Basis of neurovirulence of avirulent rabies virus variant Av01 with stereotaxic brain inoculation in mice

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Av01 is a variant of the challenge virus standard strain of fixed rabies virus that was selected with a neutralizing anti-glycoprotein monoclonal antibody, and has a single amino acid change in the glycoprotein. It is avirulent after both intracerebral and peripheral routes of inoculation in adult mice. In this study, Av01 was found to be neurovirulent with stereotaxic brain inoculation in either the striatum or cerebellum of adult mice. Mice that had been inoculated simultaneously with Av01 by the intracerebral and intrastriatal routes recovered. More infectious virus was present in the brains of mice inoculated intrastriatally than intracerebrally, and more neurons contained rabies virus antigen. However, the topographical distribution of infected neurons was similar with both routes. Serum neutralizing antibodies against rabies virus were produced later and in smaller quantities after intrastriatal inoculation. Av01 is probably neurovirulent after stereotaxic brain inoculation because this route produces both a direct site of viral entry into the central nervous system and a low level of immune stimulation.

Introduction

Rabies virus is a highly neurotropic virus that causes fatal disease in humans and animals. Experimental rabies in mice provides a convenient model for the study of virus–host interactions. Avirulent variants are useful tools in gaining an understanding of the molecular and biological bases of rabies virus neurovirulence (Coulon \textit{et al.}, 1989; Dietzschold \textit{et al.}, 1983, 1985; Jackson 1991a, b; Kucera \textit{et al.}, 1985; Lafay \textit{et al.}, 1991; Seif \textit{et al.}, 1985). Av01 is a variant of the challenge virus standard (CVS) strain of fixed rabies virus \textit{in vitro} with a neutralizing anti-glycoprotein monoclonal antibody (Coulon \textit{et al.}, 1982a). Sequencing data showed that Av01 has a single amino acid substitution of a glutamine for the arginine of CVS at position 333 of the glycoprotein (Seif \textit{et al.}, 1985). CVS causes fatal disease in adult mice after a variety of routes of inoculation and over a wide range of dosages. In contrast, Av01 does not cause neurological disease or death in adult mice after either intracerebral or peripheral inoculation (Coulon \textit{et al.}, 1982b, 1983, 1989; Flamand \textit{et al.}, 1987; Kucera \textit{et al.}, 1985; Seif \textit{et al.}, 1985). Instead, Av01 induces high levels of neutralizing antibody (Coulon \textit{et al.}, 1982b, 1983), and provides protection against a challenge with virulent strains of rabies virus (Coulon \textit{et al.}, 1982b; Flamand \textit{et al.}, 1984).

A similar avirulent variant, RV194-2, has been described by Dietzschold \textit{et al.} (1985), and it has the same amino acid substitution at position 333 of the glycoprotein.

Gillet \textit{et al.} (1986) investigated central neural transport mechanisms in rabies with stereotaxic inoculation of CVS into specific nuclei of rats. This study provided evidence that rabies virus spreads in the brain by fast axonal transport. A model of focal encephalitis in mice has been developed with stereotaxic inoculation of herpes simplex virus in the hippocampus (McFarland \textit{et al.}, 1986; McFarland & Hotchin, 1987). Stereotaxic inoculation was also used to investigate the mechanisms whereby scrapie spreads within the brain and causes disease in mice (Kim \textit{et al.}, 1987, 1990a, b, c). In the present study, Av01 was found to be neurovirulent after stereotaxic inoculation in either the striatum or cerebellum of adult mice. Studies were performed to determine why Av01 is virulent with stereotaxic inoculation in the striatum.

Methods

\textit{Virus.} Av01 was obtained from Anne Flamand (Centre National de la Recherche Scientifique, Gif sur Yvette Cedex, France), and stock virus was grown in BHK-21 cells to a titre of $3.6 \times 10^7$ p.f.u./ml.

\textit{Animals.} Six-week-old female CD-1 mice (Charles River Canada) were used. At least 16 mice were assigned to each experimental group and three mice were used for each time point. The mice were
anaesthetized for procedures by inhalation of methoxyflurane, and then inoculated intraperitoneally with pentobarbital (60 mg/kg).

**Intracerebral inoculation.** Mice were inoculated intracerebrally with 1000 p.f.u. of Av01 in 0.03 ml PBS and 2% foetal bovine serum (FBS) using a 26-gauge needle.

