TABLE 11. FLUORESCENT ANTIBODY TITERS.

Antiserum	HSV	Sq.Mo.	IBR	ERPV
Anti-HSV #309	1/128[a]	1/32	1/8	1/4
Anti-SMHV #303	1/32	1/256	1/2	1/2
Anti-SMHV #313	1/32	1/32	1/8	1/8
Anti-IBR #305	1/8	1/2	1/32	1/2
Anti-IBR #315	1/4	1/8	1/64	1/4
Anti-ERPV #307	1/4	1/8	1/2	1/32
Anti-ERPV #311	1/4	1/8	1/2	1/128

[a] Titers represent highest serum dilution resulting in a 1+ fluorescence.

sion that all four viruses are antigenically related. Controls were again negative. In addition, positive controls, carried out with a different strain of each virus and with viruses passed several times in mouse brains or cat kidney monolayers, gave identical or very similar endpoint titers with anti-SMHV antiserum (Table 12).

Intracellular Development and Localization of Cross-Reacting Antigens

Following the results obtained with the initial fluorescent-antibody tests, an attempt was made to gain some insight as to the "type" of viral antigens which were cross-reacting, i.e., "early" or "late" antigens, cytoplasmic or nuclear antigens, etc. Therefore, the development and localization of fluorescent cross-reactions were followed in a timed-sequence study of infected monolayers. Following a two-hour absorption period, infected monolayers were harvested and stained at various time intervals (3, 4, 5, 7, 12 and 26 hours after infection). The results of these tests are presented in Table 13 and in Plates 7 through 20.

Utilizing anti-HSV antiserum, weak cytoplasmic staining of cells infected with HSV was seen as early as three

TABLE 12. FLUORESCENT ANTIBODY TITERS AGAINST A DIFFERENT STRAIN OF EACH VIRUS AND AGAINST VIRUSES PASSED SEVERAL TIMES IN EITHER MOUSE BRAINS OR CAT KIDNEY TISSUE CULTURES.

VIRUS	TITER OF ANTI-SMHV #303
HSV, Watson, m4[a]	1/32
HSV, Watson, c5[b]	1/32
HSV, Bramson, c5	1/32
SMHV, Baylor, m4	1/128
SMHV, Baylor, c5	1/128
SMHV, Pb. St. Luke's, c5	1/256
IBR, Colorado, c3	1/2
IBR, L.A., c5	---[c]
EHV, EH 39, c4	1/2
EHV, Doll, m7	1/2

[a] m4 = mouse brain, pass 4.

[b] c5 = cat kidney tissue culture, pass 5.

[c] An intensity of 1+ was recorded with undiluted antiserum.

TABLE 13. FLUORESCENT ANTIBODY TESTS: DEVELOPMENT OF CROSS-REACTING ANTIGENS AT VARIOUS TIMES AFTER INFECTION.

Antiserum		HSV		SQ.MO.		IBR		ERPV	
	Hours Post Inoculation	Intensity of Staining	Location of Staining*	Intensity of Staining	Location of Staining	Intensity of Staining	Location of Staining	Intensity of Staining	Location of Staining
	3	±	C	±	C	0		±	C
	4	1+	C	1+	C&N	±	C	1+	C&N
Anti-HSV	5	2+	C&N	1-2+	C&N	1+	C&N	1+	C&N
#309	7	3+	C&N	2+	C&N	1-2+	C&N	1+	C&N
	12	5+	C&N	3+	C&N	2+	C&N	1+	C&N
	26	5+	C&N	3-4+	C&N	2+	C&N	1+	C&N
	3	N		1+	C	0		1+	C
	4	O		1-2+	C	0		1+	C
Anti-SMHV	5	T D		2+	C&N	0		2+	C
#303	7	O		3+	C&N	1+	C	2+	C&N
	12	N		4+	C&N	2+	C	2+	C&N
	26	E		5+	C&N	2+	C&N	2+	C&N
	3	0		0		1+	C	0	
	4	0		0		1-2+	C&N	0	
Anti-IBR	5	0		1+	C	2+	C&N	1+	C
#305	7	0		1+	C	2+	C&N	1+	C
	12	0		1+	C&N	2+	C&N	1+	C
	26	2+	C&N	2-3+	C&N	4+	C&N	2+	C
	3	0		0		0		1+	C
	4	0		0		0		1+	C&N
Anti-ERPV	5	0		±	C	0		2+	C&N
#311	7	0		1+	C	0		2+	C&N
	12	0		1+	C	0		2+	C&N
	26	1-2+	C&N	3+	C&N	2+	C	5+	C&N

* C = Cytoplasm
N = Nucleus

hours after infection. The intensity of staining increased each hour after infection until it reached a maximum intensity (5+) at 12 hours. Fluorescent staining remained isolated in the cytoplasm as a diffuse mass at four hours post-inoculation (Plates 7A and 7B). At five hours, fluorescence appeared in the nucleus as small granules and strands (Plate 8). The cytoplasm remained diffusely fluorescent throughout the twenty-six hour period, while the nucleus did not become diffuse until seven hours post-inoculation. At twelve hours after infection, the nuclear membrane was stained intensely (Plate 9).

Cells infected with squirrel monkey herpesvirus were also weakly stained as early as three hours after infection by anti-HSV antiserum, and also in the cytoplasm exclusively. However, at four hours post-inoculation, small granules and strands of fluorescent material appeared in the nucleus (Plate 10). These granules and strands became more evident at seven hours (Plate 11). The diffuse cytoplasmic staining increased in intensity at 5, 7, 12 and 26 hours post-inoculation, reaching an intensity of 3 to 4+ (Plate 12), while the nucleus became increasingly granular or "sandy" in appearance and stained less intense than the cytoplasm.

Appreciable staining by HSV antiserum of cells infected with IBR virus did not occur until five hours post-inoculation. The fluorescence appeared weak and diffuse in the cytoplasm with even fainter granules in the nucleus. The most intense staining was of the nuclear membrane. The cytoplasmic fluorescence increased only slightly in intensity, reaching a value of 2+ at twenty-six hours after infection. The nucleus remained weaker than the cytoplasm and became diffuse at twelve hours post-inoculation.

Very faint cytoplasmic staining occurred three hours post-inoculation in cells infected with EHV and treated with anti-HSV antiserum. The diffuse cytoplasmic staining remained very weak, reaching a value of only 1+ at twenty-six hours post-infection. Very weakly stained granules and strands appeared in the nucleus at four hours post-inoculation, and the nuclear staining remained weak, becoming "sandy" in appearance at twelve hours after infection and diffuse at twenty-six hours (Plate 13).

The staining of cells infected with squirrel monkey herpesvirus by homologous SMHV antiserum followed the same course as that of the staining of HSV-infected cells by HSV antiserum. Weak diffuse cytoplasmic staining occurred

at three hours after infection, and increased in intensity up to a maximum (5+) at twenty-six hours post-inoculation. Nuclear staining, consisting of finely dispersed granules and an intensely staining nuclear membrane, appeared at five hours after infection. The nucleus became diffuse and strongly fluorescent at seven hours post-infection and remained as such thereafter.

Cytoplasmic fluorescence in cells infected with IBR virus and treated with SMHV antiserum did not appear until seven hours post-inoculation. Staining intensity increased only slightly (to 2+) up to twenty-six hours after infection. Nuclear staining did not appear until twenty-six hours and consisted of granules.

Cells infected with EHV were stained three hours post-infection with anti-SMHV antiserum. The staining was weak and diffuse in the cytoplasm. The intensity increased slightly at five hours and remained constant thereafter. Nuclear staining appeared at seven hours and was diffuse. The staining pattern did not change thereafter.

Rabbit kidney cells infected with IBR virus and treated with IBR virus antiserum presented a staining pattern similar to the two homologous systems previously

discussed. Diffuse cytoplasmic fluorescence appeared three hours after infection and was weak. Granules and strands of fluorescent material appeared in the nucleus at four hours post-infection and became diffuse by five hours. The intensity of staining of both the nucleus and cytoplasm increased thereafter, reaching a value of 4+ at twenty-six hours.

HSV-infected cells remained unstained by IBR virus antiserum until 26 hours post-infection. At that time fluorescence was located in both the nucleus and cytoplasm and was diffuse but weak (Plate 14).

Fluorescence first appeared at five hours after infection, in the cytoplasm, in cells infected with SMHV and treated with IBR virus antiserum. The staining was weak and diffuse (Plate 15). Weak diffuse nuclear staining appeared at twelve hours and at twenty-six hours, both the cytoplasmic and nuclear staining were more intense (2 to 3+, Plate 16).

