ARTICULOS ORIGINALES COMPLETOS/ FULL ORIGINAL PAPERS

HUMORAL IMMUNOLOGICAL RESPONSE OF PATIENTS WITH EPIDEMIC NEUROPATHY AGAINST A VIRUS ISOLATED FROM THE CEREBROSPINAL FLUID OF A CLINICAL CASE

Alexis Musacchio, Luis Herrera, Pilar Rodríguez, Reinaldo Alvarez, Martha González-Griego, Eduardo Pentón and José de la Fuente.

Group for the Study of the Epidemic Neuropathy. Centro de Ingeniería Genética y Biotecnología. P.O. Box 6162, La Habana, Cuba.

Recibido en enero de 1995. Aprobado en julio de 1995.

Keywords: coxsackie, epidemic neuropathy, immunology, virus.

SUMMARY

Serum specimens from patients with epidemic neuropathy (EN) were tested in immunoblots for the presence of IgG, IgM and IgA antibodies against the structural proteins of: a virus isolated from the cerebrospinal fluid of a patient (isolate C-47), coxsackie B2 and poliovirus. Sera from blood bank donors, healthy workers from the CIGB and healthy children were used as controls. All the sera had IgG antibodies against the C-47 VP0 protein, but only sera from patients with EN had IgG and/or IgM/IgA antibodies against the C-47 VP1 protein. Immunological response to poliovirus was as expected for a vaccinated population. Practically all the sera recognized the viral VP1 structural protein, as the most important protein for protection against this virus. In the case of coxsackie virus B2 (CVB2), most of the sera recognized VP0, VP1 and VP2 structural proteins, thus indicating the previous circulation of this virus among the population. Antibodies against the C-47 isolate were detected in the CSF of the patients but not in healthy controls. The data obtained from the analysis of areas with low and high incidence of the disease showed that the virus associated with the EN circulated among the population. These results supported our hypothesis that the causal agent of the disease could be the virus in combination with nutritional deficiencies and stress that made the population more susceptible to the appearance of clinical symptoms because of the infection with the virus, although it could play the main role in the neurological damage.

RESUMEN

Muestras de suero de pacientes con neuropatía epidémica (EN) se analizaron en inmunoblots para la presencia de anticuerpos tipo IgG, IgM e IgA contra las proteínas estructurales de un virus aislado del líquido cefalorraquídeo (LCR) de un paciente (aislamiento C-47), virus coxsackie B2 (CVB2) y polio. Se emplearon como control sueros provenientes de donantes de sangre, trabajadores sanos del CIGB y niños sanos. Todos los sueros mostraron reacción frente a la proteína VP0 del C-47, pero sólo los provenientes de pacientes con EN tenían IgG y/o IgM/IgA contra la proteína VP1. La respuesta inmunológica frente al virus polio fue la esperada para una población vacunada. Prácticamente todos los sueros reconocían la VP1 como la proteína más importante para la protección frente al virus polio. Para el virus CVB2, la mayoría de los sueros reconocieron las proteínas VP0, VP1 y VP2, indicando la previa circulación de este virus en la población. Se detectaron anticuerpos tipo IgG contra el aislamiento C-47 en el LCR de pacientes y no en controles sanos. Los datos obtenidos de regiones con baja y alta incidencia de la enfermedad mostraron que el virus asociado a la EN circuló en la población. Estos resultados apoyaron nuestra hipótesis de que el agente causal de la EN puede ser el virus en combinación con deficiencias nutricionales y stress que hacen a la población más susceptible a la aparición de síntomas clínicos producto de la infección con el virus, aunque este pueda jugar el papel fundamental en el daño neurológico.

INTRODUCTION

An epidemic of neuropathy producing central and peripheral symptoms appeared in Cuba between 1991 and 1993. The clinical and epidemiological characterization of the disease has been published elsewhere (Llanos *et al.*, 1993). The disease was termed **epidemic neuropathy** (EN) and three clinical forms were identified: purely ocular (optic neuropathy), peripheric neuropathy and mixed cases. About 50 000 cases were diagnosed until October 1993. Different hypothesis about the etiology of the disease were suggested. Toxic-nutritional and biological factors were argued as being involved in the pathogenesis of the epidemic. The toxic-nutritional hypothesis was supported by laboratory evidences and may play a role as a background conditioning requirement for the disease.

