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DISSEMINATION OF HERPES SIMPLEX VIRUS

IN THE PREGNANT RABBIT

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CHAPTER I

INTRODUCTION

The pathogeneses of the diseases caused by herpes simplex virus have not been clearly determined. The exact mechanisms of dissemination of this virus from the initial site of infection to the many anatomical areas which may be involved is not completely described. In some instances herpetic inclusion bodies have been observed and the virus has been identified after isolation from autopsied tissues of human beings who died of clinically diagnosed herpetic infections. In the majority of studies the etiologic agent was not demonstrated. A survey of the literature indicates that this virus is believed to be spread along nerve pathways, by hematogenous dissemination or by a combination of these routes. The extent of the involvement of tissues and organs as sequalae to primary herpes simplex virus infection will be reviewed.

Dissemination of Herpes Simplex Virus

The groundwork for experimental infection of animals with herpes simplex virus was established in 1912 by Grüter who reported (1920) that when the scrapings from a herpetic lesion of a human being were

placed on the scarified cornea of a rabbit, the animal developed an encephalitis. He was able to maintain the virulence of the virus by rabbit to rabbit transfer with infected rabbit brain homogenate. Löwenstein (1919) infected rabbits by corneal inoculation with herpes simplex virus in material from eruptions of the face, lips and genitalia of human beings.

Marinesco and Dragonesco (1923) reported that herpes simplex virus traversed the tissue spaces and sheaths of the nerves of experimentally infected rabbits rather than progressing along the nerve. Friedenwald (1923) suggested that a keratoconjunctivitis and oftentimes an encephalitis resulted when rabbits were infected with pustular fluid from human herpetic lesions by the corneal scarification technique. He postulated that the virus was transported along the sensory nerves of the cornea and from there to the brain. Goodpasture and Teague (1923_a, 1923_b) proposed that, in rabbits, the peripheral nerves (sensory, motor, or sympathetic) were the portals of entry to the central nervous system from the site of initial infection. They demonstrated a myelitis of the lumbar region of the spinal cord when the herpes simplex virus was inoculated into any muscle innervated by the sciatic nerve. However, if this nerve were cut first, no myelitis developed. Evidence was presented by Goodpasture (1925_a, 1925_b, 1929) which suggested that the virus travelled along axis cylinders of peripheral nerves of rabbits and so entered central nervous system pathways. Abe (1926) ruled out hematogenous dissemination of the virus. He reported that when the virus was inoculated into the vitreous humor of a rabbit's eye, it left the area

by way of the optic nerve exclusively, crossed the optic chiasma and infected the other eye. Gifford and Lucic (1927) confirmed Abe's findings.

King (1940) demonstrated intranuclear inclusion bodies in nerve cells of mice which he inoculated in the vitreous humor of the eye with herpes simplex virus. From serial microscopic sections of the nerves which innervate the eye he concluded that the virus was transported from this area to the central nervous system along the fifth nerve rather than the optic nerve. Berry and Slavin (1943) and Slavin and Berry (1943_a) reported that intranasal instillation of herpes simplex virus in mice was followed by prompt invasion of the central nervous system by all available neural routes.

Wildy (1954) gave what he termed "unequivocal evidence" that a viremia played no significant part in the spread of herpes simplex virus from the periphery to the central nervous system in mice. He treated the hind foot pad with hypertonic saline and then inoculated the virus into the pad. Most of the mice developed posterior paralysis and many died. An upward progression of the virus along the nerve structures was demonstrated by means of titrations of foot, sciatic nerve, lumbar and cervical sections of the spinal cord and brain. He reported that only a low grade viremia appeared late in the disease. He did not postulate as to whether axonal or fluid spaces were involved. Boyse, et al. (1956) confirmed the findings of Marinesco and Dragonesco (1923) that herpes simplex virus was dispersed through tissue fluids which surround the nerves of rabbits rather than along axonal pathways. They used radio-iodinated

serum proteins to locate the inflammatory sites and to determine the severity of the reactions.

There is evidence to support the theory of hematogenous dissemination of herpes simplex virus to organs and to the central nervous system of animals. After evaluating their results of experimental infection of rabbits, Doerr and Vöchting (1920) proposed that the virus was spread by way of the blood stream. Doerr and Schnabel (1921) inoculated rabbits intravenously with herpes simplex virus and the animals developed an encephalitis. The investigators isolated the virus from the heart's blood of these animals by corneal inoculation of the heart's blood into normal rabbits. This second group of animals developed encephalitis and died. It was concluded that the virus must enter the bloodstream from the eye, the site of experimental inoculation, and directly invade the central nervous system as a result of hematogenous dissemination. Greenbaum and Harkins (1925) reported that whole blood from a herpes simplex infected rabbit produced a herpetic keratoconjunctivitis when it was inoculated onto the scarified cornea of normal rabbits. They were unable to produce a central nervous system involvement by this method. Fevre de Arric and Millet (1926) found that intravenous inoculation of bile salts, urea and other compounds into rabbits prior to corneal inoculation of the animals with the herpes virus caused a change in permeability of the choroid plexus. The virus could easily invade the brain to which it had been carried by the blood from the site of initial infection. They postulated that in human herpetic infections the choroid plexus might be disrupted by cellular breakdown products which result from the infection and the

invasion of the brain by the virus would be facilitated. Remlinger and Bailly (1926_a) inoculated rabbits intracerebrally or subcutaneously with blood from experimentally infected rabbits and dogs which exhibited symptoms of herpes simplex virus infections. The infected rabbits died after exhibiting central nervous system involvements. Defibrinated flood from the infected rabbits and dogs was inoculated intravenously into rabbits and results similar to those described above were obtained. They were able to cause death in only 1 of 7 rabbits inoculated intracerebrally with the serums from the infected animals. Remlinger and Bailly (1927) inoculated rabbits subcutaneously and intracorneally with herpes simplex virus. They postulated that the encephalitis which resulted from this procedure was due to the virus which had been transported to the brain by the blood. Despite the fact that they were unable to demonstrate herpes virus in the blood of experimentally infected rabbits, Urbain and Schaeffer (1927) nevertheless suggested that the virus was present in the blood at least for a short time. Gildmeister and Heuer (1929) demonstrated the virus in the blood of patients by the intracerebral inoculation of the blood into normal rabbits. The rabbits subsequently died as a result of encephalitis.

Armstrong (1943) and Zarafonetis, et al. (1944) suggested that a viremia played a role in the spread of the herpes virus from the primary lesion to the central nervous system but they were unable to isolate the virus from the blood of human beings who had been clinically diagnosed as having herpetic meningitis and encephalitis. Kipping and Downie (1948) isolated the virus from lesions of patients by the embryo-

nated egg and animal inoculation techniques and they believed that these generalized human herpes simplex infections resulted from the dissemination of the virus by the blood stream. Haymaker (1949) postulated that human herpetic encephalitis infections resulted from the spread of the virus by the blood from the site of the primary lesion to the central nervous system.

Ruchman and Dodd (1950) recovered the virus from the blood of a patient with herpetic rhinitis by intracerebral inoculation of the blood into mice. They suggested that a viremia must exist since meningitis, encephalitis and dermatitis often result from herpes simplex infections. Patients with fulminating visceral herpetic infections and herpetic hepatitis were described by Zuelzer and Stulberg (1952). These investigators postulated that the virus was disseminated by way of the blood stream to produce such an overwhelming infection. Buddingh, et al. (1953) reported studies of severe herpetic stomatitis infections in children in which the causal agent was isolated from specimens of mouth washings and stools, but not from blood. Scott (1954) described many of the clinical manifestations of herpes simplex virus infections. He concluded that a viremia occurred during the primary infection and that the dispersion of the virus throughout the body was associated with blood stream dissemination. In describing Kaposi's varicelliform eruption (eczema herpeticum) in an 11½ month old infant, Pugh, et al. (1955) suggested that there was a hematogenous spread of the virus to many organs from the site of primary infection. Beale and Hair (1956) used tissue culture methods to isolate the virus of herpes simplex from the blood

of 2 patients with Kaposi's varicelliform eruption.

A few investigators have suggested that herpes simplex virus may be distributed to various organs and tissues, including the central nervous system, by a combination of neural routes and by hematogenous dissemination. Anderson (1939, 1940) demonstrated herpetic lesions and inclusion bodies on the chorioallantoic membrane and in many of the organs of chick embryos which had been inoculated on the membrane with herpes simplex virus. She postulated that the virus was hematogenously disseminated throughout the embryo, but suggested that central nervous system involvement resulted from centripetal and centrifugal transmission of the virus. She infected rabbits by the corneal scarification method and isolated the virus from the fifth nerve and ganglion by the chorioallantoic membrane technique. By intracerebral inoculation of mice, Berry and Slavin (1940) demonstrated herpes virus in the blood of young mice which had received the virus by nasal instillation. They believed that invasion of the central nervous system by the virus was independent of blood transport. After studying visceral lesions in mice which had been inoculated intranasally or which had received the virus by intraperitoneal inoculation, Slavin and Berry (1943_b) concluded that hematogenous dissemination of the virus was responsible for its establishment in the endothelium of the organs. They maintained, however, that the central nervous system was invaded by transport of the virus along nerve pathways. Evans, et al. (1946) inoculated the foot pads of young mice and noticed that they developed paralysis of that limb which was followed by paraplegia and finally an encephalitis. Titration studies for virus

in various parts of the feet led them to conclude that the virus invaded and traveled along the peripheral nerves to the central nervous system. They thought that some of the virus was transported by the blood to other parts of the body where local foci of infection were established and that from these foci the virus invaded the nerves to gain entrance to the brain along the nerve tracts. They also found that if immune serum were given to the mice simultaneously with the virus, the mice were protected against paralysis and death. If the serum were administered more than 12 hours after the experimental inoculation of the virus, paralysis and death resulted from the infection. In re-examining the data on the development of central nervous system involvements which resulted from experimental inoculation of animals with herpes simplex virus, Field (1952) found that the virus traversed the tissue fluid spaces of nerve pathways as earlier suggested by Marinesco and Dragonesco (1923). He suggested also that a transient viremia could play an important role in some disease manifestations of the virus.

From the literature review just presented it is evident that the dissemination of herpes simplex virus throughout the body may be accomplished by a combination of routes such as by 1) the axons of nerve cells, 2) the sheaths and tissue fluids surrounding the nerve cells, and 3) hematogenous dissemination. Unfortunately, many of the reports are lacking in definite knowledge of the identity of the causal agent.

Sites of Viral Infection

There are many reports in the literature of the disease manifestations and the organs and tissues involved in herpes simplex infec-

tions in animals. In rabbits, the virus has been demonstrated in ocular secretions (Scott, et al. 1941), cerebrospinal fluid (Urbain and Schaeffer, 1927), the central nervous system (Good, 1947), and the saliva, nasal secretions, urine, feces, spleen and suprarenals (Urbain and Schaeffer, 1927). The virus has been found in the brain (Slavin and Berry, 1943_a), lungs, liver, spleen and the adrenals (Slavin and Berry, 1943_b) of mice. The establishment of a herpes simplex virus infection in the guinea pig was more difficult and not as generalized. The foot pad was susceptible (Bedson and Crawford, 1927) and infection resulted in paralysis of the inoculated foot. Dermal injection onto a scarified area of the skin resulted in a local infection but with infrequent generalized infection (Bedson and Bland, 1928). Rats can be infected by intracerebral inoculation and the virus recovered from the brain (Smith, et al. 1941). When intracerebrally inoculated with the virus, monkeys developed a chronic encephalitis resembling the disease in man (Zinsser and Tang, 1929). Remlinger and Bailly (1926_b, 1926_c) established the herpes virus in the brains of cats and dogs. The teratogenic effect of the virus on chick embryos was described by Heath, et al. (1956) and Anderson (1939, 1940) demonstrated the virus in many of the visceral organs and the brains of developing chick embryos.

Scott (1954) reviewed the disease manifestations which the herpes virus causes in human beings by describing primary lesions of the mucocutaneous junction of the mouth, herpes genitalia, ocular infections and visceral and central nervous system involvements. He also discussed the manifestations of herpetic stomatitis and recurrent herpes simplex

infections which are usually confined to the genitalia or mucocutaneous junction of the mouth and can reappear despite the demonstration of specific herpes simplex antibodies in the patient's serum.

Reports in the literature which were based on clinical and epidemiological observations describe viral infections of the newborn and imply a prenatal infection. The first clear evidence of viral infection of the fetus from the mother was reported by Gregg (1941). He correlated clinical evidence of the viral damage of the offspring with epidemiological history of exposure of the mother to rubella virus. Since then, numerous such reports particularly on the prenatal effect of rubella virus have been presented. Haas (1941) inoculated pregnant mice with the virus of lymphocytic choriomeningitis and recovered the virus from fetal tissues by mouse inoculation techniques. Among the other viruses reported to be the etiologic agents of neonatal infections are poliovirus (Torres Umana, 1951), varicella (Lucchesi, et al. 1947), infectious hepatitis (Karlova, 1958), herpes zoster (Feldman, 1952) and Coxsackie virus (Freudenberg, et al. 1952).

