STUDIES ON HERPETIC INFECTION IN MICE

V. Influence of Route of Inoculation on Susceptibility to Herpetic Infection; Effect of Age, and of Methylcholanthrene*

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In the preceding papers of this series (1–4) studies on passive immunization of mice against herpes virus inoculated intranasally or into the foot pad were reported. It was shown that antibodies acquired naturally by young mice suckled by immune mothers, or artificially by mice injected with specific antiserum from an heterologous species are effective in protecting against the virus subsequently inoculated by the nasal route. When the virus was inoculated into the foot pad, allowed to multiply locally, and enter the local nerves before antiserum was administered, a definite although incomplete protection was demonstrated. The present paper is concerned principally with the influence of the site of cutaneous inoculation of the virus on the development of herpetic infections of the nervous system of mice.

A number of investigators have reported studies in which mice were inoculated with herpes virus by one route or by several routes (5–8) but no one has previously carried out a systematic study of the influence of the site of cutaneous inoculation on the susceptibility of mice to herpes virus.

Virus was inoculated into the skin in various areas of the bodies of adult and young mice. Great differences in susceptibility have been found depending upon (1) the age of the mice, and (2) the site of the inoculation. Treatment of the skin with methylcholanthrene before inoculation of virus increased the susceptibility to herpetic infections to a limited extent.

Materials and Methods

The animals used in the present study were all Swiss mice from the stock described earlier (1).

Two strains of herpes simplex virus were used. One of these was the HF strain. It had been maintained for more than 20 years in experimental animals and at the time the present experi-

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ments were done, had been passed serially at the University of Rochester for more than 125 generations by brain-to-brain inoculation of mice.

In order to compare the activity of a newly isolated strain of herpes virus with that of the HF strain, the Klee strain was selected for study. It was isolated in 1942 from a patient with recurrent herpes of the thigh. Isolation from the human source was made by the corneal inoculation of a rabbit and the virus was maintained by alternate passages through mouse brain and rabbit cornea. By storing the mouse-brain virus in 50 per cent glycerol for periods of 2 or 3 weeks, it was possible to carry out all the experiments with virus that had been carried through not more than 3 passages following the original isolation. The brains used for inocula in the experiments reported herein were never stored in glycerol for more than 7 days. The methods of preparing the suspension of virus and the bacteriological control procedures employed were described in the first paper of the present series (1).

Rabbits inoculated on the cornea with Klee virus invariably developed encephalitis, which was usually fatal. Of 24 rabbits inoculated on the scarified skin of the flank, 19 died of herpetic encephalomyelitis and the other 5 developed a severe myelitis. Therefore, the neurotropism of the Klee virus is of the same order as that of the HF strain.

Either freshly removed mouse brains or, more often, brains that had been in glycerol for from 2 to 7 days were used as a source of virus. (The brains that had been in glycerol for a few days seemed to yield more virus than those that were freshly removed, but the data are insufficient to justify a final statement.)

Two or more mouse brains were pooled in preparing the inoculum for each experiment and the potency of each batch of virus was checked by the intracerebral inoculation of mice. An estimation of the amount of virus in each suspension was made by carrying out serial tenfold dilutions in Locke's solution and injecting 3 mice intracerebrally with 0.025 ml. of each dilution. The least concentration of the HF virus that killed all the mice was usually 10^{-3} or 10^{-4} (in one instance, 10^{-5}). The Klee virus regularly killed mice in a concentration of 10^{-3}.

The general plan of the present investigation was as follows. Groups of mice were inoculated by various peripheral routes with a virus preparation of measured potency. Following inoculation, the mice were carefully observed for evidence of local infection and for signs of involvement of the nervous system. After an observation period of 3 or 4 weeks, all the surviving mice were given intracerebrally, as a challenge inoculation, a large dose of HF virus (between 100 and 1000 minimal lethal doses by intracerebral inoculation—M.C.L.D.). All of those that exhibited evidence of immunity to the challenge inoculation were considered to have had an active infection even though no symptoms had been observed.

EXPERIMENTAL

With a few exceptions, the results of individual experiments are not given in the text. The results of all experiments are summarized in Table I. In Figs. 1, 2, and 3 the results of inoculating virus on the abdomen, tail, and foot are presented.

1. Herpetic Infections of Normal Adult Mice

Immersion of the Sectioned Tibial Nerve in Virus.—

Seven 3-month-old mice were used. The tibial nerve was exposed and sectioned with sharp scissors. Then a pool of about 0.015 ml. of a 30 per cent suspension of the HF virus was de-

posited around the cut end of the nerve and the wound closed. Four days after operation 6 of the mice showed clean healing of the incision; the seventh showed considerable suppuration.

