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Murine Forebrain Anomalies Induced by Coxsackievirus B3 Variants

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Neonatal or 7-day-old mice inoculated intracranially with either of two temperature-sensitive mutants (ts1, ts6) or the parent coxsackievirus B3 (CVB3) subsequently developed porencephaly or hydranencephaly. The forebrain anomaly induced depended upon age of the animal at inoculation and virus variant inoculated. Sections of brains from hydranencephalic mice revealed severe meningeal reactions, necrotizing encephalitis, and liquifactive necrosis in the cerebrum. No pathology was found in the pons, medulla, or cerebellum. Immunofluorescence studies with hyperimmune anti-CVB3 antiserum showed a random distribution of virus-infected cells in the cerebrum. Virus was recovered from several organs but little to no interferon and no anti-CVB3 neutralizing antibody were present in brain tissues. Availability of cells for replication of virus at the time of inoculation and replicative properties of each virus likely contributed to the outcome. Thus, forebrain anomalies resembling those found in infants can be induced in a murine model by select variants of coxsackievirus B3.

Key words: coxsackievirus, porencephaly, hydranencephaly

INTRODUCTION

Hydranencephaly is a term generally applied to a prenatal condition in which the cerebral hemispheres are almost, if not totally, replaced by membranous sacs [Crome, 1958]. Porencephaly refers to cavitation of the cerebral hemispheres and the size of the cavities varies considerably [Tardieu et al, 1981]. The wall of the membranous sac is composed of two layers, the leptomeninges and a thin shell of gliosed tissue representing remnants of the cortex [DeMyer, 1974; Kendall, 1979; Urich, 1976]. Diagnosis of hydranencephaly is currently based upon initial results from transillumination and subsequent computerized tomography tests, although the final

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diagnostic distinction between hydranencephaly and hydrocephalus rests with neuropathologic data [Dublin and French, 1980; Dyken and Miller, 1980; Friede and Mikolasek, 1978; Sutton et al, 1980]. A second type of hydranencephaly has been recognized as hydranencephaly of postnatal onset [Friede, 1975]. The cerebral lesions arise during an acute postnatal disease process within the first 18 months of life and the prenatal type occurs five times more frequently than the postnatal type [Friede, 1975].

Several viruses have been shown to induce hydranencephaly and porencephaly, ie, malformations which resulted from tissue destruction. The syndrome is induced by Akabane virus naturally in sheep and cattle and experimentally in sheep, cattle and goats, and by bluetongue virus naturally in sheep and experimentally in sheep fetuses and mice [see review by Eklund and Hadlow, 1978]. Seroepidemiologic evidence [Kurogi et al, 1975] in cattle fetuses and experimental studies in sheep suggested that Akabane virus infections were directly responsible for the destruction of undifferentiated neural cells, and that differences in severity of lesions were dependent on developmental stages of the infected fetus. Attenuated bluetongue vaccine virus induced noninflammatory cavitary anomalies limited to the ovine forebrain, which resembled porencephaly or hydranencephaly in man [Eklund and Hadlow, 1978; Kurogi et al, 1975; Johnson, 1974; Johnson, 1978; Schultz and De Lay, 1955]. Ovine fetuses inoculated intramuscularly with vaccine virus at 50 days of gestation developed hydranencephaly; those inoculated at 75 days of gestation developed porencephaly; and those inoculated at 100 days of gestation showed no gross malformation and only microscopic microglial nodules [Osburn et al, 1971a,b]. As noted above, additional work in mice with a mouse-adapted strain of bluetongue virus suggested that age dependency of the lesion was apparently determined by availability of germinal cells in the subventricular zone of the forebrain [Narayan and Johnson, 1972]. Cavitary lesions of the forebrain resembling porencephaly have been induced following inoculation of fetal monkeys with Venzuelan encephalitis virus [cited in Johnson, 1978].

Although less well documented, herpes simplex virus, cytomegalovirus, and coxsackievirus A9 have been implicated in production of hydranencephaly in humans [Friede and Mikolasek, 1978; Adams, 1976], as well as in less serious infections of the central nervous system.

The present study was undertaken to determine whether the ubiquitous coxsackieviruses of group B [Crowell and Landau, 1979] could induce either of the forebrain anomalies in mice, an excellent animal model for myocarditis induced in humans by these viruses [Woodruff, 1980; Gauntt et al, 1979; Trousdale et al, 1979]. The results show that two temperature-sensitive (ts) mutants of coxsackievirus B3 (CVB3) [Trousdale et al, 1977; Gauntt et al, 1983] can induce the forebrain anomalies of hydranencephaly and/or porencephaly in a high proportion of mice surviving inoculation as neonates.

MATERIALS AND METHODS

Animals

Young breeding pairs of CD-1 mice were purchased from Charles River Breeding Laboratories, Inc., Boston, MA, and maintained in the Laboratory Animal Resources facilities of the University of Texas Health Science Center at San Antonio.

The breeding pairs of C57 B16/J mice were purchased from Jackson Labs, Bar Harbor, ME. All animals were given fresh water daily and placed on a standard diet of laboratory mouse chow ad libitum. Neonates are defined as animals less than 24 hr at the time of inoculation with virus.