Despite the frequency of neonatal viral infections and the assumption that fetal infections may occur there is no unequivocal proof that herpetic infections of fetal tissues occur in man or in experimental animals. To date the herpes virus has not been isolated and identified from prenatal tissue. However, some reports in the literature have incriminated herpes simplex virus as the etiologic agent of fetal infections. Hass (1935) reported a fatal neonatal infection of a 12 day old baby. He demonstrated herpetic-like inclusion bodies in liver cells of

the patient. Smith, et al. (1941) investigated the causal agent of death in a 4 week old child who suffered from acute encephalitis. A brain homogenate from the child produced death in mice by intracerebral inoculation. Wenner (1944) isolated herpes simplex virus in rabbits, hamsters and guinea pigs from vesicular fluid and brain homogenates obtained from specimens of 4 infants who died of eczema.

Wildi (1951) wrote of a mother who suffered a genital herpes infection during the fifth month of pregnancy. The infant died 13 days after birth and the investigator demonstrated herpetic-like inclusion bodies in brain tissue. A fatal neonatal infection in an infant whose mother suffered an attack of herpes labialis 9 days before birth was described by Quilligan and Wilson (1951). Herpes simplex virus was isolated from skin and liver lesions of the baby. Florman and Mindlin (1952) recovered the herpes virus from one of newborn premature triplets. From the vesicular fluid of lesions of the generalized eruption and lip blisters, the virus was isolated on the chorioallantoic membrane of the developing chick embryo and in mice and rabbits. Zuelzer and Stulberg (1952) discussed their observations of herpetic visceral disease and hepatitis in 5 infants, 5 to 13 days of age. They suggested that 3 of these children were mechanically infected during the birth process from genital herpetic lesions of the mothers, and that the other 2 children were infected in utero. France and Wilmers (1953) did not isolate the etiologic agent from newborn twins who died soon after birth from clinical herpetic hepatitis or encephalitis, but they found herpes simplex virus inclusion bodies in the liver of each baby. By use of chorioallantoic membrane and

mouse inoculation techniques, McDougal, et al. (1954) isolated the herpes virus from the liver of a baby who died 11 days after birth. The infant's mother had suffered an undiagnosed "unspecific dermatitis" during the pregnancy. Colebatch (1955) demonstrated herpetic inclusion bodies in the liver cells of several newborn infants who succumbed to severe generalized herpes simplex infections. Hepatic necrosis in 2 neonatal patients who died of herpes simplex infection was reported by Williams and Jack (1955). No skin lesions were apparent in either case but microscopic sections of the liver of the children gave evidence of necrosis and herpetic inclusion bodies were found. By the suckling mouse, chorioallantoic membrane and rabbit cornea inoculation techniques, the virus was isolated from the liver of 1 child. Heath, et al. (1956) demonstrated the teratogenic effects of herpes simplex virus on early chick embryos. By inoculation of the virus on the membranes, they observed daily abnormal changes in the embryos. However, Miller, et al. (1950) noted a herpetic infection in a pregnant mother and after birth of the child it was observed for 9 months for neonatal herpetic lesions or malformations. None were seen.

Detection of Virus

Herpes simplex virus may be detected in specimens of tissues, organs and exudates by animal and tissue culture inoculation techniques. The chorioallantoic membrane method of inoculation of embryonated hen's eggs (Beveridge and Burnet, 1946) is probably the most widely used, but animal inoculation techniques are also extremely valuable. The suckling mouse has been demonstrated to be highly susceptible to the herpes virus

when inoculated by the intraperitoneal route (Kilbourne and Horsfall, 1951). The corneal route of inoculation of rabbits has long been used for demonstration of herpes virus in tissue homogenates and exudates from lesions (Löwenstein, 1919 and Grüter, 1920). Adult mice (Scott, 1956) are also used for this purpose. Guinea pigs (Bedson and Crawford, 1927), hamsters (Zarafonetis, et al. 1944) and the cotton rat (Florman and Trader, 1947) have been used less commonly for demonstration of the herpes virus. HeLa cells in culture have been widely adapted for use as an aid in the laboratory diagnosis of herpes simplex virus infections.

Direct microscopic examinations are also of assistance in the detection of this virus in tissue sections. Since the virus as a unit is submicroscopic in size, physical-optical examination of this entity has been limited to observation of the pathological lesions or to tissue staining techniques designed to demonstrate inclusion bodies formed by certain of the viruses. Diagnosis of viral diseases in general has depended upon clinical observation, serological reactions and in some cases, isolation of the virus in animals and tissue cultures as previously described. The development of a histochemical immunologic staining technique has proved to be of great assistance, not only in specific identification of viruses in tissue, but also in the rapid identification of viral, bacterial and parasitic species.

Early in the twentieth century, Bogert and Wright (1905) investigated the potentialities of nitro-derivatives of fluorescein as tools for specific labelling of antiserums to facilitate the demonstration of antigens in tissues. The next important report was that of Hop-

kins and Wormall (1933) who experimented with phenylisocyanate-protein compounds and the immunologic reactions which involved these antigens. They conjugated phenylisocyanate and p-bromophenylisocyanate to caseinogen, gelatin and horse serum. They employed the precipitin technique to demonstrate specific antigen-antibody reactions by layering the antigen and labelled antibody in a tube and observing the ring of fluorescence at the interface. Fieser and Creech (1939) conjugated amino acids with anthracene isocyanate and 1,2 benzanthracene isocyanate and described the chemical properties of the conjugated proteins. Creech and Jones (1940) conjugated 1,1 benzanthylisocyanate to horse serum and used ultraviolet light to determine if true conjugation occurred. During the years since Bogert and Wright (1905) first tested fluorescein derivatives as immunologic stains, the techniques and the compounds used were improved by each subsequent investigator.

Coons, et al. (1941) prepared beta-anthryl-carbamide derivatives and coupled them to antipneumococcal type III rabbit serum. The results demonstrated little difference between specific and nonspecific fluorescence. The report of Coons, et al. (1942) laid the foundation for the improved fluorescent antibody technique which now is widely used. They noted green fluorescence when their preparation of fluorescein isocyanate, which had been conjugated to antipneumococcal type III serum, was used to treat Diplococcus pneumoniae type III infected mouse tissue. In these preparations of infected tissue, only the microorganisms fluoresced and the background tissue did not. When uninfected tissue was treated in the same manner as the infected tissue, nonspecific blue fluo-

rescence but not specific green fluorescence was observed. The improved method of fluorescent antibody staining which was developed by Coons and Kaplan (1950) is the preferred technique now in general use. They prepared fluorescein amine fractions I and II from basic chemicals. When fraction II was converted to the isocyanate by treatment with phosgene, clearer and more consistant results were obtained than with fraction I.

Many viral agents and their resulting infections have been studied with the aid of the fluorescent antibody technique. These include mumps virus (Coons, et al. 1950, Watson, 1952_a, 1952_b), influenza virus (Watson and Coons, 1954, Liu, 1955_a, 1955_b, 1956), canine distemper virus (Moulton and Brown, 1954, Moulton, 1956, Liu and Coffin, 1956, 1957_a, 1957_b), vaccinia virus (Sawai, et al. 1955, Noyes and Watson, 1955), infectious canine hepatitis virus (Coffin, et al. 1953), psittacosis virus (Buckley, et al. 1955), primary atypical pneumonia virus (Liu and Eaton, 1955), Shope papilloma virus (Noyes and Mellors, 1957), varicella and herpes zoster viruses (Weller and Coons, 1954) and Egypt 101 virus (Noyes, 1955). O'Dea and Dineen (1957) studied the growth of virus and the resulting morphological changes in epithelial cell cultures infected with herpes simplex virus, and Lebrun (1956_a, 1956_b) used this technique to investigate cellular localization of the virus in infected mouse brains and human carcinoma cells in vitro.

Fluorescence microscopy as improved by Coons and Kaplan (1950) has been used widely. Not only has it found application in studies with viruses, but also with protozoa, bacteria, microbial products and tissues infected with these agents. The information obtained is invaluable espe-

cially because of the rapidity and accuracy of this method as compared with previous methods.

In order to study the pathogenesis of disease manifestions of herpes simplex virus in adult pregnant rabbits, a method of dissemination of this agent from the initial site of infection to the various tissues and organs had to be determined. Isolation and fluorescent antibody techniques were used to determine the extent of the viral dissemination.

CHAPTER II

MATERIALS AND METHODS

Young adult, white albino male and female rabbits which weighed approximately 8 pounds were obtained from a local rabbitry and used in this study. Virgin females were bred by males of the same pure line strain for studies of viral dissemination in fetuses. Three-weeks-old white Swiss mice were obtained from breeders who furnished pure line animals. In some instances pure line strains of mice were bred in this laboratory. The embryonated hen's eggs from Austria White and White Leghorn breeds of chickens were obtained from a local hatchery.

Three strains of herpes simplex virus (HF, HFt and K) were used in these investigations. The HF and HFt strains were obtained from the Communicable Disease Center, Montgomery, Alabama. The HF strain had been propagated 56 times on the chorioallantoic membrane (CAM) and 7 times in the yolk sac (YS) of the developing chick embryo, once in the brains of white Swiss mice (MB) and once on the cornea of the rabbit. The HFt strain had been propagated 7 times on HeLa cells in culture when it was received. Pools of virus were prepared of each of the HF and HFt strains of the herpes virus by a combination of infected allantoic fluid and homogenate of infected CAM. These pools which contained in 0.5 ml at least 10^4 LD_{50} for YS were used as the inocula throughout these studies.

The K strain was isolated in this laboratory from a patient suffering a herpetic lesion at the oral mucocutaneous junction.

Throughout the investigations, all dilutions of the virus and all blood and tissue homogenates were prepared in appropriate amounts of sterile buffered gelatin saline (BGS) according to Scott (1956). For each ml of diluent, 200 units of penicillin (prepared from crystalline penicillin G Merck, Sharpe and Dohme Company, Philadelphia) and 200 mg of streptomycin (prepared from streptomycin sulfate Squibb, E. R. Squibb and Sons, New York) were added to control possible bacterial contamination. All glassware, instruments and other materials were properly sterilized by the dry air oven method (180 C for 2 hours) or by autoclaving (15 pounds pressure, 121 C, for 15-30 minutes). Representative samples of homogenates and virus pools were tested for bacterial sterility before use by placing aliquots of the material in broths and on solid media for aerobic and anaerobic bacteriological studies.

Herpes simplex immune serum was prepared by immunization of young adult rabbits. One-half ml of allantoic fluid containing formalin inactivated herpes simplex virus was injected subcutaneously into the animals 3 times a week for 2 weeks. After a lapse of 1 week, 1 ml of the inactivated virus suspension was inoculated subcutaneously. The animals were permitted to rest for 1 week and were then bled aseptically by cardiac puncture. The serums were collected and adsorbed with dried, mouse liver powder to remove nonspecific fluorescing material.

Fluorescein isocyanate prepared according to the method of Coons and Kaplan (1950) was purchased from the Sylvania Chemical Company,

Orange, New Jersey, and it was conjugated to the herpes simplex immune serum by the following method. Ten and one-half ml of phosphate buffered saline (PBS), 3 ml of carbonate-bicarbonate buffer (0.5 M, pH 9.0), 2.5 ml of dioxane and 0.5 ml of calcium sulfate-dried acetone were placed in a 50 ml Erlenmeyer flask which was in an ice bath. When the temperature of the mixture dropped to 0 to minus 2 C, 3 ml of antiserum was added. In a test tube, 0.5 ml of fluorescein isocyanate (based on the use of 0.05 mg of fluorescein amine II per mg of protein in the gamma globulin fraction of the antiserum) was added dropwise to a mixture of 1 ml of dried acetone and 0.5 ml of dioxane which did not contain antiserum. The fluorescein isocyanate mixture was then added to the cold antiserum mixture in the flask and stirred for 18 hours. After this process, the solution was transferred to a cellophane sack and dialyzed against daily changes of PBS until no fluorescence was observed when the dialyzing saline was examined with ultra-violet light (5 to 6 days). The solution was then centrifuged to remove the precipitate which formed and merthiolate (1/10,000) was added. The conjugate was stored at 4 C in a tightly stoppered vial.

Dissemination of the Virus

Rabbits were anesthetized lightly with ether and the corneas of both eyes of each rabbit were scarified with a piece of sterile wire gauze. The wounding process penetrated the epithelial cells. One-tenth ml of the HF or K strain of herpes simplex virus was massaged into the scarifications of the left eye and 0.1 ml of BGS which contained no virus was massaged into the right eye of each animal. Blood samples were ob-

tained aseptically from the marginal ear vein 24 and 48 hours after inoculation from all rabbits studied. From the first 5 animals additional blood samples were removed every 6 hours for 102 hours; from 5 more, samples were taken every 12 hours for 132 hours and from a group of 6 more rabbits, the bleeding schedule was every 12 hours for 180 hours. Normal rabbits which had been inoculated only with BGS were treated in a similar manner to the virus inoculated animals. Specimens from these animals served as controls to determine if the plaques formed on the CAM were caused by the blood or the virus. The blood samples were inoculated onto CAM in 0.1 ml amounts according to the method of Beveridge and Burnet (1946) and incubated for 72 hours. The membranes were then harvested and examined for plaques as evidence of herpetic infection.

