1970

Chronic Infections by Human Herpesviruses

Diane Claire Halstead
Loyola University Chicago

CHRONIC INFECTIONS BY HUMAN HERPESVIRUSES

by

Diane Claire Halstead

A Thesis Submitted to the Faculty of the
Graduate School of Loyola University
in Partial Fulfillment of the
Requirement for the Degree
of Master of Science

February
1970

Diane Claire Halstead was born in Chicago, Illinois, March 21, 1940. She graduated from Niles Township High School, Skokie, Illinois, in June 1958 and from North Park College in June 1962 with the degree of Bachelor of Science.

The author began her graduate studies in the Department of Microbiology, Stritch School of Medicine, Hines, Illinois in September 1967.

ACKNOWLEDGEMENTS

I am indebted to my advisor, Dr. G. Plummer, and Dr. J. G. Masterson, Loyola University Stritch School of Medicine. My deep appreciation also to Mr. Anan Phuangsab for technical assistance.

DEDICATION

DEDICATED TO MY PARENTS AND GENE

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I.

INTRODUCTION

Human herpesviruses are capable of causing a chronic infection in a susceptible host, but little is known about the specific host-virus relationship such as the mode of persistence and the immunological response.

This thesis is based on a study of human herpesvirus infections and is presented in two parts. In part one, experimental infection of the rabbit cornea by Herpes simplex with resultant physical and immunological effects is described. In part two, a study of the comparative immunological response to the human herpesviruses in patients receiving chemotherapy for ovarian malignancy is described. Appropriate discussion and conclusions based on the experimental evidence are presented.

Chapter II.

RABBIT STUDY

Review of Literature

Herpesviruses have several characteristic properties. A mature virus particle consists of a genome of DNA surrounded by an icosahedral capsid composed of 162 capsomeres. The virus is assembled within the nucleus of the host cell. Most herpesvirus particles become enclosed in a lipid-containing envelope derived from the host cell membrane. The total diameter of an enveloped particle ranges from 150-200 mμ. Agents with these biological, physical and chemical properties have been isolated from a number of animal species; these agents are now classified together as members of the Herpesvirus Group. Included in this group are: B-virus of rhesus and cynomolgus monkeys; SA8 of vervet monkeys; Squirrel monkey herpesvirus; SA6-a cytomegalo-like virus of vervet monkeys; Equine herpesviruses-Type 1 and 2; Infectious bovine rhinotracheitis virus; Pseudorabies virus of pigs; Cat rhinotracheitis virus; Dog herpesvirus; Guinea pig cytomegalovirus; Mouse cytomegalovirus; Infectious laryngotracheitis virus of chickens. To date there are four known human herpesviruses: Herpes simplex virus -

Type 1; Herpes simplex virus - Type 2; Varicella-Zoster virus; Human cytomegalovirus.

Herpesviruses have a predilection for tissues derived from embryonic ectoderm; they frequently infect the eye, skin, mucous membranes, mucocutaneous junctions (genital and labial) and the central nervous system. Generalized infections involving all the body tissues occur in newborns, who appear to be more susceptible to infection by herpesvirus than adults.

The persistence of Herpes simplex virus following primary infection, and the ability of this agent to reappear repeatedly, even in the presence of circulating antibody, have long been recognized. Perhaps all herpesviruses can produce a chronic* infection in their host. Only 10-15% of primary infections result in clinically recognized Herpes simplex (45), yet neutralizing antibody titers to Herpes simplex virus were found in 80-90% of 591 patients, fifteen years of age or older, most of whom had no history of herpetic disease (4). Somewhat lower figures for antibody incidence were reported by Scott (45).

Some workers, in direct opposition to the theory of "chronic infection", believe reappearance of Herpes simplex,

---

* In this thesis, the term chronic infection will refer to persistence of virus (either active or inactive) within a susceptible host.

as well as persistence of antibody, is caused by reinfection with Herpes simplex virus. There is no substantial evidence to support the theory of "reinfection". In addition, one would find it very difficult to explain the reappearance of lesions at the same anatomical site.

The persistence of other herpesviruses, varicella-zoster virus, human cytomegalovirus, equine herpesviruses and infectious bovine rhinotracheitis virus has recently been reviewed (35). The etiological agent of shingles and chickenpox, two different clinical entities, is Herpes zoster (varicella) virus. The virus ordinarily causes chickenpox in susceptible children and shingles in people previously infected with Varicella-Zoster virus. Two explanations for recurrent Varicella-Zoster virus have been proposed: (1) Reactivation of virus which has lain dormant in the dorsal ganglia since childhood chickenpox, or (2) Reinfection of a partially immune adult. Evidence to support the reactivation theory includes: (1) Administration of certain drugs or the presence of certain tumors appear to be capable of precipitating clinical zoster. (2) There is no documented occurrence of zoster following contact with chickenpox, though the reverse occurs. A parallel may be drawn between the behavior of Varicella-Zoster virus with production of recurrent shingles and chronic infections with Herpes simplex virus with production of recurrent lesions.

Fifty to eighty percent of the adult population possess antibody to human cytomegalovirus without prior detectable clinical disease (51,40). Cytomegalic intranuclear inclusions were observed in the salivary glands of 10-32% of unselected autopsies of infants (57). The major clinical significance of human cytomegalovirus is invasion of the fetus resulting in abortion (47) or the clinical syndrome CID (Cytomegalic Inclusion Disease) (53,28). This syndrome CID is characterized by involvement of the central nervous system and of the reticuloendothelial and hematopoietic system. Prolonged excretion of the virus into urine of adults, especially women bearing children with CID (28) again suggests chronic infection. In the postnatal period the disease is manifested by chronic hepatitis (17) or acute hemolytic anemia (58). Pneumonia is the most commonly recognized symptomatic form of disease in an adult (56). A high prevalence of clinically detectable cytomegalovirus infections occur after renal transplantation; cytomegaloviuria was reported in 65% of renal transplant recipients and cytomegalic cells were found in the lungs and other organs of 52% of autopsied recipients (37). Immunosuppressive therapy was given to these recipients so allowing activation of persistent cytomegalovirus, or infection from an outside source.

Evidence for chronic infections of animals by herpesviruses has also been reported. The invasion of the human fetus by cytomegalovirus is analogous to fetal horse invasion by Equine herpesvirus-Type 1. Abortions have been known to occur in horses possessing circulating antibody, even in the absence of an epidemic (9). IBR (Infectious bovine rhinotracheitis) virus can cause genital, as well as other infections, in its host. Snowden (48) isolated IBR virus from the vagina of a cow on two occasions separated by eleven months. It is conceivable that IBR virus also produces chronic infections.

The form of Herpes simplex virus during its quiescent phase and the processes involved in its emergence have not been determined. The virus may persist in latent* form. By analogy with lysogenic bacteria, the viral genome may become associated with the chromosome of a susceptible cell and produce little or no complete virus. Doerr (8) and Burnet and Williams (5) suggested that herpesvirus is retained in the body after the initial infection, and recurs, with the formation of lesions, after reactivation. Evidence has accumulated of viral reactivation by such

---

* In this thesis, the term latent infection will refer to persistence of inactive virus within a susceptible host, undetectable by present day methods.

stimuli as fever, nerve injury, menstruation, etc. (6). On the other hand, continuous multiplication of Herpes simplex virus may occur. The virus may be present in a fully infective form but at sub-clinical levels, consequently the usual methods of detecting virus would be inadequate.

Localization of Herpes simplex virus in susceptible tissue in man has been ill defined. Skin, sensory ganglia or sensory nerve endings have been suggested as a possible site of persistence. Attempts to demonstrate virus in affected skin areas between recurrent episodes have been fruitless. Virus could not be demonstrated in human skin scrapings taken from sites of former lesions (12). Neither could virus be isolated from affected tissues grown in vitro (tissue culture) and exposed to ultraviolet light or hydrocortisone or alternately propagated for five months (41).

Various investigators have suggested that virus can persist in the sensory ganglia, in view of the frequent reappearance of herpetic lesions on the face and the association of post-operative herpetic lesions following nerve root sectioning (11,6). The few attempts to isolate Herpes simplex virus from the Gasserian ganglion have been negative (6,36). Evidence to support the possibility of Herpes simplex virus localization in this ganglion has been provided by laboratory animals infected intranasally. The results of this study suggest that

virus ascends the trigeminal nerve to the brain, with viral antigen subsequently appearing in the ganglion cells (21). Other observations also suggest Herpes simplex virus localization in sensory nerve ganglia following the initial infection; a zoster-like distribution of herpetic lesions can be induced in rabbits by tarring adjacent skin prior to intradermal inoculation. Virus has been demonstrated in the corresponding spinal ganglia (52).

Evidence against localization of the virus in the sensory nerve endings or skin of humans has been provided by excisional studies (50). When skin (full thickness) from a site of a primary infection was transplanted to a normal site, and a pyrogen was given parenterally, the transplant site remained unaffected while the intact skin adjacent to the primary site developed lesions. In another case, full thickness skin was removed from a primary site and a graft inserted. Two years later recurrent lesions appeared in the skin adjacent to the graft but not in the graft itself (Kibrick, unpublished).

Experimental studies of chronic infections by Herpes simplex virus appear to be confined mainly to the rabbit, because when inoculated, a primary infection develops and "reactivation" occurs similar to the infection produced in man. Grütar, for example, in 1912, demonstrated that virus isolated from dendritic ulcers of the human eye could, when inoculated

onto rabbit corneas, produce lesions similar to those seen in man (27). Studies of chronic infections in the rabbit have been confined mainly to the central nervous system and the eye; chronic encephalitis or herpetic keratitis, respectively, has been experimentally produced.

Observations of spontaneous acute encephalitis in rabbits, six months after recovery from an intratesticular inoculation with Herpes simplex virus, have been reported (32). A decade later, Good and Campbell (13,14) suggested that encephalitis could be a manifestation of a reactivated infection. Rabbits were sensitized to egg white, then immunized intramuscularly with infective virus. After one to three months, those rabbits which had completely recovered and those who had never shown signs of infection were subjected to anaphylactic shock. Encephalitis was precipitated 19 times in 16 rabbits. Isolation of virus was successful from the central nervous system after anaphylactically induced encephalitis and also during quiescence and spontaneous exacerbations of encephalitis. Using the same experimental model, Schmidt and Rasmussen (44) produced encephalitis in their animals by injection of epinephrine. They inoculated rabbits, intramuscularly and again after one month intracerebrally with Herpes simplex virus-Type 1. Epinephrine was administered to 10 rabbits, one to five months after the challenge dose; 6 rabbits developed fatal

encephalitis. The authors were able to isolate Herpes simplex virus from 6 of 8 control rabbits previously inoculated with virus but subsequently not given epinephrine. Plummer and Phuangsab (personal communication) were able to isolate virus from lower spinal cord and nerve root cultures from rabbits which were sacrificed two to three months after intramuscular inoculation with either Herpes simplex virus-Type 1 or 2, whether or not they were previously stimulated with epinephrine. Plummer *et al.* (34) report that it is possible to isolate virus from cultures of the lower spinal cord and nerve root of rabbits immediately after primary inoculation of Herpes simplex virus-Type 2, without subsequent epinephrine administration. The results appear to confirm the persistence of herpesvirus in the central nervous system, but leave much confusion. The following questions arise: Does herpesvirus continuously multiply in susceptible tissue? If so, can virus be isolated at all times using an appropriate system? Alternatively, does infective virus release occur only after an appropriate stimulation? Subsequent studies of the infected rabbit eye have attempted to answer some of these questions.

"Reactivation" of Herpes simplex virus in healed rabbit corneas was induced by a corneal Arthus reaction (1). Virus was inoculated onto the cornea of 8 rabbits. The animals were sensitized to horse serum after the initial healing of the

corneal lesion. A corneal Arthus reaction was induced in the left eye 2-3 times within a two and a half to six month period after virus inoculation. Reactivation occurred within fourteen days in 7 of 19 attempts. Spontaneous release of virus occurred in both the challenge and control eyes in 8 of 204 (approximately 4%) attempts to isolate virus, but not during the fourteen day post-Arthus period. These results support earlier reports of persistence of virus, but suggest the need to extend these studies before one can state definitely whether release of infectious virus requires a particular stimulus or whether it is a spontaneous event.

As a result of studies with Herpes simplex virus in rabbit eyes, two rival hypotheses have been formulated. Laibson and Kibrick (25,26) suggest that herpesvirus does indeed persist and requires activation by an appropriate stimulus. They found that they could rarely isolate virus from the eyes of their rabbits after the initial virus activity had subsided. But, they could isolate virus after "reactivation" with epinephrine. As also reported by Anderson *et al.* (1), spontaneous virus release occurred only occasionally. In marked contrast, Kaufman *et al.* (23) concluded that continuous virus multiplication in structures such as the lacrimal and salivary glands may cause recurrent herpetic disease; they reported frequent (at least 3 episodes per rabbit within a two month period)

spontaneous virus releases from the eyes of 15 rabbits without prior stimulation. In support of Kaufman's findings, the report by Nesburn (30) confirmed the occurrence of spontaneous release of Herpes simplex virus in rabbits.

Each new finding seems to answer one question and raise two or three others. Investigations, to date, have established the fact of persistence of Herpes simplex virus in vivo. The varied results obtained by Laibson and Kibrick (25,26) and by Kaufman (23) are indeed puzzling, particularly in light of the fact that both groups used the same strain of virus and similar experimental conditions. If Kaufman is correct, it would be difficult to demonstrate positively that epinephrine does in fact activate Herpes simplex virus as Laibson and Kibrick have reported. Kaufman has suggested (24) that epinephrine does not actually activate virus, but only causes further damage to pre-existing lesions created by the virus.

