STUDIES ON HERPETIC INFECTION IN MICE

IV. The Effect of Specific Antibodies on the Progression of the Virus within the Nervous System of Young Mice*‡

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In previously reported studies (1–3) it was found that in young mice the intranasal instillation of herpes virus (HF strain) was followed by the prompt invasion of the central nervous system (CNS) by way of all available neural routes. Death resulted regularly on the 4th or 5th day after inoculation. Specific antibodies, acquired as the result of an intraperitoneal injection of immune rabbit serum or by suckling an immune mother, protected young mice from herpetic infection of this sort. In these experiments the antibodies were present in the mice at the time the virus was administered. They presumably protected the mice by preventing the formation of peripheral foci of infection from which invasion of the CNS could occur.

In order to determine whether passively acquired antibodies can significantly influence the course of herpetic infection within the CNS of mice, further investigations have been carried out. Disturbance of the "blood-brain barrier," which might affect the penetration of antibodies into the CNS, was avoided by the use of a peripheral site of inoculation. Specific antiserum was injected at various intervals after introduction of the virus, before involvement of the nervous system became apparent. When experimental herpetic infections induced in this manner are treated by administering specific antiserum prior to the appearance of obvious signs of involvement of the nervous system, it is virtually impossible to be certain whether an efficacious result is due to the effect of antibodies at the peripheral focus or within the nervous system. In experiments included in the present report, this difficulty has been circumvented by eliminating, by amputation, the peripheral focus as a complicating factor. It was found that hyperimmune rabbit serum, injected intraperitoneally, can retard, and in some cases can completely arrest, the progress of an established herpetic infection within the nervous system of mice.

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Clinical trials of immune serum in the treatment of virus infections of the CNS have been limited almost exclusively to poliomyelitis. The results have been discouraging. Poliomyelitis virus exerts a relatively weak antigenic stimulus in human infections (4). As a result, most of the experimental and clinical trials made with convalescent human serum have been carried out with serum that was probably of low neutralizing capacity. Indeed, little is known on this score, for careful titrations of convalescent human serum have rarely been made because of the expense of titrating serum in monkeys.

Relatively few experimental studies of the effects of specific antibodies on established virus infections within the nervous system have been published. True, there are reports of experiments in which specific antiserum was injected into animals that subsequently were inoculated with a neurotropic virus. Experiments of this type have shown that specific antibodies given before the inoculation of the virus are capable of preventing infection of the nervous system with the viruses of rabies (5), poliomyelitis (6), equine encephalomyelitis (7, 8), and herpes simplex (1, 9, 10). Presumably, in these experiments, the virus was neutralized principally, or entirely, before nerve cells were infected. While such experiments are informative in regard to the general question of immunity to neurotropic viruses, they shed little light on the relationship of antibodies to virus infections established within the nervous system.

Experiments in which serum treatment has been delayed a period of hours or days after inoculation of virus have been reported with poliomyelitis (11–13), rabies (5, 14), and equine encephalomyelitis (7, 8, 15–17).

In the experiments of Schultz and Gebhardt on poliomyelitis (11), large numbers of monkeys were inoculated with the virus and, after an interval of from 1 to 6 days, the animals were treated with immune serum. Although a few treated animals survived or underwent a prolonged course of the disease, they were so few in number that the authors concluded, "specific immune serum is without demonstrable value in the treatment of experimental poliomyelitis." Kramer (12) has shown that mice infected with the Lansing strain of poliomyelitis virus by intracranial inoculation may be significantly benefited by treatment with immune serum. In one experiment the results suggested a possible effect of serum as late as 96 hours, although serum had no effect upon a group of 12 mice treated at 48 hours. Howe and Bodian (13) injected 3 chimpanzees with large amounts of serum of known antipoliomyelitic activity and subsequently infected these 3 animals by the oral route. Poliomyelitis virus appeared in the feces. Although no paralysis developed in these animals or in 3 control chimpanzees given virus but no serum, all 6 exhibited lesions of the nervous system considered characteristic of poliomyelitis.

Habel (14) has recently described experiments with rabies virus in which the quantitative aspects of passive immunization, and the time at which serum treatment may be effective, were carefully studied. Using guinea pigs inoculated intramuscularly with the virus, he found that antiserum injected at 3 hours protected 15 out of 19 animals; if serum was given at 24 hours, 10 out of 19 guinea pigs survived. With treatment at 48 and 72 hours, the number of survivors was smaller but possibly significant (4 out of 10, and 2 out of 9). In mice a 25 per cent survival rate was obtained with treatment at 24 hours; 3 of 24 animals treated at 48 hours survived. It is not clear from these experiments whether the antibody acted on virus within the nervous

system, although this is suggested by the fact that animals that succumbed after serum treatment usually died several days later than untreated mice.

Wyckoff and Tesar (16), working with young *rhesus* monkeys infected by the nasal route with Eastern or Western equine encephalomyelitis virus, found hyperimmune horse serum to be of no value if given after the appearance of fever. Zichis and Shaughnessy (7) have attributed these unfavorable results to the use of insufficient quantities of antiserum.

Zichis and Shaughnessy (15) have reported a study in which hyperimmune rabbit serum was used therapeutically in the early stages of infection with Western equine encephalomyelitis virus. Mice were inoculated intracerebrally and guinea pigs intralingually with known quantities of the virus. The serum was injected intraperitoneally into the mice either 48 hours after infection or later, at a time when they showed signs of illness. Guinea pigs were given the serum either at the onset of fever or at a time when they were visibly ill. The authors stated, "The majority of the animals treated at these stages of the disease recovered, whereas all the untreated controls died." Challenge inoculations of equine encephalomyelitis virus administered to survivors showed that the mice injected with serum at 48 hours, and the guinea pigs injected at the onset of fever, were not immune, but that the mice and guinea pigs that had recovered after treatment, begun when the animals were visibly sick, possessed an active immunity. The results of these experiments indicate a much greater therapeutic effect than has been found in the treatment of other virus diseases of the CNS.