Cell Culture and Media

HeLa cells used in this study were cultured as previously described [Trousdale et al, 1977, 1979]. Primary cultures of neonatal or 7-day-old infant murine brain cells were prepared as follows. The brain was removed and cells were dispersed by repeated slow aspirations and delivery with a large bore pipette in complete minimum essential medium (Eagle's MEM; Grand Island Biological Company (GIBCO), Grand Island, NY) supplemented with 10% calf serum (Sterile Systems, Inc., Logan, UT), 2 mM L glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 mcg/ml Fungizone (GIBCO) and 0.056% $NaHCO_3$. After dispersion, the cells were seeded onto plastic petric dishes and incubated for 24 hr at 37°C in a CO_2 incubator. Less than 5% of the total cell population attached, these cells were washed twice and incubated in the same medium for 5–7 days.

Viruses

The origins, preparations of virus stocks and plaque assay methods for the parent coxsackievirus B3 ($CVB3_m$) and the temperature-sensitive (ts) mutants derived from this parent virus have been described previously [Trousdale et al, 1977; Gauntt et al, 1983]. The ts1 and ts6 variants were determined by complementation analyses to belong to the same group (Group III) [Trousdale et al, 1977; Gauntt et al, 1983].

Assay of Organs for Infectious Virus

The organs from each individual mouse were taken up in a preweighed 1 ml volume of MEM and then weighed again to determine organ weight by difference. The organs were disrupted with 20 strokes of a tight-fitting Dounce homogenizer in an ice bath. The homogenates were frozen (−90°C) and thawed (37°C) three times and infectious virus titers were calculated as pfu/g tissue. In some adolescent survivors with obvious virus-induced hydranencephaly, fluids were removed from brains of deeply anesthesized mice by aspiration with a 27 gauge needle and stored at −70°C until assayed for interferon or anti-$CVB3_m$ neutralizing antibody.

Virus Inoculation

Neonatal or infant mice were inoculated by intracerebral (i.c.) route into the right cerebral hemisphere with 0.02–0.05 ml of a stock virus solution containing 10^8 pfu/ml. All mice were observed daily for signs of disease and mortality.

Histology

Brain tissues were fixed in situ in deeply anesthesized mice by rapidly instilling 12 ml of a phosphate-buffered 10% formalin solution, pH 7.4, into the mouse via the left ventricle of the heart. The calvarium was gently removed and the intact brain was placed in the same buffered fixative for an additional 24 hr prior to embedding in paraffin, sectioning, and staining with hematoxylin and eosin. Some sections were stained by the Alizarin red method for calcium [Pearse, 1980]. Brain sections to be studied by an indirect immunofluorescence method were obtained from mice imme-

diately after sacrifice by cervical dislocation. Brains were quickly frozen in liquid nitrogen and stored at −70°C until thin sections (6–10 μm thick) were prepared on a Bright cryostat (Hacker Co., Fairfield, NJ). Hyperimmune rabbit anti-$CVB3_m$ antiserum was obtained from a rabbit immunized with $CVB3_m$-HeLa cell lysate, mixed in equal volume with Freund's complete adjuvant. This serum had an anti-$CVB3_m$ neutralizing antibody titer of 900. The fluorescein isothiocyanate (FITC)-conjugated goat antirabbit antibody (Cappel Laboratories, West Chester, PA) was mixed with Evans blue dye (0.003%) prior to addition to the fixed tissues. The tissues were incubated with either reagent for 30 min at 37°C followed by extensive washing with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). Negative controls used in these experiments included prebleed rabitt serum or omission of the hyperimmune rabbit anti-$CVB3_m$ antiserum prior to addition of the FITC-conjugated goat antirabbit antibody. Also, thin sections of brain from control (MEM-inoculated) animals were included in the assay. The tissue sections were photographed with a Nikon Motel UFX microscope equipped with a Nikon FX-35A camera using Kodak Tri-X pan (TX 135) black and white film.

Determination of Serum or Brain Fluid Antibody and Interferon Titers by Plaque Reduction Assays

Antibody titers to ts6 virus were determined by a plaque reduction assay in HeLa cells, as previously described [Gauntt et al, 1979]. At endpoint, the reciprocal of that dilution of serum or brain fluid which resulted in a 90% or greater reduction in plaques (1,000 pfu) was taken as the titer. Serum interferon titers were measured by a 50% reduction in titer of 50–100 pfu of vesicular stomatitis virus plaques, as previously described [Gauntt et al, 1979], except that sera were not adjusted to pH 2 prior to assay. Supernatant fluids clarified from brain tissue homogenates (50–60% brain, V/V) of 4-5 pooled brains were also assayed for interferon. One unit of mouse reference standard interferon, obtained from the National Institutes of Health, NIAID Reference Reagent Branch, was assayed as 2 or 5 units/ml.