In this thesis I have attempted to answer the following questions: (1) Can infective herpesvirus be detected in chronically infected tissue, specifically the rabbit eye, without prior artificial stimulation? (2) Can epinephrine activate virus? (3) Do herpesviruses Types 1 and 2 differ in the nature of the chronic infection they cause in the rabbit eye, and (4) what fluctuations in antibody levels can be observed in the experimental rabbits?

Materials and Methods

Viruses

Herpes simplex virus-Type 1, strain 197, passage level approximately 4 (supplied by Communicable Disease Center, Atlanta, Georgia) and Type 2, strain MS, passage level approximaterly 16 (supplied by Gudnadottir; refer to Gudnadottir *et al.* [16]) were grown in rabbit kidney tissue cultures and subsequently in human lung fibroblast tissue cultures obtained from Microbiological Associates, Bethesda, Maryland. Herpes simplex virus-Type 1, strain Rodanus, passage level 27 (supplied by S. Kibrick, Boston University, Boston, Massachusetts) was grown in human amnion and subsequently in rabbit kidney tissue cultures.

Virus Assay

Virus was titered in rabbit kidney tissue cultures by the plaque technique using 60 x 15 mm sterile petri dishes, #3002 (Falcon Plastics, Los Angeles, California). Viral suspensions were serially diluted (ten-fold) in 199 maintenance medium. From each dilution, 0.2 ml was adsorbed onto a rabbit kidney tissue culture plate for 15 min, shaking once after 10 min. The plates were then overlayed with methocel (methylcellulose, 1500 centipois, Fisher Scientific, Chicago, Illinois) medium

and incubated in a 5% CO_2 atmosphere at 37° C for 48 hr. Plaques were counted microscopically. The final titers are expressed as plaque forming units (PFU) per 0.2 ml. All assays were run in duplicate.

Tissue Culture Media

A commercial preparation of Medium 199 (Grand Island Biological Company, Grand Island, New York) supplemented with amino acids, glucose, vitamins, nucleic acids, required growth factors, intermediary metabolites and phenol red, combined in an Earle's balanced salt solution base was employed. The following additions were required: 0.3 mg/ml unmodified glutamine without $NaHCO_3$ (Eli Lilly & Company, Indianapolis, Indiana), 100 units/ml buffered potassium penicillin G (Eli Lilly & Company), 100 µg/ml streptomycin sulfate (Eli Lilly & Company) and 0.5 µg/ml fungizone (E. R. Squibb & Sons, New York).

Initiator media, which supports cellular proliferation was used in preparing primary tissue cultures. The following additions to the basic media described above are required: $NaHCO_3$ solution plus lamb serum (Grand Island Biological Company) sufficient to give a final concentration of 0.1% and 10%, respectively. Maintenance media, which is designed to permit metabolism of cells but not cellular proliferation, was used to maintain tissue cultures after a monolayer was

established. The media included 0.18% $NaHCO_3$ solution and 3% fetal calf serum (Grand Island Biological Company) plus the supplemented Medium 199 previously described.

Overlay media for plaque assay included 2.5% methylcellulose in maintenance media.

Preparation of Rabbit Kidney Tissue Cultures

Primary rabbit kidney tissue cultures were prepared as follows: Approximately 3 week old New Zealand albino rabbits (supplied by Abrams Small Stock Breeders, Chicago, Illinois) were sacrificed. The kidneys were excised and finely minced. One pair of minced kidneys along with 25 ml of 2.5% pancreatic trypsin in normal saline (Difco Laboratories, Detroit, Michigan) were added to a trypsinization flask and placed on a magnetic stirrer for 30 min. The trypsin-cell suspension was then centrifuged for 10 min at 500 x g in an International Centrifuge with a swinging bucket head. The sediment was suspended in 400 ml of initiator media and dispensed as desired, 2 ml per 16 x 150 mm tube (Scientific Products, Chicago, Illinois) with a number 'O' rubber stopper (Aloe Scientific, Schiller Park, Illinois) or 5 ml per 60 x 15 mm petri dish (Falcon Plastics). Tissue culture monolayers were refed as needed with 1.5 ml of maintenance media per tube or 5 ml of maintenance media per dish.

Human Cells

Tubes of human epithelial tissue culture were obtained from Microbiological Associates.

Virus Stock

Virus stocks were grown in 500 ml capacity Blake Bottles (Bellco Glass Company, Vineland, New Jersey) containing a monolayer of either human fibroblast or rabbit kidney cells. Virus was adsorbed onto a tissue culture for 15 min, shaking once after 10 min. The monolayer was then covered with 50 ml of maintenance media and the bottle was tightly sealed with a number '5' rubber stopper (Aloe Scientific) and placed in a 36 C incubator for 48 hr. Virus particles were released from the cells by freeze-thawing. The fluid containing the virus was centrifuged for 10 min at 500 x g (International Centrifuge with a swinging bucket head). The supernatant was immediately placed in ampoules in 0.5 - 1.0 ml volumes, sealed and stored at -70 C.

Neutralization Test

Neutralization curves described by Plummer (33) were used to test for serological differences between strains of Herpes simplex virus-Types 1 and 2. Experiments were performed using Medium 199 diluent. One ml of virus suspension containing approximately 1000 PFU was mixed with 1.0 ml of antiserum

(supplied by J. Waner). The mixture was kept in a 37 C water bath and assayed for virus at 5 min, 20 min and 40 min. A control tube, consisting of 1.0 ml of the same virus suspension mixed with 1.0 ml of Medium 199 diluent, was incubated with the test preparation. The control was assayed at '0' time and again at 40 min. Rabbit kidney tissue cultures grown in 60 x 15 mm petri dishes were used for plaque assays with a 2.5% methylcellulose overlay media.

Rabbit Inoculation

Unilateral corneal infections were established in young adult, New Zealand albino rabbits weighing approximately 2 kg (Abrams Small Stock Breeders, Chicago, Illinois). Approximately 0.1 ml of virus suspension containing 5.6 Log_{10} PFU/0.2 ml was introduced into the lower cul-de-sac of the rabbit eye and the lids were held shut for 30 sec (25). The following regimen was employed: Every other day, the preinoculated eye of each rabbit was swabbed using a sterile, medium cotton tipped applicator stick (Tomac applicator sticks supplied by American Hospital Supply Company, Evanston, Illinois), and was examined for ocular damage. The rabbits rapidly became accustomed to this procedure, thus eliminating the necessity for topical anesthesia. One or two swabs were rotated in the lower cul-de-sac, then swept across the cornea into the upper cul-de-sac, removed and placed directly into a tube of rabbit

kidney and/or human epithelial tissue culture(s). The tissue cultures were examined every other day for characteristic cytopathogenic effect produced by Herpes simplex virus. An isolate was frozen at -70 C until a positive identification was made using the neutralization test. Tissue cultures were maintained for 2 weeks before they were considered negative.

Epinephrine (Adrenalin)

Epinephrine was administered after complete recovery of the primary infection, absence of inflammation and negative virus cultures. Epinephrine in peanut oil 1:500, 2 mg/ml (Parke, Davis & Company, Detroit, Michigan) was used as follows: 0.5 ml was injected intramuscularly into the hind thigh on three consecutive days. Alternatively, epinephrine, aqueous 1:1000, 1 mg/ml (Parke, Davis & Company) was administered five times within two days as follows: 0.2 ml was injected intramuscularly into the hind thigh and 0.6 ml was injected subcutaneously.

Complement Fixation Test

Rabbits, previously infected with Herpes simplex virus, were bled both before inoculation of epinephrine and at periodic intervals thereafter. Serum was either placed in ampoules and frozen at -20 C for future use or prepared for use in the complement fixation test.

The microtiter complement fixation test (46), used as a 2 unit test, was employed to measure rabbit serum antibody levels to Herpes simplex virus (Microbiological Associates). Antisheep hemolysin preserved with equal parts glycerol (Markham Laboratory) was diluted 1:50, stored at -20 C and titered before use. Lyophilized guinea pig complement (Markham Laboratory) was rehydrated with the accompanying diluting fluid, dispensed in small volumes and immediately stored at -70 C. Complement was titered before use. Sheep red blood cells stored as a 50% suspension in Alsever's solution (Microbiological Associates) were prepared as a 2% suspension in cold saline buffered with diethylbarbituric acid, 1.0 molar $MgCl_2$ and 0.3 molar $CaCl_2$ (Veronal buffer). The cells were washed three times in cold Veronal buffer; each time the cells were centrifuged at 200 x g in a table model, anglehead centrifuge (Clay-Adams, Inc., New York) for 5 min in a graduated centrifuge tube (Bellco Glass) in an attempt to pack the same volume of cells in each test.

Anticomplementary substances, which prevent complement from lysing red blood cells, have been detected in some sera. The following procedure was used to remove these substances: 1 part undiluted complement was added to 3 parts undiluted serum and incubated overnight at 10 C. The serum-complement mixture was subsequently incubated at 37 C for 30 min and

diluted 1:4 in sterile normal saline. The diluted serum was then incubated at 60 C for 30 min (43). The complement fixation tests were performed in disposable, round bottomed plates (Cooke Engineering Company, Alexandria, Virginia). Cold Veronal buffer (0.025 ml), antigen (0.025 ml), complement (0.025 ml) and hemolytic system (0.05) were distributed with calibrated dropper pipettes. The serum (0.025 ml) was investigated in a series of twofold dilutions in saline, 1:4 through 1:512, by the use of calibrated wire loops. The total volume of the test was 0.125. After the addition of saline, serum, antigen and complement, the plates were covered and placed at 4 C for 18 hr. They were then placed at room temperature for 10 min and the hemolytic system (equal volumes of 2% sheep red blood cells and adjusted hemolysin mixed and allowed to stand at room temperature for 10 min) was added. The plates were covered with tape, shaken thoroughly and immediately placed in a 37 C incubator until the complement controls showed the proper degree of hemolysis, usually within 45 min. The plates were then placed at 10 C until the remaining cells settled. The degree of inhibition of hemolysis was visually estimated from 4+ to 0; only 4+ and 3+ were considered to be positive. Horsfall and Tamm (20) was used as a reference for techniques of hemolysin and complement titrations.

Results (Virus Isolation)

The first of two experiments was performed in an attempt to determine whether infective herpesvirus could be detected in the previously infected rabbit eye without prior artificial stimulation and if epinephrine does in fact induce virus release. Also of interest was whether herpesviruses Type 1 and 2 differ in the nature of the chronic infection they cause in the rabbit eye. The left eye of each of 46 rabbits was infected with Herpes simplex virus, either Type 1, strain 197 or Type 2, strain MS. The eyes of ten of these rabbits were swabbed for a period of a year or more. The second experiment, which lasted approximately 4 months, was designed to confirm as well as to extend the results obtained in the first experiment. Thirty-four rabbits were inoculated in the left eye with one of three strains of Herpes simplex virus: Type 1, strain 197 or strain Rodanus used by Laibson and Kibrick (25) and Kaufman (23) and identified as Type 1 by the neutralization test described by Plummer (33), or Type 2, strain MS.

Virus was isolated from the eye of each rabbit from the second through the ninth day, and in some cases as long as the eighteenth day, after virus inoculation. Within 4 days of virus inoculation most rabbits developed moderate to severe keratoconjunctivitis with inflammation of the conjunctiva,

swollen eyelids, watery eyes and production of an exudate. Of interest were the distinct differences observed between the infection produced on the one hand by strains 197 and Rodanus and on the other hand by strain MS. In the first experiment, 34% of the 26 rabbits inoculated with strain MS developed encephalitis and died during the primary infection whereas only 10% of the 20 rabbits inoculated with strain 197 died during this same period. In the second experiment, 17% of the 12 rabbits inoculated with strain MS and 42% of the 12 rabbits inoculated with strain Rodanus died following inoculation of virus but all 10 rabbits inoculated with strain 197 survived. Secondly, all clinical manifestations of infection disappeared within 2 weeks of inoculation of strains 197 and Rodanus whereas within this same period of time 80% of the surviving MS rabbits in the first experiment and 50% of the surviving MS rabbits in the second experiment developed permanent, cloudy lesions of the cornea (Fig. 1, p. 23).