Olitsky, Schlesinger, and Morgan (8), when they injected the Zichis strain of equine encephalomyelitis virus intracerebrally into mice, found that immune serum given subcutaneously to infected mice at the onset of illness (3 to 5 days) failed to protect. In a similar experiment, using the Rockefeller strain of virus which brings on illness more rapidly, they found that the immune serum had no therapeutic action when given to mice at 48 hours and little or no effect at 24 hours. When, however, antiserum was given 24 or 48 hours after the inoculation of the foot pad of guinea pigs with the virus of equine encephalomyelitis, the animals survived or developed delayed reactions terminating in death. Antiserum given at 72 or 96 hours was ineffective. How much of the beneficial effect resulted from neutralization of the virus in the foot pad or elsewhere outside the nervous system, and how much from an action upon the virus within the nervous system, is unknown. The surviving animals were not actively immune.

In studying immunity to equine encephalomyelitis in rabbits, Schlesinger, Morgan, and Olitsky (19) found that vaccinated animals were resistant to an intracerebral injection of the virus only if their serums contained sufficient antibody to give a titer of 1:300 or higher. Subcutaneous injection of the virus usually gave rise to inapparent infection of the CNS which most of the animals survived. Survival was associated with the development of a level of serum antibodies of at least 1:300. The authors believe that this level is an indicator of the availability of antibodies within the CNS.

Traub (17), in experiments similar to those of Olitsky, Schlesinger, and Morgan, found immune serum, administered to guinea pigs that had been inoculated on the foot pad with equine encephalomyelitis virus, to be effective when given during the

first 2 days. In analyzing the mechanism of the protective action of the immune serum, he showed that virus free in the blood plasma was rapidly neutralized, but that virus fixed to the blood cells was more slowly inactivated. However, no direct evidence of neutralization of the virus within the nervous system was presented. Traub compared the potency of individual lots of immune serum, as determined by neutralization tests, with their effectiveness when used therapeutically in guinea pigs. The results led him to indicate that neutralization alone is not an adequate test of the therapeutic value of an antiserum in this disease.

More recently Zichis and Shaughnessy (7) have presented convincing evidence of the efficacy of adequate serum therapy in the treatment of infection with the Western strain of equine encephalomyelitis virus. Guinea pigs were infected by intralingual inoculation of the virus. Treatment of 55 animals with hyperimmune rabbit serum was started when they were definitely ill. This was usually 24 hours after the onset of fever. Most of the guinea pigs were ataxic at the time treatment was started or developed ataxia soon after. Of 41 control animals given no treatment, or injected with normal rabbit serum, 40 died. Thirty-seven, or 67 per cent, of the 55 treated animals survived. Serum therapy was also tested in monkeys. Of 21 animals injected intracranially with the virus, 11 were treated with serum at the onset of fever. Although all developed ataxia, 5 recovered. Of 10 control monkeys, given virus but no serum, none survived. The authors emphasize the importance of using large amounts of serum of high potency.

A recent report by Sulkin, Zarafonetis, and Goth (18) indicates that anesthesia may be a valuable adjunct to serum therapy in the treatment of virus diseases of the nervous system. Using mice injected intracranially with the Western strain of equine encephalomyelitis virus in a dose sufficient to kill 80 per cent of control animals, they found that with serum treatment at 18 hours, 52 per cent survived. Prolonged ether anesthesia combined with serum treatment increased the number of survivors to 90 per cent.

EXPERIMENTAL

Materials and Methods

The herpes virus used in the present experiments was the HF strain used in the earlier studies 1–3; it had been maintained for more than 125 serial passages in Swiss mice. The preparations of the virus used for inoculation were suspensions of infected mouse brain, either freshly harvested or glycerolated for not over 10 days. Only Swiss mice from the Rochester colony were employed.

The standard method of injection was the intracutaneous (and unavoidably subcutaneous) injection of the virus into the foot pad of 2-week-old mice, combined with rubbing the virus into the scarified skin of the pad. A number of preliminary experiments on the pathogenesis of herpetic infection led to the selection of this mode of inoculation. All inoculations were performed under light ether anesthesia.

The serum used in the passive immunization of mice was derived from a pool of the serums of 8 hyperimmunized rabbits. Each animal had survived injection by one of several strains of herpes virus and was given from 3 to 6 further immunizing injections of the HF virus in the form of a suspension of rabbit brain. While no attempt was made to study in detail the immune response of these rabbits, it was noted that the neutralizing power of their serums

was definitely increased by the repeated injections. This remark is based on assays of each rabbit's serum done to determine which serums were suitable for the pool.

A total of 231 mice was employed to measure the neutralizing power of the immune serums. The titration tests were carried out in the following manner. The HF virus was prepared as a 10 per cent suspension of mouse brain in Locke's solution. This suspension was centrifuged at slow speed for 5 minutes and the sediment discarded. The supernatant fluid was designated a 1:10 dilution of "virus." Further dilutions (1:100, 1:1000, 1:10,000, and 1:100,000) were then made. One-half ml. or 1 ml. of immune serum was mixed with an equal volume of the virus dilution (1:10, 1:100, and 1:1000) and the mixtures were incubated for 2 hours at 37°C. in an air incubator. Similar mixtures of higher dilutions of the virus (1:1000, 1:10,000 and 1:100,000) and normal rabbit serum were incubated at the same time. At the end of the period of incubation each mixture of serum and virus was injected intracerebrally in a dose of 0.025 ml. into each of 3 adult Swiss mice. As may be seen in Table I, the result of a titration of the pooled antiserums used in the present experiments showed that,

TABLE I

*Neutralizing Capacity of Herpes Antiserum Prepared in Rabbits**

	Normal rabbit serum			Undiluted antiserum			Antiserum diluted		
							1:10	1:40	1:160
Dilution of virus....	1:1000	1:10,000	1:100,000	1:10	1:100	1:1000	1:100	1:100	1:100
No. mice injected.....	3	3	3	3	3	3	3	3	3
Day of death...	4, 4, 5	4, 4, 6	S‡, S, S	4, 4, 6	6, S, S	S, S, S	6, S, S	4, 6, X§	4, 5, 5

* Mixtures of antiserum and virus tested by intracerebral innoculation of mice. See text.
‡ S = mouse survived for 13 days.
§X = mouse died of accidental trauma.

in contrast to a control titration using normal rabbit serum, 0.025 ml. would protect against approximately 100 minimal, cerebral, lethal doses of the virus.

