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

Fidel Ovidio Castro[1], Jorge Berlanga[1], Carlos Alfonso Sabatier[2], Pilar Rodríguez[1], Maydel Hechevarría[1], Orlando Hayes[1], Raúl Pérez[3], Dagmara Pichardo[1], Manuel Pérez[1], Ileana Dorta[1], Elisa López[2], María C. López[2], Luis Herrera[1], José de la Fuente[1]

[1]*Mammalian Cell Genetics Division, Centro de Ingeniería Genética y Biotecnología, PO. Box 6162, Havana, Cuba.* [2]*Department of Pathology, Centro de Investigaciones Médico-Quirúrgicas, PO. Box 6096, Havana, Cuba.* [3]*Department of Experimental Ophtalmology, Hospital Ramón Pando Ferrer, Havana, Cuba.*

Key words: enteroviruses, Coxsackie viruses, epidemic neuropathy, neuritis, animal models

SUMMARY

A virus previously identified as a Coxsackie A9 by plaque neutralization assay and partial nucleotidic sequence (designated C-47) was isolated from the cerebra spinal fluid of patients with epidemic neuropathy. After intracerebral challenge of newborn Balb/c mice with $10^{3.5}$ TCID$_{50}$, 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 antisera 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 cerebra spinal 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.

RESUMEN

Un virus previamente identificado como Coxsackie A9 mediante ensayo de neutralización en placa y secuenciación parcial de su genoma (nombrado C-47) fue aislado a partir del líquido cefaloraquídeo (LCR) de pacientes aquejados de neuropatía epidémica. La inoculación intracerebral de ratones Balb/c neonatos, con $10^{3.5}$ DICT$_{50}$, no provocó signos de enfermedades neurológicas en los animales, sin embargo, se logró detectar gran cantidad de antígeno viral *in situ* en diferentes tejidos y órganos mediante ensayo inmunohistoquímico. La cepa de referencia para virus Coxsackie A9 (Griggs, ATCC) produjo un típico daño neurológico, incluída parálisis en los ratones inoculados. Antisueros policlonales en conejos contra C-47 o A9 detectaron cruzadamente antígenos virales *in situ*. En otro grupo de ensayos, se infectaron experimentalmente conejos con el aislamiento C-47, mediante la inyección de virus en el humor vítreo o en la cavidad retrobulbar del ojo de los conejos. Se detectó viremia en los animales infectados y antígeno viral en cortes de tejido mediante ensayo inmunohistoquímico, empleando suero de pacientes. El virus fue hallado en el LCR de los conejos inoculados en el humor vítreo, pero no en aquellos inoculados en la cavidad retrobulbar. Dos aislamientos y la cepa de referencia Griggs fueron inyectados en el LCR de monos verdes africanos, ninguno de los animales desarrolló cuadro meníngeo o signos clínicos de enfermedad neurológica, el virus A9 pudo ser aislado en uno de los monos, 7 días después de la inoculación. Se concluye que el aislamiento C-47 causa infección subclínica en ratones y conejos infectados experimentalmente, pero no provoca encefalitis como el virus Coxsackie A9 empleado como referencia.

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 ethiology of EN has not yet been clearly defined. A multicausal ethiology 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 cerebra spinal 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 coxsackie 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 $10^{3.5}$ TCID$_{50}$ 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, illness onset, runting, and 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 groups, 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, genitals 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 5×10^5 TCID$_{50}$ 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 cerebra-spinal 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 (*Cercopithecus aethiops*) were intrathecally infected with: C-47 isolate ($10^{3.5}$ TCID$_{50}$), IG-26 (an isolate from the Center for Genetic Engineering and Biotechnology, Havana, Cuba, $10^{3.5}$ TCID$_{50}$), A9 reference Griggs strain from ATCC ($10^{3.5}$ TCID$_{50}$), 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 those 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