RESULTS

Induction of Hydranencephaly/Porencephaly by $CVB3_m$ ts Variants

Two ts mutants of $CVB3_m$ were examined for their capacity to induce either forebrain anomaly subsequent to inoculation of neonates or 7-day-old infants of the outbred CD-1 murine strain (Table I). The parent $CVB3_m$ virus was lethal as previously reported [Trousdale et al, 1979] for neonates within 3 days. However, in the week-old infant mice inoculated with $CVB3_m$, about half of the small number inoculated presented with hydranencephaly and none with porencephaly. The ts1 variant induced both porencephaly and hydranencephaly at equal frequencies in CD-1 mice inoculated as neonates. Mice inoculated at 7 days of age were little affected by this virus; only one of 19 exhibited two tiny porencephalic cavities in the right cerebral hemisphere. The ts6 variant induced no demonstrable porencephaly in 105 surviving CD-1 mice, however, 72 of the 105 mice developed hydranencephaly. Infant mice inoculated at 7 days of age with ts 6 did not develop hydranencephaly and only 2 of 16 exhibited tiny porencephalic cavities in the right cerebral hemisphere. A more detailed examination of some of the hydranencephalic brains revealed that this anomaly can present as partial destruction of either right or left hemisphere or total

TABLE I. Induction of Hydranencephaly and Porencephaly by Coxsackievirus B3 Variants Following Inoculation of Neonatal and Infant CD-1 Mice[a]

Virus variant	Age at time of inoculation	Number of mice inoculated	Number survived/total number inoculated	Number with porencephaly/ total[b]	Number with hydranencephaly/ total
$CVB3_m$	Neonates	11	0/11	—	—
	7 days	15	11/15	0/11	6/11
ts1	Neonates	48	35/48	10/35	11/35
	7 days	21	19/21	1/19[c]	0/19
ts6	Neonates	173	105/173	0/105	72/105
	7 days	17	16/17	2/16[c]	0/16

[a]Mice were inoculated intracerebrally into the left cerebral hemisphere with a 0.02 ml (neonates) or 0.05 ml (7 days of age) of virus at 1×10^8 pfu/ml. Mice were sacrificed at 10–105 days postinoculation.
[b]The porencephaly cavities appeared in either right or left cerebral hemisphere or near the corpus callosum.
[c]The porencephaly in these mice consisted of two pinpoint cavitary lesions in the right cerebral hemispheres.

destruction of both hemispheres. The latter situation was observed in most mice. Twenty neonates inoculated intracerebrally with 0.02–0.05 ml of MEM did not display any forebrain anomalies.

Gross pathologies of brains from ts6- or ts1-inoculated mice, depicting hydranencephaly or porencephaly, respectively, are shown in Figure 1. The majority of mice which developed virus-induced hydranencephaly appeared runted; they moved very little and their heads were generally in a tucked-under position. The frontal portion of the cranium of the majority of these mice was distended. Most mice showed a lack of coordination in locomotion and they rolled monotonously from side to side or repetitively turned somersaults or back-flips. Mice which subsequently exhibited ts1 virus-induced porencephaly could not be distinguished from normal mice in appearance or by any action(s).

Pathology in the Brains of Animals Inoculated With ts6 Virus

Coronal sections of brains taken from mice inoculated with ts6 virus at 15 days p.i. were stained with hematoxylin/eosin (Fig. 2). The brains of afflicted animals showed meningitis (Fig. 2b–d), necrotizing encephalitis (Fig. 2a,b), gliosis (Fig. 2c) and cavity formation (Fig. 2d). Meningeal reactions were evident in most of the cerebral cortex. Necrosis of neurons, pycnosis of neurons, and necrosis of glial cells were observed in most sections. Necrosis appeared to be a total liquifactive process involving the affected areas. Tissues were infiltrated by mononuclear cells, especially about the margins of necrosis and around adjacent blood vessels, ie, perivascular cuffing. Early reactive gliosis (gemistocytosis) to intense gliosis (Fig. 2c) was present in many sections. Similar descriptions of pathology were made for brains taken from ts6 virus-inoculated mice on days 9, 12, 18, 21, and 28 postinoculation [authors' unpublished observation].

Sections prepared from mice with ts6 virus-induced hydranencephaly at 46 days p.i. (inoculated as neonates) showed the same pathological processes as described above and also active necrosis at the margins of hydranencephalic lesions, focal lesions of mononuclear cell infiltrates, and extraordinary amounts of calcium depos-