None of the mice exhibited any sign of infection of the nervous system by the end of the 3 week period of observation. After 4 weeks the mice were given intracerebrally a challenge

TABLE I

Herpetic Infections of Mice Inoculated by Various Peripheral Routes

Route of inoculation	HF strain of herpes virus					Klee strain of herpes virus				
	No. of mice	No. paralyzed	No. died	Day of death	No. resistant to challenge inoculation	No. of mice	No. paralyzed	No. died	Day of death	No. resistant to challenge inoculation
1. Normal adult mice										
Tibial nerve	7	0	0	—	1/7*					
Sciatic nerve	6	1	2	6–10	1/4					
Cornea	4	4	4	8–20	—	3	2	2	8–15	
Skin of body	5	4	4	7–9	1/1	5	3	2	9	1/3
Foot pad	16	2	2	11–14	6/14	5	4	3	13–19	0/2
Tail	16	0	0	—	1/10	5	0	0	—	0/5
2 a. Adult mice treated by local application of methylcholanthrene										
Skin of body	5	5	5	7–9	—	4	4	4	7	—
Foot pad	5	0	0	—	5/5‡	5	5	4	11–17	1/1‡
Tail	5	2	2	8–21	1/2	5	0	0	—	0/5
2 b. Adult mice treated by local application of turpentine-acetone mixture										
Skin of body	5	5	5	7–9	—	4	3	3	8–9	0/1
Foot pad	5	0	1	5	3/4‡	5	4	2	11–17	2/3
Tail	5	0	0	—	0/5	5	0	0	—	0/4
3. 2-week-old mice										
Skin of body	5	5	5	8–9	—	5	—	1	7	1/4
Foot pad	20	20	20	5–6	—	7	6	6	6–11	0/1
Tail	6	4	4	7–14	0/2	6	4	4	7–14	0/2

* Numerator = No. of mice that survived; denominator = No. of mice injected with challenge dose of virus.

‡ Prolonged illness and delayed death indicating partial immunity. See Figs. 1, 2, and 3.

injection of HF virus. Six died and one survived. Presumably the lone surviving animal experienced a mild herpetic infection as a result of the original inoculation.

Immersion of the Sectioned Sciatic Nerve in Virus.—

Six 2-month-old mice were used. The sciatic nerve was exposed at about the midthigh level and a pool of a 20 per cent suspension of the HF virus was deposited around the nerve which

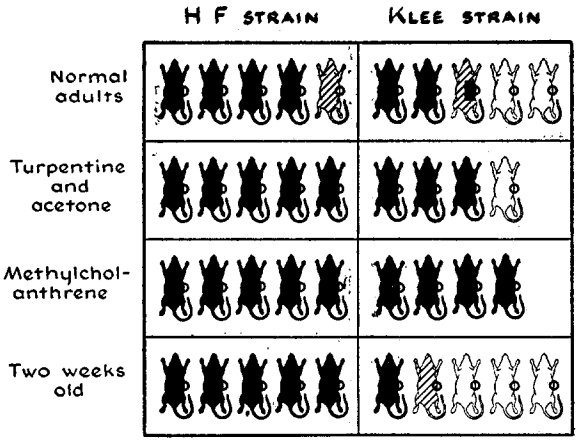

FIG. 1. Herpetic infections of mice by 2 strains of virus following inoculation of abdomen. Each figure represents one animal. Shading indicates death (entire mouse shaded) or paralysis without death (region of paralysis shaded). Diagonal lines indicate immunity to subsequent challenge inoculation intracerebrally; lack of immunity shown by absence of diagonal lines. o = site of inoculation.

