During the neuropathy epidemic in Cuba, two types of antigenically-related cytopathic agents were isolated from the cerebrospinal fluid (CSF) of patients diagnosed with neuropathy. Different strains were studied. A group of these showed a cytopathogenic effect (CPE) typical of the enterovirus and was identified as coxsackievirus A9. The other group produced a slowly progressive CPE, this pattern was referred to as light CPE (CPE-L). Previous results obtained during the characterization of these agents had suggested the presence of more than one agent in the same sample since an agent with a CPE typical of the enterovirus was obtained from a CPE-L strain. Two standard methods for virus purification, gradient centrifugation and viral cloning by plaque assay, were performed. Some physicochemical characteristics of the purified agents are described in this paper. Sensitivity to chloroform, PAA, GHCL and BRDU was assayed. Viral size and morphology were studied by electron microscopy and our results point at the presence of two variants of the same agent. No virus particle was observed in the case of CPE-L strains. These observations related to CPE-L strains are in agreement with other molecular studies that show structural virus alterations.

Keywords: electron microscopy, epidemic neuropathy, virus

Introduction

An epidemic of more than 50,000 cases of optic and/or peripheral neuropathy occurred in Cuba from late 1991 through 1993; 52,406 cases were reported by the end of 1995. The disease was associated with dietary limitations. In particular, an impairment of the protective antioxidant pathways was suggested. Smoking was a related risk factor, which intensified the effects of the nutritional deficiencies and was thought to cause injury due to oxidative damage. In addition to the clinical and epidemiological studies, virological research was carried out to exclude the possibility of an infectious agent. However, 5 isolates of Coxsackievirus A9 (CA9) and 100 isolates of the light cytopathic effect (CPE-L) agent were recovered from 105 cerebrospinal fluid (CSF) specimens tapped from 125 epidemic neuropathy patients [1]. The viral isolation of antigenically-related agents by means of independent systems that are confirmed by immunohistochemical techniques and reisolation of the “light” CPE virus in cell cultures from mice, strongly evidence that a virus was present in the CSF of patients. Also, its in vivo persistence for more than 1 month was shown by its reisolation in the CSF of 24 patients. This result further suggests that the virus may be important in the pathophysiology of this disease [2].

The CPE-L producing agents were not identified by the Lim Benyesh-Melnick (LBM) pools of antisera [3] but the specific antiserum developed in rabbits against these agents neutralized the CA9 strain in vitro. During the isolation process the typical Enterovirus CPE was identified in samples that had been showing CPE-L for several days. The results of the physicochemical characterization of the CPE-L agents were not conclusive enough to classify the strains. Enterovirus in general show no sensitivity to chloroform and their replication is inhibited by guanidinium chloride

RESUMEN

Purificación y caracterización de los agentes virales aislados durante la neuropatía epidémica en Cuba. Durante la epidemia de neuropatía ocurrida en Cuba, a partir del líquido cefalorraquídeo (LCR) de pacientes, fueron aislados dos tipos de agentes citotóxicos relacionados antigénicamente. Diferentes cepas fueron estudiadas. Un grupo de estas mostró efecto citotóxico (ECP) típico de enterovirus y se identificó como coxsackievirus A9. El otro grupo produjo un ECP de progresión lenta, al que se denominó ligero (ECP-L). Resultados previos, obtenidos en la caracterización de estos agentes han sugerido la presencia de más de un agente en la misma muestra, ya que un agente de ECP típico de enterovirus fue obtenido a partir de una cepa de ECP-L. En este trabajo se desarrollaron dos métodos convencionales de purificación viral: gradiente de centrifugación y clonaje por placas. Se describen algunas características físico-químicas de los agentes purificados. Se realizaron ensayos de sensibilidad al cloroformo, ácido fosfórico, cloro de guanidina y bromodeoxiribina. Por microscopía electrónica se estudiaron talla y morfología viral. Nuestros resultados sugieren la presencia de dos variantes del mismo agente. En las muestras correspondientes a cepas de ECP-L, no se encontraron partículas virales. Estas observaciones relacionadas con las cepas de ECP-L concuerdan con otros estudios moleculares realizados, que muestran alteraciones estructurales en el virus.