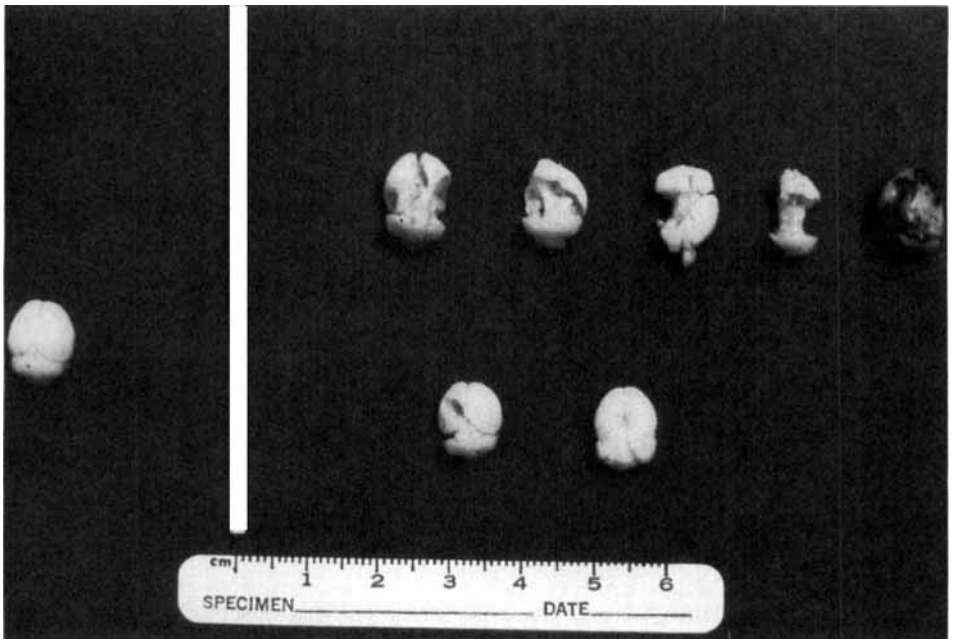

Fig. 1. Hydranencephaly or porencephalic cysts in brains of ts6 or ts1 virus-inoculated neonates. A normal brain in dorsal view is on the left. Brains in the upper row right were from ts6 virus-inoculated neonates at 10 to 105 days p.i. All are dorsal view except the middle brain. The brain on the right was taken at 10 days p.i. and demonstrates hemorrhaging that was found in about 10% of all brains. Note the complete hydranencephaly in the fourth brain from the left. Brains from ts1 virus-inoculated mice are presented in the lower row right and show large (left brain) and small midline porencephalic cysts.

ited in the tissues. Dystrophic calcification was verified by an alizarin red stain of sections which showed that the crystalline calcium deposits were present in the necrotic tissues, mostly outside of cells, and the calcium was apparently sequestered by necrotic debris.

The anterior cerebral cortex, hippocampus, and particularly the ventral aspects of the cerebral cortex adjacent to the hippocampus were areas that showed most pathology, whereas the midbrain rarely showed any evidence of pathology. No pathology was observed in the intact pons, medulla, or cerebellum of brains from more than a dozen mice which were inoculated with ts6 or ts1 as neonates and which upon sacrifice at 10–46 days p.i. had porencephalic lesions or hydranencephaly, from partial to complete bilateral.

The location of ts6 virus-infected cells in these brains was determined by immunofluorescence studies using hyperimmune rabbit antiserum to $CVB3_m$ (Fig. 3). Frozen sections of brain tissues were taken at 0, 3, 6, 9, or 12 days p.i. of neonates or 12 days p.i. of 7-day-old infants. Hematoxylin/eosin-stained fixed sections adjacent to those examined in these experiments and which were from neonate brains at 9 or 12 days p.i. showed identical pathology as described above. Normal brain and 0 day (2 hr p.i.) brain tissues were negative (data not shown). By day 3 p.i., single cells and a few groups (three–eight cells per group) of cells were positive and were randomly distributed throughout the cerebral hemispheres (data not shown). By day 6 p.i., the number of randomly distributed groups of positive cells per field had increased twofold or more and groups were found near cerebral ventricles (Fig. 3a).

