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Hemagglutinin Variants of Reovirus Type 3 Have Altered Central Nervous System Tropism

Abstract. *Variants of the Dearing strain of reovirus type 3 with antigenically altered hemagglutinin proteins are much less neurovirulent than the parental virus. When injected intracerebrally into mice these variants infected a subset of the brain neurons that were infected by the parental virus. When injected intraperitoneally, the variants did not spread to the brain. These results indicate that minor modifications of the reovirus hemagglutinin dramatically alter the ability of the virus to spread into and injure the central nervous system.*

Recent advances in the understanding of genetic and molecular aspects of viral pathogenesis have led to new approaches for studying the basis of recognition of viruses by their receptors (*1*). A striking feature of viral infection is the distribution of virus-induced injury in different tissues. Such distribution may depend in large part on the interaction of viruses with receptors on different subsets of differentiated cells. Thus, although many viruses can interact with the same tissues (for example, the nervous system), differences in the way they interact with such tissues suggest that viruses recognize distinct cell types.

We have used mammalian reoviruses as a model system for studying virus-host interactions and have determined the viral genes responsible for tropism and neurovirulence. We and others showed previously that reovirus type 3 is neurotropic since it infects neurons and causes a diffuse fatal encephalitis (*2, 3*). Reovirus type 1 is not neurotropic since it infects primarily the ependymal cells lining the cerebral ventricles resulting in a nonlethal hydrocephalus (*4*). Genetic studies show that the reovirus hemagglutinin determines the cellular tropism of the reoviruses in the brain (*3, 5*). Thus, the type 1 hemagglutinin interacts with receptors on ependymal cells and the type 3 hemagglutinin binds to receptors on neurons (*1, 6*).

To define the interaction between the reovirus type 3 hemagglutinin and receptors on neurons, we isolated a number of monoclonal antibodies directed to the viral hemagglutinin. By means of these antibodies we showed that there are at least three distinct epitopes on the hemagglutinin, one of which interacts with both neutralizing antibody and cytotoxic T lymphocytes (NT epitope) (*7, 8*). Since this site plays such a central role in determining the specific interaction of reovirus with the immune system, we selected reovirus type 3 variants that were not neutralized by the monoclonal antibodies. These variants induced characteristic cytotoxic T cell responses (*8*) and were less neurovirulent than the parental type 3 virus (*9*).

Here we outline our studies to define the basis for the reduced neurovirulence of the reovirus type 3 variants. We fo-