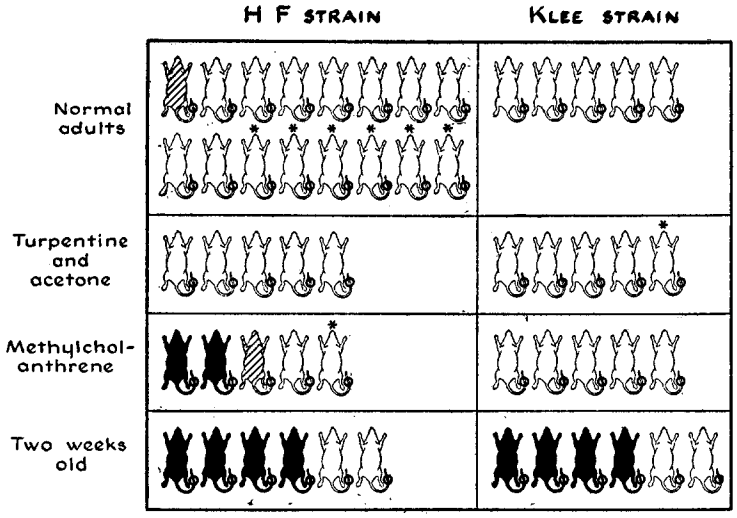

FIG. 2. Herpetic infections of mice by 2 strains of virus following inoculation of tail. See Fig. 1 for explanation of symbols.
* Not tested for immunity.

was then sectioned with sharp scissors. More inoculum was then added and the incision was closed without sutures. No bacterial infections occurred.

One mouse developed a paraplegia on the 5th day and was dead on the 6th. Another mouse was found dead on the 10th day; no previous paralysis had been noted. The 4 surviving mice

were given challenge inoculations intracerebrally 23 days after the original inoculation; 3 died. The lone survivor presumably had acquired immunity as a result of an inapparent infection.

It is evident that herpes virus (at least 100 M.C.L.D.) deposited around the cut end of a sciatic nerve lying in the intermuscular spaces caused no appreciable infection in half of the animals.

Corneal Inoculation.—

Seven 3-month-old mice were inoculated on the cornea, 3 receiving Klee virus (30 per cent suspension of mouse brain) and 4, HF virus (10 per cent suspension of mouse brain). The

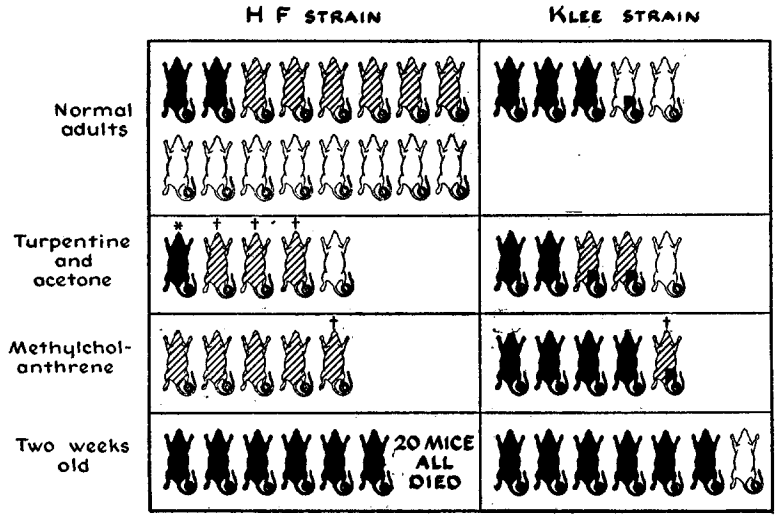

FIG. 3. Herpetic infections of mice by 2 strains of virus following inoculation of foot pad. See Fig. 1 for explanation of symbols.

* Death probably accidental.

† Partial immunity—prolonged illness and delayed death.

virus was applied to the right eye of each mouse after the cornea had been scarified with a sharp scalpel under ether anesthesia.

For 4 days all mice appeared entirely normal, no exudate being noted during this period. On the 5th and 6th days, purulent exudate was present in the inoculated eyes of 6 of the mice. Throughout the experiment, the seventh mouse, which had been inoculated with Klee virus, showed no exudate or other evidence of infection. On the 6th day none of the mice showed signs of nervous disease, but on the 7th day all 6 that developed keratitis had disturbances of equilibrium, leaning towards the right side. One of the mice given Klee virus and 3 of those given HF virus died on the 8th day. The 2 surviving, but ill mice went through a prolonged, slowly progressive illness with increasingly severe neurological disturbances until death occurred on the 15th and 20th days.

When rabbits were similarly inoculated on the scarified cornea with either of these viruses,

an exudate appeared within 24 hours, becoming abundant in 48 hours. Ataxia was usually evident on the 3rd or 4th day.

The prolonged incubation period in mice may have been the result of the relatively small number of cells injured by scarifying the small surface presented by the mouse cornea. It is clear, however, that once a corneal infection was well established, both strains of virus passed quickly to the brain.