Two types of viral cytopathic effects (CPE) were observed when the cerebrospinal fluid (CSF) from patients was inoculated into a Vero cell monolayer. Most of the samples showed a weak, slowly progressing and

delayed CPE (Rodriguez *et al.*, 1994) and some other samples showed an enterovirus-like CPE that was characterized as *Coxsackievirus* A9 (CoxA9) by a neutralization test with the Limb-Beyesh-Melnick (LBM) pool of sera and by partial determination of the virus genome sequence (Más *et al.*, 1993; Roca *et al.*, 1994; Riego *et al.*, 1994).

Coxsackievirus, which include 23 group A serotypes (CVA-1 to CVA- 22 and CVA-24) and six group B serotypes (CVB-1 to CVB-6), are common infectious agents that cause a wide spectrum of diseases, ranging from flu-like symptoms to severe infections of the central nervous system (More, 1982). They belong to the enterovirus genus of the *Picornaviridae* family. Like the other members of the family, the *coxsackievirus* are nonenveloped icosahedral viruses of approximately 28 nm (300 Å). Each virion contains one copy of a single-stranded messenger-sense RNA genome of approximately 7 500 nucleotides, enclosed in a roughly spherical capsid. A single polypeptide is post-translationally processed through two precursors (VP4+VP2 = VP0 (≈37.4 kDa) and VP1+VP3) to originate the four coat protein subunits. The capsid is composed of 60 copies of each of the four coat protein subunits VP1(≈33 kDa), VP2(≈30 kDa), VP3(≈26 kDa), VP4(≈7.4 kDa).

The development of the infection produced by the enteroviruses is characterized by a significant antibody response. During infection, antibodies to the denatured viral antigens appear before antibodies to the native antigens, and subsequently the levels of antibodies to the denatured antigens are the first to fall (Melnick, 1990).

The immune response to enterovirus infection consists mainly in the production of neutralizing antibodies as demonstrated by the severity of these infections in agammaglobulinemic patients (MacKinney *et al.*, 1987). The detection of serum antibodies to enterovirus is widely used for the diagnosis as a complement to virus isolation and more recently to virus detection by hybridization.

Although most of the CSF samples from patients with EN showed a CPE not typical for enteroviruses, these isolates presented some similarities with this genus in the size and morphology of the virus particles (Rodríguez *et al.*, 1994), in the crossreactivity of viral epitopes (Castro *et al.*, 1994), and, at least for some of them, in the genome sequence as shown by PCR analysis (Alvarez *et al.*, 1995). This fact suggested that we could be dealing with a mutant enterovirus or a new unidentified virus. However, due to the difficulties in growing these viruses and considering the existence of some degree of homology between these viruses and the coxsackievirus, the characterization of the immunological response of patients with EN was performed against a virus isolate classified as coxsackievirus A9 obtained from the CSF of a patient (isolate C-47/IPK). Here it was shown that patients with EN had a characteristic immunological response against C-47 viral proteins and that the virus circulated in the population. The relevance of these findings for the multicausal hypothesis of the etiology of the EN is discussed.

MATERIALS AND METHODS

Virus preparation

The C-47/IPK (C-47) isolate (classified as coxsackievirus A9) was obtained at the Tropical Medicine Institute "Pedro Kourí" (IPK) from the CSF of a female adult patient with EN showing essentially optical symptoms. The coxsackievirus B 2 (CVB2) and polio 1 virus strains were employed as references and were obtained from the IPK collection. Viruses were propagated twice in Vero cells prior to analysis. After propagation in Vero cells, virus were partially purified (Falcón *et al.*, 1994).

Samples of the CSF and sera from patients and controls

Samples were aseptically collected from the hospitals and transported in ice to the CIGB. At the center, samples received a code number and were aliquoted and stored at -20°C until use. Characteristics of the individuals from whom the samples were collected are summarized in tables 1 and 2.