**Stereotaxic brain inoculation.** Mice were placed in a stereotaxic apparatus (David Kopf Instruments) equipped with a mouth adapter. The skull was exposed with a longitudinal midline scalp incision, the landmarks of bregma and lambda were identified, and a hole was made on the left side of the skull with a dental drill press at coordinates of F.(1-0), L.(2.5) for the left neostriatum and F.(−6-0), L.(2-0) for the cerebellum (Slotnick & Leonard, 1975). A needle was inserted to a depth of 3 mm from the surface of the skull and then pulled back 0.5 mm. Mice were inoculated with 1000 p.f.u. of Av01 in 1.0 µl PBS and 2% FBS over 5 min, and the needle was removed after an additional 3 min. Preliminary tests using India ink showed that inocula were retained at the target sites in the striatum and cerebellum.

**Simultaneous intracerebral and intrastriatal inoculations.** Mice were inoculated both stereotaxically with 1000 p.f.u. (in 1.0 µl) of Av01 in the left striatum and intracerebrally with 1000 p.f.u. (in 0.03 ml) of Av01. In the control group, 0.03 ml of PBS was inoculated intracerebrally instead of virus.

**Tissue processing and staining.** Mice were sacrificed at scheduled time points by perfusion with buffered 4% paraformaldehyde. Brains were removed, immersed overnight in the same fixative at 4 °C, and embedded in paraffin. Coronal sections of brain and transverse sections of brainstem 6 µm thick were cut at multiple levels on a microtome. Sections were stained with haematoxylin and eosin, and immunoperoxidase staining was performed for rabies virus antigen as previously described (Jackson & Wunner, 1991).

**Serum neutralizing antibody assays.** Serum rabies virus-neutralizing antibody levels were determined using the rapid fluorescent focus inhibition test. (Smith et al., 1973). Blood samples were taken from the heart. Sera were diluted twofold and neutralization titres were expressed as the reciprocal of the highest serum dilution that reduced the number of microscope fields with fluorescing cells by 50% or more compared to infected Av01 control slides.

**Mouse brain homogenate for intracerebral inoculation.** A 10% (w/v) homogenate of brain in PBS and 2% FBS was prepared from a mouse 9 days after stereotaxic inoculation in the striatum. After clarification (500 g, 5 min), the homogenate was used for intracerebral inoculation of mice for assessment of whether the phenotype of Av01 had changed after stereotaxic inoculation.

**Virus assays.** Serial 10-fold dilutions of brain homogenates of infected mice were assayed on individual samples by plaque formation on BHK-21/S13 cells as previously described (Sedwick & Wiktor, 1967).

**Results**

**Clinical observations**

After intracerebral inoculation of 1000 p.f.u. of Av01, the mice developed only transient mild weight loss, ruffled fur and diminished activity between day 7 and 9, and all mice survived. On day 5 or 6 after stereotaxic inoculation of Av01 into either the striatum or cerebellum, the mice developed weight loss, ruffled fur, diminished activity and hunching. The mice developed limb paresis at a mean of 10.1 ± 2.4 (s.d.) days after intrastriatal inoculation, and the mean time to death was 14.1 ± 3.0 (s.d.) days. The mortality rate was 94% (15 of 16) at 28 days, and the only surviving mouse had severe triparesis. The cumulative mortality is shown in Fig. 1. In contrast, after intrastriatal inoculation of 100 intrastriatal LD₅₀ (82 p.f.u.) of CVS, the mortality rate was 100% (16 of 16) and the mean time to death was 11.0 days.

After inoculation in the cerebellum, the mean date of onset of limb paresis was 9.8 ± 1.0 (s.d.) days, and the mean time to death was 13.8 ± 2.3 (s.d.) days. Seventy-five percent (12 of 16) of the mice died by day 28 (Fig. 1), and two of the four survivors had neurological sequelae.

After simultaneous intracerebral and intrastriatal inoculations of Av01, the mice developed weight loss, ruffled fur, and hunching on day 5, and they all (16 of 16) recovered completely by day 12. In the group with intrastriatal inoculation of Av01 and intracerebral inoculation of PBS, the mean date of onset of limb paresis was 10.4 ± 2.1 (s.d.) days, the mean time to death was 13.6 ± 2.4 (s.d.) days, and all mice (16 of 16) died by day 18.

**Viral growth**

The viral growth in the brain after intracerebral and intrastriatal inoculation are compared in Fig. 2. Viral growth peaked 5 days after inoculation in both groups. On day 5 viral growth was 28 times higher after intrastriatal inoculation than after intracerebral inoculation, and 275 times higher on day 9. Virus was cleared more rapidly after intracerebral inoculation, and viral clearance was associated with the production of serum neutralizing antibodies (see below).

**Mouse inoculation test**

Sixteen mice were inoculated intracerebrally with 1000 p.f.u. (in 30 µl) of diluted brain homogenate derived from
Neurovirulence of rabies virus variant Av01

897

Fig. 2. Growth curves of virus in the brain after intrastriatal (●) and intracerebral (○) inoculation of Av01.