In general, antigen-antibody titration carried out by means of the antibody dilution method is more accurate than one in which the antigen is diluted (20). However, such titrations are not ordinarily practicable when a virus is used as the antigen, unless it is maintained in a state in which it will not change in potency. In the final assay of the pool of immune serums used in the present experiments, a titration by the antibody dilution method was made. The immune serum, diluted 1:10, 1:40, and 1:160, was mixed in equal volume with a 1:100 dilution of the virus, the highest concentration of the virus that was regularly neutralized by the undiluted immune serum. The mixtures were incubated and tested in mice as previously described. The results are also given in Table I. It may be seen that 2 of 3 mice were protected by the 1:10 dilution of the immune serum.

1. The Pathogenesis of Herpetic Infection in Young Mice Inoculated on the Foot Pad

Preliminary studies had shown that young mice inoculated on the foot pad with HF strain of herpes virus develop paralysis of the inoculated limb, followed by paraplegia and finally by encephalitis that regularly cause death on the 4th 5th, or 6th day after inoculation. As a guide to the proposed studies of im-

munity, it was necessary to carry out more detailed investigations of the pathogenesis of these infections. In order to study the time relationships of the spread of the virus to the CNS, mice were inoculated with the virus in the usual way and later subjected to amputation of the inoculated foot. Based upon exploratory experiments in which the techniques of inoculation and amputation were standardized, the following experiment was carried out.

A group of 42 2-week-old mice was inoculated on the pad of the right, hind foot. Following inoculation, the feet were amputated just above the ankle joint at intervals of 2, 12, 24, 36, 48, 60, and 72 hours. The amputations were performed on 6 mice at each time, and the feet were fixed in formalin for microscopic study.

Of the 6 mice subjected to amputation after 2 hours, 4 subsequently developed CNS disturbances, which from their inception were apparently encephalitis, and which terminated in death on the 5th (1 mouse), 6th, or 7th day after inoculation. Two mice remained well. Of those subjected to amputation after 12 hours, 1 survived and the others died on the 5th (1 mouse), 6th, or 7th day. Two of these animals had a paraplegia before the onset of encephalitis. In all 6 of the mice from which the feet were amputed after 24 hours, the initial sign of disease of the nervous system was paralysis of one or both hind legs; this was followed by death on the 4th or 5th day after inoculation.

The following interpretation of these results seems reasonable. Amputation after 2 hours removed the local focus of infection and prevented invasion of the CNS by way of the local nerves. However, some virus apparently escaped by way of the blood stream or through the lymphatics and established metastatic foci of infection from which it reached the brain by other neural routes. It has been shown (3) that, following intranasal inoculation of herpes virus, foci of infection develop regularly in the liver and less frequently in other viscera. Furthermore, it should be emphasized that infection of the CNS from such metastatic foci does not produce the posterior paralysis characteristic of infection primary in the foot pad. Moreover, the encephalitis resulting from metastatic lesions is slower to develop.

Amputation after 24 hours, or later, had no effect on the course of the infection of the CNS. All 6 animals in the 24 hour group died without delay and showed initially the same paralysis of the hind limbs as animals whose feet were not amputated after inoculation of the foot pads.

Sections of the amputated feet to be studied microscopically were stained with hematoxylin and eosin and with a modification of Shorr's stain (21). In feet removed 2 hours after inoculation, the principal changes observed were hemorrhage and moderate distention of the tissue spaces by fluid. A few infiltrating cells were present. In no instance did the sections of the amputated feet give evidence that the inoculum had extended beyond the ankle joint. In each case the amputation was seen to be 2 or 3 mm. above the distal end of the tibia. After 12 hours there were noted more extensive congestion and cellular infiltration, principally polymorphonuclear leucocytes, but also

lymphocytes. No inclusion bodies were found. All of the feet amputated after 24 hours showed foci of epithelial cells containing the typical, intranuclear inclusions of herpes simplex. These cells were located along the linear defects in the epithelium resulting from the scarification. After longer intervals of time, the sections revealed increased necrosis of the epithelium and diffuse, severe inflammation of the foot pad.

The significant point emerging from these observations is that, between 12 and 24 hours after inoculation, the primary virus infection reached maturity in epithelial cells and virus began to pass up the local nerves towards the CNS. Accordingly, it was decided, in the studies of immunity, to perform the amputation after an interval of 24 hours in order to eliminate the primary focus and yet not interfere with the progress of direct infection of the CNS from the foot pad.

2. *Passive Immunity to Herpetic Infection in Mice with Special Reference to the CNS. Peripheral Inoculation of the Virus*

In the immunological studies designed to explore the effect of specific antibodies on the invasion of the CNS of mice by herpes virus, 2 sets of experiments were carried out. In the first set (A), the effect of immune serum was investigated without the elimination of the primary focus of infection. In the second set (B), the primary focus was eliminated by amputation of the inoculated foot and the immune serum was injected at various intervals of time thereafter.

A. Passive Immunity in Mice with the Primary Lesion Intact.—Preliminary experiments involving 20 young mice showed that the largest dose of serum that could be administered at one injection to 2-week-old mice was 0.5 ml. This dose of the antiserum was effective in protecting the young mice from detectable infection by the HF virus if the serum was given before the inoculation of the foot pad. An experiment was next set up to discover the effect of the immune serum at various intervals of time after the inoculation of the virus, the primary herpetic lesion on the foot being left intact.