Palabras claves: microscopía electrónica, neuropatía epidémica, purificación, virus
immunoelectron microscopy
Fifty milliliters from each band were mixed with 50 mL of the corresponding hyperimmune serum at a 1:20 dilution, and incubated overnight at 4 °C. The virus-antibody suspension was centrifuged for 30 min (100 000 x g). The pellet was resuspended in distilled water and stained with 2% uranyl acetate [6]. The grids were examined in a JEOL JEM 100S electron microscope (Jeol, Canada) at 80 kV.

Electron microscopy
The infected Vero cell monolayers were washed with PBS and immediately fixed using 2.5% glutaraldehyde at 4 °C, post fixed with osmium tetroxide, dehydrated and embedded in spurr resin (Polyscience, Inc., USA). Thin sections were cut on an LKB Ultratome, Nova (LKB, Sweden), placed on uncoated grids, stained with uranyl acetate and lead citrate and examined with a JEOL JEM 100S electron microscope (Jeol, Canada) at 80 kV [7].

Results
Figure 1 shows two standard methods of viral purification that were used to separate the different particle populations that may be present in the same sample.

For the characterization of the purified strains we studied the sensitivity to chloroform, BDUR, PAA and GHCl, as well as the morphology, size and time of appearance of the plaques during cloning. The morphology and size of the particles were determined through electron microscopy (Tables 1 and 2).

Immunoelectron microscopy
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Purification and characterization of viral antigens

Vero cells (ATCC) were grown at 37 °C in a Parker medium 199 plus 10 % fetal bovine serum (FBS) and 1.2 g/L sodium bicarbonate.

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Virus
Isolates from the CSF of epidemic neuropathy patients were comprehensively studied. One typical strain of CA9 (CPE-E): 47/93 IPK and M26 IG. The later was isolated at the Center for Genetic Engineering and Biotechnology in Havana.

Hyperimmune sera
The hyperimmune sera were produced in New Zealand rabbits immunized with viral antigens obtained from the inoculated Vero cells supernatant.

Purification of viruses by gradient centrifugation
Vero cell cultures were seeded into plastic rollers and immediately infected at a multiplicity of infection of 1. The cultures were incubated at 37 °C until cells were harvested. The virus was aggregated by adding 7% (PEG 6000 and centrifuged at 10 000 rpm. Precipitation was enhanced by adding 0.5 M/L NaCl. The concentrated virus was centrifuged through a sucrose gradient (15-60%) in an SW40 rotor at 28 000 rpm for 2 h at 4 °C. The band formed was recovered for the electron microscopy study.

Viral cloning by plaque assay
Rapid virus plaque assay was performed as previously described [5] with certain modifications. A suspension of 2.5 x 10⁶ Vero cells/mL was seeded into 24-well plastic plates. Plates were inoculated with the 100-fold serial dilutions of virus strains and incubated at 37 °C in a 5% carbon dioxide atmosphere. After 4 h, a media overlay was added, consisting of a 2x Parker medium 199 containing 10 % FBS, 1.2 g/L sodium bicarbonate and 1.5% carboxymethyl Cellulose. Cultures were reincubated until the plaques were developed. Individual plaques were chosen, expanded and cloned twice.

Characterization of cloned virus
Sensitivity to chloroform, PAA, GHCl and 5'-bromodeoxyuridine (BDUR) were assayed following the procedure described by Guzmán et al. [4].
Strain 47/93 IPK

Strain 47/93 IPK identified as a CA9 was purified by sucrose gradient showing virus-like particles of 28 nm in the negative staining of the purification band. Virus-like particles of 28 nm were observed in the cytoplasm of infected cells studied by transmission electron microscopy (TEM) Figure 2.