Results from Rabbits Inoculated with Type 1

Figure 2, p. 24, shows the number of spontaneous virus releases obtained from 11 rabbits inoculated with strain 197 virus and the approximate day each release occurred. A total of 26 out of approximately 1500 attempts to isolate virus were successful. Nine episodes of virus release occurred in 7 rabbits and each episode averaged 4.8 days. The results are

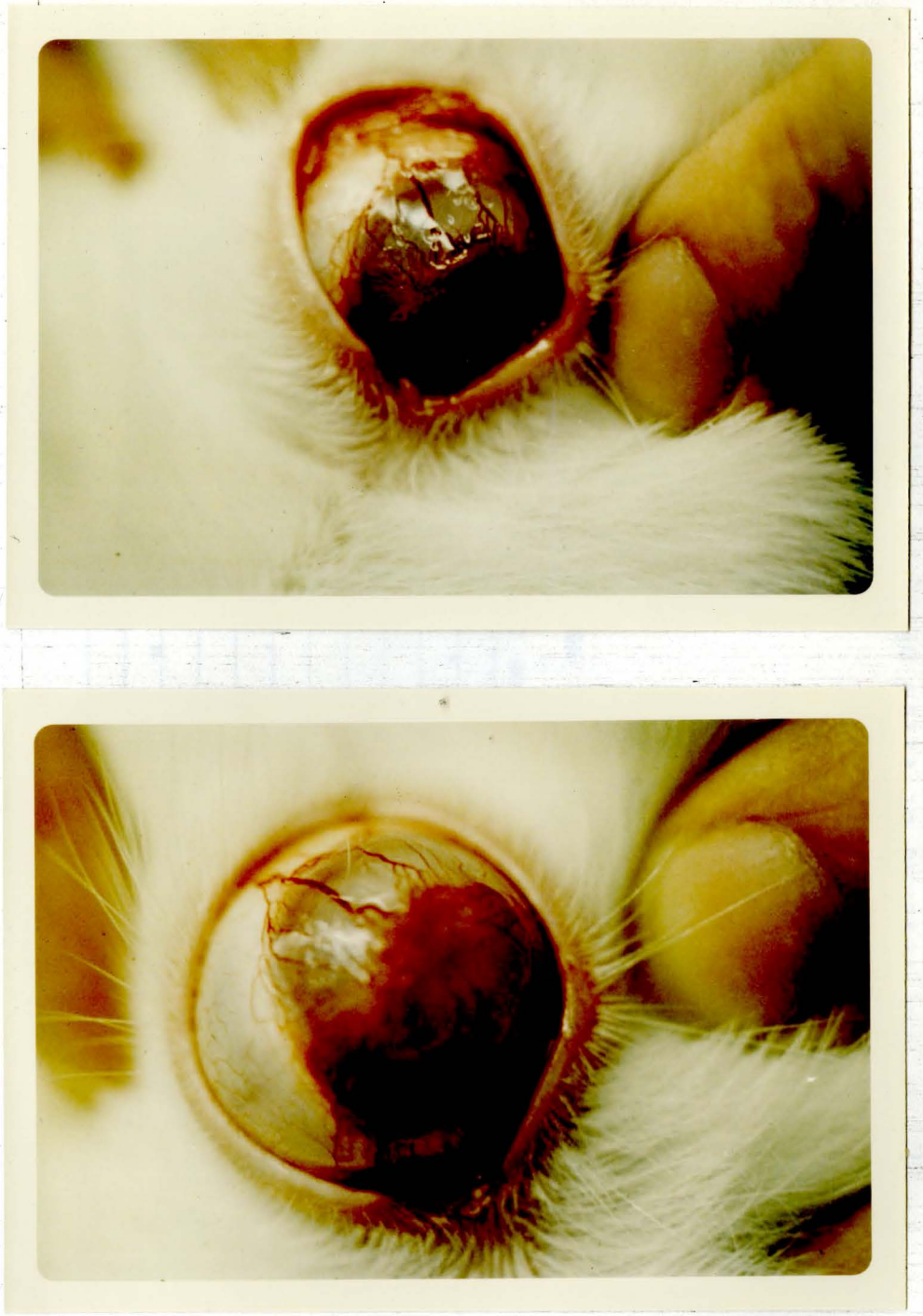


Fig. 1. Disciform keratitis produced by herpes simplex virus, Type 2, Strain MS.

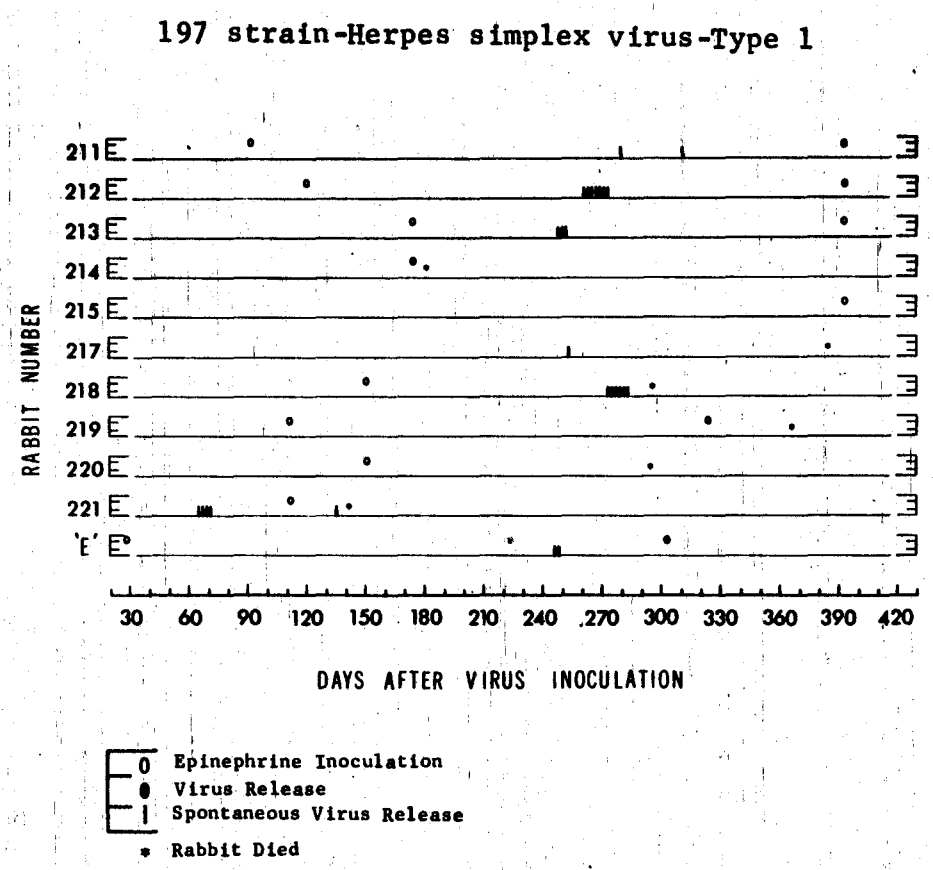


Fig. 2. The release of virus obtained from rabbits inoculated with strain 197 (Exp. 1).

summarized in Table I, p. 26. Figure 3, p. 27, presents the results obtained in the second experiment from 10 rabbits inoculated with strain 197 virus. Seventeen virus isolates were obtained out of approximately 670 attempts. Seven episodes of virus release occurred in 6 rabbits (Table 2, p. 28) and each episode averaged 3.9 days. Figure 4, p. 29, shows the results obtained from rabbits inoculated with strain Rodanus. Three spontaneous virus releases were obtained out of approximately 460 attempts. These releases occurred in 3 rabbits, R 428 = (Rabbit number 428) and R 432 on day 66 and R 431 on day 74 after inoculation with strain Rodanus.

Figures 2 (p. 24) and 3 (p. 27) indicate when epinephrine was administered in each rabbit infected with strain 197. Laibson and Kibrick (25) interpreted each positive virus culture which was obtained within 16 days after epinephrine inoculation as an induced release. In an attempt to reproduce their results, an arbitrary 16-day limit was imposed to avoid introducing a time variable. In experiments 1 and 2, a total of 23 attempts to stimulate virus release by the use of epinephrine were unsuccessful, in rabbits inoculated with Type 1, strain 197 but the following results were obtained from rabbits inoculated with another Type 1 virus, strain Rodanus: All rabbits received epinephrine, beginning on day 96. Rabbit 428 succumbed to epinephrine administration, thus Fig. 4 (p. 29)

Table I. Spontaneous Release of Virus in Rabbits Inoculated with Strain 197

Rabbit Number	Number of Days Virus was Released	Number of Days After Virus Inoculation
221	7	66
221	1	126
'E'	3	247
213	5	249
217	1	254
212	13	262
218	11	274
211	1	281
211	1	312

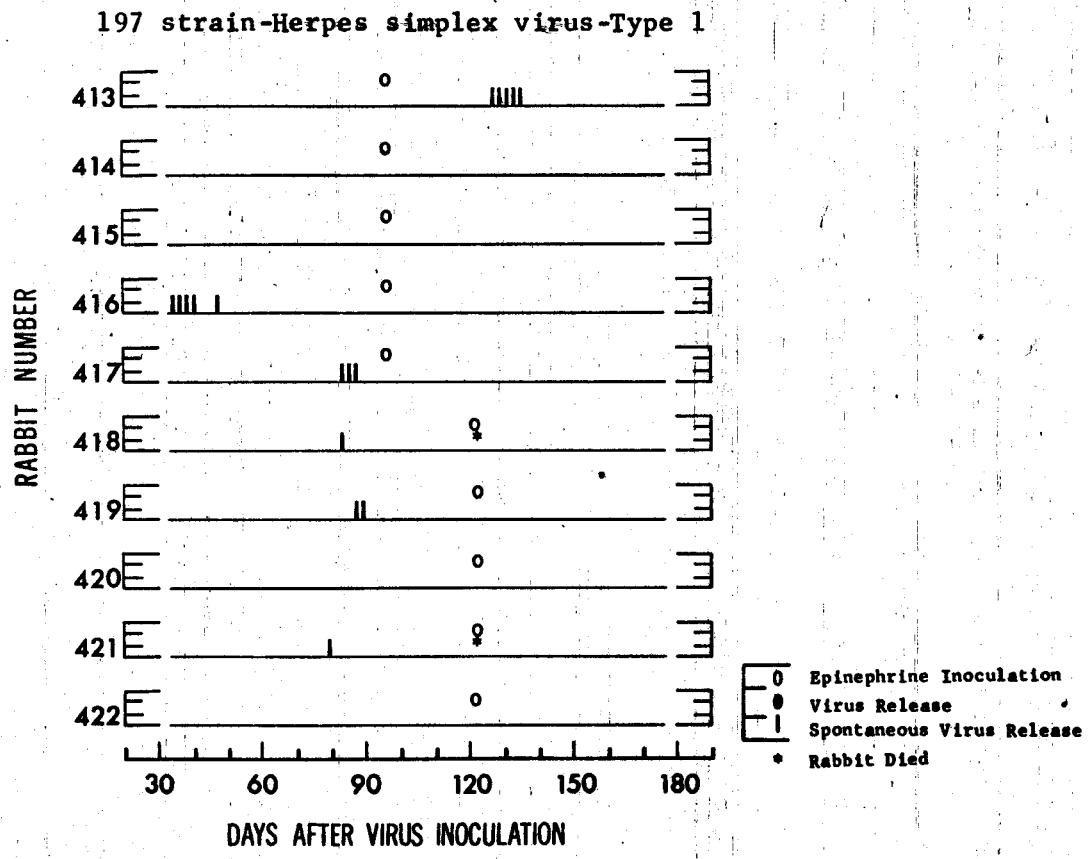


Fig. 3. The release of virus obtained from rabbits inoculated with strain 197 (Exp. 2).

Table 2. Spontaneous Release of Virus in Rabbits Inoculated with Strain 197 (Exp. 2)

Rabbit Number	Number of Days Virus was Released	Number of Days After Virus Inoculation
416	7	35
416	1	48
421	1	80
418	1	84
417	5	84
419	3	88
413	9	128

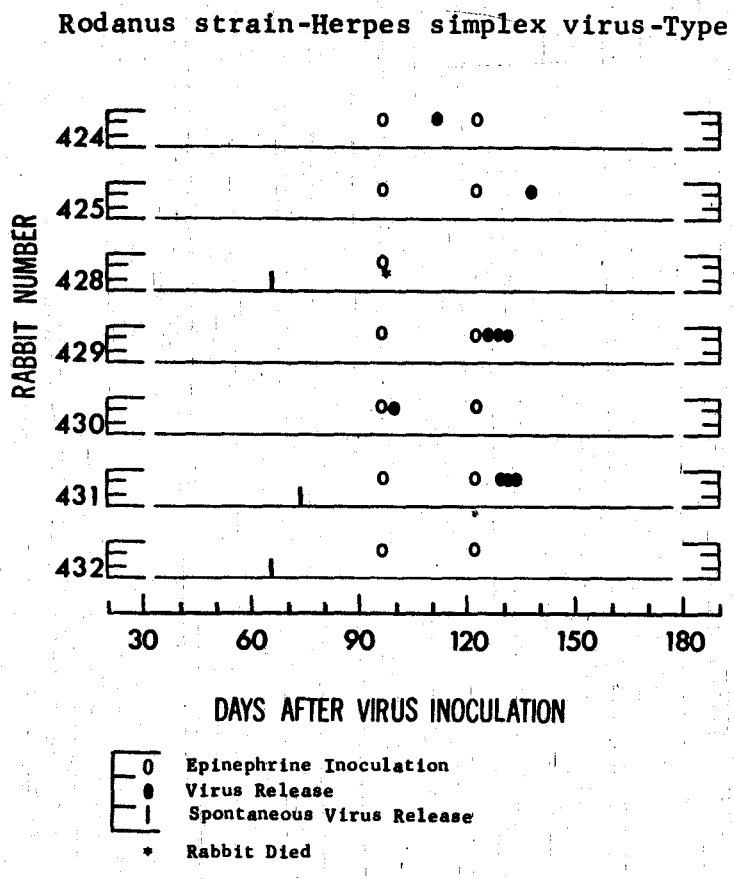


Fig. 4. The release of virus obtained from rabbits inoculated with strain Rodanus.

shows that 2 of 6 surviving rabbits, R 430 and R 424, yielded virus on day 98 and day 111 respectively. On day 122, all Rodanus rabbits received epinephrine again. Three of 6 rabbits yielded virus within 16-days post-epinephrine inoculation. Rabbit 429 released virus on day 126, R 431 released virus for 3 days beginning on day 130 and R 425 released virus on day 138. Thus a total of 5 of 12 attempts to induce virus were perhaps successful.

Results From Rabbits Inoculated with Type 2

Figure 5 (p. 31) presents the number of spontaneous virus releases obtained from 11 rabbits inoculated with Type 2, strain MS, and the approximate day each virus release occurred. Virus was recovered from 2 MS rabbits, R 207 and R 204, on the 28th and 73rd day respectively after inoculation of Herpes simplex Type 2 virus; a total of 2 of 1100 attempts at virus isolation were successful. Figure 6 (p. 32) presents the results obtained in the second experiment with rabbits inoculated with strain MS. In sharp contrast to the results obtained in the second experiment with Type 1 (strain 197), MS virus was recovered from only 1 rabbit (R 406) 130 days after virus inoculation. Thus, only 1 out of approximately 600 attempts to isolate virus was successful.