Forty-eight 2-week-old mice were inoculated with the HF virus on the pad of the right, hind foot. They were then divided into 8 groups of 6 mice each. The mice in each group were injected intraperitoneally with 0.5 ml. of normal (control), or immune, serum according to the following schedule. One group was given normal rabbit serum immediately after the inoculation of the virus and a second group received immune serum at the same time. The mice in the remaining 6 groups were given immune serum at intervals of 12 hours, the last group being injected 72 hours after inoculation of the virus. The 6 mice that received normal serum were selected from 6 litters; those in each of the other groups were from 2 or 3 liters.

The normal serum exerted no protective effect and all of the control mice died on the 4th or 5th day. The immune serum given at zero time completely protected 5 of the 6 mice; 1 died on the 6th day. At 12 hours, the immune serum exerted a definite but less marked effect. Three mice were paralyzed on

the 4th day and died on the 5th, 6th, or 7th day. Three showed no paralysis and survived. One of the mice given serum at 24 hours died of an accidental cause. Four of the remaining 5 were paralyzed on the 4th day and died on the 5th or 6th day. The fifth mouse developed, on the 6th day, a partial monoplegia which persisted to the end of the experiment, 23 days after inoculation. Immune serum injected at 36 and 48 hours seemed to retard the infection. In each case 5 of the 6 mice died between the 4th and the 7th days. All mice that received immune serum at 60 hours and at 72 hours died.

The results just described show clearly that immune serum exerts a definite effect upon herpetic infection of the type under study and that the effectiveness of antiserum decreases progressively as the time between the inoculation of the virus and the injection of the immune serum is increased.

The local lesions of the inoculated foot pads were examined grossly. The mice given the immune serum at zero time showed no evidence of herpetic infection of the feet, whereas those injected with immune serum at 12 hours had the typical encrusted lesions of an herpetic infection of the skin. Apparently, the immune serum given at zero time had neutralized the virus before it could enter susceptible epithelial cells, whereas the serum given at 12 hours did not prevent the infection of epithelial cells. In many animals it presumably acted to prevent the spread of the virus from the initial cutaneous focus to the nervous system. In view of the preceding studies on pathogenesis, it seemed probable

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CHARTS 1 to 5. Therapeutic effect of immune rabbit serum on herpetic infection of the nervous system of 2-week-old mice.

Each horizontal line represents 1 mouse. The vertical lines mark the lapse of time after inoculation of the virus. All of the animals were inoculated with herpes virus on the pad of the right, hind foot. Twenty-four hours later the inoculated feet were amputated. The control group of animals, shown in Chart 1, was subjected to inoculation and amputation but received no antiserum. The mice in the control group were litter mates of those treated with antiserum. The litter from which individual animals were obtained is indicated in each chart. Charts 2 to 5 show the effect of treatment with specific antiserum, given intraperitoneally, at various intervals after inoculation of the virus. Thus, in Chart 2, serum was administered 24 hours after introduction of the virus and 1 hour after the infected feet had been amputated; in Chart 3, serum was given 6 hours after the feet had been amputated and 30 hours after inoculation of the virus.

Paralysis involving 1 or both hind quarters is shown by shading of the region involved Encephalitis (hyperirritability, lethargy, ruffling, ataxia) is indicated by shading the area of the head. Shading of the entire mouse means that the mouse was found dead.

It may be noted in Chart 1 that the first sign of illness in all the control animals was either paralysis of the leg from which the foot had been amputed, or a posterior paraplegia. This is interpreted to indicate that, at the time the foot was amputated, the virus was in the nerves leading from the foot to the spinal cord. As is shown in Charts 2 to 5 serum therapy retarded the progress of the infection of the nervous system in many mice, while in others it completely arrested the infection.

On the basis of delayed paralysis and prolonged survival, the data shown in all 5 charts are presented in summary and analyzed statistically in Table II.

that the protective action of antiserum, given 24 hours or later after the introduction of the virus, must be the result, at least in part, of the action of the antibodies on the virus within the nervous system. To determine whether this was actually the case, a final experiment was carried out.

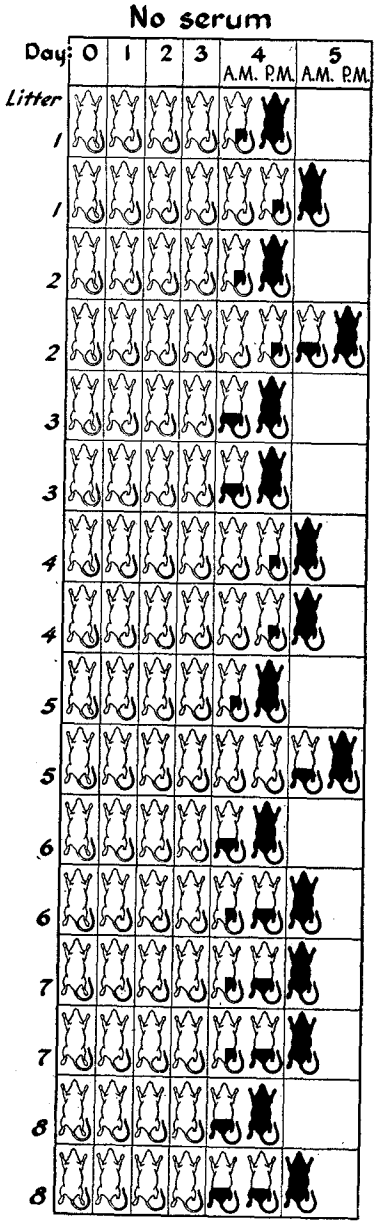

CHART 1

B. Passive Immunity in Mice with the Elimination of the Primary Lesion.—

A total of 80 2-week-old mice from 13 litters was used to test the effect of the immune serum on herpetic infections of the nervous system induced by peripheral inoculation of the virus but uncomplicated by the presence of the primary lesion. All of the mice were inoculated