Strain M26 IG

Strain M26 IG showed similar characteristics before and after purification by sucrose gradient and cloning (Tables 1 and 2). However, when the fraction obtained from the purification gradient of this strain (Fraction 11, F11/94) was treated with PAA at a low multiplicity, its CPE-L changed to a typical enterovirus CPE. The new agent was named F11PAA/94. After cloning, the plaque size it produced increased, and 28 nm particles were detectable after purification in the sucrose gradient (Figure 3).

The purified F11PAA/94 strain (Fraction 20) showed not only a different CPE to F11/94 but also a major sedimentation coefficient similar to the 47/93 IPK strain. In addition, the identification of F11PAA/94, using the LBM pools revealed an antigenic relation with echovirus (EV) 7 and 27 although a precise identification was not possible. At this point we have the F11PAA/94 strain obtained from a CPE-L strain (M26 IG) that became another agent with a typical enterovirus CPE.

Strain 44/93 IPK

Cells infected with 44/93 IPK were studied under TEM. No virus-like particles were seen in the infected cells. However, infected cells show an enhancement of granular electrodense structures when compared with the control that may be related with ribonucleoproteins (Figure 4). Infected cells in a terminal stage of degeneration were observed.

When cloning the 44/93 IPK strain, a slight CPE change was observed. The C44/94 strain produces the CPE-L in shorter time and more intensely (Figure 5). Plaques formed by C44/94 are similar to the original strain and very different to the CPE-E strains (Tables 1 and 2 and Figure 6).

After purification by the sucrose gradient the C44/94 band, showed a sedimentation coefficient similar to M26 IG (CPE-L), but lower than that of 47/93 and F11PAA/94. No virus-like particles were seen by the negative staining of the band. In this case, when the C44/94 purification band was inoculated in Vero cells at a low multiplicity, a typical enterovirus CPE was observed after two passages and identified as CA9.

Discussion

The complete characterization of the 47/93 IPK strain was accomplished. According to the neutralization test using the LBM antiserum pools, this strain may be classified as a CA9 virus. In this case, the strain is not sensitive to chloroform, it is sensitive to GHCl and particles of 28 nm were seen by electron microscopy as reported for enteroviruses [8].

It is significant that the majority of the viral isolates obtained during the neuropathy epidemic corresponded to CPE-L strains. This type of strain can not be identified with the LBM pools, as described previously by MÁs et al, 1997 [2], but its relationship with the enteroviruses has been demonstrated [1, 9].
During the study of these CPE-L agents some peculiar characteristics were observed. Contrary to our expectation, the CPE-L produced by the M26 IG differs when Vero cells are kept in a maintenance media containing PAA. In the presence of PAA a typical enterovirus CPE is obtained. Based on the viral DNA polymerase inhibitory action of PAA [10], it is difficult to postulate a molecular mechanism to explain this change to an RNA virus. However, when the M26 IG strain was grown at a low multiplicity in the absence of PAA, a typical enterovirus CPE was observed. Therefore, it is probable that the change may be favored by the low multiplicity of infection and not by the presence of PAA. In addition, similar results were obtained in the characterization of the 44/93 IPK strain. A typical enterovirus CPE appears when the purified strain of CPE-L is grown at a low multiplicity without PAA. These results may support the theory that the presence of PAA is not the cause of the change.

On the other hand, it is striking that F11PAA/94 (a new agent from the M26 IG strain) is significantly neutralized by the hyperimmune antiserum against EV 7 and EV 27. This antigenic relation is not contradictory. The major echovirus group is genetically EV 7 and EV 27. This antigenic relation is not neutralized by the hyperimmune antiserum against PAA. These results may support the theory that the great majority of EVs, CBVs, and also CA9 have diverged from each other relatively late in evolution. There are some antigenic epitopes where CA9 also belongs to this same cluster. It seems obvious that the great majority of EVs, CBVs, and also CA9 have diverged from each other relatively late in evolution. There are some antigenic epitopes where the immunological response of the host has had an effect in favor of mutation [11].