The results obtained from rabbits infected with Type 2 and subsequently inoculated with epinephrine are graphically

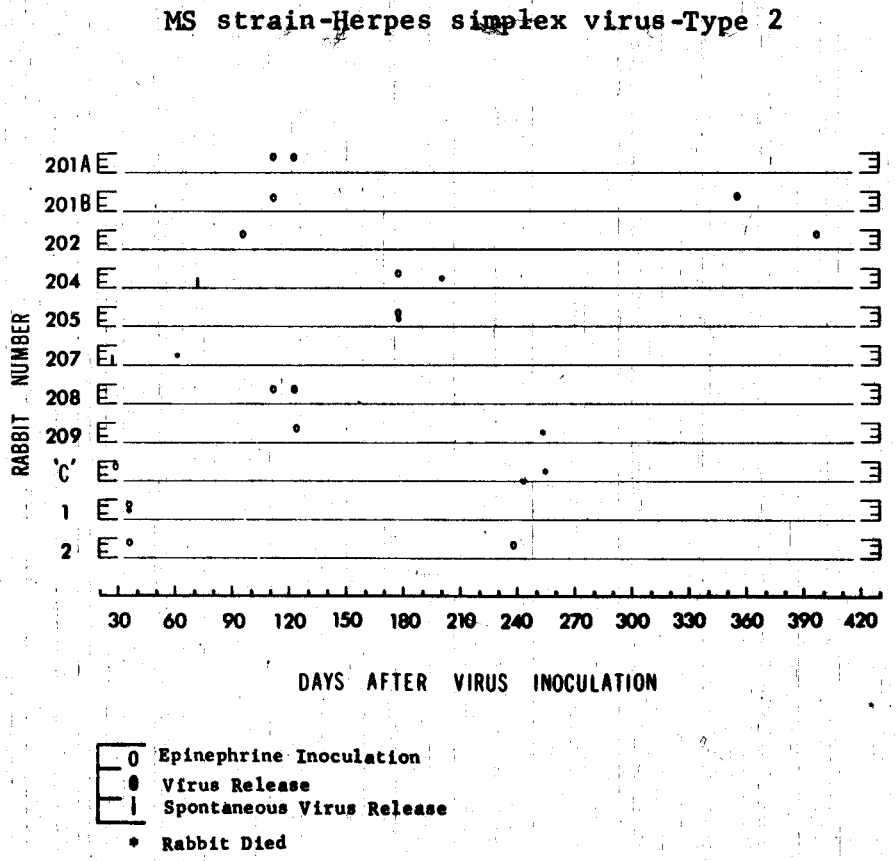


Fig. 5 The release of virus obtained from rabbits inoculated with strain MS (Exp. 1).

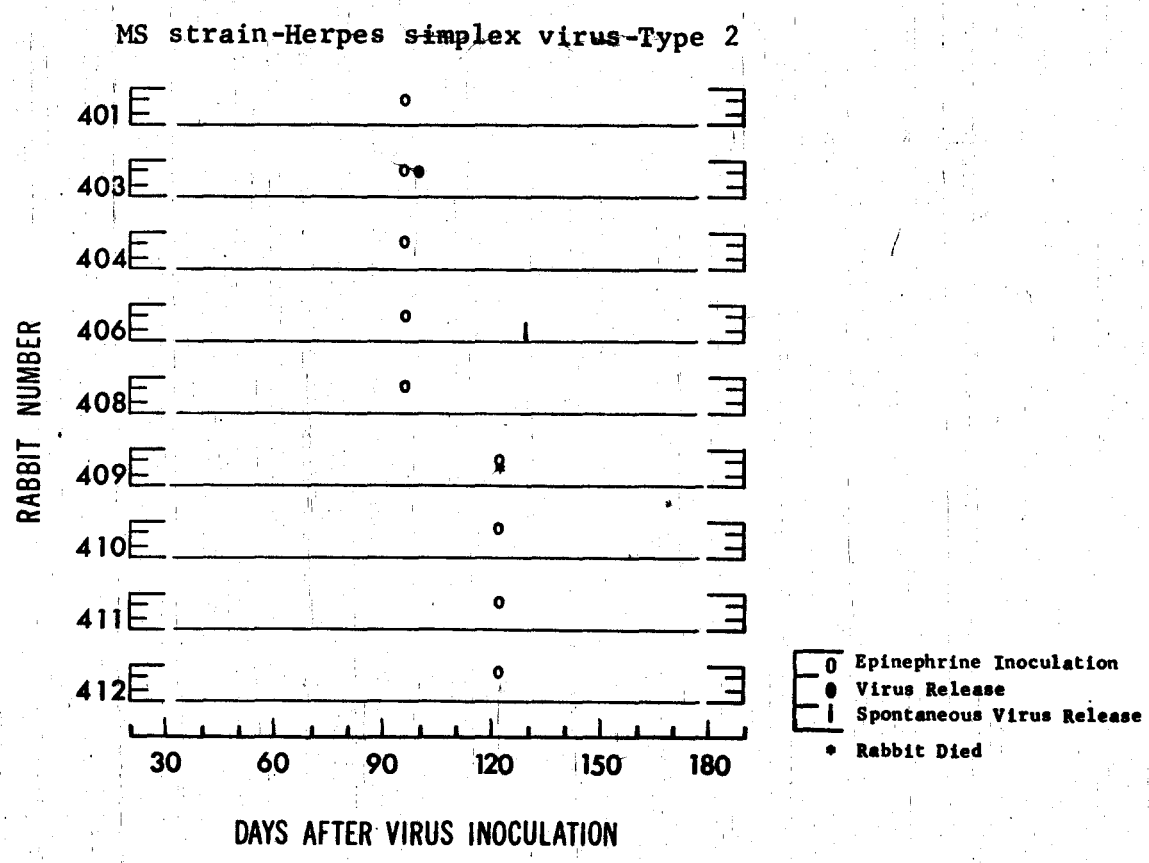


Fig. 6. The release of virus obtained from rabbits inoculated with strain MS (Exp. 2).

illustrated in Fig. 5 (p. 31). A positive virus culture was obtained from 2 rabbits (R 201A and R 208) within 16 days of epinephrine inoculation. Induction was attempted a total of 11 times in 8 rabbits. Figure 6 (p. 32) presents the data obtained from 8 rabbits subjected to epinephrine administration. Only 1 rabbit (R 403) yielded virus and this release occurred on day 100.

A summary of the results obtained from induction attempts in rabbits inoculated with strain MS follows: A total of 2 of 11 and 1 of 8 attempts to isolate virus were successful in the first and second experiment, respectively.

Discussion (Virus Isolation)

Herpes simplex virus can be recovered from the eyes, which appear to be normal, of rabbits with healed herpetic keratitis both following epinephrine inoculation and as a spontaneous event. Release of strain 197 virus from the rabbit eye was exclusively spontaneous whereas the release of strains Rodanus and MS occurred both spontaneously and following epinephrine administration.

The conflicting results obtained by various workers, using either the same or different strains of Herpes simplex virus, may be caused by one or more significant variables:

(1) strain variations, (2) size of inoculum, (3) unilateral versus bilateral inoculation, (4) passage history of the virus, (5) frequency of swabbing, (6) duration of experiment and perhaps other unknown factors. The significance of strain variations may become apparent when one surveys the literature. Anderson *et al.* (1), using an unknown strain of Herpes simplex virus obtained from a labial lesion, observed an occasional spontaneous virus release during attempts to "reactivate" virus but, the authors chose to minimize the importance of these findings. Nesburn *et al.* (30), using MacKrae strain of Herpes simplex virus isolated from a patient with recurrent keratitis, observed multiple spontaneous releases. Since the virus strain and/or experimental techniques differ from one experiment to another, direct comparisons are impossible. The procedure used in experiment #2 with strain Rodanus, closely parallels the technique used by Laibson and Kibrick (25), in an attempt to rule out at least some of the variables.

The data presented leaves little doubt that spontaneous viral release occurs. In both experiments #1 and #2, rabbits inoculated with Type 1, strain 197, shed virus spontaneously. No satisfactory explanation has been found to account for the fact that 6 of 9 rabbits in experiment #1 shed virus only in the eighth month after inoculation and 4 of 6 rabbits from which virus was isolated in experiment #2, shed virus between

days 80 and 90. Nesburn *et al.* (30) and Laibson and Kibrick (unpublished data) have documented clustering of spontaneous releases in Herpes simplex virus infected animals. These releases, rather than being spontaneous, may have been induced by some extrinsic factor. Epinephrine, which is released in excess during periods of stress in humans, could likewise be released in rabbits under stress, and thus be responsible for initiating virus release. In the present study animals were individually caged and handled to eliminate the possibility of cross contamination. There was no change in food, water or animal handling throughout the experiment. No known exogenous influences which might have caused increased release of endogenous epinephrine were found on thorough analysis of the experimental conditions; yet some inapparent stress factor may have played a part in stimulating virus release.

Three of seven rabbits inoculated with Type 1, strain Rodanus released virus spontaneously. Although Kaufman reported a much higher incidence of spontaneous release with the same strain (23), he swabbed daily (while swabbings were done only on alternate days in the present study), which in itself would increase the incidence of positive virus cultures. The techniques of viral culture are relatively insensitive; less than 0.05 ml of fluid on a swab is placed into the medium bathing a tissue culture (23). Many releases of virus might be missed

if the duration of release is 24 hr or less. The figures for virus isolation must therefore be considered minimal values. Kaufman did not state the size of his inoculum; perhaps this has some influence on the frequency of isolation. Laibson and Kibrick reported sporadic spontaneous releases with the same strain, even though they too reported they had swabbed daily. But, subsequent to their publication, Kibrick (personal communication) admitted some irregularity in the timing of swabbing.

Only a small number of rabbits inoculated with Type 2, strain MS, released virus spontaneously. No explanation for the low frequency of isolates can be given. A high percentage of these rabbits however, developed permanent, corneal lesions after primary infection. Other investigators who have attempted to culture virus from corneas afflicted with disciform keratitis have also been unsuccessful (55).

None of the rabbits inoculated with Type 1, strain 197, released virus within the arbitrary 16-day period following epinephrine administration. In contrast, viral release occurred in 5 of 6 rabbits inoculated with Type 1, strain Rodanus (out of 12 induction attempts with epinephrine) within the arbitrary period and 3 of 16 rabbits inoculated with Type 2, strain MS, out of 19 induction attempts.

At present, no definitive conclusion can be made as to the effect of epinephrine on the mechanism of viral release.

The pharmacological effects of epinephrine most certainly are limited to a few hours. Perdrau (31) has demonstrated that the infectivity of herpesvirus can be reduced or eliminated by oxidation, but may be "reactivated" by reduction. It is reasonable to speculate that temporary vasoconstriction resulting from epinephrine could produce a local hypoxia and consequent reactivation of residual herpesvirus, a process which may take several days. It is impossible to establish proper criteria to implicate epinephrine as the causative factor because the exact sequence of events leading to viral release are as yet unknown. A virus release was considered by Schmidt and Rasmussen (44) to be epinephrine induced if it occurred within 14 days post-epinephrine inoculation. They reported apparent induction of Herpes simplex encephalitis (J. R. Smith strain) in 6 of 10 rabbits with a fatal outcome within 3-8 days of epinephrine administration. The lack of spontaneous exacerbations in 25 rabbits inoculated 3.5 - 8 weeks previously, supports the premise of induction by epinephrine. Laibson and Kibrick (25,26) used an arbitrary time limit of 16 days as one of their criteria for epinephrine induced viral release. If the 16-day induction period is accepted as valid, the isolation of virus (Rodanus) in 5 of 6 rabbits would appear to be significant and would support the hypothesis of epinephrine-induced viral release. One cannot disprove the possibility of

spontaneous rather than induced "reactivations". More convincing evidence of epinephrine induction of viral release would have been provided if one or more rabbits had released virus subsequent to both courses of epinephrine.

It is unknown whether or not epinephrine is the only substance which might induce viral release. Schmidt and Rasmussen (44) were unsuccessful when using pyromen, cortisone acetate or glutathione in "reactivating" herpetic encephalitis. But, as discussed previously, they could induce encephalitis with epinephrine. Good and Campbell (13,14) were able to induce an exacerbation of encephalitis in their rabbits via anaphylactic shock. Anderson *et al.* (1) used an Arthus reaction to induce viral release in healed rabbit eyes. Perhaps further studies will incriminate epinephrine in these reactions.

Significant differences appear to exist between strains of Herpes simplex virus. A high percentage of rabbits, inoculated with either Type 1, Rodanus strain (passage level 27), or Type 2, MS strain (passage level approximately 16), succumbed to the primary infection whereas all rabbits inoculated with Type 1, strain 197 (passage level 4), survived. Laibson and Kibrick (25) also found the Rodanus strain to be neurotropic; encephalitis and subsequent death ensued in 25% of their infected rabbits. Perhaps a high passage level increases the neurovirulence of Herpes simplex virus.

Primary infection with strain MS, Type 2, produced severe disciform keratitis with permanent, corneal opacity, in contrast to Type 1, strains 197 and Rodanus, which produced mild, transient conjunctivitis. Williams *et al.* (55) reported 3 out of 4 high passaged strains of Herpes simplex virus (type unknown) produced disciform keratitis. The identification of these strains as Type 1 would eliminate the possibility of this lesion being a type characteristic. Perhaps multiple passages of a strain can significantly increase the incidence of disciform keratitis following primary herpetic keratitis.