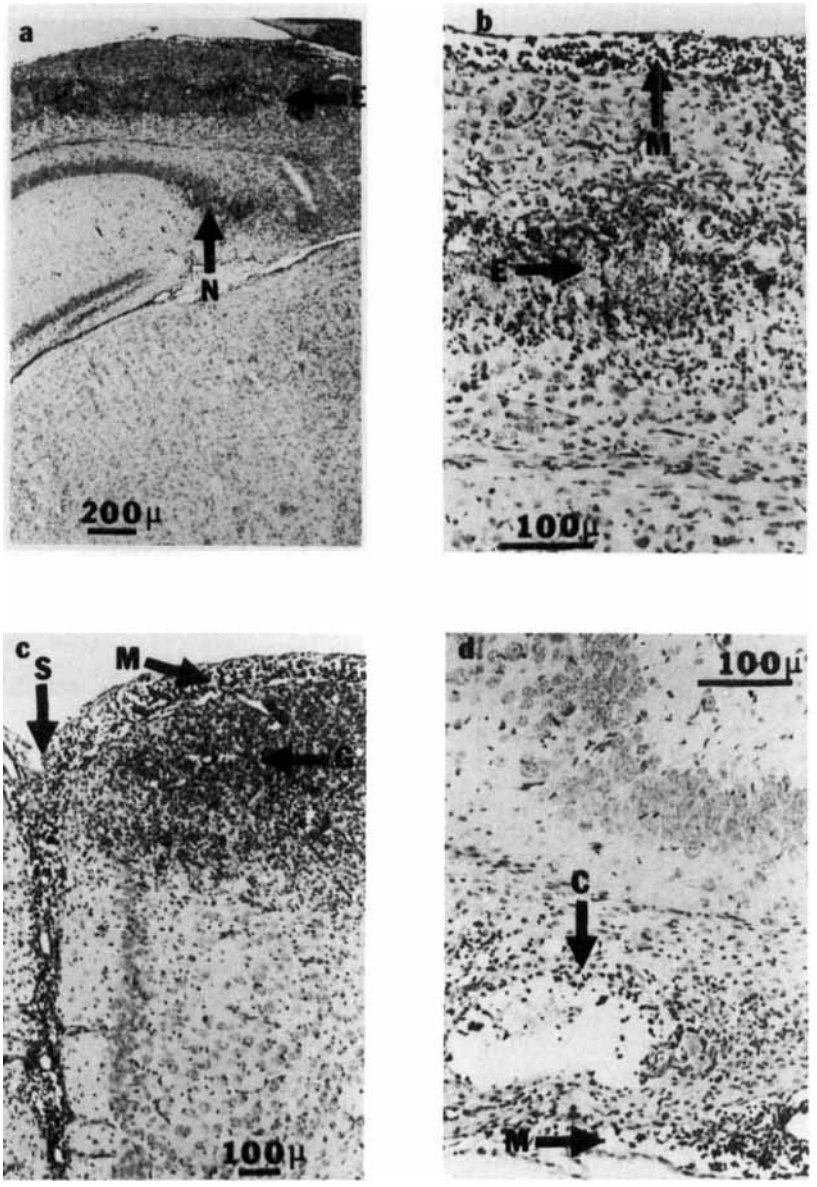

Fig. 2. Brain tissues from ts6-inoculated CD-1 neonates at 15 days p.i. a. Coronal section of dorsal hippocampus at medial portion of CA1. Laminar lesion of necrotizing encephalitis (E) in the cortex overlying the hippocampus. Destruction of neurons (N) in the hippocampus. b. Higher magnification of a section. Meningitis (M) and necrotizing encephalitis (E). c. Coronal section of superior layers of the cerebral cortex shows meningitis (M) and gliosis (G). Dorsal medial longitudinal sulcus (S). d. Coronal section of entorhinal cortex showing a cavity with cellular debris; this cavity resulted from necrotizing encephalitis with liquifaction and is likely the origin of a porencephalic cavity and/or hydranencephaly.

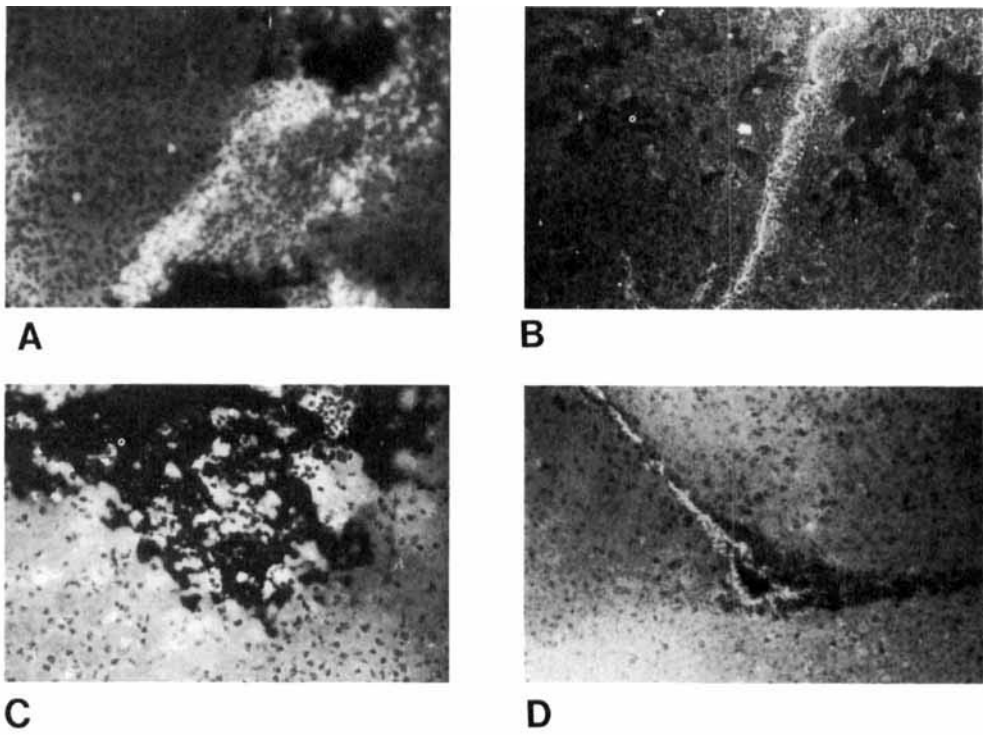

Fig. 3. Immunofluorescence assay for virus antigens on frozen sections of ts6-inoculated neonate or 7-day-old infant mouse brains. Thin sections were acetone-fixed and reacted with rabbit anti-CVB3 antiserum and then with FITC-conjugated goat antirabbit antiserum. Photographs a and b were taken of infant mice inoculated as neonates. a. Section from brain on day 6 p.i. showed clusters of positive cells near cerebral ventricles. 200×. b. Section of brain on day 6 p.i. showed antigen-positive cells lining the cerebral ventricles. c. Section of brain on day 9 p.i. showed greater destruction in the ventricular region with viral antigen-positive cells surrounding and within a virus-induced cavity. 200×. d. Section of infant brain at day 12 p.i., showed positive ependymal cells in the cerebral ventricles. 100×.