Immunobloting

SDS:polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure described by Laemmli (1970). For electrophoresis, 400 µg of the virus preparation were diluted 1:2 in 2X electrophoresis sample buffer (1x: 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% ß-mercaptoethanol, 0.01% bromophenol blue), placed in a boiling water bath for 20 min at 100°C and loaded into a 10 cm long slot. SDS-PAGE were run for 45-60 minutes with a 20 mA constant current at room temperature on a 0.75 mm thick 10% polyacrylamide gel (pH 8.8) with a 3% acrylamide concentration gel (pH 6.8). After electrophoresis, the antigens were transferred from the gel to 0.2 µm pore size nitrocellulose filters (Schleicher & Schuell, Germany) in an electroblotting buffer containing 0.025 M Tris-HCl, 0.15 M glycine, 20% methanol, pH 8.3, for 1h at 40V. After blotting, the membrane was stained with Ponceau S-red to demonstrate successful transfer of proteins to the nitrocellulose membrane. The filters were washed 3 times for 10 min in PBS and incubated for 1h at 37°C in a blocking solution containing 5% skim milk (Oxoid, England) in PBS. Later, the membrane was cut in 3 mm wide-strips. Serum samples were added (human serum 1:20) and the strips were incubated for 2h at room temperature with continuous shaking. The strips were washed 3 times, for 10 min each in PBS and horseradish- peroxidase-conjugated goat antihuman IgG, IgA or IgM antibodies were added in 5% milk in PBS. The incubation was for 1h at room temperature with shaking. The strips were then washed once for 10 min in PBS, once for 5 min in 0.02 % NP-40 in PBS and once for 5 min in PBS, and placed in a color developing solution (5 mL 4-Cl-I-naphtol dissolved in 1 mL methanol, 15 µL hydrogen peroxide and 25 mL PBS). The reaction was stopped after 15 min by dipping the blots in distilled water.

Table 1
Data from patients and controls included in these studies.

No.	Patients and Controls (Number of the clinical history)[a]	Specimen	Sex	Clinical diagnosis[b]	Region
1	4478 , from a blood donor bank	Serum	M	Healthy	C. Habana
2	4479 , from a blood donor bank	Serum	F	Healthy	C. Habana
3	4451 , from a blood donor bank	Serum	M	Healthy	C. Habana
4	1-T, from CIGB	Serum	M	Healthy	C. Habana
5	2-T, from CIGB	Serum	F	Healthy	C. Habana
6	3-T, from CIGB	Serum	M	Healthy	C. Habana
7	7-T, from CIGB	Serum	M	Healthy	C. Habana
8	11-T, from CIGB	Serum	F	Healthy	C. Habana
9	Q1082	Serum	M	ON	P. del Rio
10	Q1095	Serum	M	ON	P. del Rio
11	Q1096	Serum	F	ON	P. del Rio
12	54790	Serum	M	ON	P. del Rio
13	Q1657	Serum	M	ON	P. del Rio
14	90072	Serum	M	PN	P. del Rio
15	Q1183	Serum	M	PN	P. del Rio
16	Q1180	Serum	F	PN	P. del Rio
17	4NP	Serum	NR	PN	Stgo. de Cuba
18	5NP	Serum	NR	PN	Stgo. de Cuba
19	6NP	Serum	NR	PN	Stgo. de Cuba
20	7NP	Serum	F	PN	Stgo. de Cuba
21	8NP	Serum	M	PN	Stgo. de Cuba
22	9NP	Serum	F	PN	Stgo. de Cuba
23	161-N , child	Serum	M	Healthy	C. Habana
24	O-1-B-N , child	Serum	F	Healthy	C. Habana
25	N-17-N , child	Serum	M	Healthy	C. Habana
26	OHB8-N , child	Serum	M	Healthy	C. Habana
27	E-1 , child	Serum	F	ON	C. Habana
28	E-2 , child	Serum	F	ON	C. Habana
29	Q1207	CSF and Serum	M	ON	P.del Rio
30	Q1204	CSF and Serum	F	ON	P.del Rio
31	51094	CSF and Serum	F	ON	P.del Rio
32	N0131	CSF and Serum	F	ON	P.del Rio
33	Q1216	CSF and Serum	F	ON	P.del Rio
34	Q1222	CSF and Serum	M	ON	P.del Rio
35	Q1044	CSF and Serum	M	ON	P.del Rio
36	Q1178	CSF and Serum	F	PN	P.del Rio
37	Q184	CSF and Serum	F	PN	P.del Rio
38	Q1173	CSF and Serum	M	PN	P.del Rio
39	Q1174	CSF and Serum	M	PN	P.del Rio
40	N0143	CSF and Serum	M	ON	P.del Rio
41	FP16	CSF and Serum	F	Healthy	C. Habana
42	FP18	CSF and Serum	M	Healthy	C. Habana
43	FP19	CSF and Serum	F	Healthy	C. Habana
44	FP20	CSF and Serum	F	Healthy	C. Habana
45	FP21	CSF and Serum	F	Healthy	C. Habana
46	FP22	CSF and Serum	M	Healthy	C. Habana