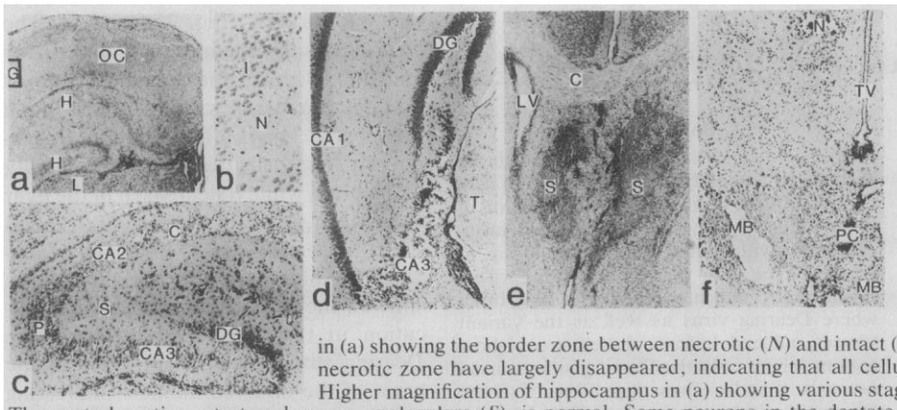

Fig. 1. (a to c) Brain sections from suckling mice injected intracerebrally with the Dearing strain of reovirus type 3. The mice were injected with 10^2 plaque-forming units of virus and killed when moribund. The brains were removed, fixed in Formalin, and sections were stained with hematoxylin and eosin by conventional techniques. (a) Coronal section slightly rostral to the brain level shown in Fig. 2c. The lack of cellular detail in the occipital cortex (*OC*) region and the lateral thalamus (*L*) is indicative of necrosis. Most of the hippocampal gyrus (*H*) is necrotic and cavitated (×33). (b) Higher magnification of region *G* in (a) showing the border zone between necrotic (*N*) and intact (*I*) portions of the occipital cortex. Nuclei in the necrotic zone have largely disappeared, indicating that all cellular types have undergone necrosis (×165). (c) Higher magnification of hippocampus in (a) showing various stages of necrosis cavitation (*C*) in the CA2 region. The central portion, stratum lacunum moleculare (*S*), is normal. Some neurons in the dentate gyrus (*DG*) and CA3 of the hippocampus are pyknotic (*P*), but these regions are relatively spared (×165). (d to f) Brain sections from suckling mice injected intracerebrally with the A variant of Dearing virus. The mice were injected with 10^5 plaque-forming units of virus and killed 15 or 21 days later. Brain sections were prepared as described for (a) to (c). (d) Coronal section at the brain level shown in Fig. 2c. Most of area CA3 of the hippocampus is necrotic and cavitated. The rest of the brain including the dentate gyrus (*DG*) and thalamus (*T*) are normal (×83). (e) Septal area from another mouse showing bilateral necrosis of the septum (*S*). Lateral ventricles (*LV*) are lateral to the septum and the corpus collosum (*C*) is above (×33). (f) Coronal section from the brain in (d) at the brain level shown in Fig. 2b. The rostral portions of the mammillary bodies (*MB*) are bilaterally necrotic and cavitated. There is also a small focus of necrosis (*N*) lateral to the third ventricle (*TV*) (×83). Abbreviations: *AC*, anterior colliculus; *C*, cingulum; *CN*, caudate nucleus; *DG*, dentate gyrus; *DNH*, dorsomedial nucleus of hypothalamus; *H*, hippocampus; *LGB*, lateral geniculate body; *LTN*, lateral thalamic nucleus; *LV*, lateral ventricle; *MB*, mammillary body; *MCN*, medial cuneate nucleus; *MSN*, medial septum nucleus; *OC*, occipital cortex; *S*, subiculum; *SN*, substantia nigra; *STN*, spinal trigeminal nucleus; *TV*, third ventricle; *ZI*, zona incerta.

cused on a detailed analysis of the anatomic distribution of injury produced by the variants and by the parental type 3 virus. The results indicate that the variants are altered in their capacity to injure selected parts of the brain and are unable to invade the central nervous system (CNS) of the mouse when inoculated into a peripheral site.

We first examined the brain pathology induced by intracerebral injection of the parental type 3 reovirus (Dearing strain). Previous reports showed that this virus causes widespread destruction of neurons in several parts of the brain (*2*, *3*). Our results agreed with these findings (Fig. 1, a to c). The Dearing strain produced bilaterally symmetric necrosis of the medial cerebral cortex, most extensively in the occipital lobes, and in the dorsal and lateral diencephalon and medulla as well as the septum, hippocampus, mammillary bodies, and midbrain tectum. Thus, the Dearing strain produced a highly lethal encephalitis with lesions widely distributed throughout the brain.

We next examined the brain pathology induced with the variant viruses. We reported previously that these viruses with antigenically altered hemagglutinin proteins are greatly attenuated and do not replicate efficiently in the brains of infected animals (*9*). We injected mice intracerebrally with variant A, F, or K (all of which were selected with the same monoclonal antibody) and killed them at various times after infection (Fig. 1, d to f). One unusual aspect of the pathology induced by the variants was the consistent bilateral destruction of regions of the pyramidal cells of the hippocampus (Fig. 1d). Neurons in the hippocampal gyrus often showed total necrosis of areas CA2, CA3, and CA4 but not CA1 or the dentate gyrus. Virus-induced lesions were also observed in other regions of the limbic system of the brain including the septum (Fig. 1e) and mammillary bodies (Fig. 1f). The tissue destruction in these areas remained focal and eventually the tissues became cavitated.