Forty-eight hours after inoculation of the rabbits, washings were obtained from the left and right eyes of each animal. Sterile BGS was dropped into each eye and the eyes were swabbed with sterile, cotton-tipped applicators. One-tenth ml of this material was used to inoculate CAM to determine if the virus were present in the washings.

Rabbits which died as a result of the experimental corneal infection were autopsied and homogenates of brain, heart, heart's blood, kidney, liver, lungs and spleen were prepared in BGS containing antibiotics and were inoculated onto CAM. The presence of the virus was determined by the production of typical herpetic plaques on the CAM.

In order to determine if the virus could spread from the site of initial inoculation on the cornea of the eye to the fetus of the rabbit, the following studies were made.

Young adult, white, virgin female rabbits were bred by males of the same strain. From 7 to 14 days after breeding, the females were lightly anesthetized with ether and inoculated on the scarified cornea with a suspension of the pooled virus. Two animals were inoculated on each of the following days after the breeding process; 7 days, 8 days, 11 days and 13 days. Three rabbits were inoculated 14 days after breeding. Blood specimens were withdrawn 24 and 48 hours following inoculation with the virus and at the time surgery was performed. Ocular washings were taken 48 hours after the corneal inoculation of the animals. These specimens were tested on the CAM for evidence of the presence of the virus.

At 54 or 96 hours following inoculation, the animals were anesthetized with ether or nembutal and surgical procedures were used to remove 1 gravid horn of the bifurcate uterus. The fetuses from the excised horns were dissected aseptically from the placental tissue and washed thoroughly in sterile physiological saline. Some of the fetuses from each rabbit were placed in sterile tubes, rapidly frozen at minus 52 C and stored at this temperature until later when microscopic sections were prepared. Fetuses from normal rabbits were removed, prepared and stored in a similar manner to those from herpes simplex virus infected animals. The remaining fetuses from each virus infected and normal rabbit were ground in a sterile tissue grinder and homogenates were prepared with BGS as the diluent. The homogenates of the fetuses from each rabbit were then inoculated onto the CAM of embryonated eggs to determine the presence of the virus.

The fluorescent antibody technique of Coons and Kaplan (1950)

was used as another means of proving that the virus had spread from the adult to its embryos. Frozen microscopic sections of fetuses from non-infected and herpes simplex virus infected animals were prepared and placed on glass slides. Some of these sections were treated with fluorescein-labelled specific immune serum in the following manner. The slides were placed in Petri dishes containing a piece of moist cotton. One drop of the labelled-antibody was placed on the section and allowed to remain for 20 minutes during which time the Petri dish was covered to prevent evaporation of the conjugate. The slides were then gently washed for 10 minutes in frequent changes of PBS. Coverslip mounts were prepared with 10 percent glycerol-phosphate buffer and sealed with Sealit (Fisher Scientific Company). A Reichert "Fluorex" 200 watt mercury vapor arc burner and the Reichert filter system for fluorescent microscopy were used as the source of illumination. The system consisted of 4 filters, 2 of which were ultra-violet transmitting filters and the others were opaque to visible light. Of the ultra-violet transmitting filters, 1 (colored black) was 1.5 mm thick and the other (colored violet) was 1 mm thick. Two blue filters were used to stop the transmission of visible light and a fifth filter, green in color, was attached to the ocular to prevent damage to the eye from the ultra-violet rays. The preparations were examined with a Bausch and Lomb monocular microscope fitted with a standard Bausch and Lomb cardioid dark field condenser and a 12 X ocular. Low power examinations were made with a 10 X objective and high, dry examinations were made with a 43 X objective. Photomicrographs were taken with an Exa camera on Anscochrome daylight color 35 mm film (Ansco

Company) and on K135 Tri X black and white 35 mm film (Eastman Kodak Company). FRX500 developer and Kodak acid stop were used to develop the negatives. Exposure time, when the low power objective was used, ranged from 30 to 45 seconds. The exposure time when the high power objective was used ranged from 1½ to 3 minutes.

As control slides for fluorescent studies, sections were prepared from the following materials and treated in the same manner as the slides of fetal sections: 1) CAM infected with virus isolated from the blood of herpes simplex virus inoculated rabbits; 2) normal CAM inoculated with sterile BGS; 3) CAM infected with vaccinia virus; 4) normal rabbit fetuses; 5) MB inoculated with virus isolated from the blood of herpes simplex virus infected rabbits; 6) normal MB inoculated with BGS. As an additional control frozen sections of the fetuses from herpes simplex infected rabbits were treated with unconjugated specific immune serum. These controls made it possible to test for the specificity of the antigen-antibody reaction under the given conditions. A reaction between specific immune serum (conjugated) and herpes simplex virus produced a yellow to yellow-green fluorescence. Areas of heavy concentration of the virus appeared to be almost white due to intense fluorescence. Nonspecific fluorescence due to reactions other than antigen-antibody were blue in color.

Representative microscopic sections from fetuses of each herpes simplex virus infected rabbit were stained with hematoxylin and eosin according to Harris (1957) and examined for the presence of herpes simplex virus inclusion bodies. Examinations were made with a Leitz light micro-

scope using the oil immersion objective (95 X). Photomicrographs were made with Kodak K135 Plus X black and white 35 mm film. Exposure time was 2 seconds with a light source of 1.6 foot candles.

To confirm the isolation of herpes simplex virus from the blood of the corneally infected rabbits by the CAM technique homogenates of the infected CAM were prepared in BGS and intracerebrally inoculated into MB. To aid in the identification of the virus isolated from the blood and fetuses of herpes simplex virus infected rabbits, neutralization tests were performed according to the method of Scott (1956). Serial dilutions of the virus (10^0 to 10^{-8}) were prepared in BGS. These dilutions were combined with herpes simplex virus specific immune serum (0.50 ml virus plus 0.50 ml antiserum). Following incubation for 1 hour, the mixtures were inoculated onto CAM. The membranes were harvested after incubation and examined for evidence of decreased infectivity of the virus for CAM in the presence of the immune serum. The results obtained were compared with those from another, parallel series in which BGS was used instead of immune serum.

CHAPTER III

RESULTS

Forty-eight hours after corneal inoculation with herpes simplex virus, the left eye of each of the rabbits exhibited severe swelling and redness with multiple vesicles on the cornea and nictitating membrane. When the 48 hour ocular washing from the left eye of each rabbit was inoculated onto the chorioallantoic membranes of the developing chick embryos (CAM), typical herpetic lesions developed. The control (right) eye of each rabbit was normal in gross appearance. No lesions were observed on the CAM which had been inoculated with the washings from the right eye of each rabbit.

Herpes simplex virus was demonstrated on the CAM after inoculation with blood samples from 16 rabbits which had been infected previously on the cornea of the left eye with this virus. A summary of the number of times the virus was isolated is shown in Table 1. The time is recorded in hours. The number of positive viral isolations is shown as the numerator and the number of isolations attempted is recorded as the denominator. On only 2 occasions was the virus not isolated from the specimens but due to the small number of animals in these groups the results are not statistically significant.

The number of hours after corneal inoculation when the virus

TABLE 1

SUMMARY OF HERPES SIMPLEX VIRUS ISOLATIONS FROM THE BLOOD OF RABBITS AFTER CORNEAL INOCULATION

	Hours at Which Blood Samples Were Taken																	
	24	48	54	60	66	72	78	84	90	96	102	108	120	132	144	156	168	180
Virus Isolations / Number of Isolations Attempted	10/16	10/16	6/7	13/16	4/5	5/16	0/2	7/16	1/5	12/16	0/4	8/11	7/11	7/11	5/6	3/6	3/5	4/5

was demonstrated in blood specimens of each individual rabbit is shown in Table 2. The virus was demonstrated in repeated samples which were obtained at different times from each rabbit. There were times when virus was not isolated from individual rabbits. In fact, certain blood specimens were obtained from each rabbit from which virus was not recovered. Typical herpetic lesions were not demonstrated on CAM after inoculation with blood specimens and ocular washings from the 3 control animals which had been inoculated with BGS.

Nine of the rabbits on which viral dissemination studies were done died after the development of symptoms indicative of a central nervous system involvement. Six of these were autopsied. The liver of each was discolored and enlarged. Other organs appeared to be normal except for the brains which were soft. Herpetic-like lesions were produced on CAM after inoculation with homogenates of brain, heart, heart's blood, kidney, liver, lungs and spleen. Isolations of herpes virus from organ homogenates of individual rabbits are recorded in Table 3. The virus was demonstrated in all homogenates of rabbit numbers 3, 13 and 14. However, the virus was not isolated from all homogenates of all the rabbits.

The time of death after corneal inoculation as shown in Table 3 is of interest. One rabbit died at approximately 96 hours, 1 at 156 hours, 3 between the twenty-first and twenty-third days and 1 at 3 months. Rabbit number 10 had apparently recovered from the corneal infection and it was being immunized with a formalin inactivated virus infected allantoic fluid suspension. This animal received 3 subcutaneous

TABLE 2

HERPES SIMPLEX VIRUS ISOLATED FROM BLOOD OF RABBITS AFTER CORNEAL INOCULATION

Animal Number	Hours at Which Blood Samples Were Taken																	
	24	48	54	60	66	72	78	84	90	96	102	108	120	132	144	156	168	180
* 1	-	+	-	+	+	+		-	+	+								
* 2	-	-	+	+	+	-		-	-	+	-							
* 3	-	-	+	-	-	+		-	-	-	-							
10	-	-	+	+	+	-	-	-	-	+	-							
11	+	+	+	+	+	-	-	-	-	+	-							
12	+	+		+		-		+		+		+	+	-				
13	+	-		-		+		+		+		+	-	-				
14	+	-		-		-		-		+		-	-	-				
** 16	-	+		+		+		-		-		+	-	+				
17	+	-		+		-		-		-		+	+	+				
19	+	+	+	+		-		+		+		+	+	+	+	-		
21	+	+	+	+		-		-		-		+	+	+	+	+	-	-
27	+	+		+		-		+		+		-	+	+	+	-	+	+
28	+	+		+		+		+		+		+	-	+	-	-	-	+
29	-	+		+		-		+		+		+	+	+	+	+	+	+
30	+	+		+		-		+		+		-	+	-	+	+	+	+
***Control 1	-	-	-	-	-	-	-	-	-	-								
Control 2	-	-	-	-	-	-	-	-	-	-								
Control 3	-	-		-		-		-		-		-	-	-				

* Data obtained from Lee H. Riley, Jr., M. D.
** K-strain of virus used with this rabbit; the HF strain used with the others.
*** Controls inoculated with buffered gelatin saline.
\+ Herpetic lesions observed on the chorioallantoic membranes.
\- No herpetic lesions observed on the chorioallantoic membranes.

TABLE 3

HERPES SIMPLEX VIRUS ISOLATED FROM ORGAN HOMOGENATES
OF RABBITS WHICH DIED OF ENCEPHALITIS

Animal Number	Time of Death After Inoculation	Presence of Virus in Organ Homogenates						
		Brain	Heart	Heart's Blood	Kidney	Liver	Lungs	Spleen
1	96 hrs.	+	-	+	-	+	-	+
3	21 days	+		+				
10	3 mos.	+	-	+	-	+	+	+
13	21 days	+	+	+	+	+	+	
14	23 days	+	+	+			+	+
19	156 hrs.	+	-	+		+	+	-

\+ Herpetic lesions observed on chorioallantoic membranes.
\- No herpetic lesions observed on chorioallantoic membranes.

injections of this suspension during a 1 week period. The day after the third injection the animal developed an encephalitis and died. Virus was isolated from tissue homogenates.

Eleven female rabbits which were in the early gestation period were inoculated with herpes simplex virus by the corneal scarification method. Typical herpetic lesions were observed on CAM which had been inoculated with blood specimens that were obtained at 24 and 48 hours after inoculation of these animals with the herpes virus. The virus was demonstrated, also, in blood specimens obtained at the time of surgical removal of the fetuses (54 hours after corneal inoculation of 9 rabbits and 96 hours after infection of 2 others), as well as in ocular washings collected 48 hours after experimental infection.

Typical herpetic lesions were produced on CAM after inoculation with homogenates of fetuses which were removed surgically at 54 or 96 hours after viral inoculation. Results of these studies are recorded in Table 4. The virus was isolated from homogenates of fetuses of 11 experimentally infected mother rabbits. The age of the fetuses at time of infection of the mother, as shown in Table 4 under the heading "Days Elapsed between Breeding and Inoculation", was varied but was not, apparently, an important factor because homogenates of fetuses from all the experimental animals contained the virus. When blood specimens, ocular washings and fetal homogenates from 2 uninfected rabbits were inoculated onto CAM, typical herpetic lesions were not observed. An interesting example of recurrent herpes simplex was observed in rabbit number 25. When she delivered 3 full term offsprings, this animal suffered a severe recurrent

TABLE 4

HERPES SIMPLEX VIRUS ISOLATED FROM FETAL HOMOGENATES
OF EXPERIMENTALLY INFECTED RABBITS

Animal Number	Days Elapsed between Breeding and Inoculation	Hours After Inoculation When Fetuses Were Removed	Presence of Virus in Fetal Homogenates
19	14	54	+
23	7	54	+
24	7	54	+
25	8	54	+
26	8	54	+
27	11	54	+
34	11	54	+
35	13	54	+
36	13	54	+
37	14	96	+
38	14	96	+

+ Herpetic lesions observed on chorioallantoic membranes.

keratoconjunctivitis of the eye which had previously healed after the initial corneal infection. Shortly after this, she developed an encephalitis and died.