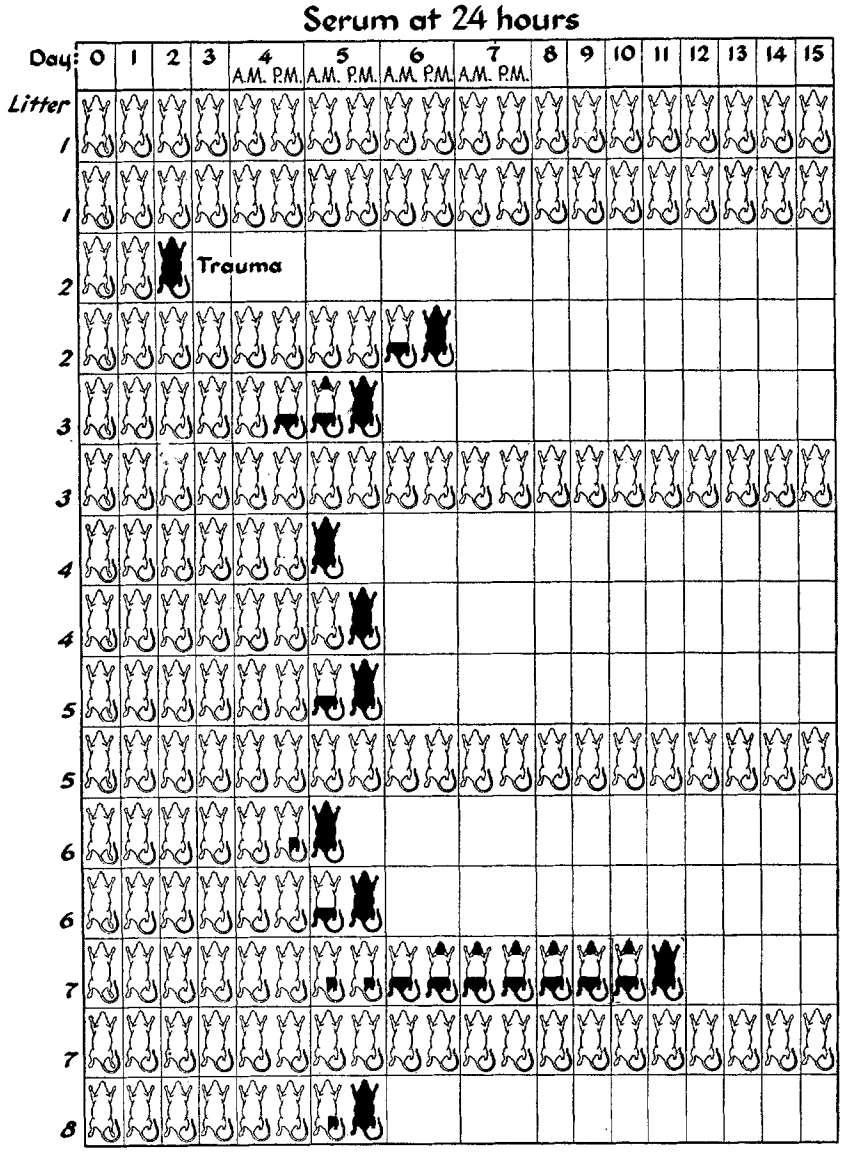

CHART 2

on the foot pad with HF virus. Twenty-four hours later the inoculated feet were amputated well above the ankle joint.

Fifteen of the mice from 5 small litters were given no further treatment. The remaining 65 mice were from 8 litters of 8 or 9 mice each. Two mice from each of these 8 litters were given no further treatment and served as litter mate controls of treated mice. Immune serum was injected intraperitoneally in a dose of 0.5 ml. into the remaining 51 mice from 8 litters, according to the following schedule.

1. Within 1 hour after amputation (24 hours after inoculation of the virus)—2 mice from each of 7 litters and 1 mouse from the additional litter.

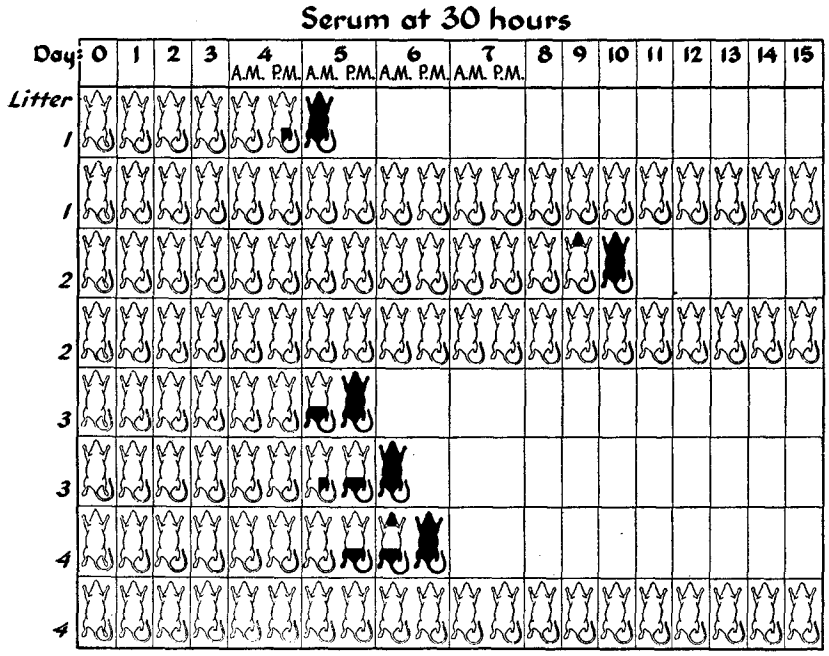

CHART 3

2. Six hours after amputation (30 hours after inoculation of the virus)—2 mice from each of 4 litters.

3. Twelve hours after amputation (36 hours after inoculation of the virus)—2 mice from each of 8 litters.

4. Twenty-four hours after amputation (48 hours after inoculation of the virus)—2 mice from each of 2 litters, and 3 mice from each of 2 litters.