Another important aspect of the pathology caused by infection with the variants was that the cerebrum and medulla rarely showed any signs of infection. These findings are in sharp contrast to the more widespread encephalitis caused by the parental Dearing virus (Fig. 1, a to c).

Composite figures showing the generalized patterns of pathology induced by the parental or variant viruses (Fig. 2, a to e) revealed that the variants infected a subset of the neurons that were infected by the parental virus. This subset is part of the limbic system of the brain which includes the hippocampus, hypothalamus, mammillary bodies, and the septum. The Dearing virus showed a broader tropism since it infected neurons in other regions of the brain including the cerebral cortex, thalamus, and medulla. It is interesting that after infection with the Dearing strain, the cortical lesions were mainly located along the medial cerebral cortex and most extensively in the occipital lobes. This cortical pathology was observed whether the virus was inoculated intracerebrally or intraperitoneally. In addition, we rarely saw evidence of viral destruction elsewhere in the cerebrum. These studies therefore indicate that the original Dearing virus does not infect neurons in all regions of the brain.

To determine whether the variants are altered in their capacity to spread to the various organs including the CNS after extraneural injection, we injected mice intraperitoneally with the Dearing strain and the variants (Fig. 2, f and g). The Dearing strain appeared early in the liver and the spleen of the animals. Little or no infectious virus was detectable in the brains until 3 days after infection. Subsequently, the virus titer increased rapidly in the brain while it decreased in the liver and spleen. Most of the mice inoculated with the Dearing strain were dead by day 14 after infection. The variant viruses showed the same basic pattern of replication in the liver and spleen as the Dearing strain but did not grow in the brain of the infected animals, indicating that they are restricted in their capacity to enter and replicate in the CNS after peripheral inoculation.

These results show that selective alterations in one antigenic domain (NT) of the reovirus type 3 hemagglutinin can change the extent and distribution of injury in the brain after intracerebral inoculation of the virus and can reduce

