Viral Interference in the Brains of Mice Infected Subcutaneously with Herpes Simplex Virus[1] (35085)

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(Introduced by S. Baron)

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Central nervous system infection with herpes simplex virus (HSV) has become recognized increasingly in man. Herpesvirus encephalitis is probably the most common cause of fatal, sporadic encephalitis in the United States at the present time (1) and accounts for 10% of well studied encephalitides (2). However, most infections with HSV are self-limited and frequently inapparent. Presumably, infection of the central nervous system (CNS) may not occur for the following reasons: (a) route of infection precludes virus from reaching the brain, (b) the virus strain is not neurotropic, or (c) host defense mechanisms, e.g., immunologic or interferon, are active in protecting the CNS.

In 1935, Magrassi (3, 4) found that an intracerebral (ic) inoculation of HSV into rabbits resulted in death after 3 to 4 days. If this ic inoculation was preceded 7 days earlier by either a corneal or subcutaneous (sc) injection of the same virus, a protective effect was demonstrable. This protective effect was not seen with shorter or longer time intervals between the two inoculations. Since this observation a number of reports have produced many examples of interference between homologous and heterologous viruses *in vivo* (5–9). Although interferon is now firmly established as an important factor in the development of nonspecific resistance (10), it is presently difficult to determine which of the early reports on viral interference were concerned with interferon production by the host or by other factors.

The present study was undertaken to investigate those host defense mechanisms which develop in the brains of mice following peripheral infection with HSV.

Materials and Methods. Animals and virus strains. Specific pathogen free male C_3H/NIH mice (18–22 g) were used for all experiments. Stocks of a Type 1 HSV (strain VR_3)[5] were grown in primary rabbit kidney (PRK) cell monolayers in 32-oz glass bottles, and the virus was titered in roller tissue culture tubes containing PRK cells (Microbiological Associates, Bethesda, Maryland). Stocks of encephalomyocarditis virus (EM CV, r^+ strain)[6] were grown and titered in primary mouse embryo cell monolayers in 32-oz glass bottles and tissue culture tubes, respectively. End points were determined on day 5 and were calculatd by the Reed–Muench

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method (11). The stocks of HSV and EMCV contained $10^{7.6}$ and $10^{7.5}$ $TCID_{50}$/1.0 ml, respectively.

Homologous virus challenge. After subcutaneous (sc) inoculation of 2000 $TCID_{50}$ (0.2 ml) of HSV, the mice were challenged intracerebrally (ic) under light ether anesthesia with either 2 or 20 $TCID_{50}$ (0.02 ml) of HSV on days 0, 3, 6, 9, and 21. Animals were observed for at least 21 days following the ic injection. Deaths occurring after the sc injection, but before the ic inoculation, were considered to be secondary to the sc treatment and were discarded.

Heterologous virus challenge. Mice were inoculated sc with 800 $TCID_{50}$ (0.2 ml) of HSV, then subsequently challenged ic with 1000 $TCID_{50}$ (0.03 ml) of EMCV on days 0, 3, 6, 9 and 21. Animals were observed for an additional 14 days following the ic injection.

Neutralizing antibody, interferon, and virus isolation assays. Animals were infected sc with 2000 $TCID_{50}$ of HSV. On days 0, 3, 6, 9, 14, and 21, at least 8 mice were harvested by exsanguination following decapitation. Blood was collected and pooled for each day in sterile tubes and allowed to clot; after centrifugation the serum was frozen at —70°. The brain, lungs, kidneys, liver, and spleen from each animal were removed aseptically, placed in a sterile vial and frozen at —70° until used.

Sera were assayed for herpes antibody by macroneutralization with 100 $TCID_{50}$ of the VR_3 strain of HSV. Serial dilutions of sera (starting at 1:4 dilution) in Eagles' minimal essential medium (MEM) containing 2% fetal calf serum (FCS) were challenged with virus, incubated at room temperature for 1 hr, and 0.2 ml of the virus–serum mixture was put into each of 4 tubes containing PRK cell cultures (12). Five days later, end points were determined by that dilution of serum which gave complete inhibition of HSV cytopathic effect.