Frozen microscopic sections from fetuses taken from 10 of the 11 rabbits were studied after they were stained with fluorescein-labelled herpes simplex virus immune serum. When these preparations were examined by ultra-violet microscopy, areas of yellow-green to green fluorescence were seen which indicated specific antigen-antibody reaction. These areas of specific reaction were within the tissues of the fetuses and indicated that herpes simplex virus was present in the tissues. The morphology of the areas of specific fluorescence varied. Small, scattered fluorescent areas were observed as well as large areas of intense fluorescence which probably represented maximum concentration of the virus. There was no correlation between the type of microscopic lesion observed and the age of the fetus at the time of infection of the rabbit. The time of surgery after the experimental infection apparently did not influence the observed results. Figures 1 through 10 are photomicrographs of sections of fetal tissues from 5 of the 11 rabbits studied. Two magnifications, 120 X and 516 X are included. The bright white appearing spots are areas of specific fluorescence while the larger scattered areas of less intensity are, in part, areas of luminescence from the intense areas of fluorescence.

No areas of specific fluorescence could be seen in frozen microsections of fetuses taken from uninfected control rabbits stained with fluorescein labelled herpes simplex immune serum (Figures 11 and 12).

FIGURE 1

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 34 which was infected with herpes simplex virus 7 days after breeding. (X 120).

FIGURE 2

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 34 which was infected with herpes simplex virus 7 days after breeding. (X 516).

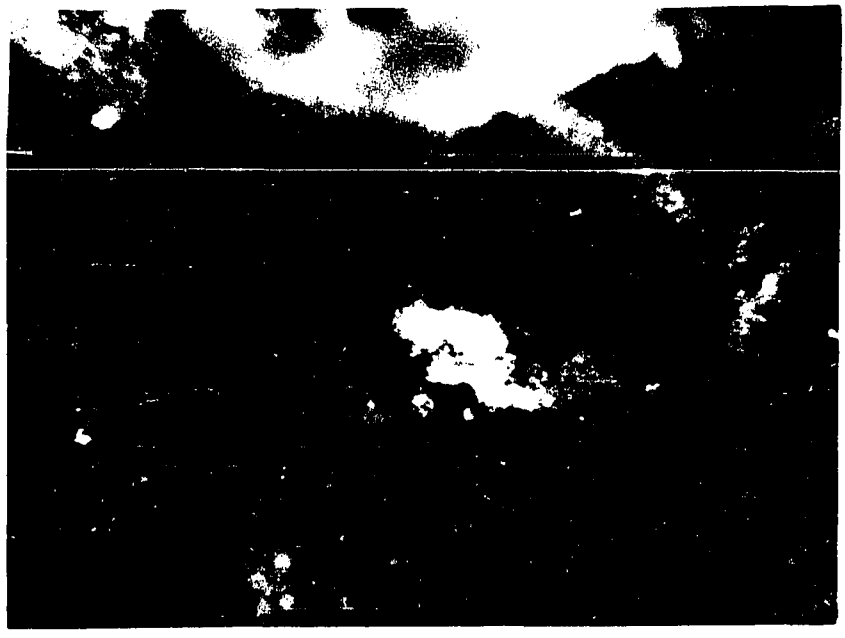

FIGURE 3

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 25 which was infected with herpes simplex virus 8 days after breeding. (X 120).

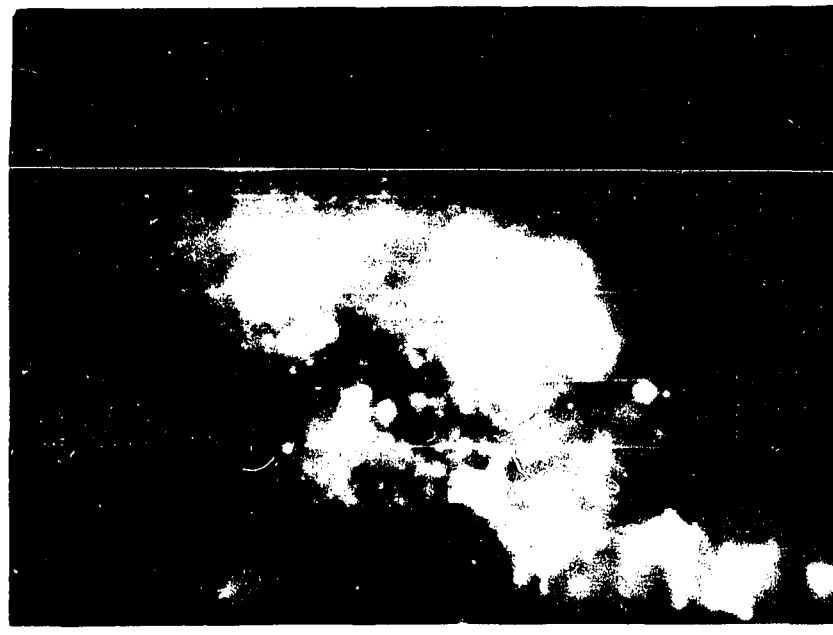

FIGURE 4

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 25 which was infected with herpes simplex virus 8 days after breeding. (X 516).

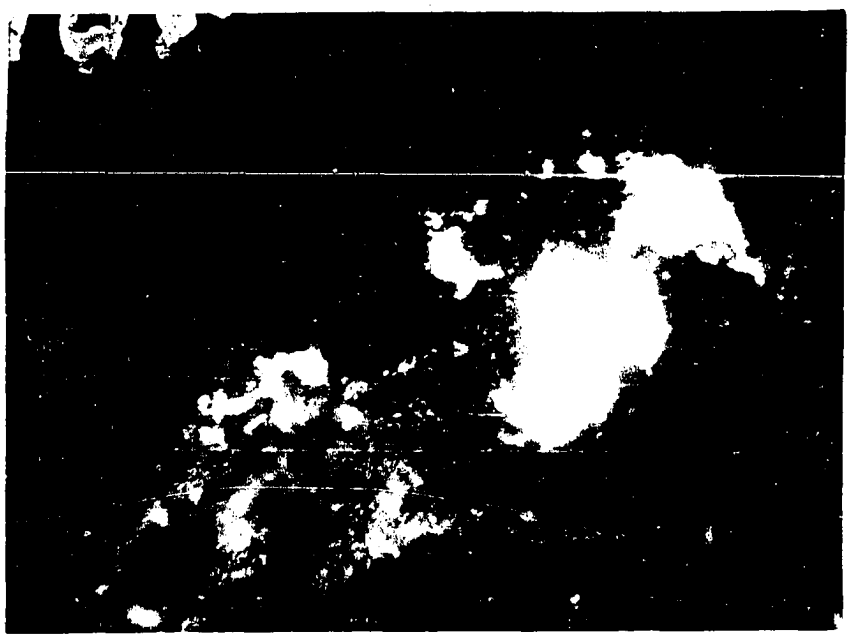

FIGURE 5

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 37 which was infected with herpes simplex virus 11 days after breeding. (X 120).

FIGURE 6

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 37 which was inoculated with herpes simplex virus 11 days after breeding. (X 516).

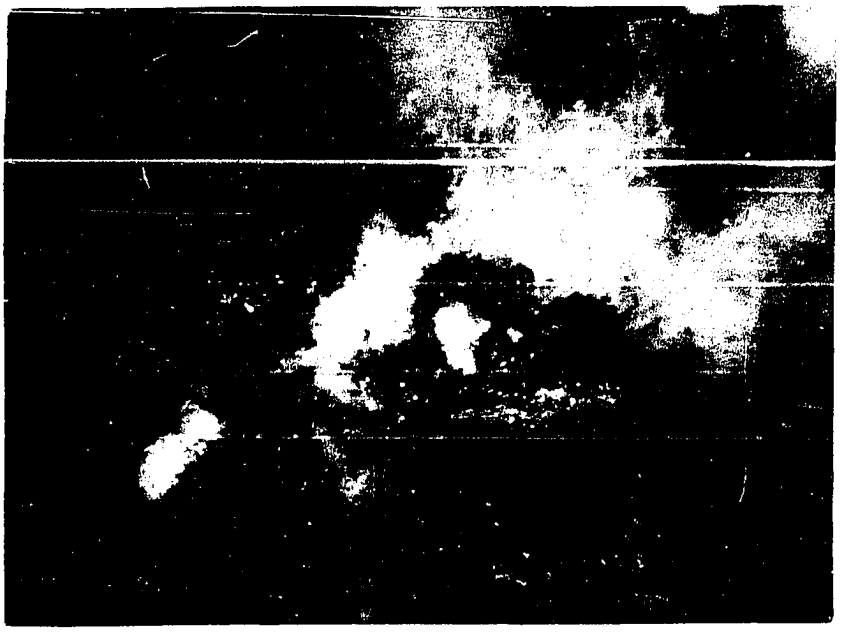

FIGURE 7

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 27 which was infected with herpes simplex virus 13 days after breeding. (X 120).

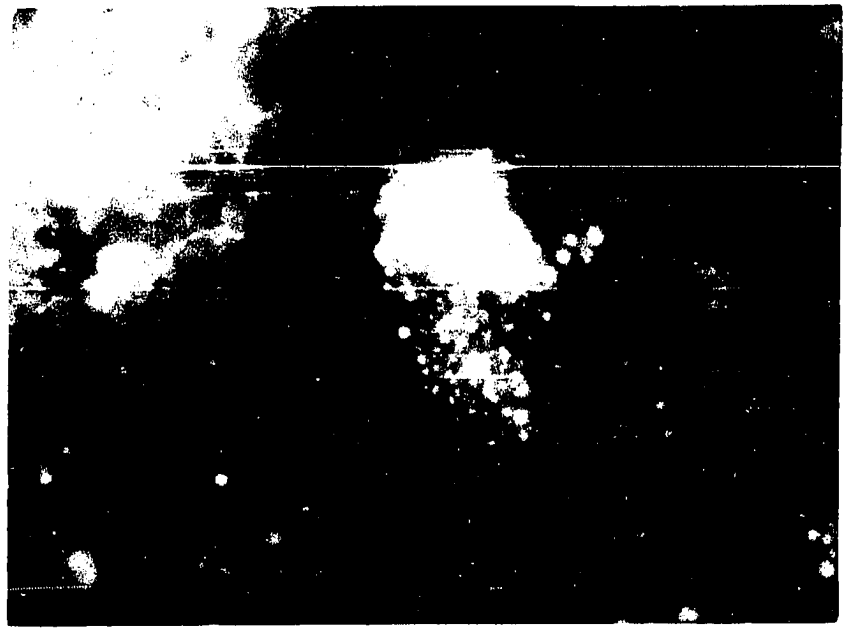

FIGURE 8

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 27 which was inoculated with herpes simplex virus 13 days after breeding. (X 516).

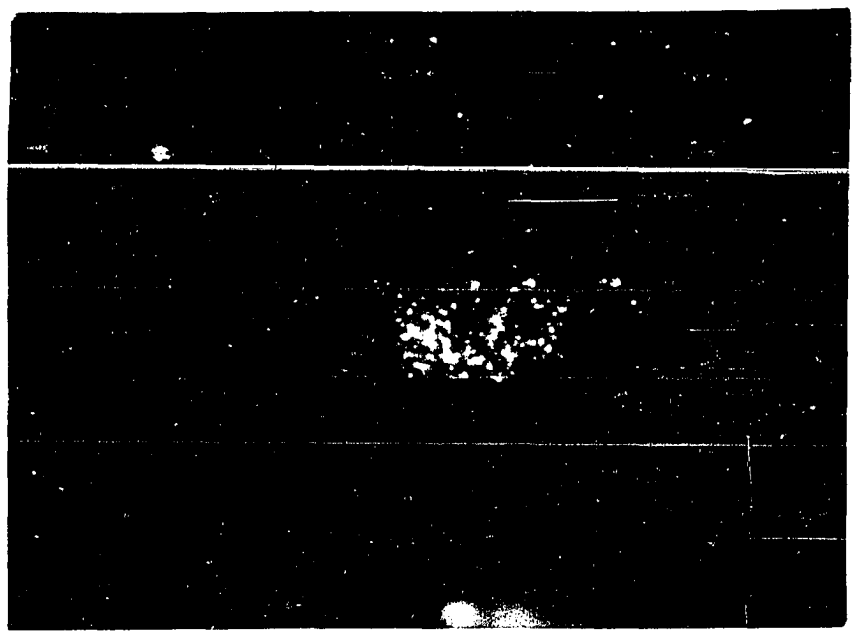

FIGURE 9

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 36 which was inoculated with herpes simplex virus 14 days after breeding. (X 120).