Cells infected with EHV were stained only in the cytoplasm with anti-IBR virus antiserum. Fluorescence first appeared at five hours post-inoculation and was weak (1+). Fluorescent intensity did not increase until

twenty-six hours post-infection, and reached a value of only 2+ (Plate 17).

Homologous staining of EHV-infected cells with EHV antiserum appeared at one hour post-infection and was weak (1+) and diffuse in the cytoplasm. Nuclear granules appeared at four hours and the nucleus became diffuse at seven hours. Staining intensity increased only slightly up to twelve hours after infection, but became very strong (5+) at twenty-six hours.

Cells infected with herpes simplex virus were stained only after twenty-six hours of infection by EHV antiserum. The staining was weak and diffuse in both the nucleus and cytoplasm (Plate 18). SMHV-infected cells were stained five hours after infection although very weakly. The cytoplasmic staining remained weak up to twelve hours post-inoculation, but increased in intensity at twenty-six hours when there was also diffuse nuclear staining (Plate 19).

Cells infected with IBR virus only showed fluorescence with EHV antiserum after twenty-six hours of infection. Staining was only in the cytoplasm (Plate 20).

Controls for all fluorescent antibody tests in the timed-sequence study were the same as those previously mentioned. Tests were not read and recorded unless all of the controls were negative.

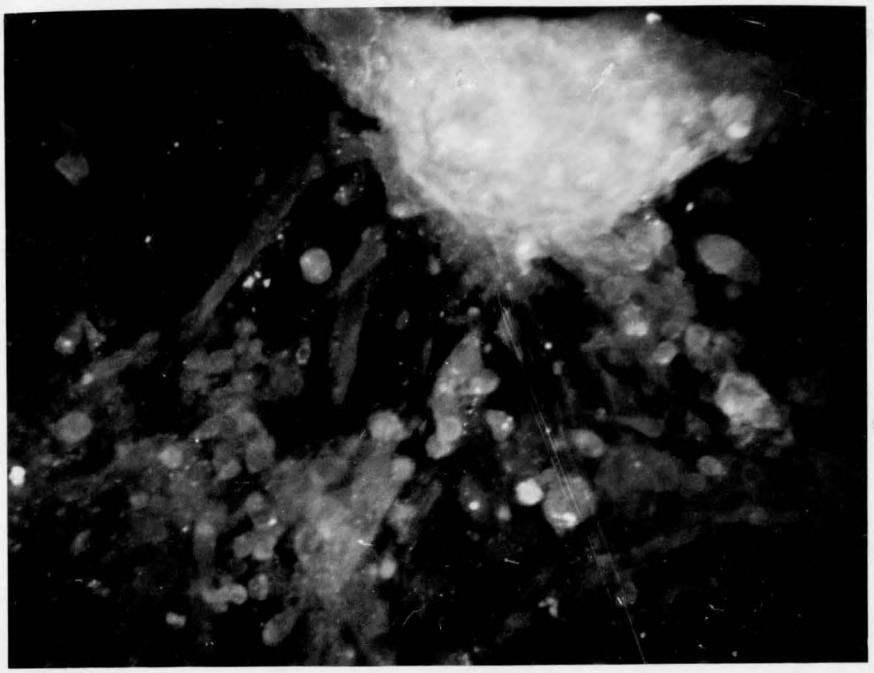

PLATE 1. Rabbit kidney cells infected with SMHV vs. HSV antiserum. x450.

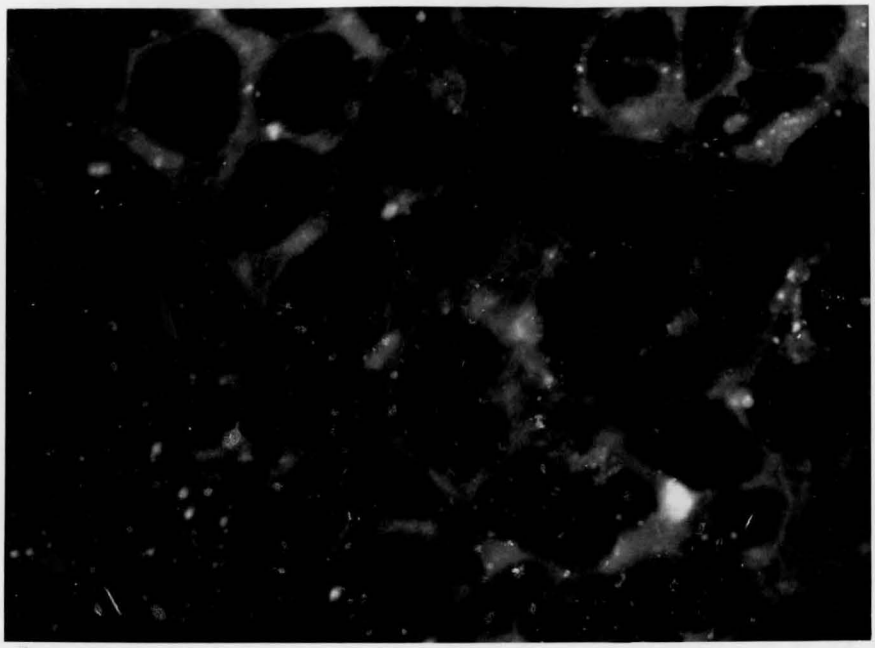

PLATE 2. Rabbit kidney cells infected with EHV vs. IBR virus antiserum. x1800.

PLATE 3. Rabbit kidney cells infected with IBR virus vs. EHV antiserum. x1800.

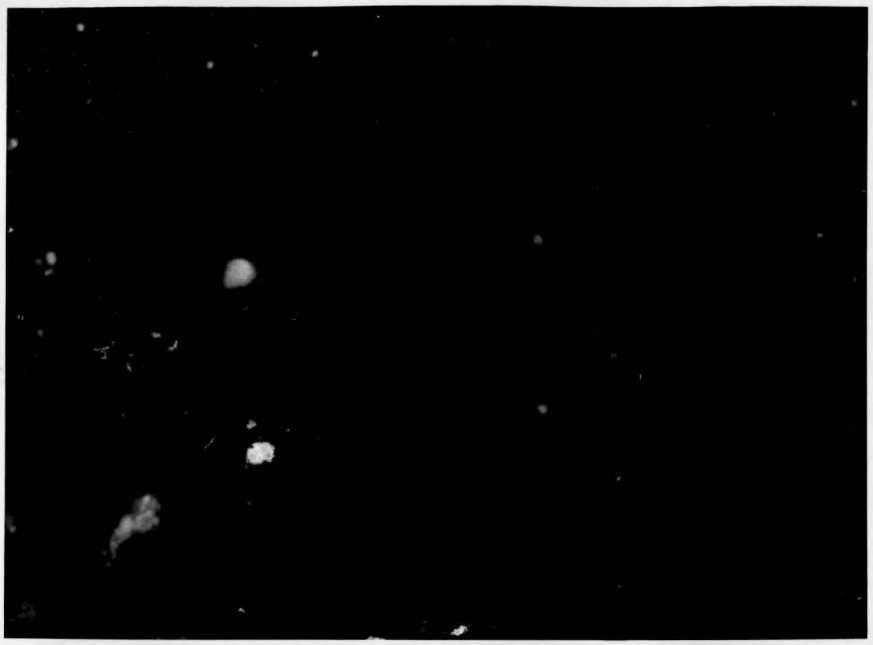

PLATE 4. Control. Uninoculated rabbit kidney cells vs. IBR virus antiserum plus fluorescent antiserum. x1800.

PLATE 5. Control. Rabbit kidney cells infected with HSV vs. fluorescent antiserum alone. x1800.