Each of the different organs was pooled for 8 mice, ground in a mortar and a 10% w/v suspension was made in MEM with 2% FCS and antibiotics (streptomycin, 100 μg/ml and penicillin 100 units/ml). After centrifugation (600g) for 10 min in a refrigerated (4°) centrifuge, 0.2-ml aliquots of each organ pool homogenate were placed in each of four PRK cell culture tubes. Media (as above) was changed 18 hr later. The tubes were observed for cytopathic effect for 7 days thereafter.

An additional sample of each organ homogenate (5 ml of a 10% suspension) and serum (0.5 ml) was acid treated (pH 2, 24 hr), neutralized to pH 7.0 and assayed for interferon. A unit of interferon was determined as the reciprocal of the highest dilution of the sample which inhibited the single cycle yield of vesicular stomatitis virus (Indiana strain) by 0.5 $\log_{10}$ in primary mouse embryo cell cultures (13).

Results. Homologous challenge. Mice were inoculated on day 0 with 2000 $TCID_{50}$ of HSV given sc in the flank; the mortality of this treatment alone was 14.3%. Brains of mice infected sc with the same dose of virus were examined histopathologically. Typical HSV intranuclear inclusion bodies were found with greatest frequency in the pontine and medullary tegmentum and ocasionally in other areas such as the hypothalamus in approximately 20% of animals treated this way. Infected mice were then challenged ic with 2 $TCID_{50}$ ("low dose") or 20 $TCID_{50}$ ("high dose") of HSV on days 0, 3, 6, 9, and 21

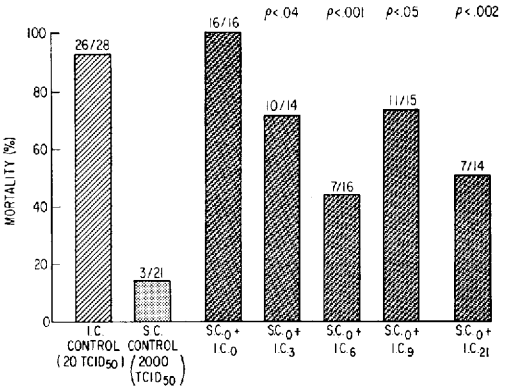

FIG. 1. High dose herpes simplex virus (HSV) intracerebral challenge; mortality of groups receiving a subcutaneous inoculation of 2000 $TCID_{50}$ of HSV on day 0, followed by intracerebral challenge with 20 $TCID_{50}$ of HSV. Subscript indicates day of challenge.

TABLE I. Effect of Subcutaneous Inoculation of 2000 $TCID_{50}$ of HSV on Subsequent Intracerebral Challenge with 20 $TCID_{50}$ ($LD_{92.9}$).

Treatment group[a]	Actual median day of death (50% mortality)	Inoculation delay	Corrected median day of death from time of ic challenge	Significance[b]	
				R_x vs control ic	R_x vs $sc_0 + ic_0$
Control ic ($LD_{92.9}$)	4.3	—	4.3	—	—
$sc_0 + ic_0$	5.5	0	5.5	NS[d]	—
$sc_0 + ic_3$	9.5	—3	6.5	NS	NS
$sc_0 + ic_6$	16.5	—6	11.0[c]	$p < .01$	$p < .01$
$sc_0 + ic_9$	14.7	—9	5.7	NS	NS
$sc_0 + ic_{21}$	28.0	—21	7.0	$p < .05$	$p < .05$

[a] Subscript following route of inoculation (*e.g.*, sc_x) indicates day of treatment.

[b] Calculated using Kolmogorov-Smirnov (nonparametric) test for difference in distribution of number of deaths (mortality).

[c] Extrapolated from probit plot of mortality vs log time, assuming >50% mortality would eventually occur (actual value at 10 days was 43.8% mortality).

[d] NS = not significant.

following the sc injection. Control animals receiving either high dose or low dose ic challenge had 92.9% and 34.5% mortality, respectively. The data presented is from one of two experiments. Similar results were observed in each.

High dose ic challenge. Mortality for each group of mice and the day of ic challenge with 20 $TCID_{50}$ of HSV are shown in Fig. 1. Fisher's exact test (2-tailed) was used to calculate p values. A significant decrease in mortality was observed for days 3, 6, 9, and 21 when compared to either the mortality observed on day 0 when both ic and sc injections were given simultaneously, or to the combined mortality of the ic control group and the sc control group; days 6 and 21 were highly significant. Table I shows the determination of the median day of death (and correction for inoculation delay) for each day following ic challenge. Significant differences were demonstrated in the distribution of the number of deaths (mortality) for the groups challenged on days 6 and 21 when compared to both the control ic treatment alone and when sc and ic treatments were given on the same days.