 Certain viruses yield a high proportion of mutants on passage in the absence of any known mutagen. These spontaneous mutations accumulate in the genome of viruses and introduce the variation in phenotype which is subject to selection pressure during the evolution of a virus [12]. In viruses with RNA genomes, high rates of spontaneous mutation, in the order of $10^{-9}$ to $10^{-4}$ per incorporated nucleotide, have been found [13]. Data from the RNA phage Qb indicated that at equilibrium a wild type dominated the population because it replicated more rapidly than the spontaneously arising mutant. Unfortunately for the geneticist, there are factors that promote disequilibrium in genome populations and these factors often favor the accumulation of mutants in a virus population. Viruses are standardly subcloned at intervals to combat this problem, but mutation often occurs during plaque formation or virus growth so that it is often difficult to produce genetically homogeneous high-titer virus stocks. In these experiments, a population with CPE-E (44/93 IPK) that had been previously cloned [12]. This type of spontaneous mutation may obviously induce the formation of defective interfering (DI) particles. Several types of interference have been noted with animal viruses. Of greatest interest to the geneticist is the homologous interference, which occurs within the cell and is exhibited only against homologous viruses or closely related viruses. One well-documented form of homologous interference is that occurring in viruses that have been passaged at a high multiplicity [14]. An interfering phenotype may be determined not by the deleted genes but by mutations in the genes that are present or by both [12].

 Purified DI particles are able to initiate a normal replication cycle but fail to synthesize capsid proteins. Several studies show that the great majority of the deletions in these DI RNA preparations are located in the genome region where capsid proteins are encoded. In addition, the population of individual DI RNAs is not static but changes during passage [15]. DI particles generated after the deletion of no more than 20% of the capsid sequences, interfere with the replication of standard particles by competing for the cellular machinery and for viral capsids [16].

 The CPE-L strains are antigenically related to enteroviruses. The CPE-E appears when the CPE-L strain grows in tissue culture at a low multiplicity. The CPE-L producing strains show a decrease in plaque size and a minor sedimentation coefficient in sucrose. In addition, the interference of 44/93 IPK had been demonstrated in the CA9 growth (Más, unpublished data 1993).

 The observations related to the CPE-L strains are in agreement with molecular studies carried out by other authors. The 44/93 IPK strain genome was amplified by PCR using primers of CA9 and B4. The results showed amplification in all regions except for the region that encodes the structural proteins [17]. Western blot assays using 44/93 IPK as an antigen revealed no bands in the size range expected for enterovirus structural proteins when rabbit hyperimmune antiserum against homologous viruses or closely related viruses. One well-documented form of homologous interference is that occurring in viruses that have been passaged at a high multiplicity [14]. An interfering phenotype may be determined not by the deleted genes but by mutations in the genes that are present or by both [12].

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postulated that they may play a role in the limitation of the progress of the disease. DI particles have also been shown to play a role in the establishment and maintenance of persistent infections in tissue culture and in vivo [18, 19, 12].

In this case, persistency was demonstrated in vivo. A second CSF specimen from 25 patients with a previous CSF positive viral isolation and continuing clinical symptoms was obtained. The second samples were collected 21 to 30 days later and in one case, one year later. Virus with CPE- was reisolated from 24 (96%) of these second CSF samples [2].

Over the years, scientists have made several attempts to understand how incomplete particles moderate virus infection in vivo. Taking into consideration the results obtained in all the research related with the epidemic neuropathy outbreak that occurred in Cuba, Más et al. formulated an integral, multifactorial hypothesis to explain a pathophysiologic mechanism by which isolated viruses could participate in the pathogenesis of the disease. According to the hypothesis, the virus isolated from patients with epidemic neuropathy might be the product of a reactivation of the enterovirus RNA which was latent in the CNS, as a consequence of an increase in oxidative stress. In very few cases the expression of viral RNA produced complete virions capable of lytic replication that is characteristic of enteroviruses. In most of the patients, the reactivation produced incomplete virions, capable of replicating without a helper virus, and producing CPE-L [20].