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

All mice were inoculated at day 1 of life by intracraneal injection of $10^{3.5}$ $TCID_{50}$
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 4-5th 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 (runting). 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
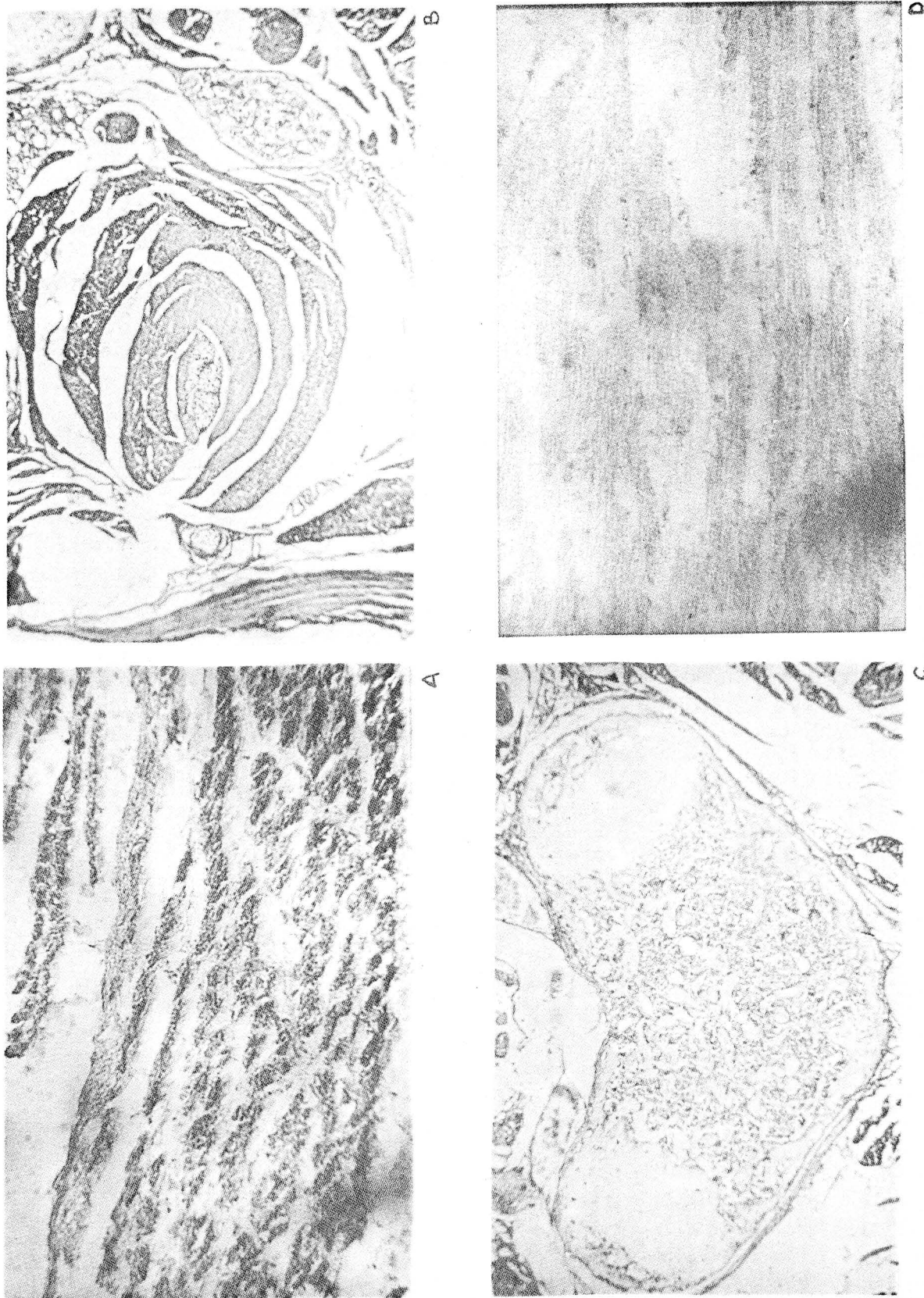
Fig. 1. Immunohistochemical assay for the presence of viral antigens in mice infected with the C-47 isolate. Embedded and fixed tissue samples were incubated with a polyclonal rabbit antiserum against the C-47 isolate, and antigen was detected with protein A-colloidal gold conjugate with silver enhancement. 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).

Table 2
Experimental infection of African green monkeys with 2 viral isolates from patients with EN, and with a Coxsackie A9 reference

Viral isolate	Route of inoculation (n)	Signs of illness	Virus found at indicated time points in:			
			blood		CSF	
			72 h	28 days	7 days	28 days
C-47	i.t (n= 2)	none	+	-	-	-
IG-26	i.t (n= 2)	none	+	-	-	-
Cox A9 (Griggs)	i.t (n= 2)	mild diarrhea	+	-	+	-

i.t = intrathecal injection of 10^5 TCD$_{50}$ of virus.