Low dose ic challenge. Figure 2 shows the mortality observed for each day following ic challenge with 2 $TCID_{50}$ of HSV. Because of the lower challenge dose, less mortality was observed, and thus it was difficult to demonstrate significant differences with the number of animals used. Similar biphasic protection was shown as with the higher ic challenge dose, but the exact timing of protection differed between the low and high challenge doses. Table II shows the day of death for each group at the time when one-half of the (final) mortality was observed for the control, low dose, ic treatment alone, *i.e.*, 17.3%.

Taken together, these findings demonstrate an early and late period of intracere-

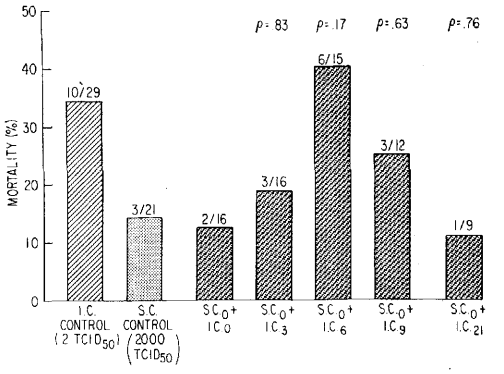

FIG. 2. Low dose herpes simplex virus (HSV) intracerebral challenge; mortality of groups receiving a subcutaneous inoculation of 2000 $TCID_{50}$ of HSV on day 0, followed by intracerebral challenge with 2 $TCID_{50}$ of HSV. Subscript indicates day of challenge.

TABLE II. Effect of Subcutaneous Inoculation of 2000 $TCID_{50}$ of HSV on Subsequent Intracerebral Challenge with 2 $TCID_{50}$ ($LD_{34.5}$).

Treatment group	Actual day of death where 50% control mortality occurred (17.3%)	Inoculation delay	Corrected day of death where 50% control mortality occurred[a]
Control ic ($LD_{34.5}$)	8.0	—	8.0
$sc_0 + ic_0$	13.0	0	13.0 (12.5%)[a]
$sc_0 + ic_3$	18.0	−3	15.0
$sc_0 + ic_6$	9.5	−6	3.5
$sc_0 + ic_9$	11.8	−9	2.8
$sc_0 + ic_{21}$	28.0	−21	9.0 (11.1%)

[a] Actual mortality in ( ) if <17.3% at end of 21 days of observation.

bral resistance to IC challenge with homologous (HSV) virus following sc infection with HSV.

Heterologous challenge. To help determine whether the observed periods of resistance to ic challenge were homologous or heterologous, a similar experiment was carried out using an antigenically unrelated virus, EMCV, for the ic challenge. Specifically, following sc infection with 800 $TCID_{50}$ of HSV, mice were challenged with 1000 $TCID_{50}$ of EMCV ic at the times shown in Fig. 3. The results are pooled from two experiments. Significant reduction in mortality was observed only 6 days after the initial sc infection with HSV indicating nonspecificity of the early resistance and specificity of the later resistance.

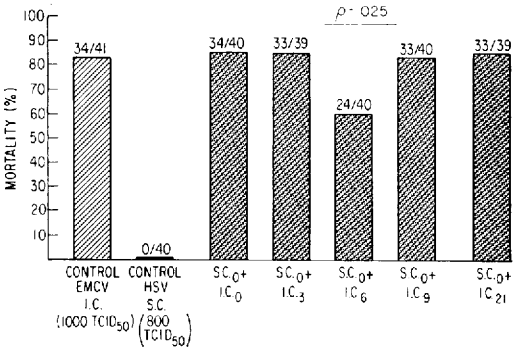

FIG. 3. Heterologous intracerebral challenge with encephalomyocarditis virus (EMCV); mortality of groups receiving a subcutaneous inoculation of 800 $TCID_{50}$ of HSV on day 0, followed by intracerebral challenge with 1000 $TCID_{50}$ of EMCV. Subscript indicates day of challenge.