Scarification of Skin of the Abdomen.—

Ten adult mice were inoculated by rubbing the virus into the prepared skin of the abdomen, 5 receiving HF virus (20 per cent suspension of mouse brain) and 5 Klee virus (30 per cent suspension of mouse brain). The hair was first plucked from an area of skin about a centimeter in diameter. Then virus was rubbed into the skin, which was immediately scarified with a scalpel and punctured 10 or 15 times with a No. 26 hypodermic needle.

All 10 mice developed encrusted lesions localized to the site of inoculation.

Inoculation of Foot Pad.—

A total of twenty-one 3-month-old mice was used in several experiments to test the susceptibility to virus inoculated on the pad of the hind foot. In the first experiment the virus was either injected intracutaneously (in which case the inoculum invariably was also subcutaneous) or applied to the surface of the foot pad, which had been prepared by multiple punctures with a No. 26 hypodermic needle and by superficial cuts with a sharp scalpel. Later, injection intracutaneously was combined with scarification in an attempt to break down the resistance of the mice to this type of inoculation. When infection occurred, the feet became appreciably swollen and developed crusted lesions with a small amount of purulent exudate in the areas scarified.

Scarification of Tail.—

HF virus (20 per cent suspension of mouse brain) was applied to the scarified tails of twelve 3-month-old mice and Klee virus was similarly applied to the tails of 5 mice of the same age. The Klee virus consisted of a mixture of a 30 per cent suspension of mouse brain plus Locke's solution that had been used to wash out the eyes of 2 rabbits with a 48-hour-old herpetic infection of the cornea. Scarification of the tail was accomplished by scraping the distal one-half inch with a sharp scalpel and making transverse incisions through the outer layers of the skin. The scarified tail was dipped into the virus suspension and allowed to dry for about a minute before the mouse was released.

Encrusted lesions formed at the site of inoculation on all mice and persisted for 2 or more weeks. No paralysis or other evidence of disease of the nervous system appeared.

2. *Herpetic Infections of Mice Treated with (a) Methylcholanthrene or (b) Turpentine-Acetone Mixture*

Friedewald (9) has shown that the susceptibility of rabbit skin to Shope's papilloma virus can be increased by preliminary treatment with methylcholanthrene or with a mixture of equal parts of turpentine and acetone. Accordingly, we decided to use these materials in an attempt to increase the susceptibility of mice to herpetic infection.

A group of 35 mice (10 weeks old) were painted with methylcholanthrene (0.3 per cent in benzene) and an equal number with a turpentine-acetone mixture. Each mouse was painted 5 or 6 times at intervals of 2 days. The tail, hind feet, and abdomen were painted on all mice with the exception of about 20 that received methylcholanthrene on the tail and hind feet only.

The visible effects of the application of the turpentine-acetone mixture were limited to a fine, dry scaliness of the abdominal skin. The mice given the methylcholanthrene showed redness, extensive loss of hair, and edema of the treated areas of the abdomen and hind feet. The tails appeared but slightly reddened. Furthermore, these mice were in poor condition as a result of the treatments, being inactive and thin by the time of the last methylcholanthrene painting.

Two or 3 days after the last painting, all the mice were inoculated with the HF or the Klee virus on either the tail, or the pad of the right hind foot, or the skin of the abdomen. The inocula consisted of 20 per cent suspensions of infected mouse brain plus, in the case of the Klee virus, washings from the eyes of a rabbit inoculated on the scarified corneas 72 hours previously. The methods of inoculation were the same as those already described in experiments with normal adult mice.

3. Herpetic Infections of 2-Week-Old Mice

It is generally known that young animals are more susceptible to certain viruses than adults. Sabin (10) has presented evidence to show that barriers preventing invasion of the CNS of adult animals by certain neurotropic viruses may be lacking in the young of the same species. In order to compare the susceptibility of young mice to herpetic infections with that of adult mice, the following experiments were carried out.

Several groups of mice, 2 weeks old (plus or minus 2 days), were inolulated with the HF or the Klee strains of herpes virus. All the young mice were left with their mothers for at least 21 days after birth.[1]

The HF virus used was a 10 per cent suspension of mouse brain. The Klee virus was a portion of the suspension used for the inoculation of normal adult mice by various routes; that is, a 30 per cent suspension of mouse brain to which the eye washings from 2 rabbits with herpetic keratitis had been added.

The young mice were inoculated by the same 3 routes used in the preceding set of experiments; *i.e.*, skin of the abdomen, foot pad, and tail.

Skin of the Abdomen.—

Because the skin of the abdomen of young mice was found to tear easily when inoculated in the usual way, it was prepared by making punctures and gently scratching with a No. 27 hypodermic needle. This preparation resulted in a less extensive and less complete scarification than was accomplished with adult mice. It seems probable that inadequate scarification accounts for the low fatility rate in the mice given the Klee virus as shown in Table I and Fig. 3.