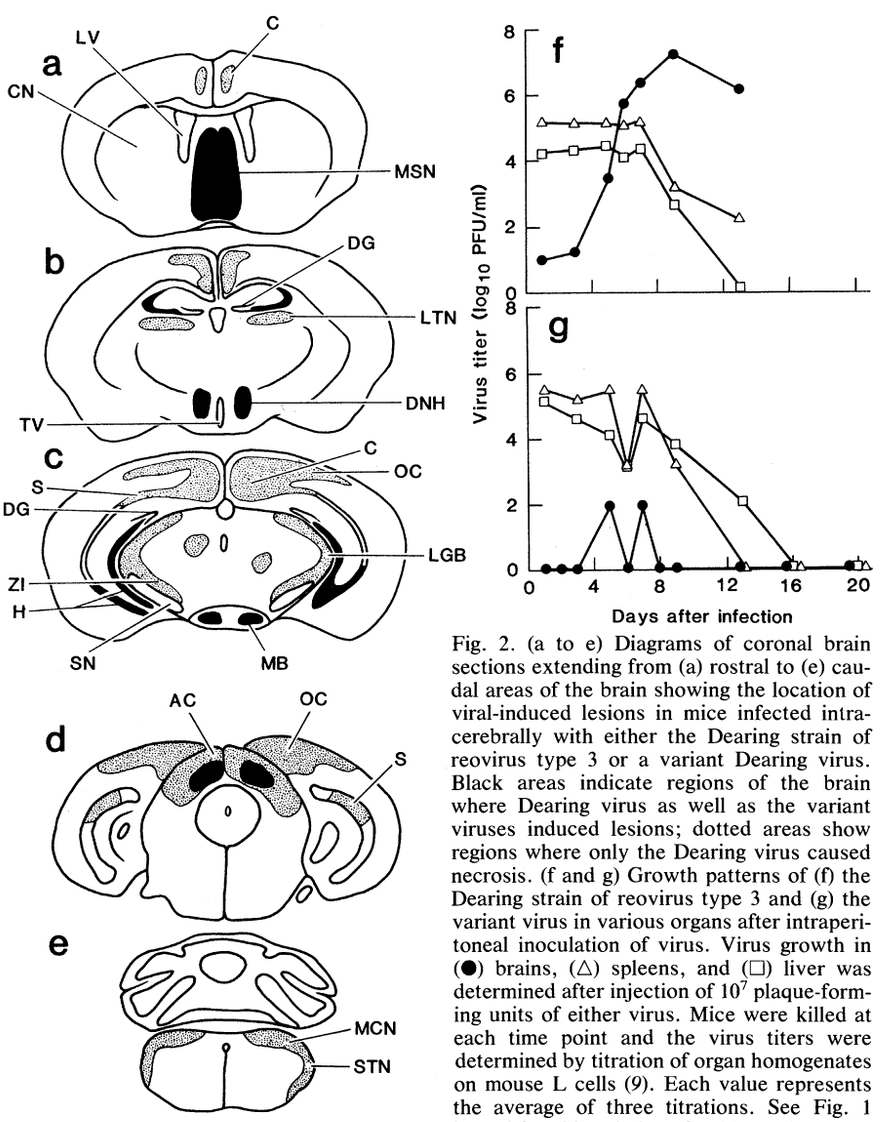

Fig. 2. (a to e) Diagrams of coronal brain sections extending from (a) rostral to (e) caudal areas of the brain showing the location of viral-induced lesions in mice infected intracerebrally with either the Dearing strain of reovirus type 3 or a variant Dearing virus. Black areas indicate regions of the brain where Dearing virus as well as the variant viruses induced lesions; dotted areas show regions where only the Dearing virus caused necrosis. (f and g) Growth patterns of (f) the Dearing strain of reovirus type 3 and (g) the variant virus in various organs after intraperitoneal inoculation of virus. Virus growth in (●) brains, (△) spleens, and (□) liver was determined after injection of 10^7 plaque-forming units of either virus. Mice were killed at each time point and the virus titers were determined by titration of organ homogenates on mouse L cells (*9*). Each value represents the average of three titrations. See Fig. 1 legend for abbreviations for (a) to (e).

the capacity of virus to enter the brain after peripheral inoculation. We showed previously that the hemagglutinin-variant viruses are extremely attenuated (*9*). We do not know whether the altered distribution of tissue injury observed in the present study is due to an altered recognition of receptors on neurons, an altered capacity of virus to spread within the central nervous system, or some other mechanism. However, the fact that the variants are altered in the region of the hemagglutinin involved in receptor binding indicates that slight changes in the viral hemagglutinin have profound effects on tissue tropism and suggest that there is an altered recognition of receptors on neurons by the viral variants. It also appears that mutations of the hemagglutinin gene occurring during the replicative cycle of a virus, perhaps even during replication within a single host, may lead to significant differences in the distribution of virus in the host. Such mutations could help explain differences in the virulence of many neurotropic viruses.

Our observations suggest that certain variant viruses might be useful as vaccines. When injected intraperitoneally, the variants did not infect the central nervous system, but did reach other organs (for example, spleen) where they could stimulate an immune response against infection with a virulent reovirus (*8–10*). The variants seem to be very stable, since we have been unable to isolate revertants from infected animals (*10*). It may therefore be possible to identify the regions of viral proteins important in virulence, select attenuated variants that are altered in their capacity to injure target tissues, and retain immunogenicity in such strains.