Fig. 3. Rabies virus antigen in the right striatum 4 days after intrastriatal (a) and intracerebral inoculation (b) of Av01. Antigen is present in many more neurons and neuronal processes in the neuropil after intrastriatal inoculation (a). Immunoperoxidase-haematoxylin staining. Bar marker represents 50 μm.

Fig. 4. Deep cerebellar nucleus 11 days after intrastriatal inoculation of Av01 showing loss of the normal cellular architecture. There are degenerating neurons with pyknotic nuclei and vacuolation of the neuropil (a) and infiltration with mononuclear inflammatory cells (b). Haematoxylin and eosin staining. Bar marker represents 25 μm.

a mouse that was sacrificed 9 days after intrastriatal inoculation with Av01. All of the mice survived during a 21-day observation period, although two mice developed persistent hindlimb paralysis.

Rabies virus antigen distribution

Rabies virus antigen was initially observed on day 3 in the cerebral cortex and striatum after either intracerebral or intrastriatal inoculation. Antigen was usually not found around the inoculation site for either route. Viral spread occurred quite rapidly. On day 4 antigen was present in all major brain regions, including the cerebral cortex, hippocampus, thalamus, striatum, cerebellum (deep cerebellar nuclei and Purkinje cells) and brainstem. The amount of antigen peaked on day 5 after both routes of inoculation. Antigen was present in a larger number of neurons after intrastriatal inoculation than after intracerebral inoculation (Fig. 3). Antigen disappeared from the brain much more rapidly after intracerebral inoculation, and with this route very little antigen was present after day 6. In contrast, antigen did not disappear after intrastriatal inoculation until after day 11.

Pathological changes

There were marked pathological changes after both intracerebral and intrastriatal inoculation (Fig. 4). Inflammatory cells were present in perivascular cuffs and there was infiltration into the leptomeninges. After day 7 there was marked infiltration in the cerebral cortex, striatum, thalamus and cerebellum. The inflammatory infiltrates consisted of lymphocytes, microglia,
macrophages and occasional polymorphonuclear leukocytes. Degenerative changes, including vacuolation in the perikaryon, nuclear hyperchromatism and cellular disintegration, were seen in many neurons, and neuronophagia was observed. After day 7 the degenerative changes were more severe after intrastriatal inoculation than after intracerebral inoculation.

Serum neutralizing antibody

Serum rabies virus neutralizing antibodies were detected earlier after intracerebral inoculation than after intrastrital inoculation (Fig. 5). The antibody response was also much greater after intracerebral inoculation.

Discussion

Rabies virus usually causes a fatal infection of the central nervous system (CNS) in humans and animals. Rabies virus neurovirulence depends on a complex interaction of both viral and host factors. Host factors include species and strain (Lodmell & Chesebro, 1984; Lodmell & Ewalt, 1985), age (Flamand et al., 1984), sex (Flamand et al., 1984) and immune status (Coulon et al., 1983; Flamand et al., 1984; Iwasaki et al., 1977; Smith et al., 1982). The route of inoculation is an important determinant of neurovirulence in many experimental models (Jackson, 1991a). After a peripheral route of inoculation, a virus may not have the ability to invade the CNS. The portal of entry of a virus into the CNS may have a major effect on the distribution of the infection, which has been shown for herpes simplex virus type 1 after four different routes of inoculation in mice (Anderson & Field, 1983).

Intracerebral inoculation is a standard method of infecting mice with neurotropic viruses. This route bypasses barriers that may be present with peripheral routes of inoculation. Intracerebral inoculation delivers a large inoculum (usually 30 µl in mice) under high pressure over a short period of time. Mims (1960) has shown in a study using India ink that the inoculum tracks back along the needle and spreads throughout cerebrospinal fluid spaces, including the subarachnoid space. The high pressure of the inoculum results in rupture of the arachnoid villi into the venous sinuses, which results in overflow of the inoculum into the bloodstream.

Inocula can be delivered to much more precise targets in the brain using a stereotaxic apparatus. Gillet et al. (1986) studied viral transport mechanisms in the CNS by inoculating CVS stereotaxically in rat brains. In recent studies mice have been infected with other viruses using stereotaxic brain inoculation (Andersson et al., 1991; Kim et al., 1987, 1990a, b, c; McFarland et al., 1986; McFarland & Hotchin, 1987). In this study the biological characteristics of an avirulent variant of rabies virus, Av01, were examined with stereotaxic brain inoculation in mice, and they were compared with inoculation by the intracerebral route.