Foot Pad.—

Five young mice were inolulated with the HF virus on the scarified pad of the right hind foot, the virus being introduced intracutaneously and subcutaneously. Seven were similarly inoculated with the Klee virus.

[1] Many of the mothers of the young mice used in these experiments were later given challenge inoculations of herpes virus; none showed resistance. In other experiments a few instances were observed in which mothers that had eaten infected young became resistant.

Tail.—

Six young mice were inoculated on the tail with each of the strains of herpes virus following scarification of the terminal half of the tail.

4. The Correlation of Neurological Disturbances with Routes of Inoculation

Goodpasture and Teague (11) have shown that, in the rabbit, herpes virus readily ascends the motor, sensory, or autonomic nerve fibers supplying an inoculated area. That the virus travels by way of nerve fibers and almost certainly within the axis cylinder is fundamental to an understanding of the pathogenesis of herpetic infection of the nervous system induced by peripheral inoculation. It is to be expected that, if herpes virus progresses to the nervous system in this manner, inoculation by the several peripheral routes that have been employed would give rise to correspondingly different neurological disturbances. This was found to be the case.

Mice subjected to corneal inoculation developed first a turning of the head towards the inoculated side, followed by a tendency to fall in that direction. Convulsions or a stuporous condition preceded death.

"Skin of the abdomen" was inoculated on the right side on an area about half way between the rib margin and the pelvis. The first sign of a neurological disturbance was a bulging of the abdomen on the homolateral side owing to a flaccid paralysis of the muscles of that area. Soon a lateral scoliosis resulted from a flaccid paralysis of the spinal muscles on the side of the inoculation, with contraction of the contralateral muscles now lacking their normal antagonists. No paralysis of the limbs was present at this stage. Usually some disturbance of either the hind legs or the front legs, or both, was evident before the terminal stages of encephalitis appeared.

In mice inoculated on the pad of the right hind foot, the first manifestation of neurological disease was invariably a disturbance of the function of that foot. Progressive paralysis of the right hind leg followed. Then the paralysis spread to the left hind leg, which was usually held forward and adducted, but which was capable of a slight pushing motion to the end of life. After inoculation of the foot pad, the stupor or convulsions of terminal encephalitis nearly always appeared before either abdominal distention or paralysis of the front legs. This finding was interpreted as indicating that the virus traversed the long nerve tracts from the lower part of the spinal cord to the brain without extensive involvement of the intervening portions of the spinal cord. In his studies on the pathology of poliomyelitis, Hurst (12) found in one instance that the virus went rapidly from a focus in the lower spinal cord to the brain without involving the cervical cord. On the other hand, Howe and Bodian (13) have recorded different observations, which indicate that poliomyelitis virus may ascend the spinal cord and involve the cervical levels before it reaches the brain.

Most of the immature mice inoculated on the scarified tail developed nervous disorders terminating in death. The first disturbance noted was often a paraplegia. Quadriplegia sometimes followed, but more often the signs of encephalitis appeared before any marked disturbances of the front limbs were detected. The first signs of illness exhibited by several of the mice were irritability and hyperactivity caused by encephalitis. These animals died without manifest evidence of involvement of the spinal cord.

RESULTS AND DISCUSSION

Immersion of the cut end of the tibial or sciatic nerve of an adult mouse in a concentrated suspension of herpes virus usually failed to induce infection. Howe and Bodian (13) found in their work with poliomyelitis in monkeys that the cut end of a large nerve was in general more readily infected than that of a small nerve. The sciatic nerve of a mouse is smaller than the nerves they were successful in infecting by this method.

Susceptibility to herpetic infection of adult mice by the other routes tested showed marked differences that followed a definite pattern determined by the site of inoculation.

Site of Inoculation	*Result*
1. Cornea	Fatal encephalomyelitis in nearly all animals.
2. Abdominal skin	Fatal encephalomyelitis in most animals. Immunizing infection in some. No symptoms and no immunity in others.
3. Pad of hind foot	Fatal encephalomyelitis in about 25 per cent of animals. Immunizing infection in 25 per cent. No symptoms and no immunity in 50 per cent.
4. Tail	No symptoms in 20 animals. Only one immunized.