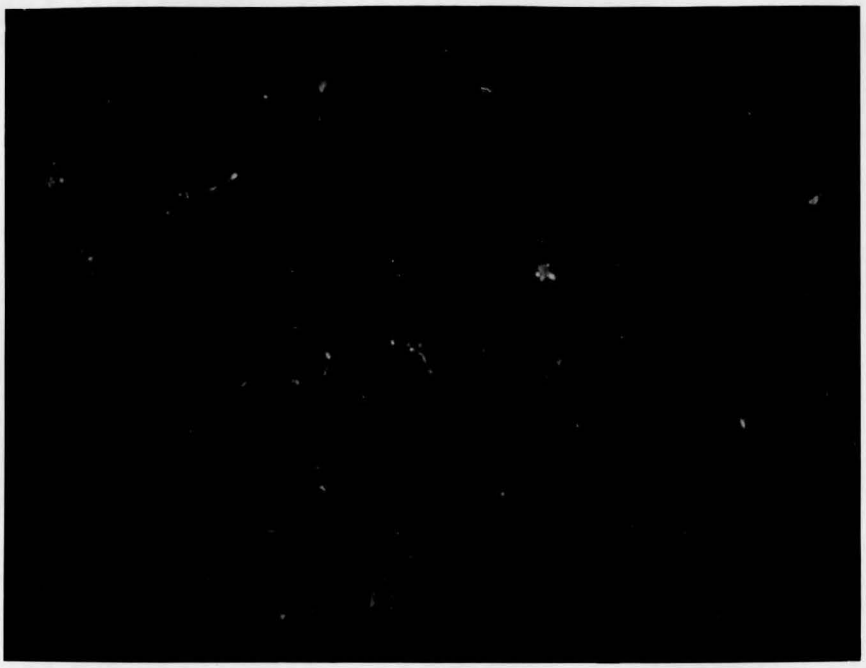

PLATE 6. Control. Rabbit kidney cells infected with vaccinia virus vs. IBR virus antiserum plus fluorescent antiserum. x450.

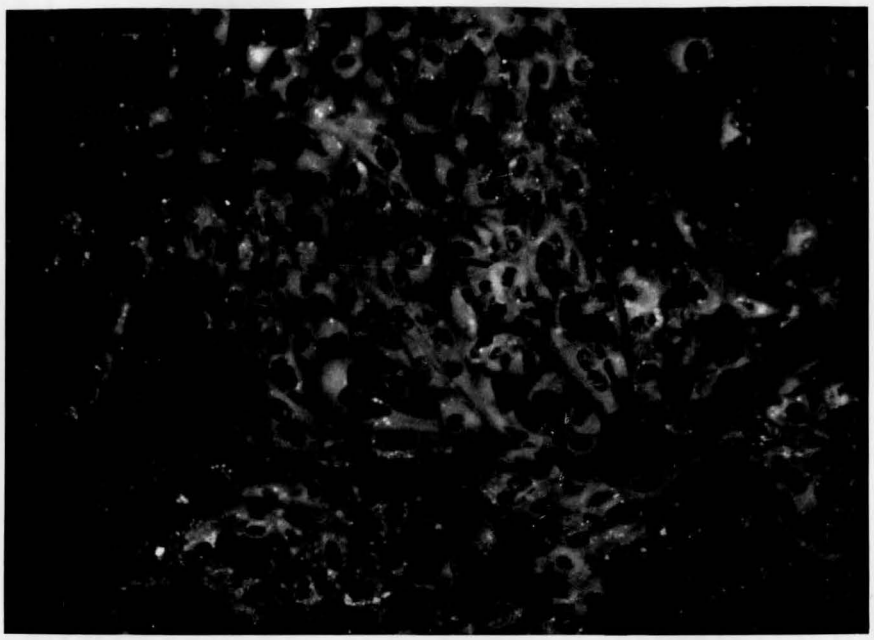

PLATE 7A. Rabbit kidney cells infected with HSV vs. HSV antiserum. Four hours post-inoculation. x450.

PLATE 7B. Same as 7A. x1800.

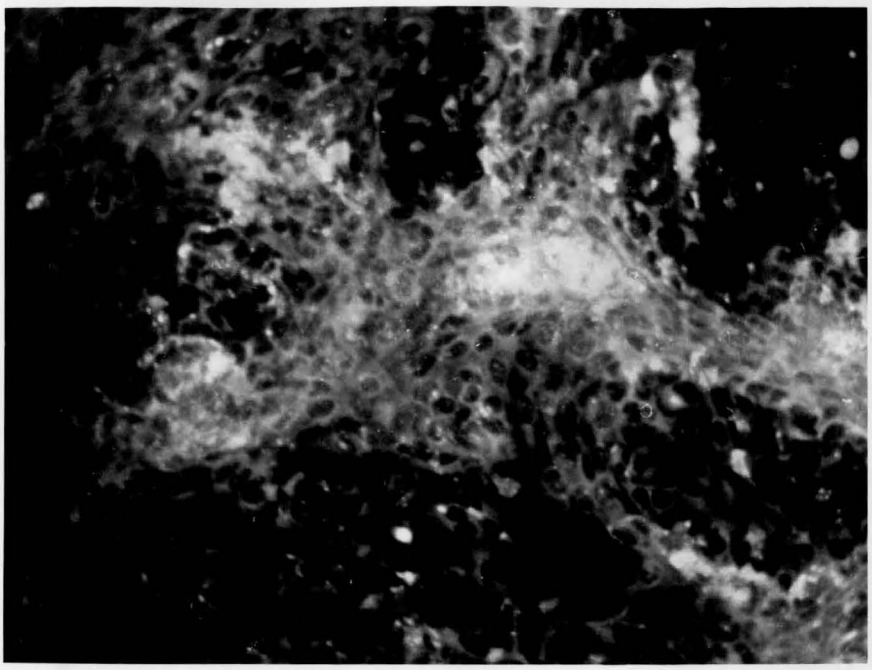

PLATE 8. Rabbit kidney cells infected with HSV vs. HSV antiserum. Five hours post-inoculation. x450.

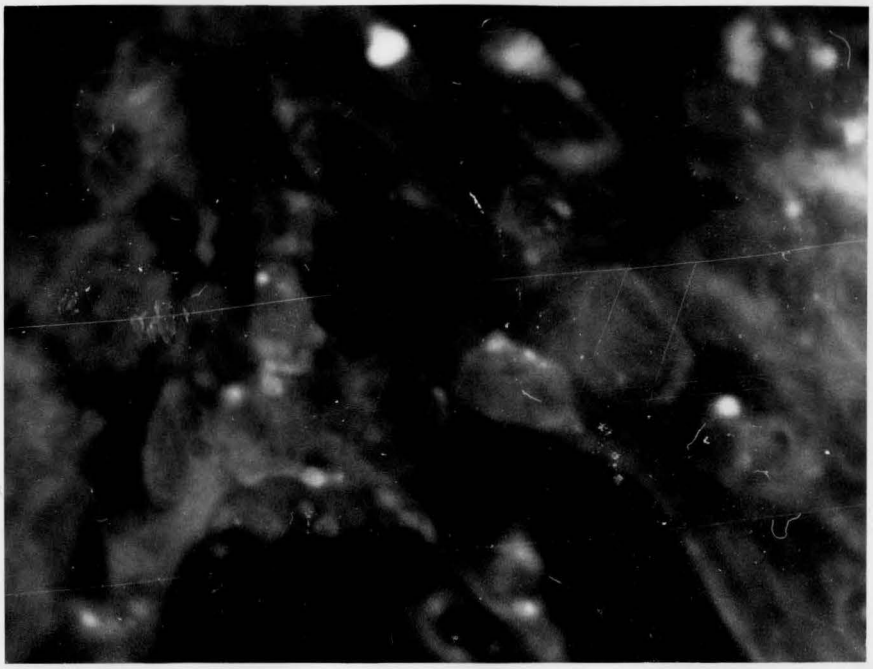

PLATE 9. Rabbit kidney cells infected with HSV vs. HSV antiserum. Twelve hours post-inoculation. x1800.

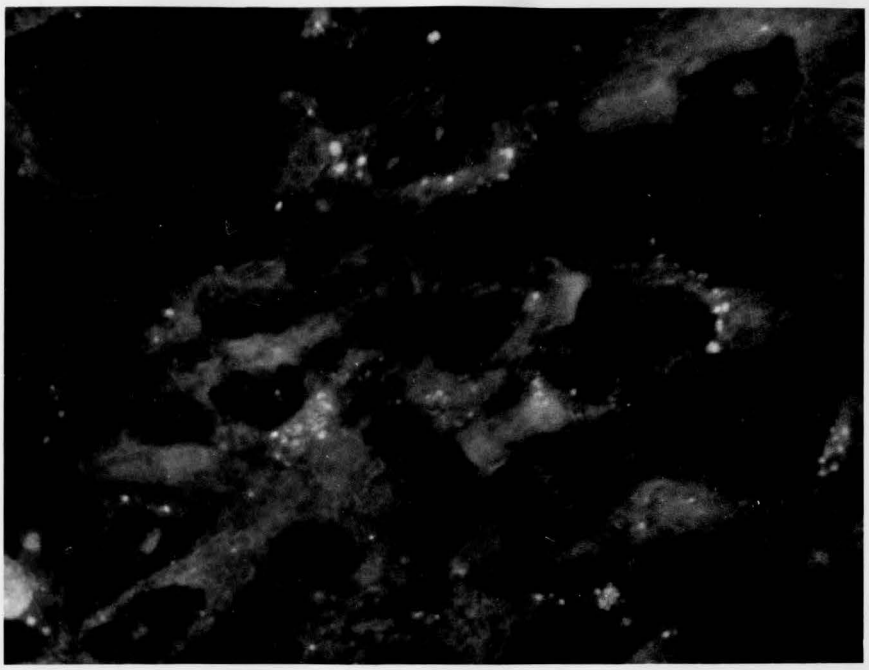

PLATE 10. Rabbit kidney cells infected with SMHV vs. HSV antiserum. Four hours post-inoculation. x1800.