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 (clioquinol) 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 ethiology of EN in Cuba was suggested in early 1992 during the beginning of the epidemic outbreak and an enterovirus was soon isolated from CSF of patients with EN (Más *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 hemato-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.
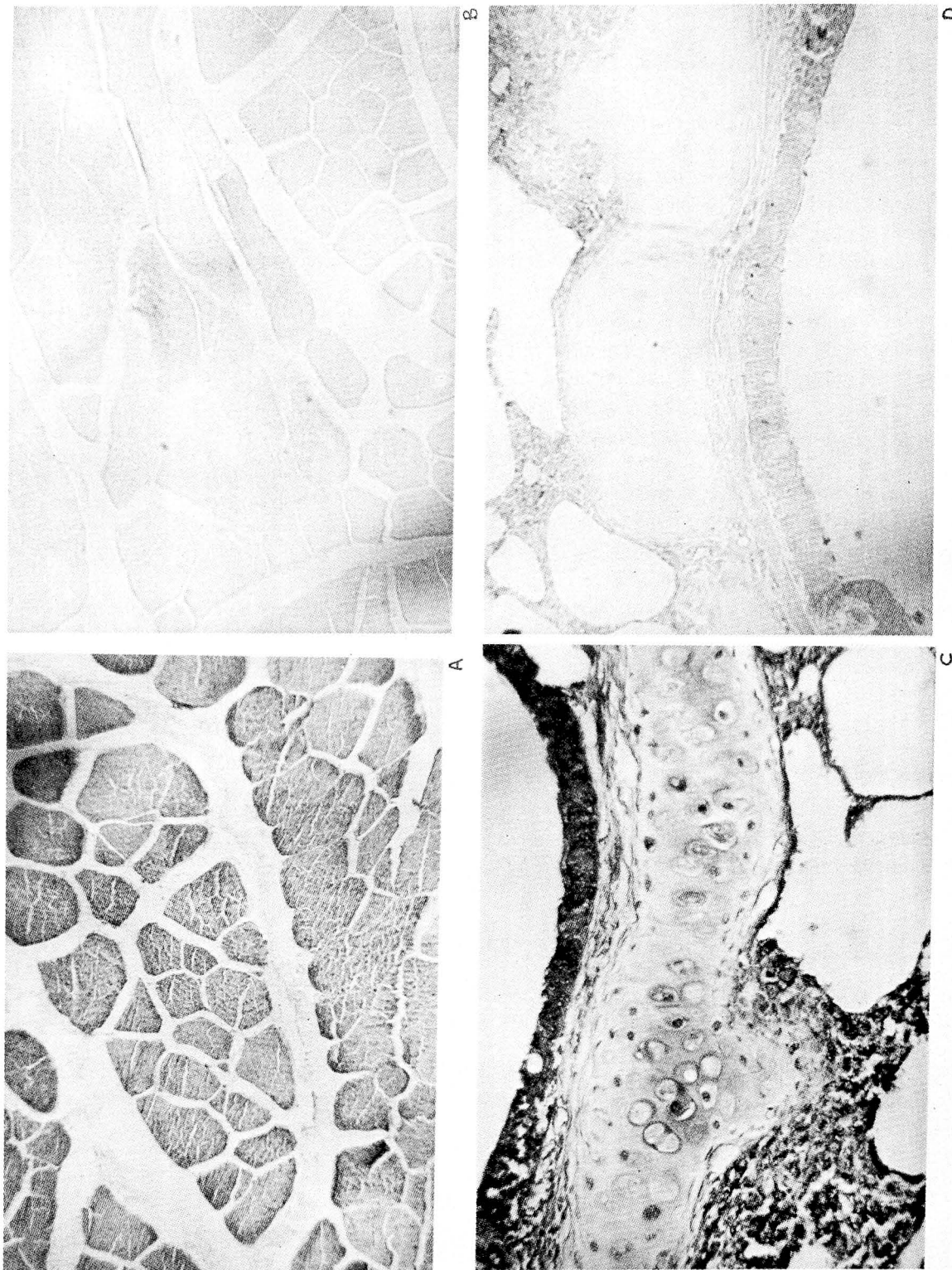
Fig. 2. Immunohistochemical assay for the presence of viral antigens in rabbits infected with the C-47, IG-26 or reference Cox A9 strain Griggs. Animals were inoculated either in the retrobulbar or in the vitreous humor of the eye. Embedded and fixed tissue samples were incubated with an immune serum from a patient with EN and revealed with protein A-colloidal gold conjugate with silver enhancement. Intense and widespread immunolabel of gold precipitate was detected in muscle (a), and in tracchea (c) of virus infected animals, but not in the same tissues of the control medium challenged rabbits (b,d).