It is noteworthy that the susceptibility of the mice to infection of the central nervous system differed so markedly with the site of inoculation in spite of the fact that in every instance the tissue inoculated was stratified squamous epithelium. That the extent of scarification was not the decisive factor is revealed by the high resistance to infection after the inoculation of the tail, which was more extensively scarified than either the cornea or the hind foot. It is probable that some animals exhibiting no symptoms of CNS involvement and no resistance underwent a limited infection of the epithelium. This interpretation seems likely in view of the extensive local lesions that were observed on the tail. Whether or not this was the case, the general picture of a graded susceptibility dependent on the site of inoculation remains unchanged.

Comparison by the chi square method of the results of inoculating normal adult mice on the tail, with results of inoculating similar mice after treatment with methylcholanthrene gives a value of $\chi^2 = 5.92$ or $P = 0.015$. Therefore, the observed differences might be expected to occur owing to chance once in approximately 67 times. These calculations are based on the number of infected animals and indicated by the occurrence of encephalomyelitis or the

development of immunity as shown by resistance to a challenge inoculation. Animals not subjected to the challenge inoculation are not included. Similar calculations based on numbers of animals dying of encephalomyelitis after inoculation of the tail or numbers infected by inoculation on feet indicate that methylcholanthrene had a significant effect in increasing sensitivity to the virus. This effect is not great. Our tests were not sufficiently extensive to detect any slight enhancing effect that turpentine may have had on infection with herpes virus.

It is interesting that the severity of the effect of methylcholanthrene on the skin of the abdomen, foot pad, and tail paralleled the susceptibility to herpetic infection in normal animals. The abdominal skin became acutely inflamed, whereas the skin of the tail showed but little reaction to the chemical.

That young mice are more susceptible to herpes virus than adults is clearly brought out by the experiments in which the virus was inoculated on the pad of the hind foot and on the tail. Two-thirds of the 2-week-old mice succumbed to herpetic encephalomyelitis after inoculation on the tail in striking contrast to the total absence of symptoms in normal adult animals inoculated by this route. Olitsky and Schlesinger (8) found 4-week-old mice to be susceptible to herpes virus applied to the abraded tail, the degree of susceptibility being greatly enhanced by a preliminary intracutaneous injection of a small amount of hypertonic salt solution. With a single exception all 2-week-old mice inoculated on the foot pad died of secondary involvement of the CNS.

The relative susceptibility of normal adult mice, mice treated with methylcholanthrene, and young mice, is revealed by the results obtained after inoculation of the pad of the hind foot. The findings can be summarized as follows:—

Condition of Host	
1. Normal adult mice	Fatal infection in 25 per cent of animals. Immunizing infection in 25 per cent. No symptoms and no immunity in 50 per cent.
2. Adult mice treated with methylcholanthrene	Fatal infection in 50 per cent of animals. Immunizing infection in 50 per cent.
3. Mice 2 weeks old	Fatal infection in nearly 100 per cent of animals.

The recently isolated Klee strain of herpes virus was compared with the HF strain which had been maintained in laboratories for more than 20 years and been carried through more than 125 brain-to-brain passages in mice prior to use in the present experiments. No important differences were discovered. This bespeaks a stability of the HF strain of virus. Both strains were fully virulent for rabbits, as shown by the invasion of the CNS after corneal or cutaneous inoculation. In view of the stability of the HF virus during prolonged passage in mice, it is of interest to recall the findings of Anderson (14), who has reported that serial passage of the HF strain through chick embryos, in one instance

resulted, after 25 transfers, in a marked decrease in the virulence of the virus for rabbits—it lost its ability to produce the usual keratitis and to invade the CNS from the cornea. Increased virulence for the chick embryo developed simultaneously with the loss of virulence for the rabbit.

In the case of the HF virus, epitheliotropism is strikingly strong in spite of prolonged neural passage. It would appear that the histotropism of herpes virus is such that factors governing virulence for cutaneous epithelium are identical with, or closely bound up with, its virulence for nervous tissues.

SUMMARY

1. The severity of herpetic infection of mice varied according to the site of inoculation, decreasing in the following order: cornea, skin of abdomen, pad of hind foot, tail.

2. The preliminary treatment of the foot pad or tail with methylcholanthrene increased the susceptibility to herpetic infection to a limited extent.

3. Two-week-old mice showed a much greater susceptibility to herpes virus inoculated on the tail or on the pad of the hind foot than did adult mice.

4. The HF strain of herpes virus, after more than 125 serial brain-to-brain passages in mice, possessed high virulence for cutaneous tissues of mice and showed no important differences in this respect from a recently isolated strain (Klee) of herpes virus.

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