EVALUATION IN ANIMAL MODELS OF THE NEUROVIRULENCE OF ISOLATES FROM PATIENTS WITH EPIDEMIC NEUROPATHY

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SUMMARY

A virus previously identified as a Coxsackie A9 by plaque neutralization assay and partial nucleotide sequence (designated C-47) was isolated from the cerebrospinal fluid of patients with epidemic neuropathy. After intracerebral challenge of newborn Balb/c mice with 10³.⁵ TCID₅₀, no signs of neurological disease appeared in the animals, but large amounts of antigen were detected in situ in several tissues and organs by immunohistochemical staining. Reference Coxsackie A9 virus (Griggs strain, from ATCC) inoculated in mice showed a typical picture of paralysis. Rabbit antiserum developed against C-47 or A9 cross-detected viral antigens in situ. In a second set of experiments, rabbits were infected with the C-47 isolate by injection of the virus in the vitreous humor or in the retrobulbar cavity of the eye. Viremia was detected in the infected animals; antigen in tissue sections was revealed by immunohistochemical staining using human immune serum. The virus was detected in the cerebrospinal fluid of rabbits inoculated in the vitreous humor, but not when inoculated in the retrobulbar cavity. Two isolates and a reference A9 Griggs strain were intrathecally inoculated in the monkeys and none developed either meningitis, or illness. It was concluded that the C-47 isolate caused subclinical infection in mice and rabbits experimentally infected, but did not cause encephalitis like the reference Cox A9 virus.

INTRODUCTION

An outbreak of epidemic neuropathy (EN) was reported in Cuba from 1991 to 1993. It affected people in all provinces with variable degrees (about 50,000 patients overall). The disease showed two well-defined clinical pictures, one with a predominance of optical symptoms and the other with peripheral neuropathic symptoms. (Más et al., 1993, Llanos et al., 1993).

The etiology of EN has not yet been clearly defined. A multicausal etiology that could involve toxic and nutritional factors was suggested and commonly accepted but not yet proved. Nevertheless, the possibility of an infectious agent (namely a virus) probably acting in association with toxic and nutritional factors was considered from the beginning of the epidemic.

Virus was isolated from the cerebrospinal fluid (CSF) of ill patients. Reverse transcription-polymerase chain reaction (RT-PCR) using primers designed for enterovirus genomic amplification followed by hybridization with
an internal probe (Rotbart, 1990) confirmed the presence of the virus in the CSF of clinically diagnosed individuals (Roca et al., 1994). In electron microscope studies, viral particles of 18-30 nm diameter resembling picornavirus particles were detected.

The virus was isolated in cultures of VERO cells inoculated with CSF from patients after several passages in vitro. Several isolates showed a typical cytopathic effect (CPE) of enteroviruses, and two, designated as 47/93-IPK (C-47) and 35/93-IPK (C-35) were further characterized at the molecular level, (Roca et al., 1994). Other isolates showed a slow progressing phenotype (IG-26, IG-28, IG-33). To investigate if the isolated virus was a Coxsackie virus, the region coding for the G-H loop of the VP1 protein was sequenced after RT-PCR. This sequence displayed 67% to 71% of homology to the reported sequences from the coxsacki B group and had 85% homology with the type A9, thus indicating that the C-47 strain was closely related to Coxsackie A9 virus.

Since the clinical symptoms of the epidemic differed from those typically observed during Coxsackie A9 infections (Melnick, 1990), a very important point was the assessment of the neurovirulence in vivo of the C-47 isolate in newborn mice, as well as the search for animal models. In this paper we describe our results in this regard.

MATERIALS AND METHODS

Mice

Groups of newborn, specific pathogen free BALB/c mice (Cenpalab, Havana, Cuba), 24-48 h old were inoculated intracerebrally with 10.5 TCID50 of the C-47 strain in a 30 μL volume of DMEM without serum. C-47 was previously titrated by the plaque assay (Cooper, 1967). As controls we inoculated mice under the same conditions and with the same dose of a reference Griggs strain of Coxsackie A9 virus (obtained from ATCC). In each experiment, mice were included that received supernatant from cultures of non-infected cells, and mice that received no inoculum at all.

Mice were kept in standard isolators (La Calhene, France) under controlled environmental conditions. Animals were observed twice a day up to day 21 post-inoculation. Record keeping included: number of animals/litter, litter size, age, health status of the animals, and any evidence of clinical signs such as ruffling of the hair, growth delay, weakness-paralysis of hind limbs, and lack of coordination during movements. At least 5 mice were necropsied from litters inoculated with the C-47 isolate or from the A9 reference strain group, and 3 animals of each control group.