Neutralizing antibody production. No detectable herpes antibody (<1:4) was found in two experiments until day 9 following sc infection with 2000 $TCID_{50}$ of HSV. A titer of 1:4 was present on days 9 and 14 and a titer of 1:4–1:8 was present on day 21.

Virus isolation studies. With the amount of virus used (2000 $TCID_{50}$), no virus was detected in two experiments from pooled organ homogenates of brain, liver, spleen, lung, or kidney on days 0 to 21 following sc infection with HSV. Virus isolation attempts were not made from mice which were found dead following infection.

Interferon production. In two experiments in which 2000 $TCID_{50}$ of HSV was given sc, no detectable interferon was present in either the pooled serum (<10 units/ml of serum) or organ homogenates (<100 units/gm of tissue) except in one instance. In one experiment, 300 units/g of interferon was found in the pooled homogenate of lung on day 6 following sc infection with HSV. Interferon was not detected in brain homogenates from either experiment (<10 units/g of tissue).

Discussion. The data from the homologous virus challenge experiments with HSV demonstrate two periods of intracerebral resistance to ic challenge with virus subsequent to sc infection with HSV. The early protective effect occurred between days 0–6. (The differences in the time of development of early resistance in the high and low challenge dose groups is considered below). The

late period of resistance occurred about day 21. The possible underlying mechanisms of resistance may be divided into those due to immune factors and those due to nonimmune factors.

The early intracerebral resistance was characterized by nonspecificity (resistance to heterologous ic challenge with EMCV) and absence of serum neutralizing antibody to HSV. These findings support the interpretation that nonimmune mechanisms were responsible for the early period of resistance. In contrast, the late period of intracerebral resistance to ic HSV infection was specific (no heterologous protection against EMCV ic infection) and associated with immune mechanisms since serum HSV neutralizing antibody was present.

The possible nonimmune mechanisms of the early, nonspecific resistance in the brain could be activation of the interferon system or direct interference (3–9) between peripherally inoculated virus which may have reached the brain, and the subsequent ic challenge virus. Neither of these possibilities could be directly confirmed by experimentation. Interferon was not detected in the mouse tissues (except for one occasion in the lung) and HSV was not isolated from the brain homogenates. In fact, virus was not isolated from any of the organs of asymptomatic mice after subcutaneous HSV infection with the small doses of virus used. It has been pointed out, however, that low levels of virus can be prsent in an organ and not be detectable in tissue homogenates but can be detected by cultivation of infected tissues *in vitro* (14). This type of isolation method was not employed in the present study. Indirect evidence obtained in the present experiments indicates that the small sc dose of HSV used was capable of replicating in at least some of the mice and reaching the brain. Approximately 15% of sc infected mice died with a characteristic encephalytic death with the doses of virus employed. Nearly the same percentage of sc infected mice which were sacrificed prior to the onset of symptoms had histopathologic evidence of CNS infection with HSV. Production of antibody to HSV and viral interference following small sc infecting doses of HSV provide evidence that HSV did replicate in the mice following peripheral infection.

If HSV was present in the brain it is possible that it induced localized interference or that it induced small amounts of interferon in the brain. It has been demonstrated that subdetectable levels of interferon may result in an antiviral state both *in vivo* and *in vitro* (15–17). It has also been shown that induction of serum interferon by polyinosinic·polycytidylic ribonucleic acid is capable of protecting the mouse brain against subsequent ic challenge with HSV (18). Furthermore, large doses of HSV can induce interferon in the mouse (19, 20), guinea pig (21), and man (Bellanti, Catalano, and Chambers, unpublished data) under other conditions. However, direct evidence is needed to finally establish the actual cause of the early intracerebral resistance.

A possible explanation for the differences in the time of development of early homologous interference when large and small challenge doses of HSV were used may come from a consideration of the times of development of lethal amounts of virus in the brains under the different dose conditions. The time of peak interference from the sc inoculation is assumed to occur at a constant time after sc inoculation for the infecting dose given. The larger ic challenge dose reached lethal levels more rapidly than did the lower challenge dose as evidenced by the median day of death which occurred 4 to 5 days earlier in the high dose challenge group. Therefore, a very early ic challenge with a large dose of virus could allow early development of lethal amounts of virus before the sc infection could establish a protective level of interference in the brain. Conversely, a very early challenge with a small dose of virus would result in late development of lethal levels of virus, thereby allowing enough time for the establishment of the interference induced by the sc infection. The expected results would be an apparent earlier resistance to low challenge doses of virus (Fig. 2, days 0–3) and apparent later resistance to high challenge

doses of virus (Fig. 1, day 6).