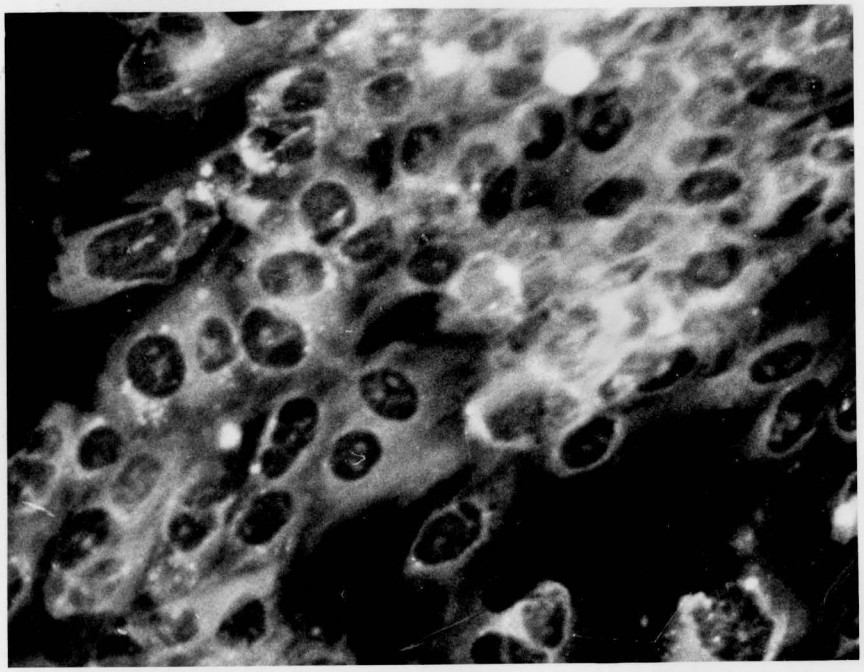

PLATE 11. Rabbit kidney cells infected with SMHV vs. HSV antiserum. Seven hours post-inoculation. x1200.

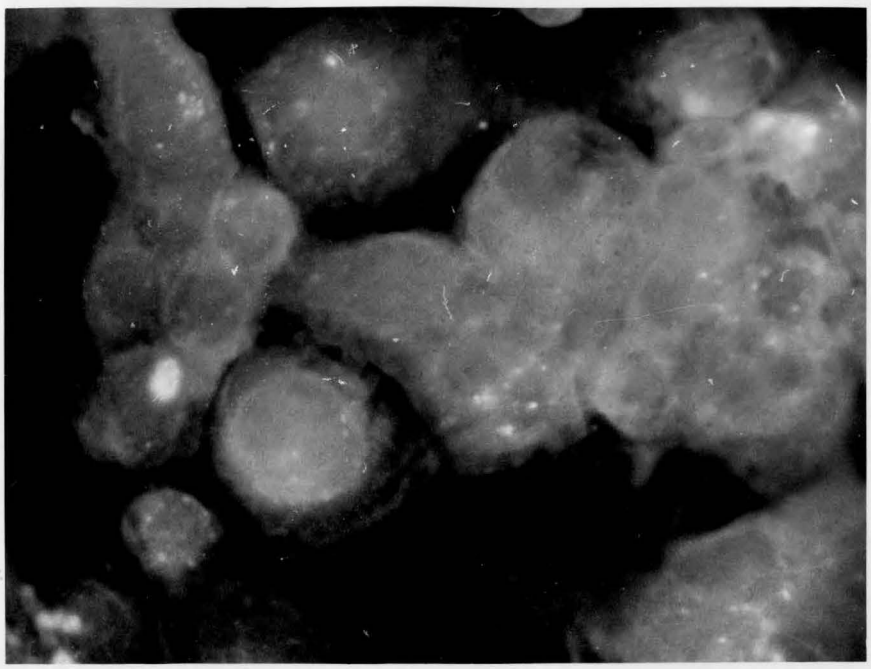

PLATE 12. Rabbit kidney cells infected with SMHV vs. HSV antiserum. Twenty-six hours post-inoculation. x1800.

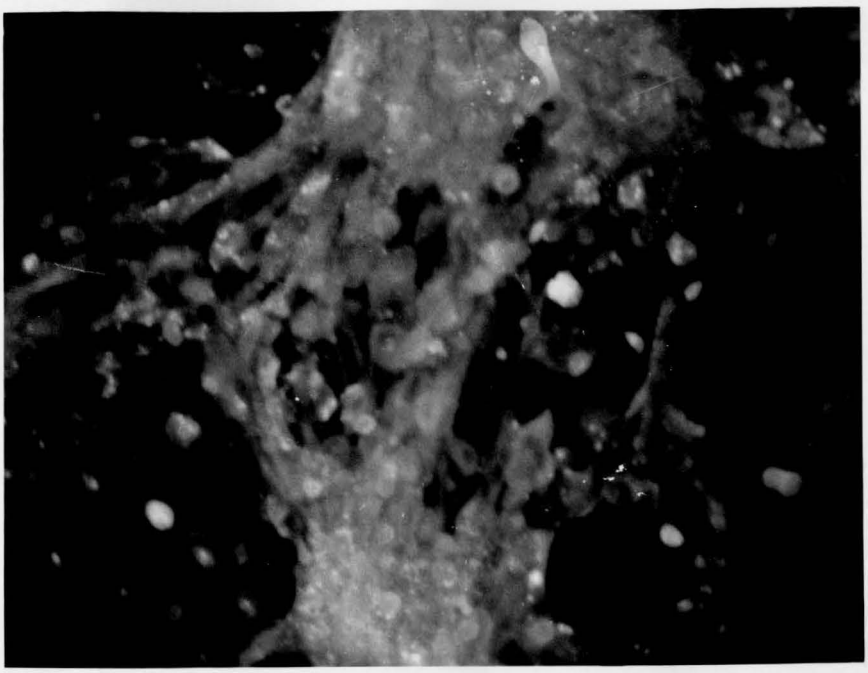

PLATE 13. Rabbit kidney cells infected with EHV vs. HSV antiserum. Twenty-six hours post-inoculation. x450.

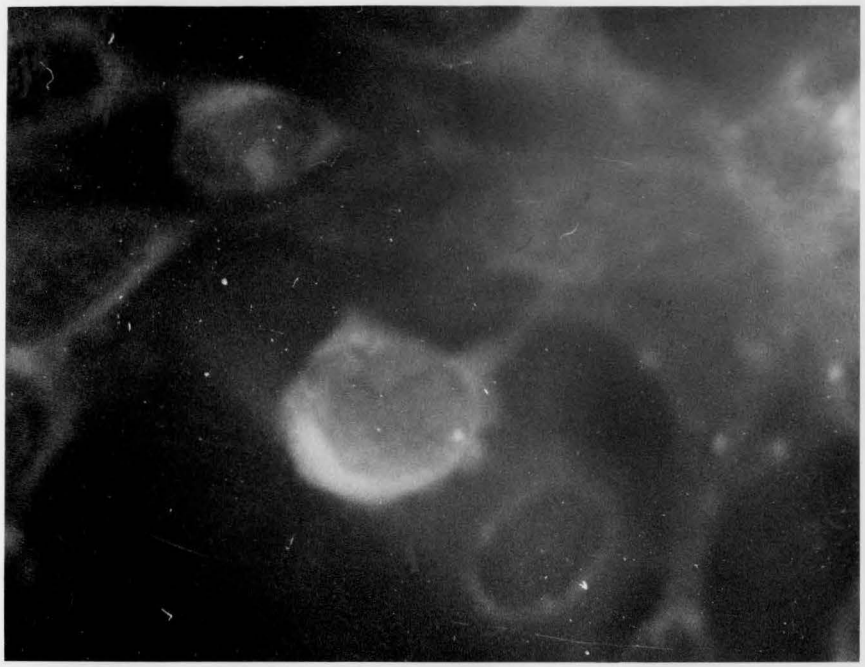

PLATE 14. Rabbit kidney cells infected with HSV vs. IBR virus antiserum. Twenty-six hours post-inoculation. x1800.