Avirulent variant Av01 was found to be neurovirulent with stereotaxic brain inoculation in either the striatum or cerebellum [RV194-2 (Dietzschold et al., 1985) was also virulent with inoculation in the striatum; data not shown]. The higher mortality rate (100%) and earlier mean time to death after intrastrital inoculation of CVS indicate that Av01 remains less virulent than its parent virus after stereotaxic inoculation. Simultaneous intrastrital and intracerebral inoculations of Av01 were performed to determine whether the addition of intracerebral inoculation provides a protective effect. This was found to be the case, which suggests that stimulation of host defences may be responsible for recovery of mice with intracerebral inoculation. Inadequate stimulation of host defences with stereotaxic brain inoculation could explain the fatal outcome of infection.

An immune response can modulate the course of rabies virus infection, and the outcome of an experimental infection depends on the strain of virus, the dose, the route of infection, and the species or strain of the host (Nathanson & Gonzalez-Scarano, 1991). Host defences include non-specific mechanisms, including scavenging monocyte–macrophages and induction of interferon, and both humoral and cell-mediated immunity (Wunner, 1987). The CNS is an immunoprivileged site under normal conditions, which makes clearance of infections more difficult than at other locations. Antibody can neutralize rabies virus and cause lysis of rabies virus-infected cells, which is probably a major mechanism for immune clearance. Helper T cells are essential for the
induction of antibody, and cytolytic T cells are important for recovery from CNS infection (Nathanson & Gonzalez-Scarano, 1991; Wunner, 1987). Immune enhancement of disease may occur under certain experimental conditions (Prabhakar & Nathanson, 1981).

In the present study, there was more infectious rabies virus in the brain after intrastriatal inoculation than after intracerebral inoculation. The difference in the amounts of infectious virus after inoculation by these routes was small at day 1 and subsequently increased, suggesting that the greater amount of virus with intrastriatal inoculation was not simply the result of more virus being retained in the brain after inoculation. The studies using immunoperoxidase staining indicated that there was a greater number of infected neurons after intrastriatal inoculation, and that the topographical distribution of the infections was similar. After day 5 infectious virus and viral antigen were cleared much more rapidly after intracerebral than after intrastriatal inoculation. Resistance to rabies is associated with the presence of rabies virus-neutralizing antibody (Wunner, 1987). Neutralizing antibody is important for protection of animals and humans from rabies, and also for recovery of animals from rabies (Jackson et al., 1989). Mice inoculated in the striatum with Av01 developed serum neutralizing antibody later and they had lower levels of antibody than mice inoculated intracerebrally. This reflects greater immune stimulation with intracerebral inoculation. The associated intravenous entry of virus with intracerebral inoculation, which is due to rupture of the arachnoid villi into the venous sinuses (Mims, 1960), may explain the enhanced immune stimulation with this route of inoculation.

Flamand and coworkers found that Av01 stimulates a more vigorous immune response than CVS after either intracerebral or peripheral inoculation of mice (Coulon et al., 1982b; Flamand et al., 1984). Av01 stimulates the production of circulating antibodies, but CVS does not (Coulon et al., 1982b). Av01 is also a remarkable inducer of early interferon and a strong stimulator of natural killer cell and cytotoxic T cell activities (Flamand et al., 1984). Av01 was virulent with stereotaxic brain inoculation, but is avirulent with all other known routes of inoculation in immunocompetent adult mice. This is probably because there is greater immune stimulation with intracerebral and peripheral routes of inoculation than with stereotaxic brain inoculation.

Av01 causes fatal infection in suckling and nude mice and mice immunosuppressed with cyclophosphamide (Coulon et al., 1983; Flamand et al., 1984). Virulent virus has been isolated from the brains of dying mice, indicating reversion of Av01 to the parental phenotype (Flamand et al., 1984). In the present study, biological evidence of phenotypic reversion was not found in a mouse brain 9 days after intrastriatal inoculation. However, this does not entirely exclude the possibility that either revertants or pseudorevertants played a role in the virulence of Av01 after stereotaxic brain inoculation.

With most routes of inoculation, Av01 stimulates host defences that prevent it from spreading efficiently in the CNS and infecting a sufficient number of neurons to cause fatal disease. CVS spreads more efficiently than Av01, although the basis for this has not yet been defined (Coulon et al., 1989; Dietzschold et al., 1985; Jackson, 1991b). The neurovirulence of Av01 in adult mice is greater with stereotaxic brain inoculation than with all other known routes of inoculation, perhaps because the virus is delivered directly into the CNS by a route that produces a low level of immune stimulation. The virulence of other avirulent neurotropic viruses or variants should also be examined with stereotaxic brain inoculation.

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