Details of the distribution of treated mice in various litters are shown in Charts 1 to 5.

The mice were examined regularly in the morning and in the evening. In most instances there was an interval of 10 hours between the two inspections. The interval was never less than 8 hours, or longer than 12 hours. Survivors were discarded after 15 days.

The following data were considered significant in interpreting the results of the experiment: (1) the time at which definite paralysis first appeared; (2) the

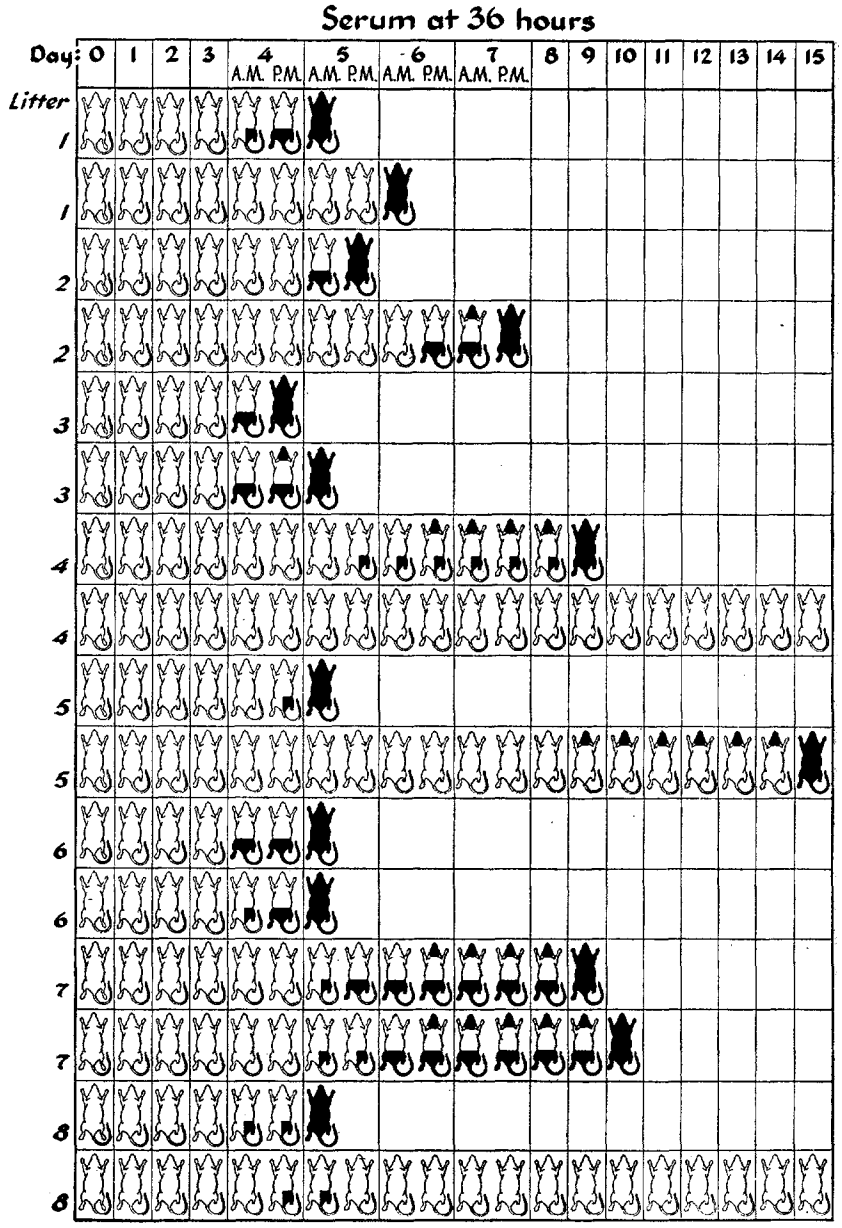

CHART 4

time of death; (3) the number of mice that survived. Inspection of Charts 1 to 5 shows that, in general, mice treated with immune serum became paralyzed later and lived longer than their litter mate controls. The difference is most

striking in the case of mice treated at 24 hours and is progressively less marked as treatment was delayed.

The significance of the findings is revealed by statistical analysis, using the χ^2 method (22). In Table II, data obtained by a comparison of treated mice with their litter mate controls are presented. It is apparent that the delay in paralysis and the prolongation of life of treated animals is highly significant in groups treated at 24, 30, and 36 hours. The evidence that serum given at 24 hours caused a delayed paralysis and a prolonged survival of mice is over-