The late (day 21) period of resistance to ic challenge with HSV appeared to be related to specific immunological responses. Previously published reports on the kinetics of antiherpes virus antibody formation (19, 22, 23), and the demonstration of the ability of circulating HSV antibody to afford protection against large ic challenge doses of herpes virus (22, 23), appear to support this interpretation.

Summary. Two periods of homologous intracerebral resistance to ic challenge with HSV were demonstrated following sc inoculation of HSV. The early protective effect occurred between days 0–6; the late period of resistance occurred about day 21. The early intracerebral resistance was characterized by nonspecificity (resistance to heterologous ic challenge with EMCV) and absence of serum neutralizing antibody to HSV. The late period of intracerebral resistance to ic HSV infection was specific (no resistance to heterologous ic challenge with EMCV) and associated with serum HSV neutralizing antibody. Indirect evidence indicated that the small sc dose of HSV used was capable of replicating in some mice and reaching the brain. However, interferon and virus were not detected in brain homogenates of apparently healthy mice with the small doses of HSV inoculated subcutaneously. The inability to explain the Magrassi phenomenon may be due to the relative insensitivity of the techniques employed. However, the availability of a model system in the mouse may permit further investigations of this phenomenon.

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1. Editorial, N. Engl. J. Med. **277**, 1315 (1967).

2. Olsen, L. C., Buescher, E. L., Artenstein, M. S., and Parkman, P. D., N. Engl. J. Med. **277**, 1271 (1967).

3. Magrassi, F., Z. Hyg. Infektionskr. **117**, 573 (1935).

4. Magrassi, F., Bull. Ist sieroter. Milan. **14**, 773 (1935).

5. Henle, W., J. Immunol. **64**, 203 (1950).

6. Schlesinger, R. W., *in* "Viral and Rickettsial Infections of Man" (T. Rivers and F. Horsfall, eds.), 3rd ed., p. 145. Lippincott, Philadelphia (1959).

7. Findley, G. M., and MacCallum, F. O., J. Pathol. Bacteriol. **44**, 405 (1937).

8. Duffy, C. E., and Morgan, P. N., Proc. Soc. Exp. Biol. Med. **84**, 298 (1953).

9. Seamer, J., Barlow, J. L., Gledhill, A. W., and Hotchin, J., Virology **21**, 309 (1963).

10. Finter, N. B., ed., "Interferon." Saunders, Philadelphia (1966).

11. Reed, L. J., and Muench, H., Amer. J. Hyg. **27**, 493 (1938).

12. Schmidt, N. J., "Diagnostic Procedures for Viral and Rickettsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.), 3rd ed., p. 78. Amer. Pub. Health Ass., New York (1964).

13. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, R. M., Proc. Soc. Exp. Biol. Med. **125**, 843 (1967).

14. Booss, J., and Hsiung, G. D., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 636 (1970).

15. Buckler, C. E, Wong, K. T., and Baron, S., Proc. Soc. Exp. Biol. Med. **127**, 1258 (1968).

16. Henderson, J. R., and Taylor, R. M,. Virology **13**, 477 (1961).

17. Wagner, R. R., and Snyder, R. M., Nature (London) **196**, 393 (1962).

18. Catalano, L. W., Jr., and Baron, S., Proc. Soc. Exp. Biol. Med. **133**, 684 (1970).

19. Sydiskis, R. J., and Schultz, I., J. Infec. Dis. **116**, 455 (1966).

20. Bostandzhyan, M. G., and Bikbulatov, R. M., Acta Virol. **11**, 159 (1967).

21. Tokumaru, T., Arch. Virusforsch. **21**, 61 (1967).

22. Anderson, W. A., and Kilbourne, E. D., Proc. Soc. Exp. Biol. Med. **107**, 518 (1961).

23. Chu, L., and Warren, G. H., Proc. Soc. Exp. Biol. Med. **105**, 396 (1960).

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