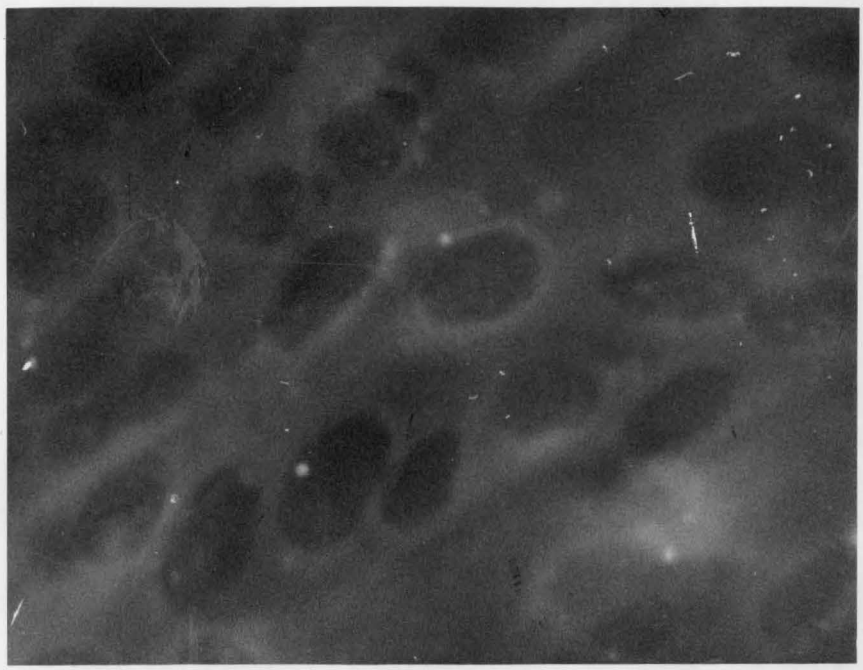

PLATE 15. Rabbit kidney cells infected with SMHV vs. IBR virus antiserum. Five hours post-inoculation. x1800.

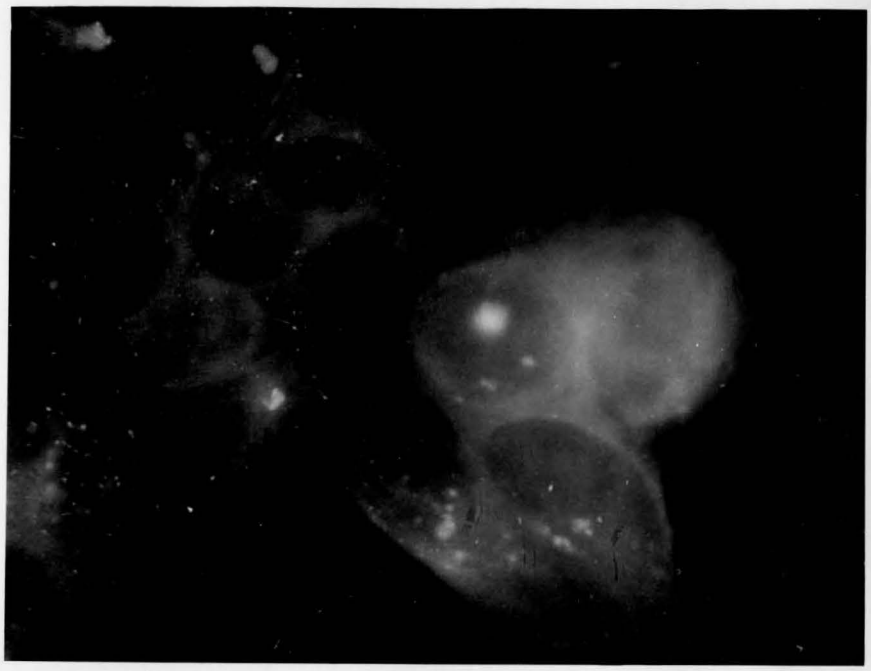

PLATE 16. Rabbit kidney cells infected with SMHV vs. IBR virus antiserum. Twenty-six hours post-inoculation. x1800.

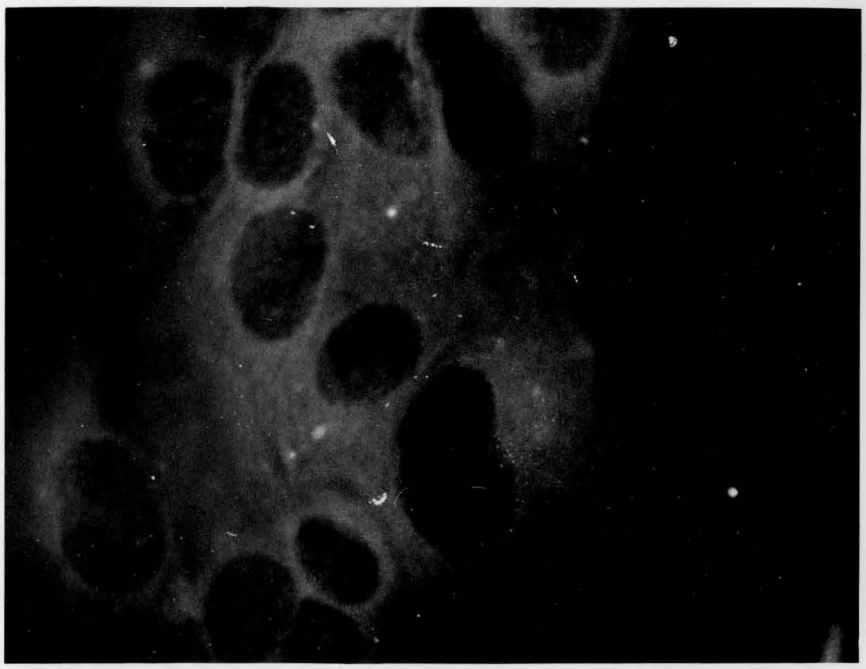

PLATE 17. Rabbit kidney cells infected with EHV vs. IBR virus antiserum. Twenty-six hours post-inoculation. x1800.

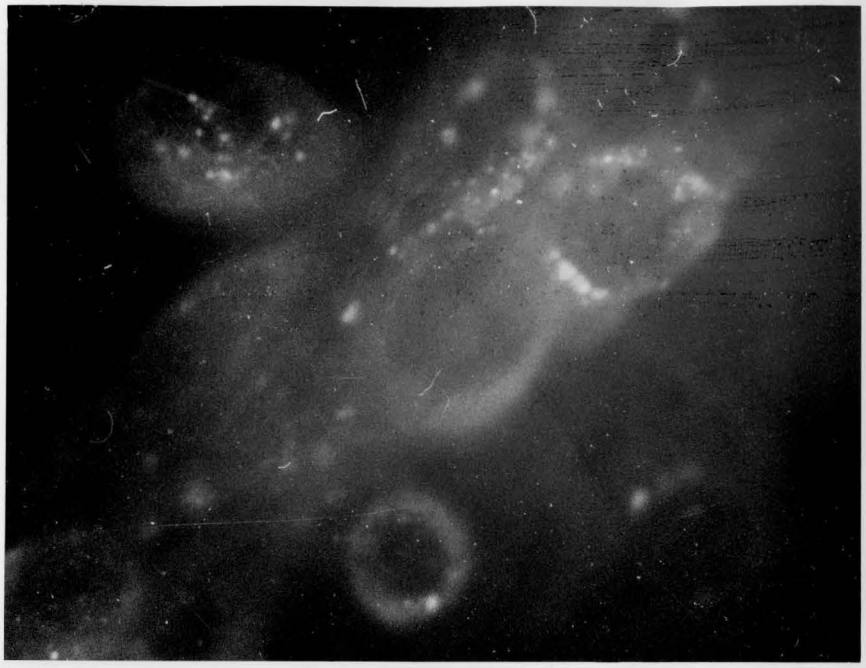

PLATE 18. Rabbit kidney cells infected with HSV vs. EHV antiserum. Twenty-six hours post-inoculation. x1800.