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 somatosensorial 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.

ACKNOWLEDMENTS

REFERENCES

BERLANGA, J.; J. INFANTE; V. CAPÓ; J. DE LA FUENTE and F.O. CASTRO (1993). Characterization of transgenic mice lineages. I. Overexpression of hGH causes the formation of liver intranuclear pseudoinclusion bodies and renal and hepatic injury. *Acta Biotechnologica*, 13:361-371.

COOPER, P.D. (1967). The plate assay of animal viruses. In. *Methods in Virology*. Edited by K. Maramorosch and H. Koprowski. New York: Academic Press. 1:243-311.

DALLDORF, G. and J.L MELNICK (1965). Coxsackie viruses. In: *Viral and Rickettsial Infections of M.* Edited by F.L.Horsfall Jr and I. Tamm. Philadelphia: Lippincot. pp 117-159.

INOUE, Y.K.; Y. NISHIDE; Y. NAKAMURA (1971). Virus associated with SMON in Japan. *Lancet*, i:853-854.

LLANOS, G.C; D. ASCHER; D.C. BROWN (1993). Epidemic neuropathy in Cuba. *Epidemiological Bulletin PAHO* 14:1-4.

MAS, P., M.P. RODRÍGUEZ; G. GUZMAN; M. ALVAREZ; V. MUZIO; O. ANCHETA; A. GOYENECHEA; H. ROCA; L. MORIER; R. ALVAREZ; M. MARRERO; A. MUSACCHIO; A. CASTILLO; V. FALCÓN; J.L. PELEGRINO; L. LUACES; A. BALMASEDA; M. BARRO; L. RODRIGUEZ; D. GARCÍA DEL BARCO; M. SOLER, B. GARCÍA; M.A. RIVAS; R. LLEONART; S. RESIK; G. PADRÓN; L. JOMARRON; M. CANDELARIO; M. MUNE; A. MARTÍN; D. ROSARIO; M.G. MORALES; J. LAFERTE; J. REYES; C. SARIOL; R. RICARDO; J.L. MAESTRE; A. RODRÍGUEZ; N. BARANOSKY; M. GONZÁLEZ GRIEGO; P. LÓPEZ; E. PENTÓN; M. LIMONTA; J. DE LA FUENTE; L. HERRERA; A. LLOPy G. KOURÍ (1993). Resultados preliminares de laboratorios virológicos en estudios de casos de Neuropatía Epidémica Cubana. IPK *Boletín Epidemiológico* 1:7-8.

MEADE, T.W. (1975). Subacute myelo-optic neuropathy and clioquinol. An epidemiological case-history for diagnosis. *British Journal of Preventive and Social Medicine*, 29:157-169.

MELNICK, J.L. (1990). Enteroviruses: Polioviruses, Coxsackieviruses and newer Enteroviruses. In: *Virology* Edited by B.N. Fields and D.M. Knipe. New York: Raven Press Ltd. 1:594-606.

ROCA, H.; H. MARRERO; E. RIEGO; V. FALCÓN; P. MAS; P. RODRÍGUEZ; R. ALVAREZ; V. MUZIO; B. GARCÍA; R. LLEONART; G. KOURÍ; L. HERRERA and J. DE LA FUENTE (1994). Molecular characterization of a virus associated with the outbreak of polyneuropathy epidemic in Cuba. *Miami Biotechnology Winter Simposia. Miami Short Reports* 4:89.

ROMAN, G.C., C. SPENCER; B.S. SCHOENBERG (1985). Tropical myeloneuropathies: The hidden epidemies. *Neurology*, 35:1158-1172.

ROTBART, H.A. (1990). Enzimatic RNA amplification of Enteroviruses. *Journal of Clinical Microbiology* 28:438-442.

WENNER, H.A. (1962). The ECHO viruses. *Annals of the New York Academy of Sciences* 101:398-412.