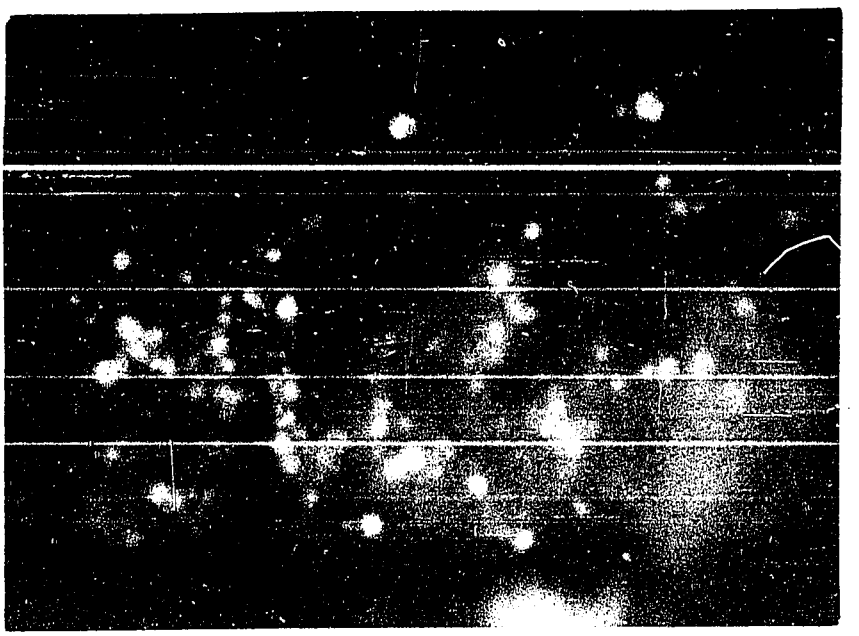

FIGURE 10

A fluorescein-labelled specific antiserum stained section of fetus from rabbit 36 which was inoculated with herpes simplex virus 14 days after breeding. (X 516).

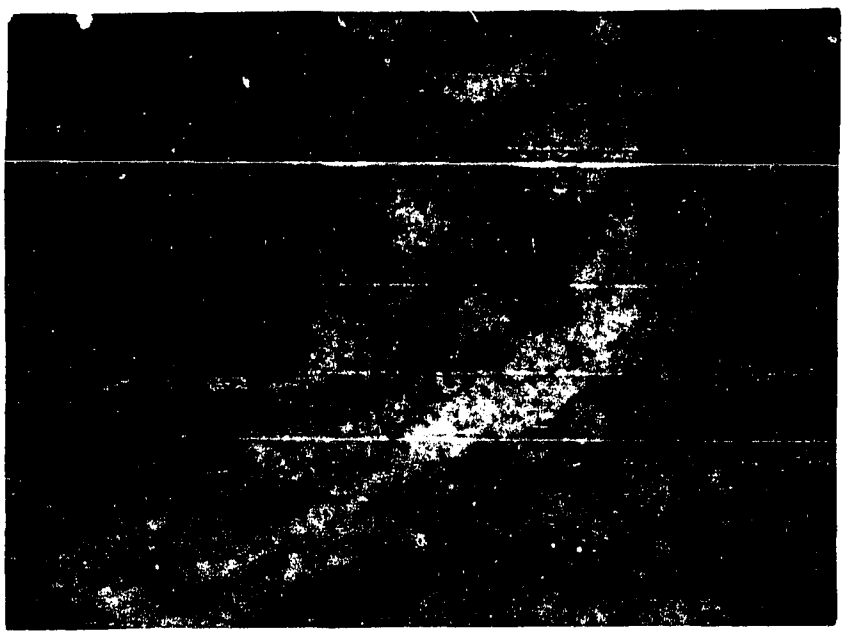

FIGURE 11

A fluorescein-labelled specific antiserum stained section of fetus from an uninfected rabbit. (X 120).

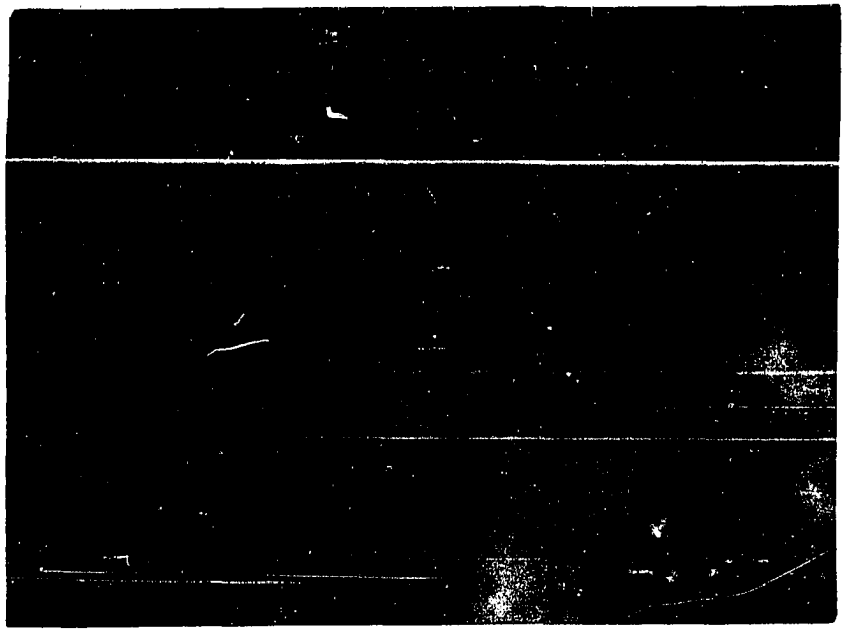

FIGURE 12

A fluorescein-labelled specific antiserum stained section of fetus from an uninfected rabbit. (X 516).

This test then indicated the absence of virus in the tissues of the control animals.

In addition to the fetuses from rabbits which were not infected other controls were included. White Swiss mice were inoculated intracerebrally with herpes simplex virus which had been isolated on CAM from the blood of an experimentally infected rabbit. This virus produced death in mice in a manner typical of herpes simplex infection. Frozen microscopic sections of the brains of uninfected as well as those of infected mice were prepared, stained with fluorescein-labelled antibody and examined by ultra-violet light microscopy. Areas of specific fluorescence, similar to those in the infected fetal sections, were observed in sections of infected, but not of uninfected, mouse brains (Figures 13 through 16). The chorioallantoic membranes of embryonated hen's eggs which had been infected with herpes simplex virus isolated from the blood of experimentally infected rabbits, vaccinia virus infected CAM and uninfected CAM were also examined by the fluorescent antibody technique. Whereas areas of specific fluorescence were observed in the herpes simplex virus infected membranes, no specific fluorescence was observed in the vaccinia virus infected or uninfected membranes. As an additional control, sections of fetuses from herpes simplex virus infected rabbits stained with unconjugated specific immune serum yielded no evidence of a specific antigen-antibody reaction in the form of green fluorescence (Figure 17).

Representative microscopic sections of fetuses of herpes simplex virus infected rabbits were stained by the hematoxylin and eosin

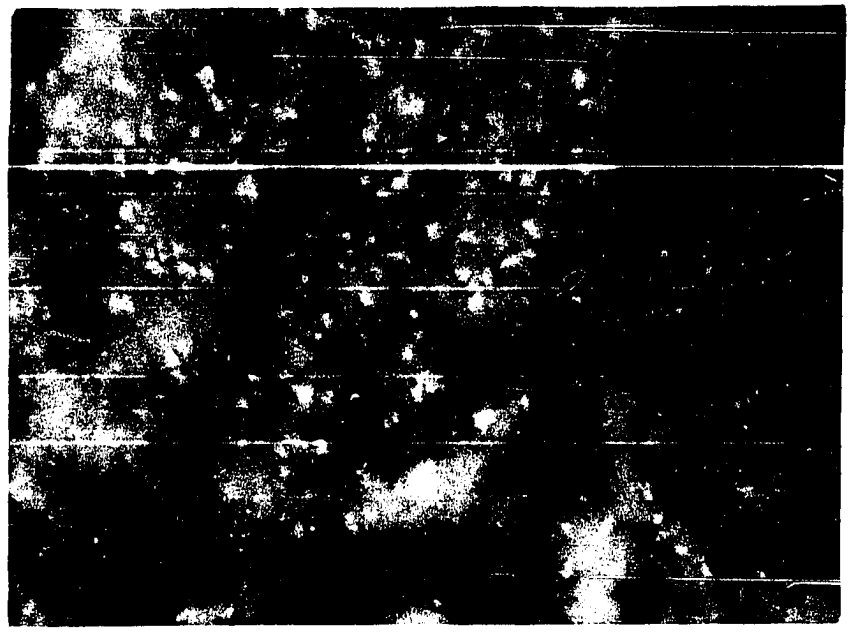

FIGURE 13

A section of mouse brain infected with herpes simplex virus and stained with fluorescein-labelled specific antiserum. (X 120).

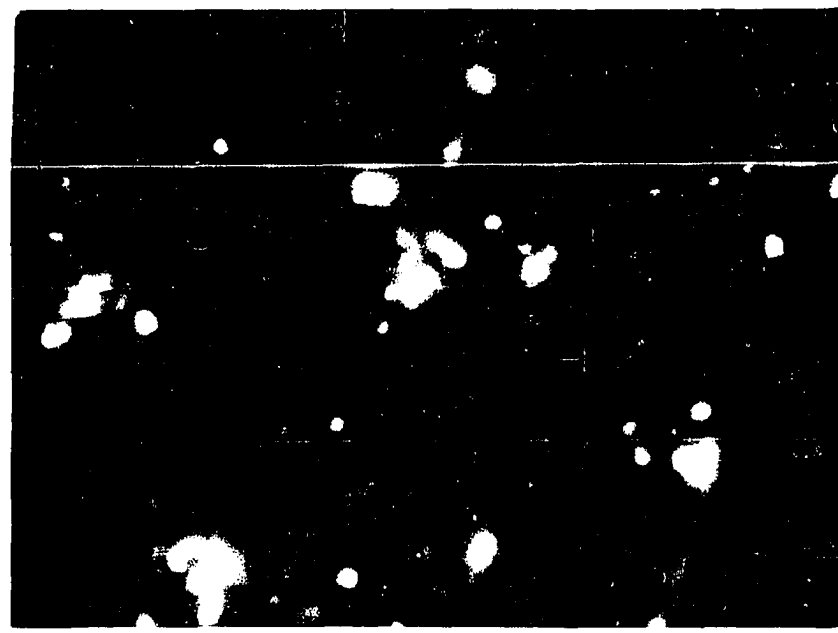

FIGURE 14

A section of mouse brain infected with herpes simplex virus and stained with fluorescein-labelled specific antiserum. (X 516).

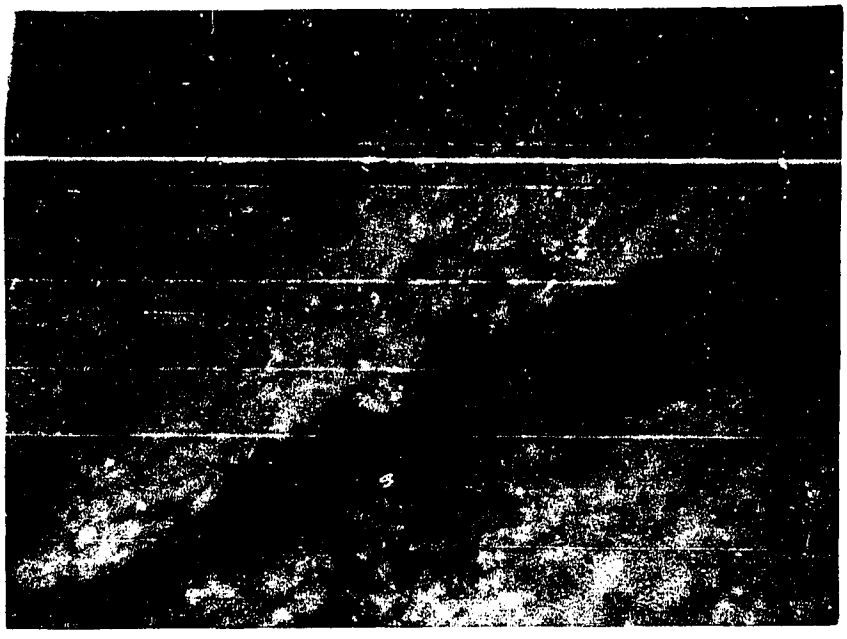

FIGURE 15

Section of uninfected mouse brain which was stained with fluorescein-labelled specific antiserum. (X 120).