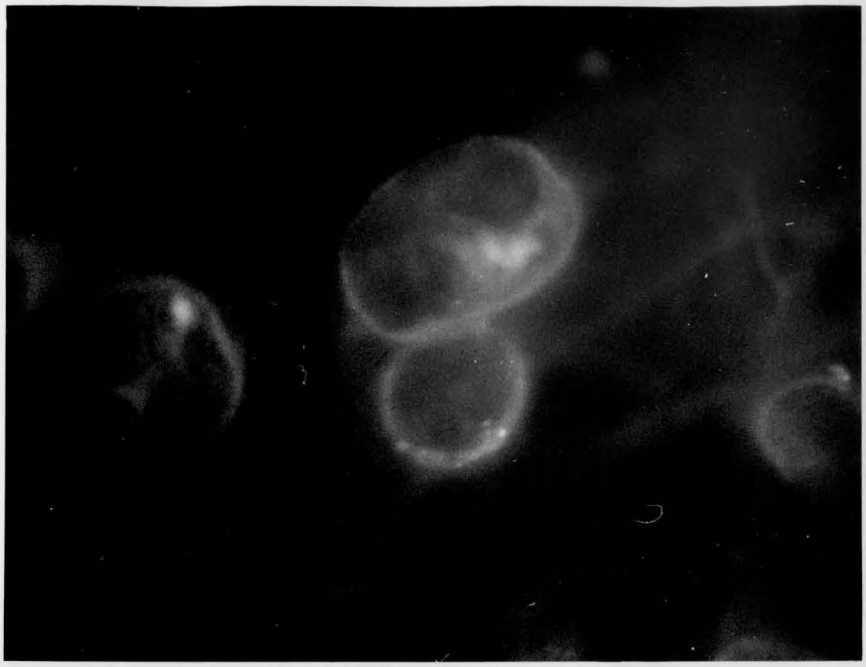

PLATE 19. Rabbit kidney cells infected with SMHV vs. EHV antiserum. Twenty-six hours post-inoculation. x1800.

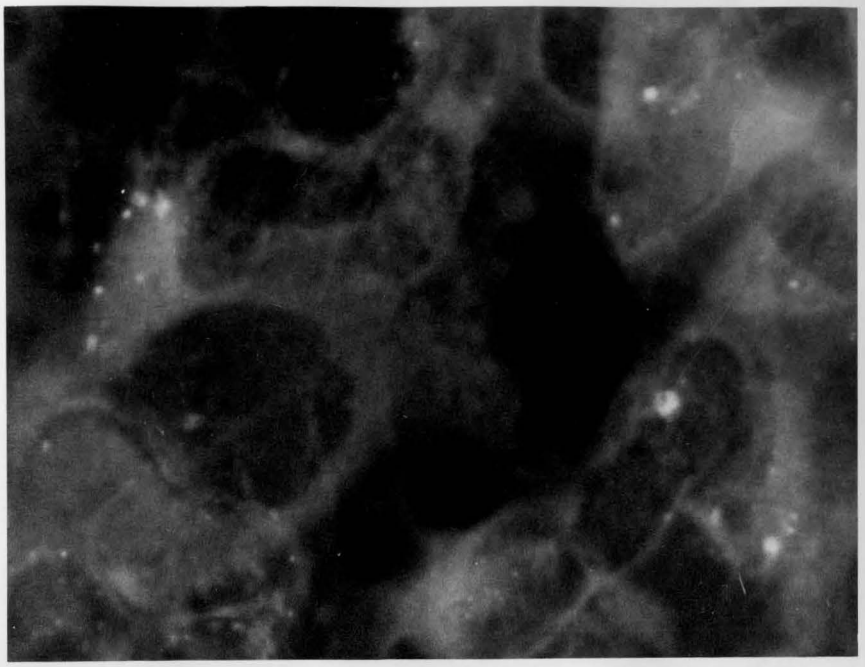

PLATE 20. Rabbit kidney cells infected with IBR virus vs. EHV antiserum. Twenty-six hours post-inoculation. x1800.

CHAPTER V

DISCUSSION

The results presented herein have indicated that the four viruses chosen for serological comparison -- HSV of man, SMHV of monkeys, IBR virus of cattle, and EHV of horses -- are related. The two most closely related of the four viruses studied are HSV and SMHV. In the neutralization tests, definite reciprocal cross-neutralization was observed.

The reciprocal cross-neutralization between HSV and IBR virus was much weaker than that between HSV and SMHV. None of the four anti-HSV antisera neutralized EHV. In fact, of the ten antisera made against HSV, SMVH, and IBR virus, none was found to have neutralizing activity against EHV. However, one EHV antiserum preparation did have weak neutralizing activity against both HSV and SMHV (#307, Table 6). Also, weak but definite reciprocal cross-fluorescence was seen between EHV and HSV, between EHV and SMHV, and between EHV and IBR virus (Tables 7 and 11). Squirrel monkey herpesvirus and infectious bovine rhinotracheitis virus also showed weak but definite anti-

genic relatedness by both the neutralization test and fluorescent antibody test (Tables 3, 4, 8, 9 and 11).

It seems evident from the data presented here, and from the work of others (2,14,16,56,64,68), that complement plays a role in enhancing the neutralization of herpesviruses by specific antibodies. Yoshino and Taniguchi (68) have reported that the first neutralizing antibodies appearing in rabbits, guinea pigs and human infants, following infection with herpes simplex virus, are actually complement-requiring. Non-complement requiring neutralizing antibodies subsequently appear, although serum containing such antibodies are still enhanced by the addition of complement. Early antibodies to herpesviruses have been found by several workers to be more susceptible to the potentiating effect of complement than are late, or hyperimmune antibodies. However, Graham et al. (14) have found that hyperimmune antisera prepared in monkeys against two strains of cytomegalovirus contain chiefly complement-requiring neutralizing antibodies.

The exact method by which complement enhances antibody neutralization of herpesviruses has not been completely elucidated. Taniguchi and Yoshino (60) have postulated that complement acts merely to accelerate the

rate of virus inactivation. Thus, early antibodies are enhanced to a higher degree than late antibodies because they have a much slower rate of virus inactivation. Heineman (16), however, has proposed that different classes of neutralizing antibodies are produced in response to infection with herpesviruses -- some totally dependent, some totally independent, and some potentiated by complement. Wallis and Melnick (64) have presented evidence which indicates that complement acts in the neutralization of herpesviruses by causing immunoaggregation. According to them, complement-requiring antibodies (CRA) differ from non-complement-requiring antibodies (NCRA) in that CRA act as if they were monovalent, unable to cause aggregation alone, while NCRA are typical bivalent antibodies. Thus, early antisera appear to be made up chiefly of complement-requiring, monovalent antibodies which attach to herpesviruses but cannot cause neutralization unless complement is present to aid in aggregation. Hyperimmune antisera, on the other hand, contain chiefly bivalent antibodies which can cause immunoaggregation alone, although Wallis and Melnick determined that a certain fraction of hyperimmune antiserum is complement requiring. It seems reasonable to conclude

that when performing neutralization tests with herpesviruses, using either primary or hyperimmune antiserum, the normal procedure of heat-inactivating the serum to destroy so-called non-specific inhibitors should always be followed by a re-addition of complement. In the case of primary antiserum, this would allow for the expression of otherwise undetectable antibodies, while with hyperimmune antiserum the result would be a boost in neutralizing titer. This procedure could be especially important in cross-neutralization tests, since as shown by Stevens et al. (56) and by this work, complement also enhances the cross-neutralization of antigenically related herpesviruses.

The fluorescent antibody test proved to be more sensitive than the neutralization test in detecting antigenic sharing between the four viruses. The cross-fluorescent titers were slightly higher than the observed cross-neutralization titers. In addition, the fluorescent antibody test revealed weak antigenic sharing between IBR virus and EHV, which was not exposed by the neutralization test. The fact that all of the controls utilized in the fluorescent antibody tests were negative lends support to the conclusions made concerning the antigenic relation-

ships between the four herpesviruses. Neither antisera plus fluorescent antibody, nor fluorescent antibody alone, following absorption with rabbit kidney cells, stained uninoculated monolayers. Pre-inoculation sera and antisera made against normal rabbit kidney cells were similarly ineffective in staining either uninfected or virus infected cells. Vaccinia virus, a DNA virus from the poxvirus group, was not stained by any of the hyper-immune antisera. In addition, SMHV antiserum gave exactly the same or similar staining titers against different strains of HSV, SMHV, IBR virus and EMV which had been passed several times in either cat kidney cells or mouse brains, thus almost completely ruling out the possibility that the cross-fluorescence was due to a common contaminant in all of the virus preparations.