47	FP6	CSF and Serum	F	Healthy	C. Habana
48	FP8	CSF and Serum	F	Healthy	C. Habana
49	FP9	CSF and Serum	M	Healthy	C. Habana
50	FP11	CSF and Serum	M	Healthy	C. Habana
51	FP12	CSF and Serum	M	Healthy	C. Habana
52	SJ1	Serum	F	Healthy	San Juan
53	SJ2	Serum	F	Healthy	San Juan
54	SJ3	Serum	F	Healthy	San Juan
55	SJ4	Serum	F	Healthy	San Juan
56	SJ5	Serum	M	Healthy	San Juan
57	SJ6	Serum	F	Healthy	San Juan
58	SJ7	Serum	F	Healthy	San Juan
559	SJ8	Serum	F	Healthy	San Juan
60	SJ9	Serum	F	Healthy	San Juan
61	SJ10	Serum	F	Healthy	San Juan
62	SJ11	Serum	F	Healthy	San Juan
63	SJ12	Serum	M	Healthy	San Juan
64	SJ13	Serum	F	Healthy	San Juan
65	SJ14	Serum	F	Healthy	San Juan
66	SJ15	Serum	M	Healthy	San Juan
67	SJ16	Serum	F	Healthy	San Juan
68	SJ17	Serum	F	Healthy	San Juan
69	SJ18	Serum	F	Healthy	San Juan
70	SJ19	Serum	M	Healthy	San Juan
71	SJ20	Serum	F	Healthy	San Juan
72	SJ21	Serum	F	Healthy	San Juan
73	SJ2	Serum	F	Healthy	San Juan
74	SJ23	Serum	M	Healthy	San Juan
75	SJ24	Serum	M	Healthy	San Juan
76	SJ-E-1	Serum	F	PN	San Juan
77	SJ-E-2	Serum	F	PN-ON	San Juan
78	SJ-E-3	Serum	F	ON	San Juan
79	SJ-E-4	Serum	F	ON-PN	San Juan
80	SJ-E-5	Serum	M	PN	San Juan
81	SJ-E-6	Serum	M	PN	San Juan
82	C-1	Serum	M	Healthy	Candelaria
83	C-2	Serum	F	Healthy	Candelaria
84	C-3	Serum	M	Healthy	Candelaria
85	C-4	Serum	F	Healthy	Candelaria
86	C-5	Serum	F	Healthy	Candelaria
87	C-6	Serum	F	Healthy	Candelaria
88	C-7	Serum	F	Healthy	Candelaria
89	C-8	Serum	M	Healthy	Candelaria
90	C-9	Serum	F	Healthy	Candelaria
91	C-10	Serum	M	Healthy	Candelaria
92	C-11	Serum	F	Healthy	Candelaria
93	C-12	Serum	F	Healthy	Candelaria
94	C-13	Serum	M	Healthy	Candelaria
95	C-14	Serum	F	Healthy	Candelaria
96	C-15	Serum	F	Healthy	Candelaria

97	C-16	Serum	F	Healthy	Candelaria
98	C-17	Serum	F	Healthy	Candelaria
99	C-18	Serum	F	Healthy	Candelaria
100	C-19	Serum	M	Healthy	Candelaria
101	C-20	Serum	M	Healthy	Candelaria
102	C-30	Serum	M	Healthy	Candelaria
103	C-E-1	Serum	M	ON	Candelaria
104	C-E-2	Serum	F	ON	Candelaria
105	C-E-3	Serum	F	ON	Candelaria
106	C-E-4	Serum	F	ON	Candelaria
107	C-E-5	Serum	F	ON	Candelaria
108	C-E-6	Serum	F	ON	Candelaria
109	C-E-7	Serum	F	ON	Candelaria
110	C-E-8	Serum	M	ON	Candelaria
111	CI1	Serum	NR	Healthy	Caimanera
112	CI2	Serum	NR	Healthy	Caimanera
113	CI3	Serum	NR	Healthy	Caimanera
114	CI4	Serum	NR	Healthy	Caimanera
115	CI5	Serum	NR	Healthy	Caimanera
116	CI6	Serum	NR	Healthy	Caimanera
117	CI7	Serum	NR	Healthy	Caimanera
118	CI8	Serum	NR	Healthy	Caimanera
119	CI51	Serum	NR	Healthy	Caimanera
120	IM299	Serum	NR	Healthy	Imias
121	IM300	Serum	NR	Healthy	Imias
122	IM301	Serum	NR	Healthy	Imias
123	IM302	Serum	NR	Healthy	Imias
124	IM303	Serum	NR	Healthy	Imias
125	IM304	Serum	NR	Healthy	Imias
126	IM305	Serum	NR	Healthy	Imias
127	IM306	Serum	NR	Healthy	Imias
128	IM307	Serum	NR	Healthy	Imias

[a]Adult individuals (>20 years old) unless otherwise stated. Individuals included in the focal studies are in Table 2.