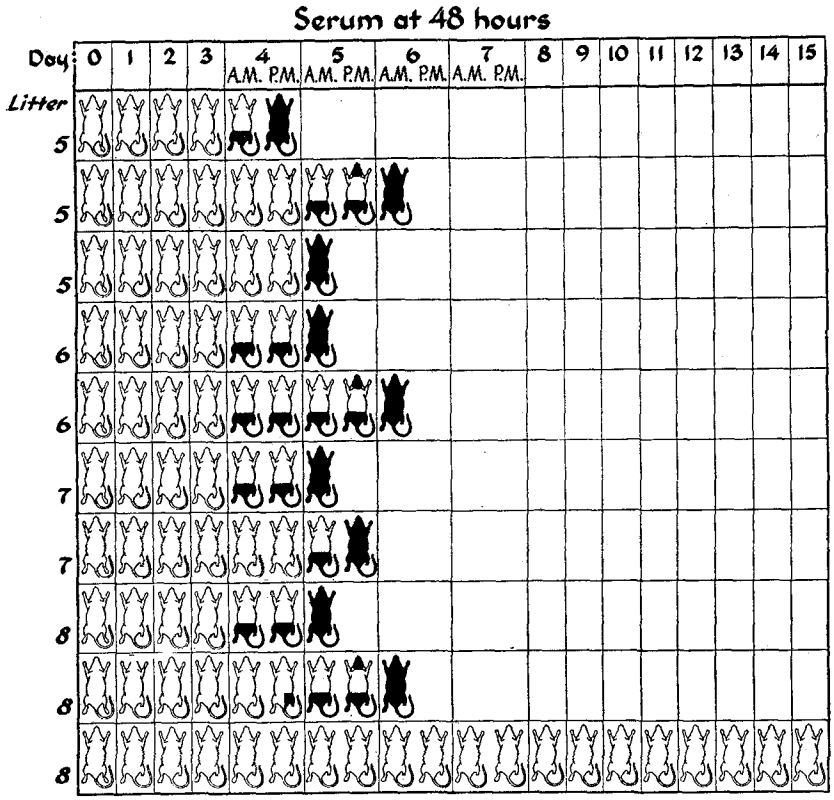

CHART 5

whelming. Such results could be expected on the basis of chance not once in 100,000 times. The results, when serum was given at 30 or at 36 hours, are significant in that the difference between treated and untreated animals could not be expected to occur as a result of chance one in 1,000 times. It should be emphasized that the data in Table II are based on a comparison of each treated mouse with 2 litter mate controls but that the same 2 litter mate controls are compared with more than 1 treated animal. Inasmuch as 2 mice in each of the 8 litters served as untreated controls, there was a total of 16 in the control group.

According to their origin from various litters, the mice treated at various time intervals are compared with all or a part of this same group of control animals.

It will be recalled that 15 mice from 5 small litters were inoculated and subjected to amputation. None of their litter mates was treated with serum.

TABLE II

Therapeutic Effect of Immune Serum on Herpetic Infection of the Nervous System of 2-Week-Old Mice. Comparison of Treated Mice with Their Litter Mate Controls on the Basis of Delayed Paralysis and Prolonged Survival‡*

	No. litter mate controls	No. mice treated with serum	No. treated mice that	
			Became paralyzed later than controls	Lived longer than controls
A. Serum injected 24 hrs. after inoculation of virus	16	14	13	11
Probability that difference from controls is due to chance			1 in more than 1 million (χ^2 = 26.24)	1 in more than 100,000 (χ^2 = 19.85)
B. Serum injected 30 hrs. after inoculation of virus	8	8	7	7
Probability that difference from controls is due to chance			1 in more than 1,000 (χ^2 = 12.44)	1 in more than 1,000 (χ^2 = 12.44)
C. Serum injected 36 hrs. after inoculation of virus	16	16	9	9
Probability that difference from controls is due to chance			1 in more than 1,000 (χ^2 = 12.52)	1 in more than 1,000 (χ^2 = 12.52)
D. Serum injected 48 hrs. after inoculation of virus	8	10	3	5
Probability that difference from controls is due to chance			1 in 10 (χ^2 = 2.9)	1 in 50 (χ^2 = 5.54)

* All animals inoculated on right, hind foot pads with the virus. Feet amputated 24 hours later. Treated mice given immune serum intraperitoneally.

‡ Comparison of treated mice with their 16 litter mate controls is also shown in Charts 1 to 5.

Therefore, they are not represented in Charts 1 to 5 and Table II, in which a comparison of serum-treated mice with untreated litter mates is presented. By grouping together all mice treated at a given time and comparing them with all untreated mice irrespective of litter, it is possible to include these 15 mice in the calculations. In Table III, the results of an analysis of this method are presented. It was found convenient to compare the treated mice with the

control group on the basis of the number of mice paralyzed at 4 days, and the number dead at 5 days.

The data in Table III show clearly that many of the treated mice and few of the untreated controls were free from paralysis after the 4th day. Similarly, nearly all control mice died on or before the 5th day, but many of the treated

TABLE III

Therapeutic Effect of Immune Serum on Herpetic Infection of the Nervous System of 2-Week-Old Mice. Comparison of Treated Mice with All Control Animals Irrespective of Litter on the Basis of Paralysis by 4th Day and Death by 5th Day after Inoculation of the Virus‡*

I. Paralysis by 4th Day

	No. paralyzed	No. not paralyzed	Probability that difference from controls is due to chance	(χ^2)
Mice treated with serum at				
24 hrs.	2	12	1 in 1 million	25.1
30 hrs.	1	7	1 in 100,000	20.1
36 hrs.	8	8	1 in 500	9.6
48 hrs.	6	4	1 in 25	4.3
Control mice	28	3		

II. Deaths by 5th Day

	No. dead	No. not dead	Probability that difference from controls is due to chance	(χ^2)
Mice treated with serum at				
24 hrs.	7	7	1 in 1,000	11.4
30 hrs.	2	6	1 in 50,000	18.3
36 hrs.	8	8	1 in 2,000	12.0
48 hrs.	6	4	1 in 100	7.3
Control mice	29	2		

* All animals inoculated on right, hind foot pad with the virus. Feet amputated 24 hours later. Treated mice given immune serum intraperitoneally.

‡ In addition to 16 litter mates, the control animals in this table include 15 mice from 5 litters in which none was treated with antiserum.

mice lived beyond this time. The difference between treated and control mice is most striking in the groups given antiserum at 24, 30, or 36 hours. The data in Table III, because they include a larger number of control animals than those in Table II, raise the χ^2 value of observed differences to the point where the beneficial effects of treatment, even as late as 48 hours after inoculation of the virus, may be significant.

Up to this point, results have been analyzed in terms of a retardation of the

herpetic infection of the nervous system (delayed paralysis and prolonged survival) as a result of treatment with immune serum. It is readily seen from Charts 1 to 5 that in an appreciable number of treated mice the infection was not only retarded, it was completely arrested; the mice survived. In Table IV, the numbers of surviving mice in the several groups are tabulated. A total of 11 of the 48 mice given serum treatment survived, whereas none of the 31 control mice remained alive. The difference is shown to be statistically significant. The numbers of mice employed were not sufficient to justify an

TABLE IV

Therapeutic Effect of Immune Serum on Herpetic Infection of the Nervous System of 2-Week-Old Mice as Indicated by Survival of Treated Animals

	No. mice treated	No. survivors at 15 days	Probability that difference from controls is due to chance
Mice treated with serum at			
24 hrs.	14	5	
30 hrs.	8	3	
36 hrs.	16	2	
48 hrs.	10	1	
Totals, 24–48 hrs.	48	11	1 in 250 (χ^2) 8.3
Controls* (no serum)	31	0	

* As in Table III, these include all control mice, both litter mates and non-litter mates of treated animals.

attempt to evaluate the efficacy of treatment as a life-saving measure at the different time intervals.