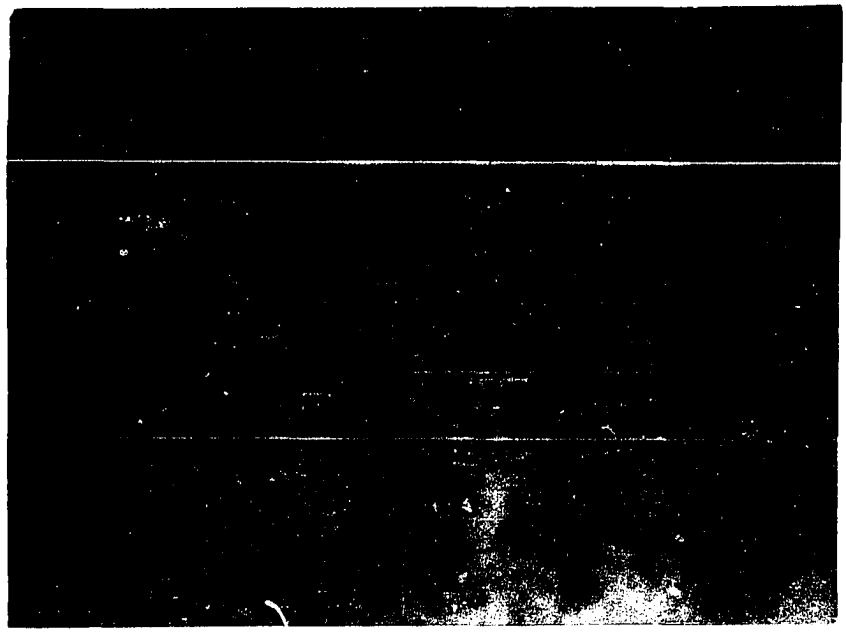

FIGURE 16

Section of uninfected mouse brain which was stained with fluorescein-labelled specific antiserum. (X 516).

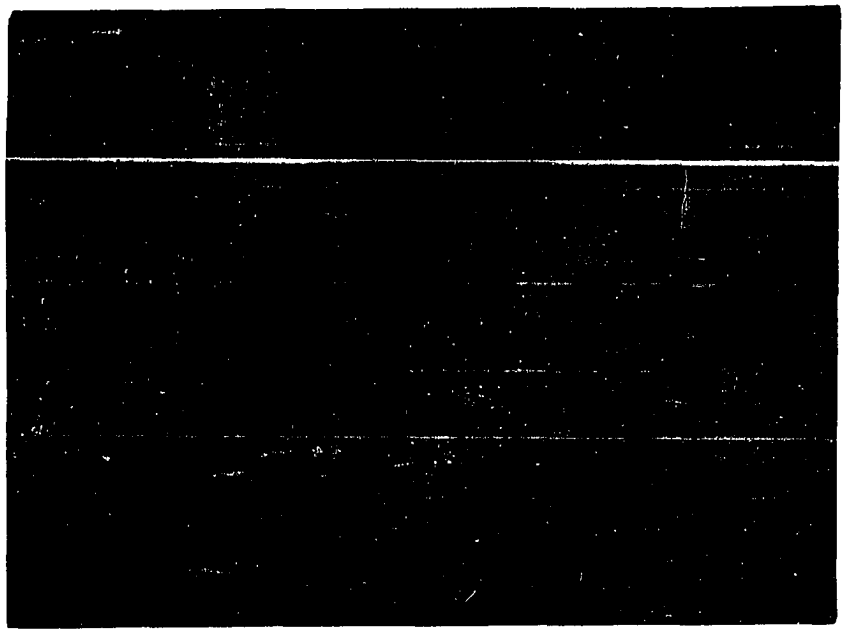

FIGURE 17

Unlabelled specific antiserum stained section of fetus from rabbit 37 which was infected with herpes simplex virus 11 days after breeding. (X 120).

method and examined for herpetic inclusion bodies. These preparations were stained first with fluorescein-labelled specific antiserum and later stained with hematoxylin and eosin. Typical herpetic inclusion bodies appear in the nucleus and the cytoplasm of cells as pink masses enclosed in a clear halo. Such inclusion bodies as seen in Figures 18 through 20 were observed in representative samples of fetuses from each of the 10 rabbits studied. Multinucleated cells and vacuolated cells were observed as well as intranuclear and intracytoplasmic inclusion bodies. A multinucleated cell with several inclusion bodies is shown in Figure 18.

Neutralization tests were performed to aid in the identification of the virus isolated from the blood and from the fetuses of experimentally infected rabbits. The infectivity for the CAM of the virus which was isolated from the blood was greatly reduced in the presence of specific immune serum as compared with the infectivity of the virus diluted in BGS. The comparative infectivity titers of the viruses isolated from the blood of rabbit numbers 16 and 17 are shown in Table 5. The comparative infectivity titers of the viruses isolated from the fetuses from rabbit numbers 26 and 35 are shown in Table 6. The reduction of infectivity in the presence of specific immune serum identifies the virus isolated from the blood and fetuses of infected animals as herpes simplex virus.

When homogenates of CAM infected with the herpetiform plaque producing agent, which was isolated from the blood of infected rabbits, was injected intracerebrally into mice, symptoms of encephalitis were observed. This central nervous system involvement was typical of that produced by herpes simplex virus.

FIGURE 18

A hematoxylin and eosin stained section of herpes simplex virus infected fetus from rabbit 34. Note the multinucleate cells and the presence of intranuclear inclusion bodies. The slide was first stained with fluorescein-labelled specific antiserum for fluorescent studies (Figures 1 & 2). (X 950).

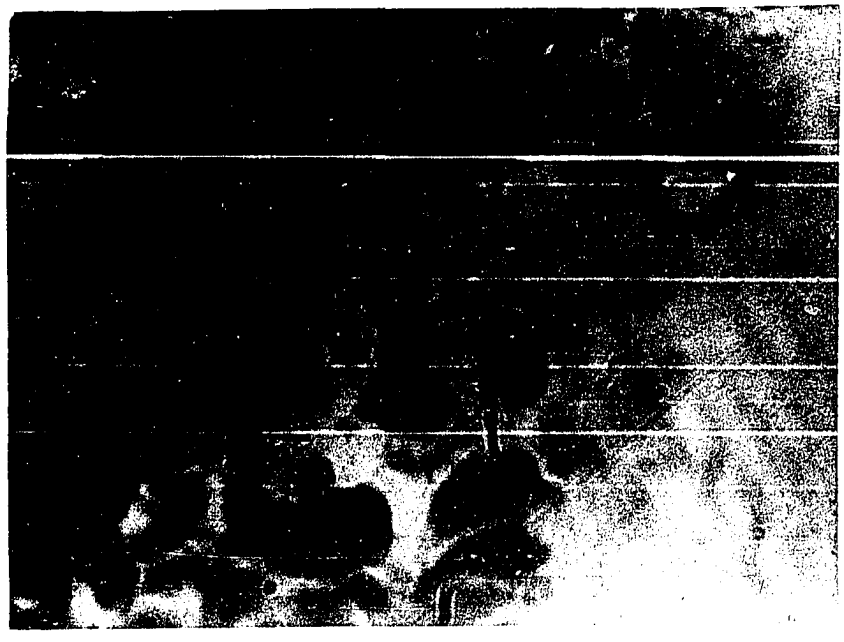

FIGURE 19

A hematoxylin and eosin stained section of herpes simplex virus infected fetus from rabbit 27. Note the presence of the intranuclear inclusion body. The slide was first stained with fluorescein-labelled specific antiserum for fluorescent studies (Figures 7 & 8). (X 950).

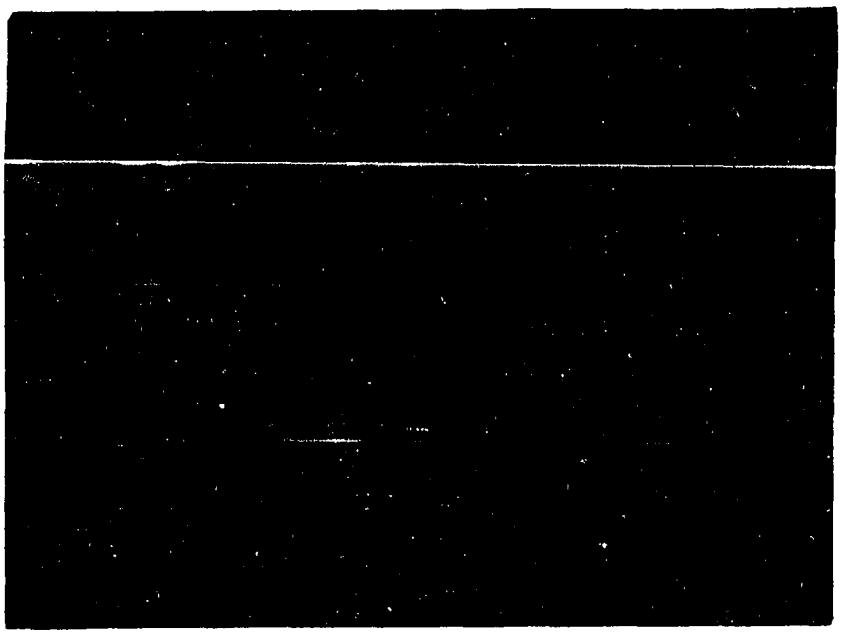

FIGURE 20

A hematoxylin and eosin stained section of herpes simplex virus infected fetus from rabbit 37. Note the presence of the intranuclear inclusion bodies. The slide was first stained with fluorescein-labelled specific antiserum for fluorescent studies (Figures 5 & 6). (X 950).

TABLE 5

IDENTIFICATION OF HERPES SIMPLEX VIRUS ISOLATED FROM BLOOD OF RABBITS BY COMPARATIVE INFECTIVITY TITRATIONS

Animal Number	Inoculation on CAM	Dilution of Virus								
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
16	Virus-Antiserum Mixture	++++	++++	+++	++	+	-	-	-	-
	Virus-Saline Mixture	++++	++++	++++	++++	+++	++	+	-	-
17	Virus-Antiserum Mixture	++++	+++	+++	++	-	-	-	-	-
	Virus-Saline Mixture	++++	++++	+++	++	++	+	-	-	-

CAM Chorioallantoic membranes.
++++ More than 25 plaques observed on CAM.
+++ From 16-25 plaques observed on CAM.
++ From 6-15 plaques observed on CAM.
+ From 1-5 plaques observed on CAM.
- No plaques observed on CAM.

TABLE 6

IDENTIFICATION OF HERPES SIMPLEX VIRUS ISOLATED FROM FETAL HOMOGENATES OF RABBITS BY COMPARATIVE INFECTIVITY TITRATIONS

Animal Number	Inoculation on CAM	Dilution of Virus								
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
26	Virus-Antiserum Mixture	++++	++++	++++	+++	++	+	-	-	-
	Virus-Saline Mixture	++++	++++	++++	++++	++++	+++	++	+	-
35	Virus-Antiserum Mixture	+++	+++	++	+	+	-	-	-	-
	Virus-Saline Mixture	++++	++++	+++	++	++	+	+	-	-

CAM Chorioallantoic membranes.
++++ More than 25 plaques observed on CAM.
+++ From 16-25 plaques observed on CAM.
++ From 6-15 plaques observed on CAM.
+ From 1-5 plaques observed on CAM.
- No plaques observed on CAM.

CHAPTER IV

DISCUSSION

The demonstration of the virus in the blood of 16 rabbits after establishment of experimental infection with herpes simplex virus proves conclusively that a regularly occurring viremia can exist during certain stages of the disease. An optimal time for the demonstration of the viremia cannot be stated because of the variation of the numbers of isolation attempts at the various time intervals. This lack of statistical validity can only be overcome by the use of large numbers of specimens at each time interval. The results indicate that while the viremia may be transient, it is not accidental. The total duration of the viremia is not known; the virus was present in the 180 hour blood specimens of 4 of 5 rabbits tested. It is possible that the eventual disappearance of the virus from the blood of these experimentally infected animals can be correlated with the appearance of an effective concentration of specific herpes simplex antibodies in the blood.

When Bedson (1931) used a formalized suspension of herpes simplex virus to immunize guinea pigs, antibodies appeared in 7 to 8 days. Burnet and Lush (1939) reported that antibodies to herpes simplex virus appeared on the seventh day after intracerebral inoculation of mice with the virus. By use of the neutralization test Florman and Trader (1947)

determined that a significant titer of herpes virus antibody occurred from 3 to 6 weeks in rabbits which had been infected with the virus by the corneal scarification method. Buddingh, et al. (1953) examined the serums from blood specimens of children who were suffering from a severe stomatitis on admission to the hospital. The investigators demonstrated the appearance of herpes simplex virus antibodies from the fourth to the seventh day after the appearance of the clinical lesion. The exact time when these antibodies first appear has not been established; that is, the duration of time from inoculation (incubation period plus acute phase in a clinical disease) until antibodies are demonstrable. Such a study would aid in explaining the disappearance of virus from the blood.

There is evidence derived from studies of laboratory animal and human infections that other viruses are spread by the blood. Cheatham (1953) confirmed the idea that the virus of herpes zoster is spread by the blood stream from local lesions to areas throughout the body. Kilham (1949) postulated that mumps virus encephalitis resulted from the hematogenous dissemination of the virus from the parotid glands to the central nervous system. Shaffer, et al. (1941) infected children by inoculating them with blood from patients in the early stages of rubella infection. Bodian (1954) and Horstman (1952) reported the existence of a viremia stage in animals which had been experimentally infected with the poliomyelitis virus. The reports of these investigators together with the evidence submitted in this report emphasize the fact that the hematogenous dissemination of viruses from the site of primary infection to other parts of the body is of great importance in some of the disease

manifestations which result from viral infections.