At necropsy, external and internal inspection were made under magnification, and samples for histopathologic study were taken from the central nervous system (CNS), heart, thymus, liver, kidneys, small and large intestine, genitalia, and skeletal muscles. Samples were fixed in 10% buffered formalin, paraffin-embedded, and 5 μm sections were made and stained with hematoxylin eosin.

Specimens were blindly evaluated by two separate experienced pathologists.

Immunohistochemical staining

For immunohistochemistry, the primary antibody was an immune polyclonal serum raised in rabbits against the C-47 isolate; the antiserum specificity was previously tested by Western-blot (not shown). The reaction was revealed with a protein A-colloidal gold conjugate with silver enhancement as described (Berlanga, et al., 1993).

In these series of experiments, reactivity of C-47 anti-serum was assessed on specimens of animals inoculated with the C-47 isolate, and Coxsackie A9 reference strain. Reaction negative controls were obtained by incubating the rabbit anti-serum with tissue samples from animals inoculated with supernatant of culture cells, and by replacing the C-47 anti-serum with a rabbit pre-immune serum.

Rabbits

Adult albino F1 (New Zealand White x Spanish Semigiant) rabbits were inoculated with 5x10^5 TCID50 of the C-47 isolate. The virus was given diluted in PBS, by injection either in the vitreous humor (n = 4) or in the retrobulbar cavity of the eye (n = 4). As controls, 2 rabbits per route of administration were inoculated with PBS alone in the same way as experimental animals. At definite time points (24, 72 and 120 h post administration of the virus), samples of cerebrospinal fluid, optic nerve and blood were taken from the inoculated rabbits. Viremia, and RT-PCR of CSF and optic nerve were assayed by conventional methods (Roca et al., 1994). After euthanasia, samples from different organs and tissues were taken for immunohistochemical assays, using immune serum from a patient, and revealed as described for mice in the previous section.

Monkeys

African green monkeys (Cercopithicus aethiops) were intracerebrally infected with C-47 isolate (10.5 TCID50), IG-26 (an isolate from the Center for Genetic Engineering and Biotechnology, Havana, Cuba, 10.5 TCID50), A9 reference Griggs strain from ATCC (10.5 TCID50), or supernatant of VERO cells cultured in DMEM medium. Prior to inoculation, samples of CSF and blood were taken from each monkey. All animals received the inocula in 1 mL of DMEM medium without serum. At days 7, 15 and 30, samples of blood and CSF were taken to study the viral persistence in these body fluids. Rectal temperature was scored at 12 h intervals during the first week after viral inoculation, and the general health status and behavior carefully observed during the whole period of study.

RESULTS

Mice

Although previous molecular studies had shown that the C-47 isolate was homologous to Coxsackie A9 virus (Roca et al., 1994) and, despite the demonstration of the typical enterovirus CPE on Vero cells inoculated with C-47, our results suggested consistent differences between C-47 and the reference A9 strain, in relation to the virulence effect observed on newborn mice (Table 1).

Newborn mice that died within the first 48 h after virus inoculation were scored as inoculation-related deaths and were excluded from the statistics. Out of 501 mice inoculated with the isolate C-47, no mice...
Table 1
Comparisson of the neurovirulence of the C-47 isolate and a reference A9 strain in mice

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>Number of mice inoculated</th>
<th>Day of the onset of clinical signs</th>
<th>Number of ill or died mice</th>
<th>Percent of illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-47</td>
<td>501</td>
<td>no onset after 21 days</td>
<td>2</td>
<td>0.4%</td>
</tr>
<tr>
<td>reference A9 (Griggs)</td>
<td>50</td>
<td>4/5 days</td>
<td>43</td>
<td>85.0%</td>
</tr>
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</table>

All mice were inoculated at day 1 of life by intracranial injection of $10^{15}$ TCID<sub>50</sub>
Animals were observed daily for clinical signs of neurological and general illness.

developed clinically identifiable signs during 21 days of observation. After this period, some animals were kept under study for an additional 2 weeks and all exhibited normal somatic growth and undamaged neurological behavior.

Groups of clinically healthy animals inoculated with the C-47 isolate were sacrificed for histopathologic study at weekly intervals. Mice exhibited no histopathologic lesions in any sampled tissue on days 7, 14 and 21 after viral challenge. Mice inoculated with supernatant of non-infected cells in culture or not inoculated were healthy by clinical observation, and showed no lesion at the anatomo-pathological level (not shown).