The results of fluorescent antibody tests carried out with virus-infected cells which were stained at various times after infection provided additional, confirmatory evidence of the antigenic sharing between the herpesviruses (Table 13). Cells infected with squirrel monkey herpesvirus fluoresced very early after infection following treatment with anti-HSV antiserum, and increased in intensity thereafter. This could reflect

either the presence of multiple shared antigens between the two viruses, some of which are produced early in infection and others later, or a very close antigenic relationship between one antigen, presumably the same antigen which is involved in cross-neutralization.

Antiserum to IBR virus and to EHV did not stain cells infected with herpes simplex virus until twenty-four hours after infection. The intensity of staining only reached a value of 1+ or 2+, indicating that these antisera only weakly recognize an HSV antigen which is either made, or accumulates, late in the course of infection. HSV antiserum, on the other hand, reacted with cells infected with IBR virus or EHV much earlier following inoculation. The intensity of the fluorescence remained very weak throughout the course of the infection, however.

The cross fluorescence observed between IBR virus and EHV was interesting in light of the lack of cross-neutralization between the two viruses. Cross-fluorescence occurred generally late after infection, and was confined completely to the cytoplasm. In all other crosses, fluorescence first appeared in the cytoplasm but later localized in the nucleus as well, which supports

the concepts proposed by others (4,12,36,46,54,57) that herpesvirus antigens are synthesized in the cytoplasm and migrate into the nucleus. The reason why no nuclear cross-fluorescence was observed between IBR virus and EHV may be the same reason why a lack of cross-neutralization exists between the two viruses. The shared antigen may reside in the inner core of the virions and would thus not be susceptible to neutralizing antibodies. Following its synthesis in the cytoplasm, it would be susceptible to fluorescent antibodies, but after migrating into the nucleus, the antigen may be quickly assembled into whole virus and consequently not vulnerable to the same antibodies.

The fluorescent antibody test, as performed, could not, of course, reveal either the type or the number of antigens which are shared between the herpesviruses. Such an analysis could be made only after isolating the various antigens and testing for cross-reactions in a system such as immunodiffusion. An attempt was made to set up an immunodiffusion system, but a variety of technical problems caused it to be abandoned. Further analysis of the antigenic relationships between the herpesviruses could be done if such problems are overcome.

Recently inventigators have obtained evidence indicating that several herpesviruses share a group-specific antigen. Kirkwood et al. (27), utilizing micro-immunodiffusion, determined that herpes simplex viruses types 1 and 2, human cytomegalovirus, EB virus, Lucke' herpesvirus and Marek's disease virus contain an identical group-specific antigen, located on their nucleocapsids. The antigen sedimented at 80,000 g and natural antibodies to it were found in 5-10% of normal humans, frogs, rabbits, dogs, cats and cows. Also, Ross et al. (45) found cross-immunofluorescent staining between herpes simplex viruses types 1 and 2, pseudorabies virus, Marek's disease virus, EB virus and the herpesvirus of turkeys. There thus appears to be a good possibility that all of the herpesviruses share at least one antigen. The results obtained in this study would tend to support such a concept.

In the study by Ross et al. (45), gradations were noted in the intensity of staining between the six viruses. Such gradations were also observed in this study, with the strongest relationship being between HSV and SMHV, followed by the relationships between IBR virus and the two primate herpesviruses. The weakest relationships occurred

between the horse herpesvirus and the other three. It may or may not be significant that HSV, SMHV and IBR virus have G+C contents in the range of 67% to 72%, while EHV has a G+C content of only 56%.

BIBLIOGRAPHY

1. Abodeely, R.A., E. Palmer, L.A. Lawson and C.C. Randall. 1971. The proteins of enveloped and deenveloped equine abortion (herpes) virus and the separated envelope. Virology 44:146-152.

2. Andersen, H.K. 1972. The influence of complement on cytomegalovirus neutralisation by antibodies. Arch. ges. Virusforsch. 36:133-140.

3. Aurelian, L. 1968. Demonstration by plaque reduction technique of immunologic relationship between canine herpes virus and herpes simplex virus. Proc. Soc. Exp. Biol. Med. 127:485-488.

4. Ben-Porat, T., H. Shimono, and A.S. Kaplan. 1969. Synthesis of proteins in cells infected with herpesvirus. II. Flow of structural viral proteins from cytoplasm to nucleus. Virology. 37:56-61.

5. Black, P.H., J.W. Hartley, and W.P. Rowe. 1963. Isolation of a cytomegalovirus from African green monkey. Proc. Soc. Exp. Biol. Med. 112:601-605.

6. Bryans, J.T. 1969. On immunity to disease caused by equine herpesvirus 1. J. Amer. Vet. Assoc. 155:294-301.

7. Buchan, A., and D.H. Watson. 1969. The immunological specificity of thymidine kinases in cells infected by viruses of the herpes group. J. Gen. Virol. 4:461-463.

8. Burnet, F.M., D. Lush, and A.V. Jackson. 1939. The relationship of herpes and B viruses: immunological considerations. Aust. J. Exp. Biol. Med. 17:41-51.

9. Diwan, A., and W.H. Prusoff. 1968. The effect of 5-iodo-2-deoxyuridine 5' triphosphate, an allosteric inhibitor, on deoxycytidine deaminase "induced" by herpes simplex virus. Virology 34:184-186.

10. Doll, E.R., and J.H. Kintner. 1954. A comparative study of the equine abortion and influenza viruses. Cornell Vet. 44:355-357.

11. Fink, M.A., G.S. Kind, and M. Mizell. 1968. Preliminary note: identity of a herpesvirus antigen from Burkitt lymphoma of man and the Lucke' adenocarcinoma of frogs. J. Nat. Cancer. Inst. 41:1477-1478.

12. Fujiwara, R., and A.S. Kaplan. 1967. Site of protein synthesis in cells infected with pseudorabies virus. Virology 32:60-68.

13. Gerder, L., and L. Vaczi. 1968. Localization of nuclear and cytoplasmic herpes simplex antigens in infected cells by immunofluorescence. Acta Virol. 12:97-105.

14. Graham, B.J., Y. Minamishima, G.R. Dreesman, H.G. Haines, and M. Benyesh-Melnick. 1972. Complement-requiring neutralizing antibodies in hyperimmune sera to human cytomegaloviruses. J. Immunol. In press.

15. Hamada, C., T. Kamiya, and A.S. Kaplan. 1966. Serological analysis of some enzymes present in pseudorabies virus-infected and noninfected cells. Virology. 28:271-281.

16. Heineman, H.S. 1967. Herpes simplex neutralizing antibody-quantitation of the complement-dependent fraction in different phases of adult human infection. J. Immunol. 99:214-222.

17. Hinze, H.C. 1971. New member of the herpesvirus group isolated from wild cottontail rabbits. Infection and Immunity, 3:350-358.

18. Hsiung, G.D., K.Y. Fond, and K.M. Lam. 1971. Guinea pig leukocytes: in vivo and in vitro infection with a herpes-like virus. J. Immunol. 106:1686-1693.

19. Ito, M., and A.L. Barron. 1972. Surface antigen produced by herpes simplex virus. J. Immunol. 108:711-718.

20. Kaplan, A.S., and T. Ben-Porat. 1968. Metabolism of animal cells infected with nuclear DNA viruses. Ann. Rev. Microbiol. 22:427-450.

21. Kaplan, A.S., and T. Ben-Porat. 1970. Synthesis of protein in cells infected with herpesvirus. VI. Characterization of the proteins of the viral membrane. Proc. Nat. Acad. Sci. 66:799-806.

22. Kaplan, A.S., and A.E. Vatter. 1959. A comparison of herpes simplex and pseudorabies viruses. Virology 7:394-407.

23. Keir, H.M., and E. Gold. 1963. Deoxyribonucleic acid nucleotidyltransferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. Biochim. Biophys. Acta 72:263-276.

24. Keir, H.M., H. Subak-Sharpe, W.I.H. Shedden, D.H. Watson, and P. Wildy. 1966. Immunological evidence for a specific DNA polymerase produced after infection by herpes simplex virus. Virology 30:154-157.