From the present study further evidence of the significance of the hematogenous spread of the herpes virus is obtained from demonstration of the virus in homogenates of tissues and organs of rabbits which died of herpetic encephalitis. However, it should be pointed out that, although the virus was isolated from these homogenates, its presence in foci of infection within the tissue cells was not confirmed. It is possible that the virus was present in the residual blood in the organs. Reports of fatal hepatitis infections of infants and demonstration of herpetic inclusion bodies in the liver cells of these children, together with the gross pathological involvement of the livers of the rabbits studied here, seem to indicate that the virus was carried to the organs by the blood stream.

The role of circulating antibodies in the prevention of recurrent attacks of this disease has not been adequately studied. Brain (1932) and Jawetz, et al. (1952) have shown that there is no correlation between the titer of neutralizing antibody and the severity of recurrent infections. The titers of antibody of rabbit numbers 10 and 25 were not determined; however, a recurrent infection occurred in each as demonstrated by viral isolation. The recovery from the primary infection and the healthy appearance of these animals between the primary and secondary attacks gave no evidence of continuing infection. The herpes virus must have been dormant in the tissues of these rabbits after recovery from the primary infection. The immunization process which may have involved a hypersensitization to the virus inoculum may have been the precipita-

ting factor in the recurrent disease in rabbit number 10 while the stress of the natural birth process may have played a similar role with rabbit number 25. These same factors have been implicated in recurrent infections in human beings along with other triggering mechanisms such as fever, shock, menstruation and emotional disturbance.

Herpes simplex virus was recovered from homogenates of fetuses of experimentally infected pregnant rabbits by the chorioallantoic membrane inoculation method. To prove that the virus was present in the fetal tissue, histochemical techniques were used. Fluorescent antibody studies produced evidence that the virus was present in sections of the tissues of the infected fetuses. Sites of specificity were observed to be yellow-green to green fluorescent areas where antigen-antibody reactions occurred. Certain controls demonstrated the specificity of the fluorescence. Sections of herpes simplex virus infected mouse brain, when treated in the same manner as the sections of infected fetuses, gave similar results. No evidence of specific fluorescence was seen in sections of uninfected fetuses and mouse brains. No areas of specific fluorescence were observed when vaccinia infected and uninfected chorioallantoic membranes were treated with fluorescein-labelled herpes simplex immune serum and examined under ultra-violet microscopy. Significantly, herpes simplex virus infected chick embryo membranes showed areas of specific antigen-antibody reaction by this technique. To demonstrate further the specificity of this technique, sections of herpes virus infected fetuses were treated with unconjugated specific antiserum. Examination of these sections revealed no fluorescence. Thus the reliability

of this method as a research tool and diagnostic aid is definitely established. Although the virus was demonstrated to be present in the tissues of the fetuses, the type of cell or tissue infected was not determined.

The demonstration of the invasion and infection of fetal tissue by this virus was further substantiated by the demonstration of herpetic inclusion bodies in restained sections of fetuses in which specific fluorescence had been observed. Thus, the classical methods of isolation and identification of a virus (the neutralization tests, the demonstration of herpetic inclusion bodies and the isolation of the virus on the chorioallantoic membranes of developing chick embryos) have been satisfied and the results obtained by the fluorescent antibody technique have been confirmed.

The demonstration of the invasion of fetal tissues of pregnant rabbits by herpes simplex virus gives further evidence of the role which hematogenous dissemination of the virus may play. It also suggests another possible etiologic agent of disease, including congenital malformations, in newborn infants. Fetal anomalies which resulted from the infection of pregnant mothers with other viruses have been described. Gregg (1941, 1945), Swan (1944, 1949), Swan, et al. (1944), and Swan, et al. (1943) and many other workers have presented overwhelming clinical and epidemiological evidence which has incriminated the rubella virus as a major cause of congenital anomalies. Fox, et al. (1948) and Holowach, et al. (1957) reported that maternal measles, mumps and chickenpox infections during pregnancy cause abortion and unilateral hairlip in infants.

Freudenberg, et al. (1952) isolated Coxsackie virus from a myositis of the hip of an infant undergoing repairative surgery to correct a bilateral hip malformation. With this evidence it is possible to postulate that some of the abortions and malformations of infants might result from herpes simplex virus recurrent infections of the mother. These recurrences may not be evident to the patient since, in general, the population is unaware that "fever blisters" are manifestations of a viral infection. Clinical and epidemiologic studies might yield further information on the effect of maternal herpes simplex infections on the unborn child. If herpes simplex virus is responsible for congenital anomalies, the demonstration of the type of fetal tissue for which it may have an affinity could prove of value in forecasting the type of malformation to expect.

CHAPTER V

SUMMARY

A regularly occurring viremia was demonstrated in 16 rabbits, which had been inoculated on the scarified cornea with herpes simplex virus. The presence of the virus in the blood of the animals was proved by chorioallantoic membrane (CAM) and mouse inoculation techniques. No virus was recovered from the blood of control rabbits inoculated with buffered gelatin saline.

Typical herpetic lesions were observed on CAM after inoculation with homogenates of organs obtained from 6 rabbits which died from herpetic encephalitis.

Eleven pregnant rabbits were inoculated with the herpes virus on the scarified cornea of the eye. Fetuses from these animals were removed surgically and examined for the presence of the virus. The virus was demonstrated by the formation of herpes simplex plaques on the CAM and by specific fluorescence observed in fluorescein-labelled antibody stained tissue sections of fetuses from 10 of the rabbits. In the same fluorescein stained sections the presence of typical herpetic inclusion bodies in hematoxylin and eosin stained sections demonstrated that foci of viral infection existed in the tissue. The homogenates of fetuses from uninfected rabbits did not produce lesions on the CAM and fluores-

cein-stained sections of uninfected fetuses did not contain areas of specific fluorescence. The infectivity for the CAM of the viruses which were isolated from the blood and fetuses were greatly reduced in the presence of specific immune serum. This proved that the viruses isolated were herpes simplex virus.

BIBLIOGRAPHY

Abe, T. 1926 Experimenta über die durch Herpes Virus erzeugta sympathische ophthalmie (v. Szily) unter besonderer Berücksichtigung der Fortleitungsbahn. Arch. Ophth. 117: 375-398.

Anderson, K. 1939 The encephalitogenic property of herpes virus. Science 90: 497.

Anderson, K. 1940 Pathogenesis of herpes simplex virus infection in chick embryos. Am. J. Path. 16: 137-155.

Armstrong, C. 1943 Herpes simplex virus recovered from spinal fluid of a suspected case of lymphocytic choriomeningitis. Pub. Health Rep. 58: 16-21.

Beale, A. J. and Hair, H. C. 1956 Rapid diagnosis of the herpetic variety of Kaposi's varicelliform eruption by tissue culture methods. Canad. M. A. J. 74: 443-448.

Bedson, S. P. 1931 Immunization with killed herpes virus. Brit. J. Exper. Path. 12: 254-260.

Bedson, S. P. and Bland, J. O. W. 1928 On the supposed relationship between the viruses of herpes febrilis and vaccinia. Brit. J. Exper. Path. 9: 174-178.

Bedson, S. P. and Crawford, G. L. 1927 Immunity in experimental herpes. Brit. J. Exper. Path. 8: 138-147.

Berry, G. P. and Slavin, H. B. 1940 The role of humoral immunity in the protection of the central nervous system of suckling mice against herpes virus given intranasally. J. Bact. 39: 66.

Berry, G. P. and Slavin, H. B. 1943 Studies on herpetic infection in mice. I. Passive protection against virus inoculated intranasally. J. Exper. Med. 78: 305-314.

Beveridge, W. I. B. and Burnet, F. M. 1946 The cultivation of viruses and rickettsiae in the chick embryo. Med. Res. Council, Special Report. Series #256, p. 16 and pp. 30-33.

Bodian, D. 1954 Viremia in experimental poliomyelitis. II. Viremia and the mechanism of the "provoking effect" after injection or trauma. Am. J. Hyg. 60: 358-370.

Bogert, M. T. and Wright, R. G. 1905 Some experiments on the nitro derivatives of fluorescein. J. Am. Chem. Soc. 27: 1310-1316.

Boyse, E. A., Morgan, R. S., Pearson, J. D. and Payling Wright, G. 1956 The spread of a neurotropic strain of herpes virus in the cerebrospinal axis of rabbits. Brit. J. Exper. Path. 37: 333-342.

Brain, T. R. 1932 The demonstration of herpetic antibody in human sera by complement-fixation and the correlation between its presence and infection with herpes virus. Brit. J. Exper. Path. 13: 166-171.

Buckley, S. M., Whitney, E. and Rapp, F. 1955 Identification by fluorescent antibody of the developmental forms of psittacosis virus in tissue culture. Proc. Soc. Exper. Biol. & Med. 90: 226-230.

Buddingh, B. J., Schrum, D. I., Lanier, J. C. and Guidry, D. J. 1953 Studies on natural history of herpes simplex infection. Pediatrics 1: 595-610.

Burnet, F. M. and Lush, D. 1939 Studies on experimental herpes infection in mice using the chorioallantoic technique. J. Path. & Bact. 49: 241-259.

Cheatham, W. J. 1953 The relation of heretofore unreported lesions to pathogenesis of herpes zoster. Am. J. Path. 29: 401-411.

Coffin, D. L., Coons, A. H. and Cabasso, V. J. 1953 A histological study of infectious canine hepatitis by means of fluorescent antibody. J. Exper. Med. 98: 13-20.

Colebatch, J. H. 1955 Clinical picture of severe generalized viral infection in the newborn. M. J. Australia 1: 377-382.

Coons, A. H., Creech, H. J. and Jones, R. N. 1941 Immunologic properties of an antigen containing a fluorescent group. Proc. Soc. Exper. Biol. & Med. 47: 200-202.

Coons, A. H., Creech, H. J., Jones, R. N. and Berliner, E. 1942 The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. 45: 159-170.

Coons, A. H. and Kaplan, M. H. 1950 Localization of antigen in tissue cells. II. Improvement in methods for detection of antigen by means of fluorescent antibody. J. Exper. Med. 91: 1-13.

Coons, A. H., Snyder, J. C., Cheever, F. S. and Murray, E. S. 1950 Localization of antigen in tissue cells. IV. Antigens of rickettsiae and mumps virus. J. Exper. Med. 91: 31-38.

Creech, H. J. and Jones, R. N. 1940 The conjugation of horse serum with 1,2 benzanthryl isocyanate. J. Am. Chem. Soc. 62: 1970-1975.

Doerr, R. and Schnabel, A. 1921 Das Virus des Herpes febrilis und seine Bezeihungen zum Virus der Encephalitis epidemica (lethargica). Ztschr.f.Hyg.u.Infektioskr. 94: 29.

Doerr, R. and Vöchting, K. 1920 Etudes sur le virus de l'herpes febrile. Rev. gen. d'opht. 34: 409.

Evans, C. A., Slavin, H. B. and Berry, G. P. 1946 Studies on herpetic infection in mice. IV. The effect of specific antibodies on the progression of the virus within the nervous system of young mice. J. Exper. Med. 84: 429-447.

Feldman, G. V. 1952 Herpes zoster neonatorum. Arch. Dis. Child. 27: 126-127.

Fevre de Arric and Millet. 1926 Localisation de virus herpetique sur le nevraxe par voie sanguine sous l'influence d'agents modificateurs de la barriere hematoencephalique. Compt. rend. Soc. de biol. 94: 782-784.

Field, E. J. 1952 Pathogenesis of herpetic encephalitis following corneal and massateric inoculation. J. Path. & Bact. 64: 1-11.

Fieser, L. F. and Creech, H. J. 1939 The conjugation of amino acids with isocyanates of the anthrocene and 1,2 benzanthracene series. J. Am. Chem. Soc. 61: 3502-3506.

Florman, A. L. and Mindlin, R. L. 1952 Generalized herpes simplex in an eleven-day-old premature infant. Am. J. Dis. Child. 83: 481-486.

Florman, A. L. and Trader, F. W. 1947 A comparative study of pathogenicity and antigenicity of four strains of herpes simplex. J. Immunol. 55: 263-275.

Fox, M. J., Krumbiegel, E. R. and Teresi, J. L. 1948 Maternal measles, mumps and chickenpox as a cause of congenital anomalies. Lancet 1: 746-749.

France, N. E. and Wilmers, M. J. 1953 Herpes simplex hepatitis and encephalitis in newborn twins. Lancet 1: 1181-1183.

Freudenberg, E., Roulet, F. and Nicole, R. 1952 Kongenitale Infektion mit Coxsockie-virus. Ann. Paediat. 178: 150-161.

Friedenwald, J. S. 1923 Studies on the virus of herpes simplex. Arch. Ophth. 52: 105-131.

Gifford, S. R. and Lucic, L. H. 1927 Sympathetic uveitis caused by the virus of herpes simplex. J. A. M. A. 88: 465-472.

Gildmeister, E. and Heuer, G. 1929 Über den Nachweis von Herpesvirus im Blute bei experimentell infizierten Kaninchen und über experimentellen Hautherpes. Deutsche med. Wchnschr. 55: 905-907.