The frequency of spontaneous viral release was higher in rabbits inoculated with Type 1 strains than with the Type 2 strain. Inoculation of epinephrine was relatively ineffective for inducing virus release in the rabbit eye. During non-epinephrine periods, strain 197 was released spontaneously more frequently than the other two strains, whereas this same strain consistently failed to be released following epinephrine administration. The incidence of viral release was slightly increased after epinephrine inoculation in "Rodanus rabbits". Epinephrine may act as an inducing agent for this strain. Although a few instances of viral release occurred following epinephrine inoculation in "MS rabbits", they were too few in number to comment upon.

Results (Virus Serology)

The rabbits in the second experiment which had not yielded virus within 2 weeks of inoculation were reinoculated in the opposite eye. These rabbits subsequently released virus within the following 2 weeks and were thus included in this study.

Levels of complement-fixing antibody in the strain 197 group of rabbits were remarkably consistent (see Table 3, p. 41). The highest titer reached was 1:128. Four rabbits reached a titer of 1:64 and four rabbits a titer of 1:32. None of these rabbits required re-inoculation and all peak titers were found at the first bleeding (two weeks post-inoculation). Seven episodes of viral release occurred during the study, but no significant changes in antibody levels ensued.

In the Rodanus group, four of the seven rabbits required re-inoculation. The highest titer was 1:64 and the lowest titer 1:16. Eight episodes of viral release occurred, and as noted with strains 197 and MS, no antibody response was apparent (see Table 4, p. 42).

In the MS group of rabbits, five of nine required re-inoculation. The highest titer was 1:256 and the lowest titer was 1:16. Two viral releases occurred, and as mentioned above, no antibody response was observed (see Table 5, p. 43).

Table 3. The complement-fixing antibody levels to Herpes simplex antigen, in rabbits inoculated with Herpes simplex strain 197, Type 1

Rabbit No.	Titer on Day 14*	Titer on Day 45*	Titer on Day 83*	Titer on Day 140*
413	1:128	1:32	1:32 (VR-days 128-136)	1:64
414	1:64	1:32	1:16	1:16
415	ND	1:32	1:16	1:16
416	1:32 (VR-day 35)	1:16 (VR-day 48)	1:32	1:16
417	1:32	1:32	1:16 (VR-days 84-88)	1:32
418	1:64	1:16	1:32 (VR-day 84)	RD
419	1:64	1:32	1:32 (VR-days 88-90)	1:32
420	1:64	1:16	1:16	1:16
421	1:32	1:16 (VR-day 80)	1:32	RD
422	1:16	1:16	1:16	1:16

* = after 1st virus inoculation
VR = virus release
RD = rabbit dead
ND = not done

Table 4. The complement-fixing antibody levels to Herpes simplex antigen, in rabbits inoculated with Herpes simplex strain Rodanus, Type 1

Rabbit No.	Titer on Day 14*	Titer on Day 45*	Titer on Day 83*	Titer on Day 140*
(R)424	1:16	1:64	1:32 (VR-day 111)	1:32
425	1:16	1:16	1:16 (VR-day 138)	1:8
428	1:64	1:32 (VR-day 66)	RD	RD
(R)429	1:8	1:64	1:64 (VR-days 126-130)	1:64
(R)430	1:4	1:16	1:8 (VR-day 98)	1:16
431	1:16	1:32 (VR-day 74)	1:32 (VR-days 130-134)	RD
(R)432	1:4	1:8 (VR-day 66)	1:16	1:8

(R) = reinoculated
* = after 1st virus inoculation
VR = virus release
RD = rabbit dead

Table 5. The complement-fixing antibody levels to Herpes simplex antigen, in rabbits inoculated with Herpes simplex strain MS, Type 2

Rabbit No.	Titer on Day 14*	Titer on Day 45*	Titer on Day 83*	Titer on Day 140*
401	1:4	1:8	1:128	1:128
403	1:4	1:8	1:32 (VR-day 100)	1:32
(R)404	1:4	1:64	1:64	1:128
406	1:4	1:8	1:16 (VR-day 130)	1:16
(R)408	1:4	1:64	1:64	1:64
(R)409	1:4	1:16	1:32	RD
410	1:32	1:128	1:32	1:64
(R)411	<1:4	1:128	1:256	1:256
(R)412	1:8	1:32	1:32	1:64

(R) = reinoculated
* = after 1st virus inoculation
VR = virus release
RD = rabbit dead

Discussion (Virus Serology)

Complement-fixing and neutralizing antibody to Herpes simplex virus are found, usually in high titer, in the sera of humans who have recurrent Herpes simplex infections. The level of antibody does not appear to fluctuate in response to recurrent episodes of virus release (54,3). Herpesvirus complement-fixing antibodies persist for life in a given individual (51). This persistence of antibody may be due to reboosting of antibody titer caused by continual antigenic stimulation.

The rabbits in this study had a significant antibody response to Herpes simplex antigen. Antibody appeared in the serum of most rabbits within 2 weeks. In fact, rabbits inoculated with strain 197 virus reached their peak antibody levels during this time. The antibody levels remained relatively constant throughout the course of the study, once the peak level was reached. The lack of antibody response to subsequent virus release is especially interesting but as yet unexplainable. Perhaps the immune mechanism is in some way altered, thus enabling the virus to establish a persistent, recurring disease. Circulating antibodies appear to be incapable of completely eradicating the virus, although they may limit viral activity to small areas (54).

Summary

In experiment 1, the left eye of 46 albino rabbits was inoculated with Herpes simplex virus, either Type 1, strain 197 or Type 2, strain MS. In experiment 2, the left eye of 34 albino rabbits was inoculated with one of three strains of Herpes simplex virus: Type 1, strains 197 or Rodanus, or Type 2, strain MS. The rabbit eyes were swabbed on alternate days for isolation of Herpes simplex virus. The rabbits were injected with epinephrine at intervals during the study. All rabbits (experiment 2) were bled intermittently and complement-fixation tests were performed to measure the antibody level to Herpes simplex antigen.

Herpes simplex virus may be recovered from the normal appearing eyes of rabbits with healed herpetic keratitis, both following epinephrine inoculation and prior to epinephrine inoculation, as a spontaneous event. There appears to be strain, rather that type, differences, notably the greater neurovirulence exhibited by strains MS and Rodanus, production of permanent corneal lesions by strain MS and the apparent inability to stimulate virus release in rabbits inoculated with strain 197 by the use of epinephrine. The rabbits in experiment 2 had a significant antibody response to Herpes simplex virus following primary infection. Although multiple

instances of viral release occurred with all three strains of virus, no antibody response was elicited in the rabbits.

Discussion of pertinent results, some speculations about the study and a review of the literature are included.

Chapter III.

HUMAN STUDY

Review of Literature

A unique opportunity for investigation of human herpetic infections was presented by the high frequency of women with Herpes zoster (40%) treated for ovarian malignancy with chlorambucil (Masterson, personal communication). The development of shingles was an ominous prognostic sign. The relationship between the tumor, the chlorambucil and the appearance of shingles in unknown. Two explanations for this phenomenon can be proposed: (1) The tumor or the chlorambucil may have a depressant effect upon the host's immunological mechanism and render her more susceptible to either a primary infection (in a partially immune host) or "reactivation" of a previous herpetic infection. The latter explanation regarding "reactivation" may hold true if antibodies do in fact retain the virus within a localized area. (2) The tumor or the chlorambucil may have a direct stimulating effect on persistent Herpes zoster virus. Bichel and Thorling (2) suggest that Herpes zoster is provoked by chemicals, roentgen rays, heat, cold, physical trauma or disease involving the spinal cord or its adjacent structures,

i.e. tumors which have metastisized. The latter suggestion may be valid if Varicella/Zoster (V/Z) virus persists in the dorsal ganglia. Dayan *et al.* (7) postulate that some patients bearing malignant tumors may have an unusual response to a herpetic infection because of hypersecretion of corticosteroids and disordered immunity caused by the tumor. Chlorambucil is known to have immunosuppressive effects (16). If patients so afflicted either developed high titers of circulating antibodies or maintained the antibody levels which persisted prior to therapy or development of the tumor, immunosuppression could be ruled out as the cause of the appearance of shingles.

In this study, a group of patients undergoing chemotherapy with chlorambucil were bled at monthly intervals and antibody titers were determined not only to V/Z virus, but also to Herpes simplex (HS) virus and Cytomegalovirus because antigenic similarities exist between members of the Herpes group of viruses. In a serological study of Herpes simplex patients, V/Z antibody levels rose in response to antigenic stimulation by HS virus (22). Schaap and Huisman (42) also found, in a serological study of V/Z patients, that antibodies to HS virus rose simultaneously with V/Z antibody, if the patient had a prior Herpes simplex infection. The present study was therefore designed to measure the serum antibody levels (to three human herpesviruses) in the sera

obtained from women under treatment with chlorambucil for ovarian tumors. The results of this study were compared with those obtained from sera of normal women of the same age, taken only one time, in an attempt to determine whether or not the patient's circumstance predisposed her to shingles accompanied by seroconversion. The study was performed with the hope of answering the following questions as well: Has a V/Z infection occurred and, can antibody levels to V/Z virus be used to prognosticate in patients with tumors of the ovary who are receiving chlorambucil.

Materials and Methods

Nine females, ranging in age from 33 to 68, who were under treatment, by Dr. John G. Masterson, with chlorambucil (Leukeran, Burrough & Wellcome & Co., Tuckahoe, New York), for malignant ovarian tumors, were bled at approximately monthly intervals. Serum complement-fixing antibody levels were measured, according to the method of Sever (46) to Herpes simplex virus, Varicella/Zoster virus and Cytomegalovirus (Microbiological Associates). Nineteen serum specimens obtained from normal, healthy females, ranging in age from 30 to 53, were used as controls (supplied by Oak Park Hospital, Oak Park, Illinois). Because herpesvirus complement-fixing antibodies persist for life in an individual (51) and tend to

attain constant levels (45), only one serum sample was collected from each control. Refer to Materials and Methods, Chapter II, for the procedure used to perform the complement fixation test.

Results

Table 6. The titers obtained from sera of women undergoing chlorambucil treatment for ovarian malignancy. Antibodies to Varicella/Zoster, Herpes simplex and Cytomegalovirus were measured by the micro-titer complement fixation test.

Patient	Date of Specimen	Antigens V/Z	HS	Cytomegalo
(1)	12/30/68	ND	1:4	ND
Age 33	1/6/69	ND	1:4	ND
	1/16/69	ND	1:4	ND
	1/28/69	1:32	1:32	<1:4
	2/18/69	1:32	1:32	<1:4
	3/10/69	1:32	1:32	<1:4
(2)	8/27/68	1:16	1:4	<1:4
Age 35	9/23/68	1:16	1:4	<1:4
	10/20/68	1:16	1:4	<1:4
	12/9/68	1:8	1:4	<1:4
	12/23/68	1:8	1:4	<1:4
	1/6/69	1:16	1:4	<1:4
	1/14/69	ND	1:4	ND
	2/4/69	1:16	1:4	<1:4

Table 6 - cont.

Patient	Date of Specimen	Antigens V/Z	HS	Cytomegalo
	2/25/69	1:16	1:8	<1:4
	3/10/69	1:16	1:4	<1:4
(3)	7/22/68	1:4	1:8	1:128
Age 46	9/16/68	1:4	1:8	1:128
	10/14/68	1:4	1:8	1:128
	12/10/68	1:4	1:16	1:128
	2/11/69	1:4	1:64	1:64
(4)	7/22/68	<1:4	<1:4	<1:4
Age 46	9/16/68	<1:4	<1:4	<1:4
	10/14/68	<1:4	<1:4	<1:4
	12/9/68	<1:4	<1:4	<1:4
	2/11/69	<1:4	<1:4	<1:4
(5)	12/10/68	1:8	1:4	1:4
Age 47	12/30/68	1:4	1:4	1:4
	1/7/69	ND	1:4	ND
	2/27/69	1:16	1:32	1:64
(6)	7/22/68	1:256	<1:4	<1:4
Age 49	8/26/68	1:128	ND	ND
	9/23/68	1:128	<1:4	<1:4

Table 6 - cont.