At this time, cells lining the ventricles were highly immunofluorescent-positive (Fig. 3b). Cells surrounding these areas of immunofluorescence were negative. By day 9 p.i., cell necrosis in large areas had resulted in cavitation. Immunofluorescent-positive cells emarginated the porencephalic cavities and were found within the cavities (Fig. 3c). A large number of small groups of immunofluorescent-positive cells were also scattered throughout the tissues and positive cells lined the ventricles. Sections examined at 12 days p.i. (data not shown) had larger cavities of tissue destruction emarginated by immunofluorescent-positive cells and areas devoid of any cells or nuclei, surrounded by immunofluorescent-negative cells. Also, at this time, the meninges in the cerebellum were immunofluorescent-positive as well as a few large cells between the molecular and granular layers. These positive cells may have been Purkinje cells. Sections taken at 12 days p.i. of 7-day-old infants with ts6 revealed only immunofluorescent-positive prominent ependymal cells within cerebral ventricles (Fig. 3d).

Temporal Appearance of Infectious Virus in Organs of Infant Mice Inoculated Intracranially With ts6

In data not presented, we found that titers of ts6 virus in brain tissues of ten mice inoculated as neonates and exhibiting hydranencephaly at 10 days p.i. varied from 5×10^5 to 1×10^8 pfu per gram of tissue. Furthermore, neonatal mice (four) inoculated with ts6 which did not show hydranencephaly gave much lower titers ($< 10^2$ to 1×10^3 pfu per gram) at 10 days p.i. No virus (<3 pfu/g) was found in brain tissues taken at 15, 21, or 62 days p.i. from hydranencephalic infants inoculated as neonates. Quantitative studies of the entire organs of mice similarly inoculated are shown in Table II. Infectious virus was recovered within 3–4 hr p.i. from all organs examined and titers increased over 100-fold in each of these organs by day 1. Except for the brain and blood where virus titers remained increased on days 2 and 3 p.i., virus titers decreased in other organs by day 2 or 3 p.i. Thus virus did spread to other organs and some replication occurred in the spleen, liver, and heart.

In a subsequent experiment, brain tissues from individual neonatal or 7 day-old mice inoculated with ts6 virus were examined for infectious virus up to 12 days p.i. (Table III). A neonatal mouse brain weighs 0.02–0.03 g at birth, thus we recovered approximately what was inoculated on day 0. By day 3 p.i., virus titers had increased in neonatal brains by an average of 2,700-fold above the levels on day 0, and virus recovery on day 6 p.i. was approximately 15,000-fold above the titer found on day 0 in 4 of five mice. Virus titers by day 9 p.i. decreased sixfold below those on day 6 p.i. and were at very low levels by day 12 p.i. Titers of ts6 recovered from 7-day-old infants were considerably lower than titers from neonatal mice at all times examined (Table III), although the temporal appearance of virus in brain tissues was similar.

Virus recovered at 6 and 9 days p.i. from brain tissues of two mice inoculated as neonates was as temperature-sensitive as the stock ts6 virus inoculated, ie, leakiness values [Trousdale et al, 1977] for the stock virus and virus in the brain samples were $1.1\text{–}5.6 \times 10^{-4}$.

Neutralizing Antibody to ts6 Virus and Interferon Levels in Brain Tissue Homogenates From ts6 Virus-Inoculated Infants

Neutralizing antibody (< 10 units/ml) was not detected in fluids taken from the brains or the sera of mice inoculated with ts6 as neonates which were hydranence-

TABLE II. Virus Titers in Organs of Neonatal CD-1 Mice at Times Postintracranial Inoculation With ts6 Virus*

Organ examined	Virus titers at days p.i. (pfu/g) tissue $\times 10^7 \pm$ S.E.M.)+			
	0†	1	2	3
Brain	1.1 ± 0.5[a]	130.3 ± 67.4[a]	52.5 ± 37.7	138.4 ± 79.4
Blood	0.004 ± 0.002[b]	0.1 ± 0.05[b,c]	0.04 ± 0.01	0.3 ± 0.2[c]
Liver	0.8 ± 0.4[d]	89.5 ± 73.1[d,e,f]	0.9 ± 0.4[e]	10.1 ± 9.2[f]
Spleen	0.2 ± 0.1[g]	23.5 ± 17.5[g]	46.3 ± 25.2[h]	12.8 ± 6.8[h]
Heart	0.02 ± 0.01[i]	9.5 ± 3.2[i,j]	3.1 ± 1.1	1.8 ± 0.5[j]

*Neonates were inoculated intracranially with 2.5×10^6 pfu of ts6 virus in 0.01 ml.

+Ten neonates were sacrificed on each day; 0-day samples were taken 3–4 hr p.i.; mean titers for ten neonates in a given organ at a particular time.

†Zero-day virus titers in blood are pfu/ml and samples on this day were taken between 3 and 4 hr p.i.