Animals inoculated with the A9 reference strain, showed nearly uniform clinically-well noticeable picture of neurological illness around the 4-5<sup>th</sup> day post-challenge. At this moment, affected animals showed a marked degree of asthenia, unilateral or bilateral hind limbs paralysis, postural accommodation incapability and impaired sensitivity response. Afflictions also encompassed ruffling of the hair and impaired feeding (runtng). By the seventh day post-inoculation mortality was 80% (Table 1).

We further assessed the persistence of viremia and corporeal distribution of the C-47 antigens by immunohistochemical staining. Immunohistochemical studies of samples from clinically healthy mice inoculated with the C-47 isolate, revealed an intense reactivity in several of the tissues studied such as skeletal muscle, heart, spinal cord (figure 1a-c), intestinal villi, and brain. No label was detected in tissues of non-inoculated mice (not shown), and the possibility of false positive results was ruled out by using a preimmune serum as the primary antibody (figure 1d).

This finding suggested that although there was no evidence of clinical illness induced by the viral challenge, the virus did multiply in the host organism as judged by the wide distribution of immunolabel of viral antigens identified by the rabbit anti-serum. Viremia was detected in samples of C-47 infected animals sacrificed on day 14, but not on day 21 (not shown).

Rabbits
Viral nucleic acid was detected in the CSF only from rabbits that were injected in the vitreous humor, but not from those injected in the retrobulbar cavity. Viral nucleic acid was detected by RT-PCR of CSF samples, at 72 and 120 h post-inoculation (no longer time points were assayed). Viremia was detected in all experimentally infected rabbits despite the route of inoculation, at least during 120 h post-inoculation.

In no case, we detected virus in the RT-PCR samples from the optic nerve of the infected rabbits, also virus was not present in any of the fluid samples from PBS-injected animals.

At the immunohistochemical level, antigen was demonstrated in several organs and tissues of infected rabbits from both experimental groups, these included lungs, trachea, striated and smooth muscle, and guts (figure 2).

Monkeys
None of the injected monkeys developed signs of meningitis, as revealed by the profiles of rectal temperature (not shown). Also no clinical illness was detected in the animals, with the exception of a mild diarrhea in the monkeys inoculated with the A9 reference strain of Coxsackie A9 viruses.

All the animals proved to be free of virus in their CSF, as judged by the absence of CPE in VERO cells. Viremia was detected in the CSF of 1 monkey inoculated with A9 strain, at day 7 (Table 2), but not after. All the remaining monkeys were negative for the correspondent viruses in their CSF at the studied time points.

DISCUSSION
The C-47 viral isolate of patient with Epidemic Neuropathy did not behave like a Coxsackie A9 in newborn mice

Neuropathy syndromes associated with severe malnutrition, alcoholism or as a consequence of infectious diseases have been described and published elsewhere (Román et al., 1985). Between 1955 and 1970, about 10000 cases of subacute
Fig. 1. Immunohistochemical assay for the presence of viral antigens in mice infected with the C-47 isolate. Embedded and fixed tissue samples were incubated with a polyclonal rabbit antiserum against the C-47 isolate, and antigen was detected with protein A-colloidal gold conjugate with silver enhancement. Intense and widespread immunolabel of gold precipitate was detected in skeletal muscle (a), and in transverse sections of heart (b), and the spinal cord (c). No label was detected when the rabbit pre-immune serum was used as the primary antibody as shown for the skeletal muscle (d).
myelo-optic neuropathy (SMON) were diagnosed in Japan. Clinical characteristics of the EN in Cuba largely resembled the characteristics of SMON, except for the absence of abdominal pain. A drug (cloquinol) was considered as a possible cause of the SMON epidemic (Meade, 1975), and a virus (virus of Inoue-Melnick) was isolated at the end of the epidemic (Inoue et al., 1971), but the role of this agent remains obscure.

The possibility of a virus involved in the etiology of EN in Cuba was suggested in early 1992 during the beginning of the epidemic outbreak and an entero virus was soon isolated from CSF of patients with EN (Mais et al., 1993). Using RT-PCR methodology, 35.3% of the analyzed CSF of patients with EN, was shown to be positive for enteroviruses sequences (Alvarez et al. unpublished).

A similar pattern of viral distribution in the immunohistochemical assays was found in the tissues of ill mice sacrificed after infection with Coxsackie A9 reference strain (not shown).