Patient	Date of Specimen	Antigens V/Z	HS	Cytomegalo
	10/20/68	1:128	<1:4	<1:4
	11/18/68	1:32	<1:4	<1:4
	2/11/69	1:64	1:4	<1:4
	3/10/69	1:32	1:4	<1:4
(7)	10/20/68	<1:4	1:4	<1:4
Age 49	12/9/68	1:4	1:8	<1:4
	12/23/68	1:4	1:8	<1:4
	Expired 1/8/69			
(8)	9/23/68	<1:4	1:8	1:8
Age 58	10/8/68	<1:4	1:8	1:16
	10/20/68	1:64	1:32	1:8
	10/29/68	1:64	1:16	1:8
	12/10/68	1:64	1:16	1:64
	12/30/68	1:64	1:16	1:64
	2/18/69	1:32	1:128	1:128
	3/18/69	1:16	1:64	1:64
(9)	7/22/68	1:16	1:16	1:16
Age 68	Expired 8/69			

ND = not done

Table 7. The titers obtained from sera of presumably normal women to serve as controls. Antibodies to Varicella/Zoster, Herpes simplex and cytomegalovirus were measured by the microtiter complement fixation test

Control	Age	Antigens V/Z	HS	Cytomegalo
(1)	30	1:4	1:32	1:8
(2)	31	<1:4	1:4	1:8
(3)	32	1:4	1:8	<1:4
(4)	32	1:8	1:32	1:16
(5)	35	1:16	1:32	1:4
(6)	36	<1:4	1:8	1:16
(7)	38	<1:4	1:16	1:16
(8)	38	1:8	1:8	<1:4
(9)	39	<1:4	1:16	1:32
(10)	45	1:4	1:16	1:16
(11)	45	1:8	1:32	1:16
(12)	46	1:8	1:32	1:16
(13)	48	1:4	1:4	<1:4
(14)	48	<1:4	<1:4	<1:4
(15)	49	<1:4	1:16	1:8
(16)	49	<1:4	1:4	1:32
(17)	49	<1:4	<1:4	1:16
(18)	52	<1:4	1:16	<1:4
(19)	53	<1:4	1:32	<1:4

Fig. 7. The percentage of patients versus controls possessing complement-fixing antibody titers to Varicella-Zoster virus

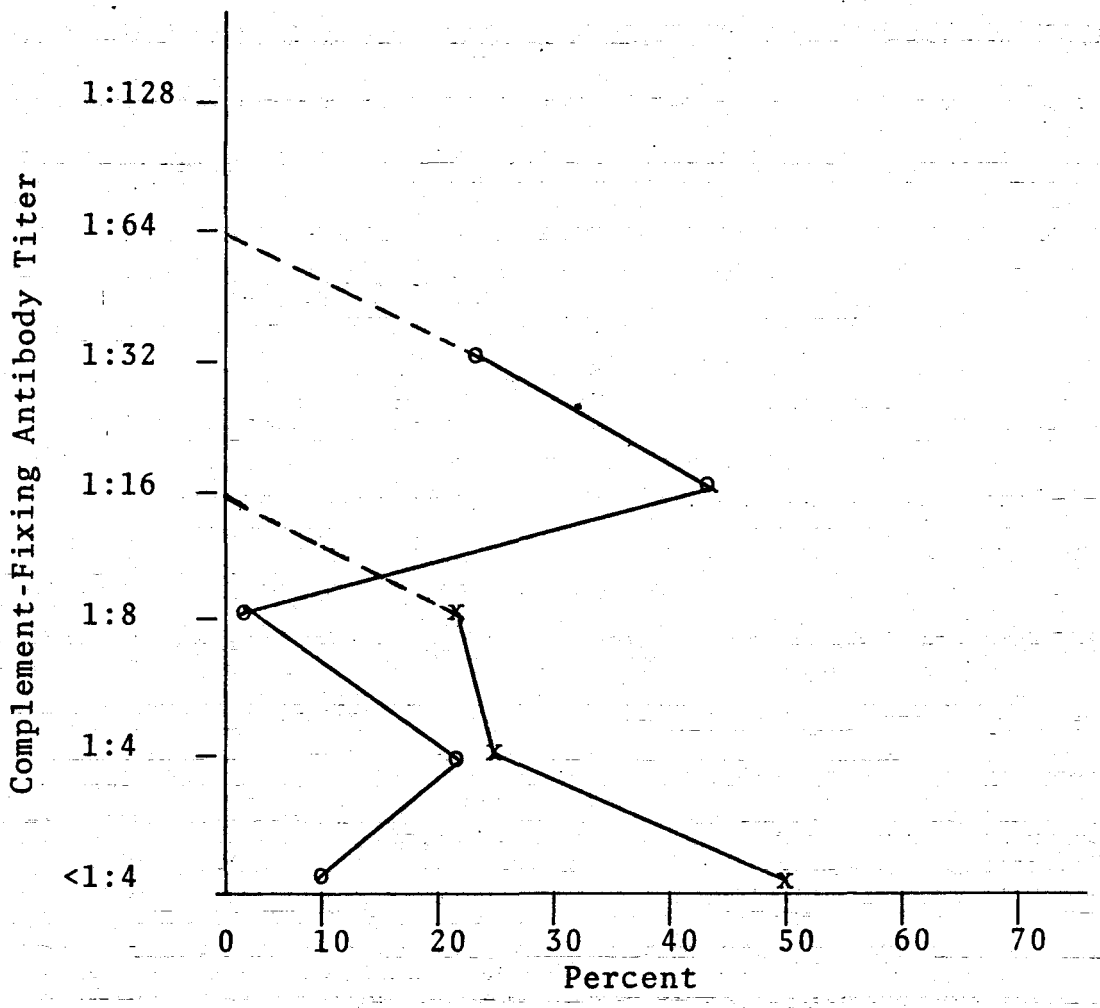


Fig. 8. The percentage of patients versus controls possessing complement-fixing antibody titers to Herpes simplex virus

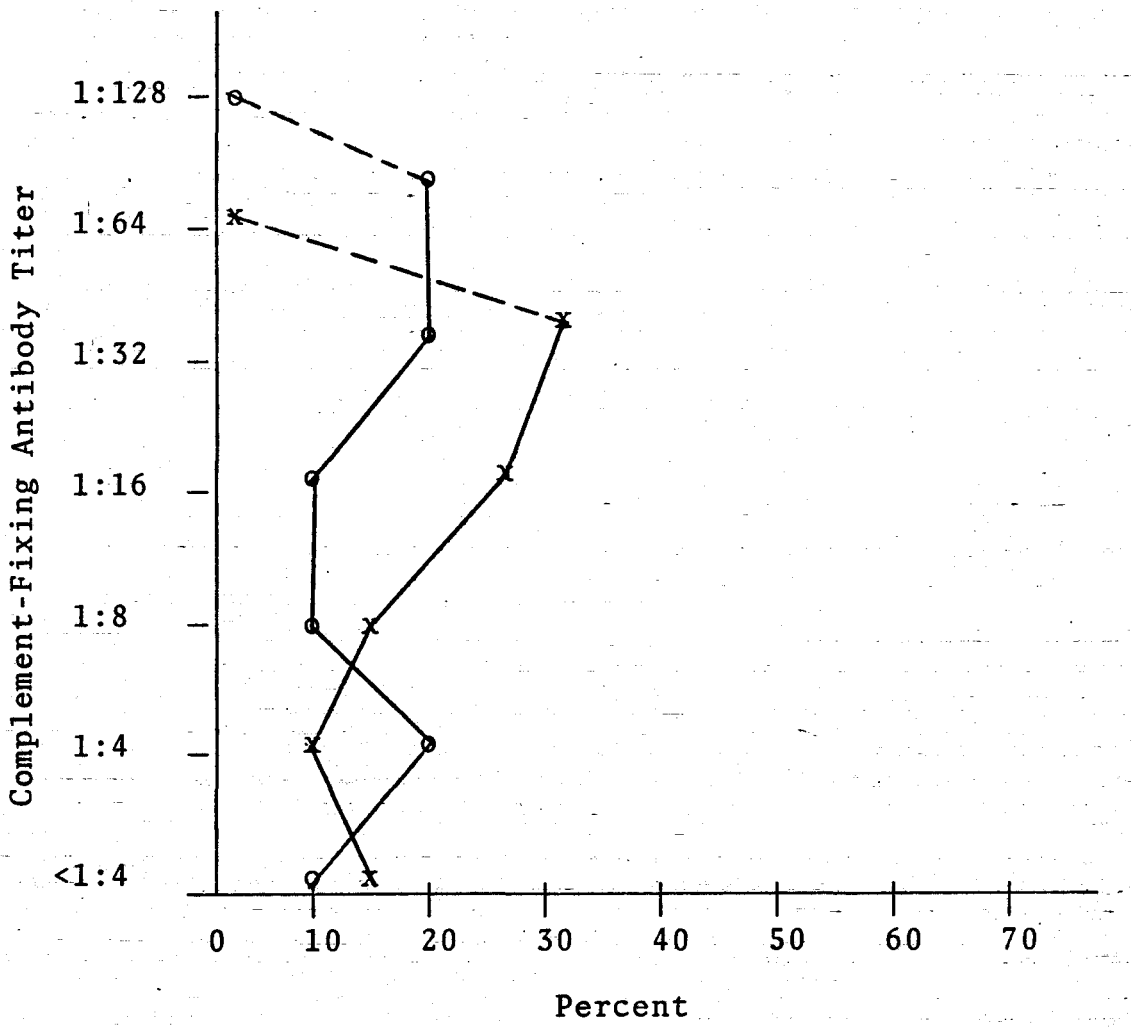


Fig. 9. The percentage of patients versus controls possessing complement-fixing antibody titers to Cytomegalovirus

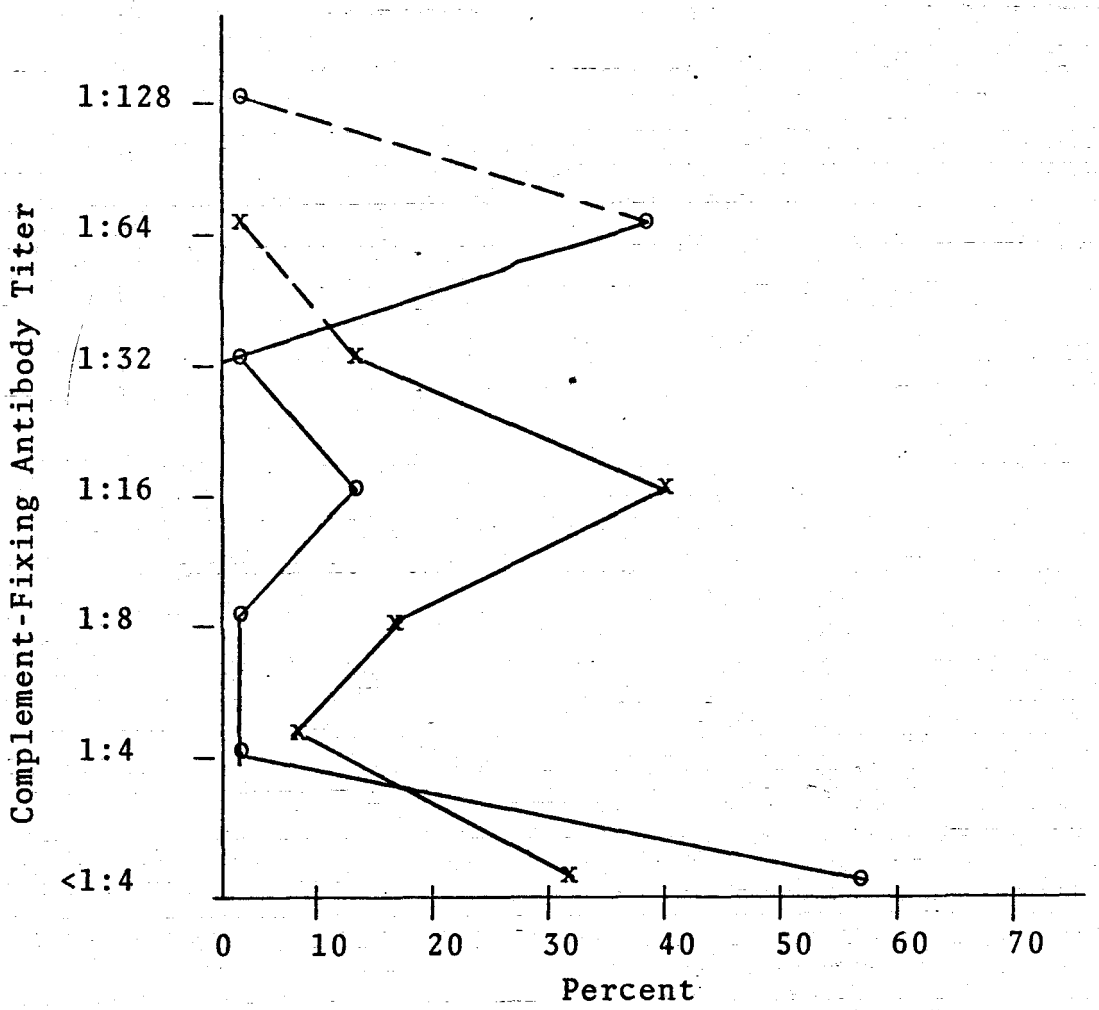


Fig. 10. The distribution by age of complement-fixing antibodies to Varicella-Zoster virus