Good, R. A. 1947 Recovery of herpes simplex virus from rabbit brain nine months after inoculation. Proc. Soc. Exper. Biol. & Med. 64: 360-362.

Goodpasture, E. W. 1925_a The axis-cylinders of peripheral nerves as portals of entry to the central nervous system for the virus of herpes simplex in experimentally infected rabbits. Am. J. Path. 1: 11-28.

Goodpasture, E. W. 1925_b The pathways of infection of the central nervous system in herpetic encephalitis of rabbits contracted by contact with a comparative comment on medullary lesions in a case of human poliomyelitis. Am. J. Path. 1: 29-46.

Goodpasture, E. W. 1929 Herpetic infection with especial reference to involvement of the nervous system. Medecine 8: 223-243.

Goodpasture, E. W. and Teague, O. 1923_a Experimental production of herpetic lesions in organs and tissues of rabbits. J. Med. Res. 44: 121-138.

Goodpasture, E. W. and Teague, O. 1923_b Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits. J. Med. Res. 44: 139-184.

Greenbaum, S. S. and Harkins, M. J. 1925 Experimental studies on immunity in herpes simplex. Arch. Dermat. & Syph. 11: 789-803.

Gregg, N. M. 1941 Congenital cataract following German measles in the mother. Tr. Ophth. Soc. Australia (British Empire) 3: 35-46.

Gregg, N. M. 1945 Rubella during pregnancy of the mother with its sequellae of congenital defects in the child. M. J. Australia 1: 313-315.

Grüter, W. 1920 Experimentelle und Klinische Untersuchungen über den Sag. Herpes corneae. Klin. Augenheilk. 65: 398-407.

Haas, V. H. 1941 Studies on the natural history of the virus of lymphocytic choriomeningitis in mice. Pub. Health Rep. 56: 285-292.

Harris, B. D. 1957 Manual of Histologic and Special Staining Technics. Armed Forces Inst. of Path., Washington, D. C., Chap. 4, pp. 30-36.

Hass, G. M. 1935 Hepato-adrenal necrosis with intranuclear inclusion bodies. Am. J. Path. 11: 127-142.

Haymaker, W. 1949 The pathology of herpes simplex encephalitis in man. With a report of three cases. J. Neuropath. & Exper. Neurol. 8: 132-154.

Heath, H. D., Shear, H. H., Imagawa, D. T., Jones, M. H. and Adams, J. M. 1956 Teratogenic effects of herpes simplex, vaccinia, influenza -A (NWS) and distemper viruses infections on early chick embryos. Proc. Soc. Exper. Biol. & Med. 92: 675-682.

Holowach, J., Thurston, D. L. and Becker, B. 1957 Congenital defects in infants following mumps during pregnancy. J. Pediat. 50: 689-694.

Hopkins, S. J. and Wormall, A. 1933 Phenyl isocyanate protein compounds and their immunologic reactions. Biochem. J. 27: 740-753.

Horstman, D. M. 1952 Poliomyelitis virus in blood of orally infected monkeys and chimpanzees. Proc. Soc. Exper. Biol. & Med. 79: 417-419.

Jawetz, E., Allende, M. F., and Coleman, V. R. 1952 Studies on herpes simplex virus. IV. The level of neutralizing antibodies in human sera. J. Immunol. 68: 655-661.

Karlova, A. N. 1958 Epidemic hepatitis in the newborn. Absts. Sov. Med. 2: p. 153, #162.

Kilbourne, E. D. and Horsfall, F. L., Jr. 1951 Studies of herpes simplex virus in newborn mice. J. Immunol. 67: 321-329.

Kilham, L. 1949 Mumps meningoencephalitis with and without parotitis. Am. J. Dis. Child. 78: 324-333.

King, L. S. 1940 Experimental encephalitis. Some factors affecting infection with certain neurotropic viruses. J. Exper. Med. 72: 573-593.

Kipping, R. H. and Downie, A. W. 1948 Generalized infection with the virus of herpes simplex. Brit. M. J. 1: 247-249.

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

Lebrun, J. 1956_b Etude de la localisation cellulaire du virus de l'herpes simplex par la methode des anticorps fluorescents. Bull. Microscop. Appl. 6: 94-99.

Liu, C. 1955_a Studies on influenza infection in ferrets by means of fluorescein-labeled antibody. I. The pathogenesis and diagnosis of the disease. J. Exper. Med. 101: 665-676.

Liu, C. 1955_b Studies on influenza infection in ferrets by means of fluorescein-labeled antibody. II. The role of "soluble antigen" in nuclear fluorescence and cross reactions. J. Exper. Med. 101: 677-686.

Liu, C. 1956 Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein-labeled antibody. Proc. Soc. Exper. Biol. & Med. 92: 883-887.

Liu, C. and Coffin, D. L. 1956 A study of canine distemper in ferrets and dogs by means of fluorescent antibody. Federation Proc. 15: 600.

Liu, C. and Coffin, D. L. 1957_a Studies on canine distemper infection by means of fluorescein-labeled antibody. I. The pathogenesis, pathology and diagnosis of the disease in experimentally infected ferrets. Virology 3: 115-131.

Liu, C. and Coffin, D. L. 1957_b Studies on canine distemper infection by means of fluorescein-labeled antibody. II. The pathology and diagnosis of the naturally occurring disease in dogs and the antigenic nature of the inclusion body. Virology 3: 132-145.

Liu, C. and Eaton, M. D. 1955 Study and isolation of primary atypical pneumonia virus in chick embryos by means of fluorescein-labeled antibody. Bact. Proc. 61.

Löwenstein, A. 1919 Aetiologische Untersuchungen über den fieberhalten Herpes. München. med. Wchnschr. 66: 769.

Lucchesi, P. F., La Boccetia, A. C. and Peale, A. R. 1947 Varicella neonatorum. Am. J. Dis. Child. 73: 44-54.

Marinesco, G. and Dragonesco, S. 1923 Recherches experimentales sur la neurotropisme du virus herpetique. Ann. Inst. Pasteur 37: 753.

McDougal, R. A., Beamer, P. R. and Hellerstein, S. 1954 Fatal herpes simplex hepatitis in a newborn infant. Am. J. Clin. Path. 24: 1250-1258.

Miller, O. B., Arbesman, C. and Baer, R. L. 1950 Disseminated cutaneous herpes simplex (Kaposi's varicelliform eruption). Arch. Dermat. & Syph. 62: 477-490.

Moulton, J. E. 1956 Fluorescent antibody studies of demyelination in canine distemper. Proc. Soc. Exper. Biol. & Med. 91: 460-464.

Moulton, J. E. and Brown, C. H. 1954 Antigenicity of canine distemper inclusion bodies as demonstrated by fluorescent antibody technic. Proc. Soc. Exper. Biol. & Med. 86: 99-102.

Noyes, W. F. 1955 Visualization of Egypt 101 virus in the mouse brain and in cultured human carcinoma cells by means of fluorescent antibody. J. Exper. Med. 102: 243-247.

Noyes, W. F. and Mellors, R. C. 1957 Fluorescent antibody detection of the antigens of the Shope papilloma virus in papillomas of the wild and domestic rabbit. J. Exper. Med. 106: 555-562.

Noyes, W. F. and Watson, B. K. 1955 Studies on the increase of vaccinia virus in cultured human cells by means of the fluorescent antibody technique. J. Exper. Med. 102: 237-241.

O'Dea, J. F. and Dineen, J. K. 1957 Fluorescent antibody studies with herpes simplex virus in unfixed preparations of trypsinized tissue culture. J. Gen. Microbiol. 17: 19-24.

Pugh, R. C. B., Dudgeon, J. A. and Bodian, M. 1955 Kaposi's varicelliform eruption (eczema herpeticum) with typical and atypical visceral necrosis. J. Path. & Bact. 69: 67-80.

Quilligan, J. J., Jr. and Wilson, J. L. 1951 Fatal herpes simplex infection in a newborn infant. J. Lab. & Clin. Med. 38: 742-746.

Remlinger, P. and Bailly, J. 1926_a Le Sang dans l'encephalite herpetique du Lapin. Compt. rend. Soc. de biol. 94: 112-114.

Remlinger, P. and Bailly, J. 1926_b Transmission de l'encephalite herpetique au chien. Compt. rend. Soc. de biol. 94: 1064-1068.

Remlinger, P. and Bailly, J. 1926_c Le virus herpetique dans ses passages de chat a chat. Compt. rend. Soc. de biol. 94: 734-736.

Remlinger, P. and Bailly, J. 1927 Sur la receptivite du tissu cellulaire souscutane au virus herpetique. Compt. rend. Soc. de biol. 96: 101-103.

Ruchman, I. and Dodd, K. 1950 Recovery of herpes simplex virus from the blood of a patient with herpetic rhinitis. J. Lab. & Clin. Med. 35: 434-439.

Sawai, Y., Tanaka, H. and Yasaki, Y. 1955 Studies on vaccinia virus by fluorescence microscope. Japanese J. Exper. Med. 25: 71-73.

Scott, T. F. McN. 1954 Infection with the virus of herpes simplex. New England J. Med. 250: 183-187.

Scott, T. F. McN. 1956 Diagnostic Procedures for Virus and Rickettsial Diseases. 2nd Ed., Amer. Pub. Health Assoc., N. Y. C., p. 313.

Scott, T. F. McN., Steigman, A. J. and Convey, J. H. 1941 Acute infectious gingivostomatitis; etiology, epidemiology and clinical picture of a common disorder caused by the virus of herpes simplex. J. A. M. A. 117: 999-1005.

Shaffer, M. F., Rake, G., Stokes, J., Jr. and O'Neil, G. C. 1941 Studies on measles. II. Experimental disease in man and monkey. J. Immunol. 41: 241-257.

Slavin, H. B. and Berry, G. P. 1943a Studies on herpetic infection in mice. II. The pathways of invasion of the central nervous system after intranasal instillation of virus in suckling mice. J. Exper. Med. 78: 315-320.

Slavin, H. B. and Berry, G. P. 1943b Studies on herpetic infection in mice. III. The visceral lesions in suckling mice. J. Exper. Med. 78: 321-326.

Smith, M. G., Lennette, E. H. and Reames, H. R. 1941 Isolation of the virus of herpes simplex and the demonstration of intranuclear inclusions in a case of acute encephalitis. Am. J. Path. 17: 55-68.

Swan, C. 1944 A study of three infants dying from congenital defects following maternal rubella in early stages of pregnancy. J. Path. & Bact. 56: 289-295.

Swan, C. 1949 Rubella in pregnancy as an aetiologic factor in congenital malformations, stillbirth, miscarriage and abortion. J. Obst. & Gynaec. (British Empire) 56: 341-363.

Swan, C., Tostevin, A. L., Mayo, H. and Barham Black, G. H. 1944 Further observations on congenital defects in infants following infectious diseases during pregnancy with special reference to rubella. M. J. Australia 1: 409-412.

Swan, C., Tostevin, A. L., Moore, B., Mayo, H. and Barham Black, G. H. 1943 Congenital defects in infants following infectious diseases during pregnancy. M. J. Australia 2: 201-210.

Torres Umana, C. 1951 Un caso probable de enfermedad de Heine Medine innata. Rev. Colomb. Pediat. y Puericult. 10: 175-176.

Urbain, A. and Schaeffer, W. 1927 Sur la repartition du virus herpetique dans les tissus et les humeurs. Compt. rend. Soc. de biol. 97: 1279-1280.

Watson, B. K. 1952_a Distribution of mumps virus in tissue cultures as determined by fluorescein-labeled antiserum. Proc. Soc. Exper. Biol. & Med. 79: 222-234.

Watson, B. K. 1952_b Fate of mumps virus in embryonated egg as determined by specific staining with fluorescein-labelled immune serum. J. Exper. Med. 96: 653-654.

Watson, B. K. and Coons, A. H. 1954 Studies of influenza virus infection in the chick embryo using fluorescent antibody. J. Exper. Med. 99: 419-428.

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

Wenner, H. A. 1944 Complications of infantile eczema caused by the virus of herpes simplex. Am. J. Dis. Child. 67: 247-264.

Wildi, E. 1951 Encephalite herpetique du nouveau-ne. Rev. neurol. 84: 201-209.

Wildy, P. 1954 Path of herpes virus infection from the periphery to the central nervous system in mice. Quoted in "Principles of Animal Virology". F. M. Burnet. Academic Press, Inc. N. Y. 1955, Chap. IX, p. 226.

Williams, A. and Jack, T. 1955 Hepatic necrosis in neonatal herpes simplex infection. M. J. Australia 1: 392-394.

Zarafonetis, C. J. D., Smadel, J. E., Adams, J. W. and Haymaker, W. 1944 Fatal herpes simplex encephalitis in man. Am. J. Path. 20: 429-445.

Zinsser, H. and Tang, F. 1929 Further experiments on the agent of herpes. J. Immunol. 17: 343-355.

Zuelzer, W. W. and Stulberg, G. S. 1952 Herpes simplex virus as the cause of fulminating visceral disease and hepatitis in infancy. Am. J. Dis. Child. 83: 421-439.