Student's t test, significant difference found for data paired by the same letter: a,c,f,g,i, $p < 0.01$; b,h,j, $p < 0.05$; d,e, $p < 0.001$.

TABLE III. In Vivo Replication of ts6 Virus in the Brains of CD-1 Mice Inoculated as Neonates or Infants*

Time of sacrifice (day p.i.)	Mouse number	Titer of virus (pfu/g brain tissue × 10^5)† Neonate brains Individual titers	Neonate brains Mean titer ± S.E.M.	Infant brains Individual titers	Infant brains Mean titer ± S.E.M.
	1	0.65		0.18	
	2	0.78		0.70	
0‡	3	0.44	0.54 ± 0.08	0.27	0.26 ± 0.17
	4	0.50		0.04	
	5	0.33		0.10	
	1	280		0.17	
	2	5200		0.006	
3	3	890	1468 ± 940	4.16	2.10 ± 1.07
	4	640		2.64	
	5	330		5.56	
	6	ND[a]		0.046	
	1	3400		0.003	
	2	5200		19.64	
6	3	11,000	7960 ± 1644	45.33	10.84 ± 8.33
	4	12,000		0.001	
	5	8200		0.018	
	6	ND		0.018	
	1	720		4.11	
	2	830		15.69	
9	3	750	1358 ± 538	<0.00002	⩽3.96 ± <3.07
	4	990		0.0002	
	5	3500		0.0001	
	1	3.9		<.00002	
	2	0.37		<.00002	
12	3	6.4	3.06 ± 1.10	<.00004	<.00003 ± <.000004
	4	0.93		<.00004	
	5	3.7		<.00002	

*Neonates (<24 hr of age) or infants (7 days of age) were inoculated intracerebrally via a 27-gauge needle with 2 × 10^6 pfu in 0.02 ml.

†Brains were removed aseptically, weighed, placed in a 10% suspension, frozen and thawed three times and virus titers were determined by a plaque method on Hela cells in duplicate at 34°C. Mean titers ± standard deviation (S.D.).

‡On day 0, mice were sacrificed within ten minutes of virus inoculation.

[a]ND, not done.

phalic at 10–105 days p.i. The capacity of ts6 to induce interferon was examined in mice inoculated with this virus as neonates or 7-day-old infants [authors' unpublished observations]. No interferon was detected (< 10 units/ml) in supernatant fluids from pooled neonatal brain tissue homogenates at 0 (within 3–4 hr p.i.), 1, 2, or 3 days p.i. Serum pooled from five neonatal animals was negative (< 10 units/ml) for interferon on days 0, 2, and 3, but on day 1 the serum contained 20 units/ml of interferon. Supernatant fluids from pooled 7-day-old infant brain tissue homogenates also revealed very little if any interferon; titers of 10, 10, < 10, 10, and < 10 units/ml were detected from pooled brain tissues at 0, 3, 6, 9, or 12 days p.i., respectively. These low interferon titers are not compatible with a role for interferon being a

factor, since replication of $CVB3_m$ is not effected at these concentrations [Gauntt et al, 1979]. Replication of ts6 virus in HeLa cells was similarly as insensitive to interferon as $CVB3_m$: at least 100 units/ml were required to effect a 50% reduction in yield [authors' unpublished observations].

Replication of ts1 and ts6 Variants in Murine Infant Brain Tissues

Although ts1 and ts6 mutants belong to the same complementation group [Trousdale et al, 1977,1979], obvious differences in their pathogenicity were found prompting the following experiments. Measurements of virus yields in primary cultures of neonatal mouse brain cells (Table IV) showed that ts6 virus replicated slightly better than ts1, especially at 37°C. As expected, replication of ts1 or ts6 did not occur at 39.5°C. Replication of all virus variants, including the parent $CVB3_m$ virus, in brain cell cultures occurred in absence of any cytopathology. The capacity of cells cultured from brain tissues of neonatal versus 7-day-old infant mice for replicating these three virus variants at 34 °C is shown in Table V. Cells cultured from neonatal brains replicated the three variants to higher titers than did cells from 7-day-old infant brains. Again, cytopathology was not evident in either cell culture producing virus.

DISCUSSION

Intracranial inoculation of infant mice with either of two ts mutants or parent $CVB3_m$ virus induced hydranencephaly or porencephaly, depending upon age of the infant at the time of inoculation and genotype of the virus variant. The parent $CVB3_m$

TABLE IV. Replication of ts1, ts6, and $CVB3_m$ in Murine Neonatal Brain Cells

	Virus Titers* (pfu/ml × 10^6)		
Virus	34°C	37°C	39.5°C
ts1	2.0	0.0024	0.003
ts6	8.7	1.2	0.046
$CVB3_m$	7.5	25.0	12.0

*Cell cultures harvested at 1 hr p.i. gave titers ranging from 1.0–7.8 × 10^4 pfu/ml.