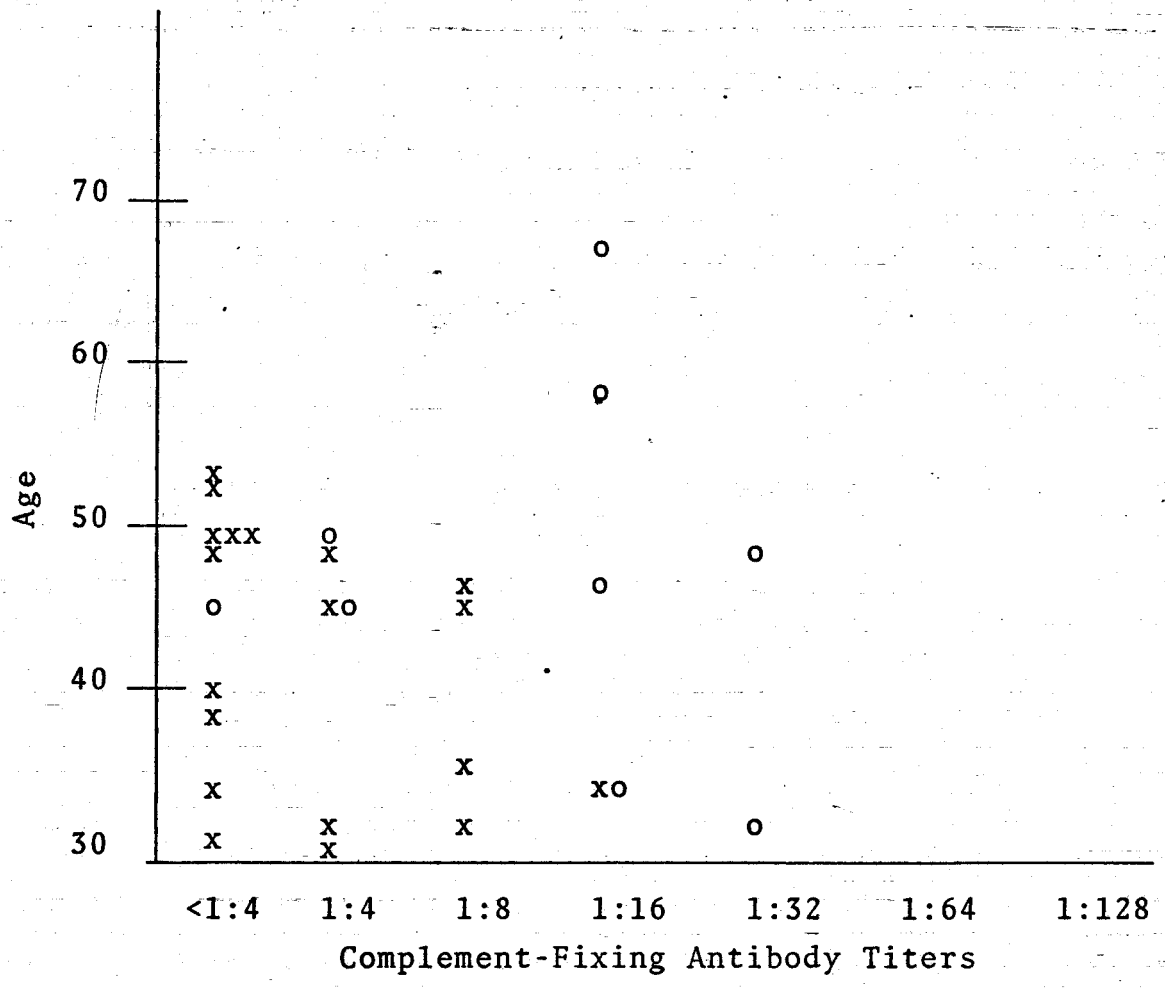


Fig. 11. The distribution by age of complement-fixing antibodies to Herpes simplex virus

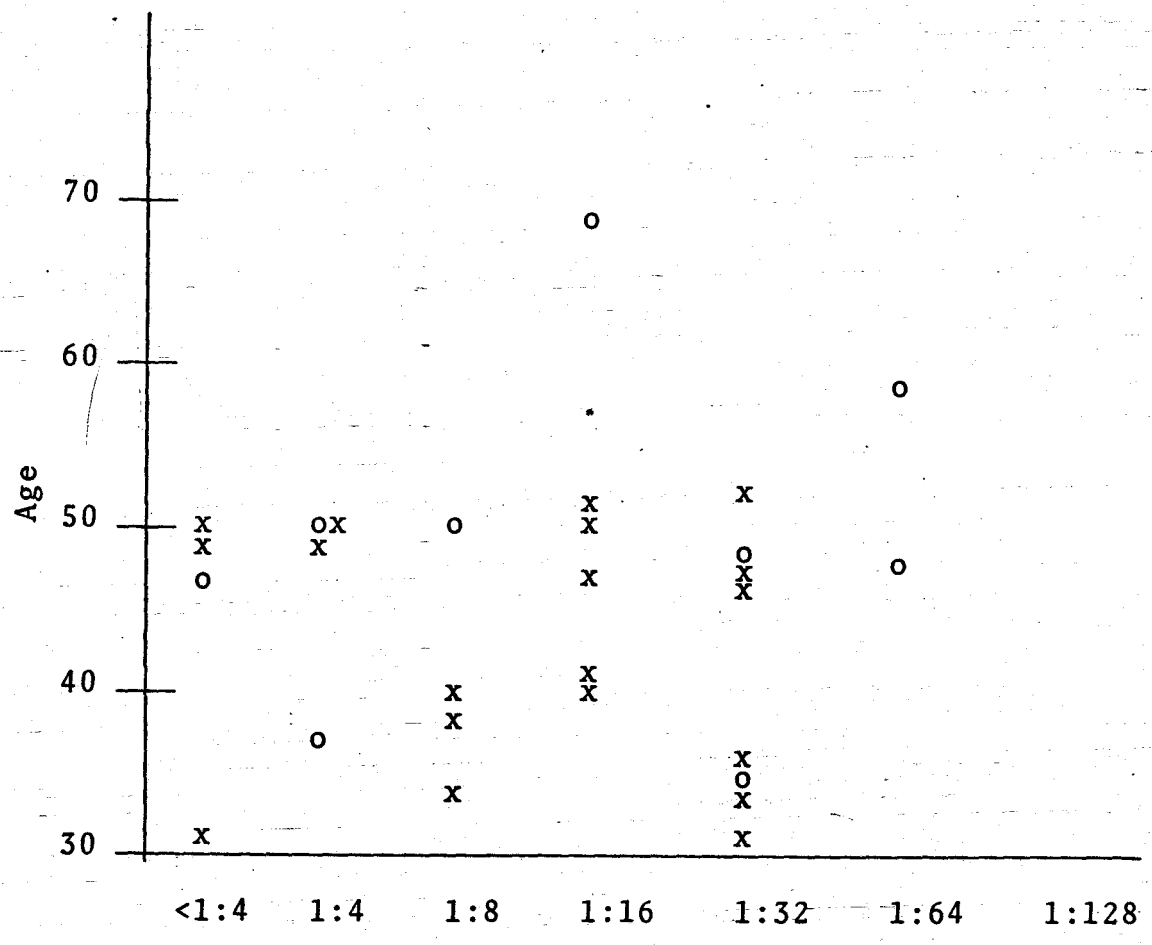
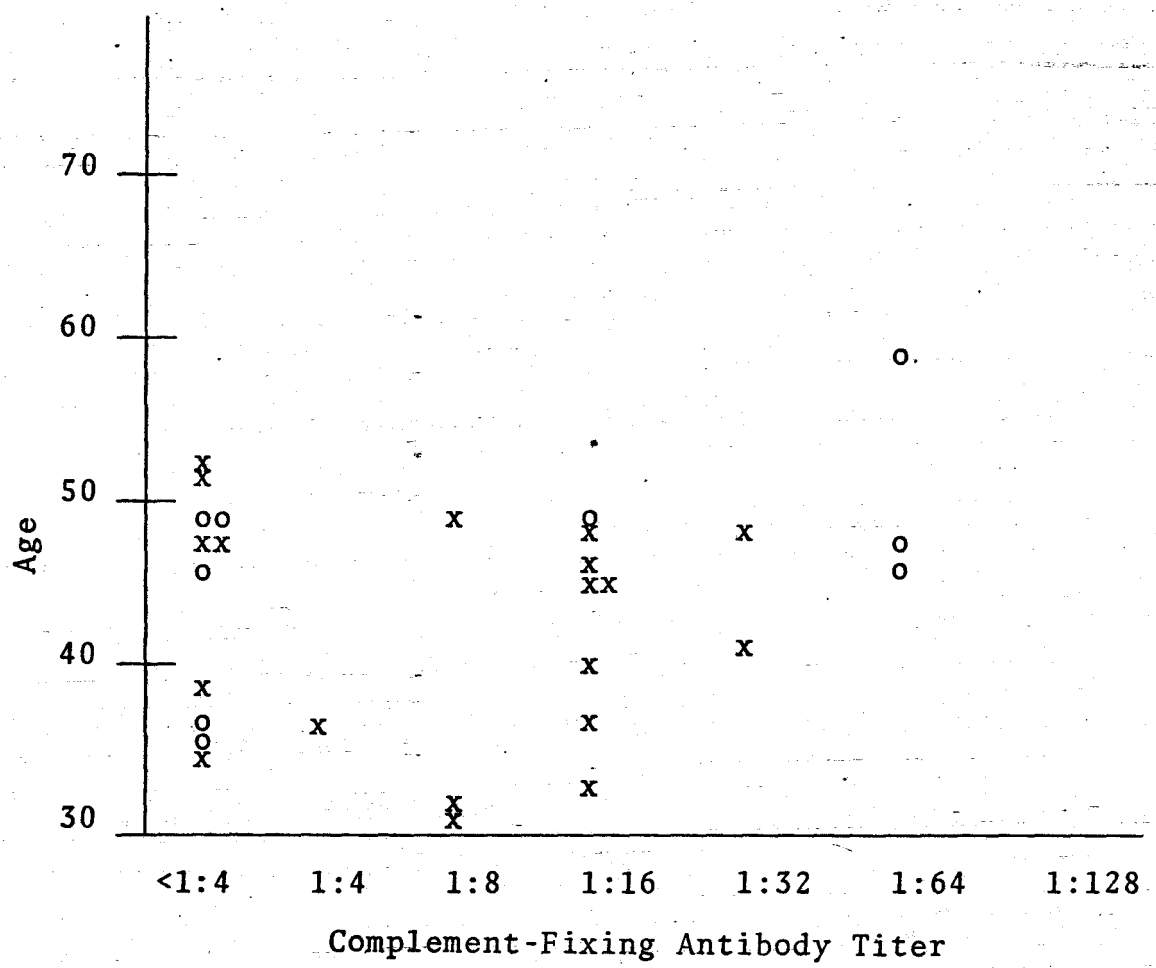


Fig. 12. Distribution by age of complement-fixing antibodies to Cytomegalovirus



Discussion

Sokal and Firat (49) reported an 8% incidence of Herpes zoster in a group of patients with Hodgkins Disease. Disseminated Herpes zoster (HZ) indicated a poor prognosis with expectant survival of less than a year whereas the localized form was not so ominous. The authors attributed HZ to a suppression of the host immune mechanism associated with advanced Hodgkins Disease.

Other modes of immunosuppression have been studied extensively. Many drugs, including the nitrogen mustards, adversely affect the immune mechanism when used in amounts adequate to treat neoplastic disease. Chlorambucil is a derivative of the nitrogen mustard group and is capable of immunosuppression. Even though several of the patients in this study had rising antibody titers, partial immunosuppression is possible.

Antibodies to Varicella-Zoster (V/Z), Herpes simplex (HS) and Cytomegaloviruses are prevalent in the adult population. Hayward (18) reported a comparative study of 95 sera from normal adults. The sera were tested for complement-fixing and neutralizing antibodies to HS virus. Eighty percent of the sera contained both types of antibody and 20% contained neither. Burnet and Williams (5) reported the presence of herpes antibodies in 93% of 55 hospitalized adult patients. Holzel *et al.* (19) reported the distribution of complement-

fixing (CF) antibodies to HS virus in 304 sera from patients of varying ages. Eighty-six percent of sera obtained from 35 patients over 15 years of age possessed Cytomegalovirus CF antibodies with titers ranging from 1:4 to 1:32. Stern and Elek (51) reported the incidence of antibodies to Cytomegalovirus in a normal British population: Under 5 years of age only 4% of the children had antibodies. By 10 years of age the incidence was 15%, by 15 years it was 21% and a maximum of 54% by 25-35 years which was maintained in the older age groups. Other authors have found significantly higher percentages of Cytomegalovirus CF antibody in the general population (40).

None of the patients in our study developed Herpes zoster during the study, precluding an evaluation of its prognostic significance. No patient had either a history of recurrent herpetic lesions or a Herpes simplex infection during the study.

Patient #1 in the present study had antibodies to V/Z and HS viruses (See Table 6, pp. 51-53) with a rising titer to HS virus. Unfortunately, an insufficient amount of serum was obtained in the first three bleedings to ascertain whether or not the V/Z titer had risen from a previously lower level. Schaap and Huisman (42) reported a simultaneous rise in CF antibodies to HS and V/Z viruses in patients with either

chickenpox or shingles. Kapsenberg (22) suggested a minor antigenic relationship between these viruses. An apparent pre-requisite to a simultaneous rise in antibodies to HS virus during antigenic stimulation by V/Z virus is a pre-existing immunity to HS virus (42). Ross *et al.* (38) studied the cross-reactions of these 2 viruses and found the homologous rise in titer to V/Z virus was generally higher than the heterologous rise to HS virus. The rise in titer to HS virus in patient #1 might have been caused by either a primary infection or cross-reactivity. A recurrent infection is excluded because of the rise in HS virus antibody (10,19,39).

Patient #2 had a constant titer to V/Z and HS viruses throughout the course of the study. Patient #3 had a constant low titer to V/Z virus, a constant high titer to Cytomegalovirus and a significant rise in titer to HS virus late in the course of the study. This rise in CF antibody to herpes probably represents a primary infection by a type or strain not previously encountered by the patient. Patient #4 had no significant titer to any of the three viruses during the course of the study. Patient #5 had initial titers of 1:8 to V/Z virus and 1:4 to HS and Cytomegaloviruses. Two months later, her titer to Cytomegalovirus rose to 1:64, while titers to HS and V/Z viruses rose to 1:32 and 1:16 respectively. A Cytomegalovirus infection, with heterologous stimulation of

HS and V/Z antibodies may have occurred. Information concerning cross-reactivity of HS and V/Z antibody in patients with Cytomegalovirus infection is sparse. Patient #6 was recovering from a HZ virus infection when she entered the study. No heterologous rise in CF antibodies to HS virus and Cytomegalovirus was observed. Patient #7 had no significant antibody changes during the 2 months of study prior to expiration. Patient #8 had a significant rise in antibodies to V/Z virus with a concomitant rise to HS virus early in the study. Two months later, she had a significant rise in titer to Cytomegalovirus. Both the HS and Cytomegalovirus titers reached higher levels than the V/Z titer. In mid-November, the patient complained of mouth pain. On subsequent phlebotomy in mid-December, her Cytomegalovirus antibody titer rose from 1:8 to 1:64 and in late December, a generalized petechial rash appeared. Phlebotomy in February revealed a rise in HS antibody from a titer of 1:16 to 1:128. The rising titers and the patient's clinical course are presumably related. Although the magnitude of the titer elevations imply primary infections, a sequence of infections by Herpes zoster, Cytomegalo and HS viruses would seem unlikely. An altered immune mechanism could predispose the patient to multiple infections. However, this appears unlikely since the patient appeared quite capable of antibody response. If heterologous cross-reactivity were

involved, the antibody levels to HS and Cytomegaloviruses would not be expected to exceed the level of V/Z antibody. No explanation for this interesting phenomenon is presently available. Patient #9 was bled only once prior to her death. She had titers of 1:16 to all three viruses. Six of 19 controls had CF antibody titers to all three viruses, but none had CF antibody titers of 1:16 or greater to all three antigens. Further bleedings of more terminal patients will be necessary to evaluate the significance of this patient's titers.