Differences in the clinical signs of disease observed in mice, inoculated with C-47 and A9 reference strain could reflect subtle differences at the molecular level between these virus isolates.

The picture for A9-infected mice is well in agreement with the typical lesions for the Coxsackie A9 type (Dalldorf and Melnick, 1965). However, it is well known that some strains of Coxsackie A9 viruses lack pathogenicity in mice (Wenner, 1962), resembling ECHO viruses. We can not rule out the possibility of C-47 isolate to be a non-pathogenic isolate for mice. On the other hand, the clinical symptoms of illness in humans during the course of EN, differed from those typically observed in Coxsackie A9 epidemics. Remarkable was the fact that the rate of ill children, was almost negligible (8.17 per 10^5, for children below 15 years, and 1676.18 x 10^5 for adults between 45 to 64 years-old) while Coxsackie A9 infections normally include a high percent of children affected.

There are two possible explanations for these differences of behavior of C-47 isolate both in mice and humans with respect to coxsackie A9. First, the C-47 isolate is not the only isolate present in the CSF of patients, but other virus(es), namely those showing the slow progressing CPE (Rodríguez et al., unpublished), could be present in minor quantities while causing most of the symptoms, or acting in synergism with the C-47 isolate, and second, the C-47 even though was classified as Cox A9 virus, is rather a Cox A9 like, and does not share the same neurovirulence properties with the common Cox A9 viruses. Experiments are in progress to clarify these issues, and new animal models in primates are being tested to ascertain the actual role of virological factors in the outbreak of epidemic neuropathy that affected Cuba during the last two years.

Infected rabbits showed marked viremia and viral nucleic acid in CSF, without developing clinical signs of illness

The presence of the virus in the CSF of rabbits inoculated in the vitreous humor could involve the eye as one of the entry sites for the C-47 isolate to gain access into the CSF of infected patients. It would be of interest to correlate the incidence of optic neuritis with recent or past conjunctivitis in ill patients with epidemic neuropathy.

In the case of the animals inoculated in the retrobulbar cavity, one can speculate that the injection was not fully accurate in terms of deposition of the virus inside the hemat-encephalic barrier that the cavity gives to the optic nerve, and therefore the virus was cleared to the blood stream, and did not gain access to the CSF of the rabbits.

Table 2

<table>
<thead>
<tr>
<th>Viral isolate</th>
<th>Route of inoculation (n)</th>
<th>Signs of illness</th>
<th>Virus found at indicated time points in:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>blood</td>
</tr>
<tr>
<td>C-47</td>
<td>i.t (n = 2)</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>IG-26</td>
<td>i.t (n = 2)</td>
<td>none</td>
<td>+</td>
</tr>
<tr>
<td>Cox A9 (Griggs)</td>
<td>i.t (n = 2)</td>
<td>mild diarrhea</td>
<td>+</td>
</tr>
</tbody>
</table>

i.t = intrathecal injection of 10^5 TCD50 of virus.
Fig. 2. Immunohistochemical assay for the presence of viral antigens in rabbit infected with the C-47, 10-26, or reference Cox-A19 strain Griggs. Animals were inoculated either in the retrobulbar or in the vitreous humor of the rabbit eye. Tissue samples were inoculated with an immune serum from a patient with EN and revealed with protein A-colloidal gold conjugate with silver enhancement. Intense and dispersed gold precipitate was detected in muscle (A), and in trachea (C) of virus infected animals, but not in the same tissues of the control medium challenged rabbits (B).
The absence of virus in the optic nerve of both groups of rabbits is unexplained; it is possible that the C-47 isolate is unable to penetrate and multiply inside the nervous cells of the optic system.

The viral inoculae used in monkeys did not cause meningitis in the animals

In the experiment with monkeys, we focused on two objectives: 1) to evaluate if the monkeys could be challenged with high titers of viral inoculae, without signs of meningitis, and 2) to study if the viral inoculation had any direct effect on the neurological behavior of the animals.

The first part of the experiment was designed as a basic step in anticipation of further studies of the ethiology of the outbreak of EN, using monkeys as models. At present we have several monkeys with epidural electrodes implanted surgically, which can be coupled to recorder computers for the measurement of evoked visual and somatosensory potentials.

It was very important therefore, to evaluate if a viral inoculation with different isolates from patients, caused meningitis in the animals, and could interfere with the measurements of the evoked potentials.

The results obtained showed that monkeys do not develop either meningitis or clinical illness after direct intrathecal inoculation of 2 viral isolates from CSF of patients with NE, and that the viruses were quickly removed from the CSF of the animals.

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