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A Cytokinin (Isopentenyl-Adenosyl-Mononucleotide) Linked to Ecdysone in Newly Laid Eggs of *Locusta migratoria*

Abstract. *Newly laid eggs of the insect* Locusta migratoria *contain high concentrations (50 nanomoles per gram) of an ecdysone conjugate of maternal origin; 3 milligrams of this conjugate were isolated by conventional techniques, and the structure was established by mass spectrometry and ^{1}H, ^{13}C, and ^{31}P nuclear magnetic resonance as the 22-N^{6}-(isopentenyl)-adenosine monophosphoric ester of ecdysone.*

Ovaries of vitellogenic females of the orthopteran insect *Locusta migratoria* synthesize large quantities of ecdysteroids, which accumulate inside the oocyte predominantly in conjugated form and are bound to the major yolk protein, vitellin (*1–4*). These conjugated ecdysteroids are present in the newly laid eggs and are progressively metabolized during embryonic development; their hydrolysis accounts for the presence of several peaks of free ecdysone in the eggs and embryos, two of which are monitored before the differentiation of any endocrine glands (*3, 5*). Ecdysteroid conjugates are also present in ovaries or eggs (or both) of several other insect species (*6–9*).

Two maternal conjugated ecdysteroids are predominant in newly laid eggs of *Locusta* at high concentrations (*3*); these are conjugated 2-deoxyecdysone (approximate concentration, 100 nmole/g) and conjugated ecdysone (approximate concentration, 50 nmole/g). We have identified the predominant 2-deoxyecdysone conjugate donated by the female to its offspring as the 22-adenosine monophosphoric ester of 2-deoxyecdysone (*10, 11*), and now report the isolation of the predominating maternal conjugate of ecdysone and its identification as the 22-N^{6}-(isopentenyl)-adenosine monophosphoric ester of ecdysone (Fig. 1, inset).

From about a million newly laid eggs we extracted the major yolk protein vitellin (*12, 13*); the ecdysteroid conjugates bound to vitellin (*4*) were extracted with 50 percent aqueous methanol and this extract was partitioned between hexane and 50 percent aqueous methanol. The methanol phase was subjected to reversed-phase C8 (Merck) liquid chromatography and the elution (gradient of pure water to pure methanol) of the ecdysteroid conjugates was monitored by ultraviolet absorption at 250 nm and by radioimmunoassay (*14*)—after hydrolysis by a *Helix pomatia* enzyme mixture (*2, 3*) of samples of the eluted fractions; the ecdysteroid-containing fractions were rechromatographed under the same conditions, and 3 mg of a conjugate was obtained. The hydrolysis by a *Helix pomatia* enzyme mixture of this conjugate yielded an ecdysteroid presumed to be ecdysone on the basis of thin-layer chromatography (TLC) on silica gel and reversed-phase high-performance liquid chromatography (HPLC).

The identity of ecdysone as the genin of this conjugate was ascertained by subjecting the steroid to ^{1}H nuclear magnetic resonance (NMR) analysis and to gas-liquid chromatography–mass spectrometry after treatment with trimethylchlorosilane; these results were identical to those obtained under the same conditions for reference ecdysone and were in agreement with earlier ^{1}H NMR (*15*) and mass spectrometry (*16, 17*) studies of ecdysone.

The unhydrolyzed ecdysone conjugate showed an ultraviolet absorption in methanol with a peak at 250 nm. The ^{31}P NMR spectra presented a signal at $\delta = 2$ ppm relative to phosphoric acid, indicating the presence of a phosphate group. The ^{13}C NMR spectrum of the unhydrolyzed conjugate showed a signal at $\delta = 80.5$ ppm, which is present as a doublet ($J = 5$ Hz) in proton-noise decoupled spectra; this signal, corresponding to a carbon carrying a phosphate substituent, can be attributed to C-22 which has undergone a downfield shift of 6 ppm as a result of the presence of an electronegative phosphate group (*18*). In contrast, the signals at $\delta = 68.5$ ppm, 68.6 ppm, 73.5 ppm, and 86.5 ppm (Table 1), which are attributed in the ^{13}C NMR spectra of reference ecdysone, respectively, to car-