TABLE V. Replication of ts1, ts6, and $CVB3_m$ Viruses in Cultures of Brain Cells From Neonatal or Infant CD-1 Mice

Virus variant	Time of harvest (hr p.i.)	Virus yields[a] (pfu/ml × 10^3)	
		Neonatal cells	Infant cells
ts1	1	0.83	0.22
	18	7000	410
ts6	1	0.36	0.27
	18	8300	85.5
$CVB3_m$	1	0.15	0.80
	18	5200	106

[a]At the time of virus challenge, there were 2.15 and 1.28 × 10^5 cells per well for neonate and infant cell cultures, respectively. Cells were incubated at 34°C for 18 hr.

was uniformly fatal to neonates [present data; Trousdale et al, 1979], however, 7-day-old infants inoculated with this virus did develop hydranencephaly. Inoculation of either ts mutant resulted in fatalities in approximately two-thirds of neonates but less than 10% of 7-day-old infants. A high proportion (70%) of neonates inoculated with ts6 virus gave evidence of hydranencephaly; however, only 12% of 7-day-old infants presented with porencephaly. The ts1 variant induced both forebrain anomalies in equal proportions (approximately 33%) in neonates, whereas 7-day-old infants developed only porencephaly and infrequently (~5%). An increased resistance to viral infections which paralleled increasing age from birth has been reported for several picornavirus-animal model systems. A decreased infection of the gastrointestinal tract following peroral inoculation with coxsackievirus B4 resulted when mice of increasing age were used [Loria et al, 1976]. Coxsackievirus B4 induced an intense myopericarditis with associated valvular changes in suckling mice, a less severe disease in weanling mice, and no disease in adult mice [El-Khatib et al, 1978]. Newborn mice inoculated with echovirus type 9 developed a flaccid paralysis and died, whereas similar inoculation into mice at 2 to 6 days of age resulted in transient paresis at most and survival [Bültmann et al, 1983]. In the reoviridae, studies with a bluetongue virus vaccine strain in sheep [Osburn et al, 1971a,b] and mice [Richard and Cordy, 1967] also showed an age-dependence on severity of forebrain anomaly induced, ie, hydranencephaly was induced in younger fetuses and porencephaly in older fetuses.

Age-dependent resistance to virus-directed tissue destruction which resulted in these forebrain malformations could not be attributed to increased neutralizing antibody titers nor interferon production. Specific immunofluorescent studies of brain tissues from ts6 virus-inoculated neonates or 7-day-old infants suggested that fewer cells were available in the cerebrum of the older infant mice for replication of the virus. Cultures of brain cells from neonates and 7-day-old infants showed a similar age-dependent resistance as did brain tissues from infants of the two ages: Titers of ts6 virus were approximately 100-fold higher from neonatal brain cells than from 7-day-old infant brain cells.

Temperature-restriction of replication of ts6 and ts1 likely played a role in the outcome, as $CVB3_m$ induced hydranencephaly in 7-day-old mice, whereas this severe form of forebrain anomaly was not induced in infants of this age by either ts mutant. In vitro experiments with cultures of neonatal murine brain cells confirmed the temperature restriction of replication of both ts mutants. The severity of forebrain anomaly induced, ie, hydranencephaly versus porencephaly might in part be attributable to an increased capacity of ts6 over ts1 for replication at 37°C, as shown by the in vitro experiments with brain cells cultured in vitro at 34, 37, and 39.5°C. The inability of neonatal mice to maintain a body temperature of 37°C and exhibiting temperatures down to 35°C [Green, 1966; Teisner and Haahr, 1974] could favor replication of ts6 over ts1.

The ability of ts mutants of several different viruses to produce serious infections is well documented. A ts mutant of reovirus induced runting and a brain degeneration in newborn rats which resembled communicating hydrocephalus [Fields, 1972; Raine and Fields, 1974]. Specific ts mutants of measles virus induced encephalitis or hydrocephalus following inoculation of newborn Syrian golden hamsters [Haspel et al, 1975; Breschkin et al, 1976]. Several ts mutants of herpes simplex virus type 2 were found to be lethal for newborn mice [Koment and Rapp, 1975]. A ts mutant of

foot-and-mouth disease virus was found to be lethal in infant mice [Richmond, 1975]. ts mutants of vesicular stomatitis virus which belong to two different complementation groups induced a spongiform myelopathy following inoculation into adolescent (3-week-old) mice [DalCanto et al, 1976]. Factors which may contribute to virulence in ts mutants of several viruses, including some of the above systems, have been reviewed [Richman and Murphy, 1979].

In the present study, induction of hydranencephaly/porencephaly by ts variants of coxsackievirus B3 utilized the intracranial route of virus inoculation. In preliminary experiments [authors' unpublished observations], peroral feeding of neonatal mice with 10^6 pfu of ts6 in 0.01 ml resulted in 100% survival and induction of porencephaly in 2 of 43 infants and none in 46 control neonates similarly inoculated with only MEM. In other preliminary experiments, inoculation of 10^8 pfu i.p. into gravid females in the second trimester resulted in induction of porencephaly in five offspring of the 110 survivors from the total of 120 delivered by the 12 mothers. Thus natural routes of infection would appear to result in one of the forebrain anomalies and additional studies are in progress to establish the frequency of induction of this forebrain anomaly.

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