Figures 7 (p. 55), 8 (p. 56) and 9 (p. 57) graphically illustrate the percentage of patients versus controls possessing various titers of CF antibody to the three viruses. As seen in Table 7 (p. 54) and Fig. 7 (p. 55), nearly 50% of the controls had no titer to V/Z virus, whereas nearly 67% of the patients had titers of 1:16 or higher. As seen in Fig. 9 (p. 57), 56% of the patients had no titer to Cytomegalovirus whereas only 31% of the controls were negative. No significant difference was found between patients and controls in regard to titers to HS virus (Fig. 8, p. 56). Figures 10-12 (pp. 58-60) graphically illustrate the distribution by age of antibodies to these three viruses. In Fig. 10 (p. 58) there is no apparent influence of age on antibody to V/Z virus in patients and controls (thirty years of age or older). This graph demonstrates quite well, however, the distribution of higher titers

in patients as compared to controls. Figure 11 (p. 59) confirms the random distribution of patients and controls in antibody levels to HS virus. Again age appears to be unimportant in women 30 years of age or older. Figure 12 (p. 60) demonstrates that there is no significant influence of age on antibody levels to Cytomegalovirus. It is interesting to note, though it is of questionable significance, the random distribution of control titers compared to the selective distribution of patient titers into a negative and a high titer group (with one exception).

It is difficult to draw conclusions about the nature of antibody changes in these patients because of their small numbers, and the extreme variability in their clinical conditions, duration of treatment and other variables. There does appear to be significant differences in the V/Z virus antibody titers between patients and controls and a few differences between the two groups when considering Cytomegalo and HS antibody titers.

Summary of Human Study

Nine females, ranging in age from 33-68, who were under treatment with chlorambucil for malignant ovarian tumors, were bled at approximately monthly intervals and complement-fixing antibody levels to Herpes simplex, Varicella-Zoster and Cytomegaloviruses were measured. Sera obtained from nineteen healthy females, ages 30-53, were used as controls.

All patients, and nearly all controls, had titers to 1, 2 or all 3 antigens at the first bleeding. Several of the patients had rising titers to one or more antigens during the course of the study. Antigenic cross-reactivity between the three viruses apparently occurred. No significant difference in antibody titers to Herpes simplex and Cytomegaloviruses were found between patients and controls. In contrast, Varicella-Zoster antibody titers appeared higher in patients than controls. Age had no apparent influence on antibody distribution. None of the patients developed shingles or a Herpes simplex infection during the study, therefore prognostic evaluation could not be determined.

Discussion of the results and review of the literature are included.

BIBLIOGRAPHY

1. Anderson, W.A., B. Margruder, and E.D. Kilbourne. 1961. Induced reactivation of herpes simplex virus in healed rabbit corneal lesions. Proc. Soc. Exp. Biol. Med. 107:628-632.
2. Bichel, J. and K. Thorling. 1967. Herpes zoster in Hodgkin's disease. Danish Med. Bull. 14:68-70.
3. Blank, H. and G. Rake. 1955. "Viral and Rickettsial Disease of the Skin, Eye and Mucous Membranes of Man" Little, Brown & Co., Toronto. 285 pp., pp. 44-70.
4. Buddingh, G.J., D.I. Schrum, J.C. Lanier and D.J. Guidry. 1953. Studies on the natural history of herpes simplex infections. Pediatrics 11:595-610.
5. Burnet, F.M. and S.W. Williams. 1939. Herpes simplex: a new point of view. Med. J. Australia 1:637-642.
6. Carton, C.A. and E.D. Kilbourne. 1952. Activation of latent herpes simplex by trigeminal sensory-root section. New Eng. J. Med. 246:172-176.
7. Dayan, A.D., I. Bhatti and J.V.T. Gostling. 1967. Encephalitis due to herpes simplex in a patient with treated carcinoma of the uterus. Speculations about herpes simplex as a cause of carcinomatous encephalitis. Neurology (Minneapolis) 17:609-613.

8. Doerr, R. 1938. Herpes febriles. In Doerr, R. and C. Hallauer: Handbuch d. Virusforchung. Wien, Springer 1:41-43.

9. Doll, E.R. and J.T. Bryans. 1962. Incubation periods for abortion in equine viral pneumonitis. J. amer. vet. med. Ass. 141:351-354.

10. Dudgeon, J.A. 1950. A complement fixation test for HS infections. J. clin. Path. 3:239-247.

11. Ellison, S.A., C.A. Carton and H.M. Rose. 1959. Studies of recurrent herpes simplex infections following section of the trigeminal nerve. J. Infect. Dis. 105:161-167.

12. Findlay, G.M. and F.O. MacCallum. 1940. Recurrent traumatic herpes. Lancet 1:259-261.

13. Good, R.A. and B. Campbell. 1945. Potentiating effect of anaphylactic and histamine shock upon herpes simplex virus infection in rabbits. Proc. Soc. Exp. Biol. Med. 59:305-306.

14. Good, R.A. and B. Campbell. 1948. The precipitation of latent herpes simplex encephalitis by anaphylactic shock. Proc. Soc. Exp. Biol. Med. 68:82-87.

15. Gudnadottir, M., H. Holgadottir and O. Bjarnason. 1964. Virus isolated from the brain of a patient with multiple sclerosis. Exp. Neurol. 9:85-95.

16. Goodman, L.S. and A. Gillman. 1968. The Pharmacological Basis of Therapeutics. The MacMillan Co., New York. pp. 1345-1357.

17. Hanshaw, J.B., R.F. Betts, G. Simon and R.C. Boynton. 1965. Acquired cytomegalovirus infection. Association with hepatomegaly and abnormal liver-function tests. New Eng. J. Med. 272:602-610.

18. Hayward, M.E. 1950. Serological diagnosis of herpes simplex infections. Lancet 1:856-858.

19. Holzel, A., G.V. Feldman, J.O. Tobin and J. Harper. 1953. Herpes simplex. A study of complement fixing antibodies at different ages. Acta Paediat. 42:207-214.

20. Horsfall, F.L. and I. Tamm. 1965. "Viral and Rickettsial Infections of Man." J. B. Lippincott, Co., Philadelphia. pp. 1217-1222.

21. Johnson, R.T. 1964. The pathogenesis of herpes virus encephalitis. 1. Virus pathways to the nervous system of suckling mice demonstrated by fluorescent antibody staining. J. Exp. Med. 120:359-374.

22. Kapsenberg, J.G. 1964. Possible antigenic relationship between varicella/zoster virus and herpes simplex virus. Arch. ges. Virusforsch. 1:67-73.

23. Kaufman, H.E., D.C. Brown and E.M. Ellison. 1967. Recurrent herpes in the rabbit and man. Science 156:1628-1629.

24. Kaufman, H.E. 1968. Read before the Finland committee. (Personal communication.)

25. Laibson, P.R. and S. Kibrick. 1966. Reactivation of herpetic keratitis by epinephrine in rabbit. Arch. Ophthal. 75:254-260.

26. Laibson, P.R. and S. Kibrick. 1967. Reactivation of herpetic keratitis in rabbit. II. Repeated reactivations in the same host. Arch. Ophthal. 77:244-248.

27. Löwenstein, C. 1919. Aetiologische Untersuchungen über den fieberhaften Herpes. Münchener Medizinische Wochenschritt. 66:769-770.

28. Medearis, D.N. 1964a. Observations concerning human cytomegalovirus infection and disease. Johns Hopk. Hosp. Bull. 114:181-211.

29. Medearis, D.N. 1964b. Mouse cytomegalovirus infection. II. Observations during prolonged infections. III. Attempts to produce intrauterine infections. Amer. J. Hyg. 80:103-120.

30. Nesburn, A.B., J.H. Elliott and H.M. Leibowitz. 1967. Spontaneous reactivation of experimental herpes simplex keratitis in rabbits. Arch. Ophthal. 78:523-529.

31. Perdrau, J.R. 1931. Inactivation and reactivation of the viruses of herpes. Proc. Roy. Soc., London, s. B. 109:304-308.

32. Perdrau, J.R. 1938. Persistence of the virus of herpes in rabbits immunized with living virus. J. Pathol. Bacteriol. 47:447-455.

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

34. Plummer, G., P.H. Cleveland and C. Stevens. 1967. Herpes simplex virus and paralysis of rabbits-Activation of the paralysis by adrenalin. Brit. J. exp. Path. 48:390-394.

35. Plummer, G. 1967. Comparative virology of the herpes group. Progr. med. Virol. 9:302-340.

36. Richter, R. 1944. Observations bearing on presence of latent herpes simplex virus in human Gasserian ganglion. J. Nerv. and Ment. Dis. 99:356-358.

37. Rifkind, D., N. Goddman and R.B. Hill. 1967. The clinical significance of cytomegalovirus infection in renal transplant recipients. Ann. Int. Med. 66:1116-1127.

38. Ross, C.A.C., J.H. Subak Sharp and P. Perry. 1965. Antigenic relationship of varicella/zoster and herpes simplex. Lancet, pp. 708-711, Oct. 9.

39. Ross, C.A. and J. Stevenson. 1961. Herpes simplex meningoencephalitis. Lancet 2:682-685.

40. Rowe, W.P., J.W. Hartley, S. Waterman, H.C. Turner and R.J. Huebner. 1956. Cytopathogenic agent resembling

human salivary gland virus recovered from tissue cultures of human adenoids. Proc. Soc. exp. Biol. N.Y. 92:418-424.

41. Rustigian, R., J.B. Smulow, M. Tye, W.A. Gibson and E. Shindell. 1966. Studies on latent infection of skin and oral mucosa in individuals with recurrent herpes simplex. J. Invest. Derm. 47:218-221.

42. Schaap, G.J.P. and J. Husiman. 1968. Simultaneous rise in complement-fixing antibodies against herpesvirus hominis and varicella-zoster virus in patients with chickenpox and shingles. Arch. ges. Virusforsch. 25:52-57.

43. Schmidt, N.J.S. and E.H. Lennette. 1965. Appendix: Basic Techniques for Virology. Refer to Horsfall, F.L. and I. Tamm: "Viral and Rickettsial Infections of Man." pp. 1217-1222.

44. Schmidt, J.R. and A.F. Rasmussen. 1960. Activation of latent herpes simplex encephalitis by chemical means. J. Infect. Dis. 106:154-158.

45. Scott, T.F.M. 1957. Epidemiology of herpetic infections. Am. J. Ophthal. 43:4 Pt. 2, pp. 134-147.

46. Sever, J.L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. 88: 320-329.

47. Smith, M.G. and F. Vellios. 1950. Inclusion disease or generalized salivary gland virus infection. Arch. Path. (Chicago). 50:862-884.

48. Snowden, W.A. 1964. Infectious bovine rhinotracheitis and infectious pustular vulvovaginitis in Australian cattle. Austr. vet. J. 40:277-281.

49. Sokal, J.E. and D. Firat. 1965. Varicella-Zoster infection in Hodgkin's disease. Amer. J. Med. 39:452-463.

50. Stalder, W. and S. Zurukzoglu. 1936. Experimentelle Untersuchung über Herpes Transplantation herpes-infizierter Hautstellen, Reaktivierung von abgeheilten, künstlich infizierten Hautstellen. Herpesbehandlung VI. Mitteilung. Zbl. Bakt. (1 Abt. Orig.), 136: 94-97.

51. Stern, H. and S.D. Elek. 1965. The incidence of infection with cytomegalovirus in a normal population. A serological study in Greater London. J. Hyg., Camb. 63:69-87.

52. Teague, O. and E.W. Goodpasture. 1923. Experimental herpes zoster. J. Med. Res. 44:185-200.

53. Weller, T.H. and J.B. Hanshaw. 1962. Virologic and clinical observations on cytomegalic inclusion disease. New Eng. J. Med. 266:1233-1244.

54. Wheeler, C.E. 1960. Further studies on the effect of neutralizing antibody upon the course of herpes simplex infections in tissue culture. J. Immunol. 84:394-403.

55. Williams, L.E., A.B. Nesburn and H.E. Kaufman. 1965. Experimental induction of disciform keratitis. Arch. Ophthal. 73:112-114.

56. Wong, T.W. and N.E. Warner. 1962. Cytomegalic inclusion disease in adults. Report of 14 cases with review of the literature. Arch. Path. (Chicago) 74:403-422.

57. Wyatt, J.P., J. Saxton, R.S. Lee and H. Pinkerton. 1950. Generalized cytomegalic inclusion disease. J. Pediat. 36:271-294.

58. Zuelzer, W.W., C.S. Stulberg, R.H. Page, J. Teruya and A.J. Brough. 1966. Etiology and pathogenesis of acquired hemolytic anemia. Transfusion 6:438-461.

APPROVAL SHEET

The thesis submitted by Diane Halstead has been read and approved by members of the Department of Microbiology.

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.

1/16/70
Date

Signature of Adviser