DISCUSSION

Immune serum may protect young mice against herpetic infection in three distinct ways. First, immune serum administered before or very soon after the inoculation of the virus prevents even a relatively large dose of the virus from infecting epithelial cells at the site of inoculation. No visible lesion develops beyond that resulting from trauma. Second, immune serum injected several hours after the inoculation of the virus (12 hours in the present study) does not prevent a local lesion from developing. Despite this, the virus is prevented, in many of the animals, from invading the nervous system sufficiently to cause symptoms. Probably, neutralization of the virus takes place principally after the virus leaves infected epithelial cells and before it enters nerve fibers. Thus, while infection of the epithelial cells occurs, significant

involvement of the nervous system is prevented in many animals. Third, immune serum exerts a protective action by combatting the activity of the virus within the nervous system. In order to demonstrate the action of antibodies on the virus within the nervous system, it was necessary to eliminate the second type of protective activity. This was achieved by the amputation of the site of peripheral infection, the foot.

It is worthy of emphasis that, in all the control animals whose feet were amputated at the appropriate time, the first sign of neurological disturbance was a posterior monoplegia or paraplegia. It is difficult to interpret this in any other way than that, at the time the foot was amputated, the virus was in the nerves leading from the foot to the central nervous system if not already in the spinal cord. It follows, therefore, that the beneficial effect of serum therapy, under the conditions of this experiment, was an effect on the virus in the nervous system. It is almost inconceivable that antibodies could reach virus present in axis cylinders of the sciatic nerve before it has attained cell bodies. It is more likely that they exerted their effect within the central nervous system. Metastatic lesions in the viscera were probably present in some mice, but it is unlikely that they could have been responsible for the focal paralyses so regularly observed. Therefore, their presence does not interfere with the significance of the effect of serum in delaying or preventing paralysis.

The question arises whether normal rabbit serum could have exerted a nonspecific protective effect. This seems unlikely. Normal serum has been shown to have no effect in neutralization tests using small amounts of herpes virus, and, in the preliminary experiments in which mice were inoculated on the foot pad and given normal serum intraperitoneally, infection progressed at the same rate as in untreated mice.

It is of interest to speculate on the relative efficacy of humoral antibodies in combatting virus infections of the nervous system as compared with similar activity of antibodies in opposing virus infections in other organs, such as the liver or skin. The existence of a barrier between the blood stream and brain tissue is well established (23) on the basis of experiments on the diffusion of dyes and other substances. It has been postulated (24) that the "blood-brain barrier" acts to interfere with the passage of antibodies into the nervous system, thus limiting the effectiveness of circulating antibodies in combatting virus infections of these tissues, the implication being that this situation is peculiar to the central nervous system. The experiments described in the present paper show that antibodies can be effective in spite of the "blood-brain barrier" if given during the first quarter or third of the incubation period. This is roughly comparable to the period during which effective serum therapy is possible in measles.

Studies on herpetic infections of man (25–29) have an important bearing on the question of the relative efficacy of antibodies in controlling virus infections

of nervous and non-nervous tissues. Those human beings whose serum contains a relatively high titer of neutralizing antibodies for herpes virus may be susceptible to recurrent attacks of herpes simplex. There is no apparent correlation between the presence of such antibodies and resistance to this type of cutaneous infection. From the work of Gildemeister and Ahlfeld (29) and from other studies (30), it seems probable that, in many cases, human beings susceptible to recurrent attacks of herpes simplex possess specific antibodies in a concentration approximating that obtained in the present experiments in mice. These facts suggest that humoral antibodies may be no more effective in preventing the spread of herpes virus from one epithelial cell to another in human skin than in preventing the spread of this same virus from one nerve cell to another in the brain of a young mouse. It seems possible that the "blood-brain barrier" in young mice exerts no greater restraint upon humoral antibodies than is imposed by the natural barriers between the blood and epidermis in man.

Until more information of a quantitative nature is available, it would seem unwise to assume that the "blood-brain barrier" makes the central nervous system peculiarly susceptible to virus infections by imposing an unusual obstruction to the passage of antibodies. It seems not unlikely that the "blood-epidermis" barrier may prove to be as formidable as the "blood-brain barrier."

SUMMARY

Two-week-old mice inoculated with herpes virus on the pad of a hind foot regularly developed paralysis of the infected limb followed by paraplegia and encephalitis terminating fatally 5 or 6 days after inoculation.

Hyperimmune rabbit serum given intraperitoneally at the time virus was inoculated on the foot pad prevented the formation of an herpetic lesion of the foot pad. When the antiserum was given 12 hours after inoculation of the virus, a typical infection of the epithelium of the foot pad developed, but the virus was prevented from causing obvious signs of infection of the nervous system in many of the animals.

Amputation of the foot 2 hours after the inoculation of the virus prevented the paralysis of the hind leg. Some of the mice died of a delayed encephalitis. Amputation of the foot at 24 hours neither prevented nor delayed the sequence of paralysis of the hind leg, encephalitis, and death.

In order to study immune serum therapy of an infection of the nervous system uncomplicated by a peripheral focus of infection or by traumatic disturbance of the central nervous system, 2-week-old mice were inoculated on the foot pad, the infected feet were amputated 24 hours later, and the immune serum was administered at varying intervals thereafter.

Using litter mate controls and statistically significant numbers of mice, it

was shown that hyperimmune rabbit serum, administered during the first one-third of the incubation period, retards and, in some cases, arrests the progress of herpetic infection within the nervous system.

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