25. Kendrick, J.W., and O.C. Straub. 1967. Infectious bovine rhinotracheitis-infectious pustular vulvovaginitis infection in pregnant cows. Amer. J. Vet. Res. 28:1269-1278.

26. Kemeny, L., and J.E. Pearson. 1970. Isolation of herpesvirus from equine leukocytes-comparison with equine rhinopneumonitis virus. Can. J. Comp. Med. 34:59-65.

27. Kirkwood, J., G. Geering, and L.J. Old. 1971. Demonstration of group and type-specific antigens of herpesviruses. IN: Oncogenesis and herpesviruses. International Agency for Research on Cancer. Lyon. p. 479.

28. Klemperer, H.G., G.R. Haynes, W.I.H. Shedden, and D.H. Watson. 1967. A virus-specific thymidine kinase in BHK 21 cells infected with herpes simplex virus. Virology 31:120-128.

29. Koller, U., E. Gonczol, and L. Vaczi. 1963. Study of the multiplication of varicella-zoster virus by the fluorescent antibody test. Acta Microbiol. 10:183-188.

30. Krech, U., and M. Jung. 1971. Antigenic relationship between human cytomegalovirus, herpes simplex and varicella zoster virus studied by complement-fixation. Arch. ges. Virusforsch. 33:288-295.

31. Lehrun, J. 1956. Cellular localization of herpes simplex virus by means of fluorescent antibody. Virology 2:496-510.

32. Malherbe, H., R. Harwin, and M. Ulrich. 1963. The cytopathic effects of vervet monkey viruses. Sth. Afr. Med. J. 37:407-411.

33. McKercher, D.G., and E.M. Wade. 1964. The virus of infectious bovine rhinotracheitis as a cause of abortion in cattle. J. Amer. Vet. Med. Assoc. 144:136-142.

34. Nohara, H., and A.S. Kaplan. 1963. Induction of a new enzyme in rabbit kidney cells by pseudorabies virus. Biochem. Biophys. Res. Commun. 12:189-193.

35. Olshevsky, U., and Y. Becker. 1970. Herpes simplex virus structural proteins. Virology 40:948-960.

36. Olshevsky, U., J. Levitt, and Y. Becker. 1967. Studies on the synthesis of herpes simplex virions. Virology 37:56-61.

37. Perera, P., and J.M. Morrison. 1970. Evidence for the induction of a new deoxycytidine kinase in cells infected with herpes virus. Biochem. J. 117:21-22.

38. Plowright, W., R. Macadam, and J. Armstrong. 1963. Growth and characterization of the virus of bovine malignant catarrhal fever in East Africa. J. Gen. Microbiol. 39:253-259.

39. Plummer, G. 1964. Serological comparison of the herpes viruses. Brit. J. Exp. Path. 45:135-141.

40. Plummer G., C.R. Goodheart, D. Henson, and C.P. Bowling. 1969. A comparative study of the DNA density and behavior in tissue cultures of fourteen different herpesviruses. Virology 39:134-137.

41. Robinson, D.J., and D.H. Watson. 1971. Structural proteins of herpes simplex virus. J. Gen. Virol. 10:163-171.

42. Roizman, B. 1969. The herpesviruses a biochemical definition of the group. Current Topics in Microbiology and Immunology. 39:1-79.

43. Roizman, B., S.B. Spring, and P.R. Roane, Jr. 1967. Cellular compartmentalisation of herpesvirus antigens during viral replication. J. Virol. 1:181-192.

44. Ross, C.A.C., J.H. Subak-Sharpe, and P. Ferry. 1965. Antigenic relationship of varicella-zoster and herpes simplex. Lancet 2:708-711.

45. Ross, L.J.N., J.A. Frazier, and P.M. Biggs. 1971. An antigen common to some avian and mammalian herpesviruses. IN: Oncogenesis and herpesviruses. International Agency for Research on Cancer. Lyon. pp. 480-484.

46. Ross, L.J.N., D.H. Watson, and P. Wildy. 1963. Development and localization of virus-specific antigens during multiplication of herpes simplex virus in BHK 21 cells. J. Gen. Virol. 2:115-122.

47. Russel, W.C., D.H. Watson, and P. Wildy. 1963. Preliminary chemical studies on herpesvirus. Biochem. J. 87:16p.

48. Sabin, A.B. 1934. Studies on the B virus. I. The immunological identity of a virus isolated from a human case of ascending myelitis associated with visceral necrosis. Brit. J. Exp. Path. 15:248-268.

49. Sabin, A.B. 1934. The immunological relationships of pseudorabies (infectious bulbar paralysis, mad itch). Brit. J. Exp. Path. 15:372-380.

50. Schmidt, N.J., E.H. Lennette, and R.L. Magoffin. 1969. Immunological relationship between herpes simplex and varicella-zoster viruses demonstrated by complement-fixation, neutralization and fluorescent antibody tests. J. Gen. Virol. 4:321-328.

51. Sharma, J.M., D. Burger, and S.G. Kenzy. 1972. Serological relationships among herpesviruses: cross-reaction between Marek's disease virus and pseudorabies virus as detected by immunofluorescence. Infection and Immunity. 5:406-411.

52. Shipkey, F.H., R.A. Erlandson, R.B. Baily, V.I. Babcock, and C.M. Southman. 1967. Virus biographies. II. Growth of herpes simplex virus in tissue culture. Exp. Mol. Path. 6:37-67.

53. Spear, P.G., J.M. Keller, and B. Roizman. 1970. Proteins specified by herpes simplex virus. II. Viral glycoproteins associated with cellular membranes. J. Virol. 5:123-131.

54. Spear, P.G., and B. Roizman. 1968. The proteins specified by herpes simplex virus. I. Time of synthesis, transfer into nuclei, and properties of proteins made in productively infected cells. Virology 36:545-555.

55. Spear, P.G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. 9:143-159.

56. Stevens, D.A., T. Pineus, M.A.K. Burroughs, and B. Hampar. 1968. Serological relationship of a simian herpes virus (SA8) and herpes simplex virus: heterogeneity in the degree of reciprocal cross-reactivity shown by rabbit 7S and 19S antibodies. J. Immunol. 101:979-983.

57. Sydiskis, R.J., and B. Roizman. 1966. Polysomes and protein synthesis in cells infected with a DNA virus. Science 153:76-78.

58. Taniguchi, K., and S. Yoshino. 1965. Studies on the neutralization of herpes simplex virus. I. Appearance of neutralizing antibodies having different grades of complement requirement. Virology 26:44-53.

59. Taniguchi, K., and S. Yoshino. 1965. Studies on the neutralization of herpes simplex virus. II. Analysis of complement as the antibody potentiating factor. Virology 26:54-60.

60. Taniguchi, K., and S. Yoshino. 1965. Studies on the neutralization of herpes simplex virus. III. Mechanism of the antibody potentiating action of complement. Virology 26:61-72.

61. Thouless, M.E., and G.R. Skinner. 1971. Differences in the properties of thymidine kinase produced in cells infected with type 1 and type 2 herpes virus. J. Gen Virol. 12:195-197.

62. Takumaru, T. 1965. Studies of herpes simplex virus by the gel diffusion technique. I. Distribution of precipitating antibodies among human sera. J. Immunol. 95:181-188.

63. Trilifajova, J., J. Sourek, and M. Ryba. 1971. Antigenic relationship between varicella-herpes zoster and herpes simplex viruses studied by the gel precipitation reaction. Acta Virol. 15:293-300.

64. Wallis, C., and J.L. Melnick. 1971. Herpesvirus neutralization: the role of complement. J. Immunol. 107:1235-1242.

65. Watson, D.H., W.I.H. Shedden, A. Elliot, T. Tetsuka, P. Wildy, D. Bourgaux-Ramoisy, and E. Gold. 1966. Virus specific antigens in mammalian cells infected with herpes simplex virus. Immunology 11:399-408.

66. Watson, D.H., P. Wildy, B.A.M. Harvey, and W.I.H. Shedden. 1967. Serological relationships among viruses of the herpes group. J. Gen. Virol. 1:139-141.

67. Weller, T.H., and A.H. Coons. 1954. Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. Proc. Soc. Exp. Biol. Med. 86:789-794.

68. Yoshino, K., and S. Taniguchi. 1964. The appearance of complement requiring neutralizing antibodies by immunization and infection with herpes simplex virus. Virology 22:193-201.

APPROVAL SHEET

The thesis submitted by William T. Blue has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis, and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

1/3/73
Date

Signature of Advisory Committee Director

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