[b]Healthy controls were obtained from blood donors, CIGB workers and surgical patients. Patients with EN were classified according to the predominant symptoms in optic neuropathy (ON) and peripheric neuropathy (PN).

NR, not recorded.

RESULTS AND DISCUSSION

The C-47 virus isolate elicited a characteristic differential response in patients with EN

In an attempt to find out if the immunological response against the C-47 isolate was masked by the presence in the sera of the population of antibodies against enteroviruses that have previously circulated in the country (P. Más, personal communication) or by the response against the poliovirus vaccine, immunoblots were performed with the sera of patients and controls against C-47, CVB2 and polio viruses. As shown in figure 1A, all the sera examined had IgG against the C-47 VP0 protein because of the crossreactivity of this protein among the enteroviruses. However, the recognition of the C-47 VP1 protein was achieved only in the sera of patients or in that of people exposed to the virus (fig.1A and table 1). The IgA (fig.1B) and IgM (fig.1C) response against the C-47 isolate was also detected in the sera of adult patients and children, including two healthy children (fig.1A, C and table 1).

Table 2
Data from individuals included in the focal studies.

Focus	No.	Sex	Age	Living together[a]	Relationship with the patient	Suspected by interview[b]	Diagnosis confirmed[c]	Observations[d]
I	1	F	36	*	Patient	*	*	ON-PN
	2	M	17	*	Son			Normal
	3	F	9	*	Daughter			Normal
	4	F	36		Neighbor			Normal
	5	F	39		Neighbor			Normal
II	6	F	40	*	Patient	*	*	PN
	7	F	71	*	Mother			Normal
	8	F	14	*	Daughter			Normal
	9	M	6	*	Son			Normal
	10	M	47		Sexual			Normal
	11	F	20		Neighbor			Normal
III	12	F	30	*	Patient	*	*	PN
	13	M	30	*	Husband			Normal
IV	14	F	47	*	Patient	*	*	ON-PN
V	15	F	23	*	Patient	*	*	ON-PN
	16	F	NR	*	Mother			Normal
	17	M	59	*	Father			Normal
	18	F	26		Friend	*	*	ON-PN
	19	F	47		Coworker			PN
VI	20	M	73	*	Patient	*	*	PN
VII	21	M	35	*	Patient	*	*	ON
	22	F	31	*	Wife			Normal
VIII	23	F	24	*	Patient	*	*	PN
	24	F	46	*	Mother			PN
	25	M	52	*	Father	*	*	PN
	26	F	27	*	Brother	*		Normal
	27	M	20	*	Brother			ON-PN
	28	F	52		Neighbor	*		PN
	29	F	22		Neighbor	*		PN
IX	30	M	11	*	Patient	*	*	ON-PN
	31	F	2	*	Brother			Normal
	32	F	30	*	Mother	*		PN
	33	M	36	*	Father	*	*	PN
X	34	F	13	*	Patient	*	*	ON-PN
	35	F	12	*	Patient	*		ON-PN
	36	F	6	*	Brother			Normal
	37	F	32	*	Mother	*		ON-PN
	38	M	63		Father	*	*	PN
	39	M	14		Brother			Normal

[a]Living in the same house.
[b]Clinical symptoms of EN referred by the pacient and observed during the focal study.
[c]Diagnosis confirmed according to the clinical specifications of the disease.
[d]Patients were classified according to the predominant symptoms in optic neuropathy (ON) and peripheric neuropathy (PN).

Similar studies were performed by using CVB-2 (fig. 2A) and polio viruses (fig. 2B) (IgG response). For CVB-2 a recognition of the VP0 and VP1 proteins was observed in most of the analyzed sera, thus confirming the previous circulation of CVB-2 in the population. For the polio virus, the main reaction was against VP1. These results prompted the suggestion that the C-47 isolate was different from CVB-2 and polio viruses. The VP0 protein from C-47 was recognized by all the individuals (fig. 1A), whereas the same protein in CVB-2 was recognized only

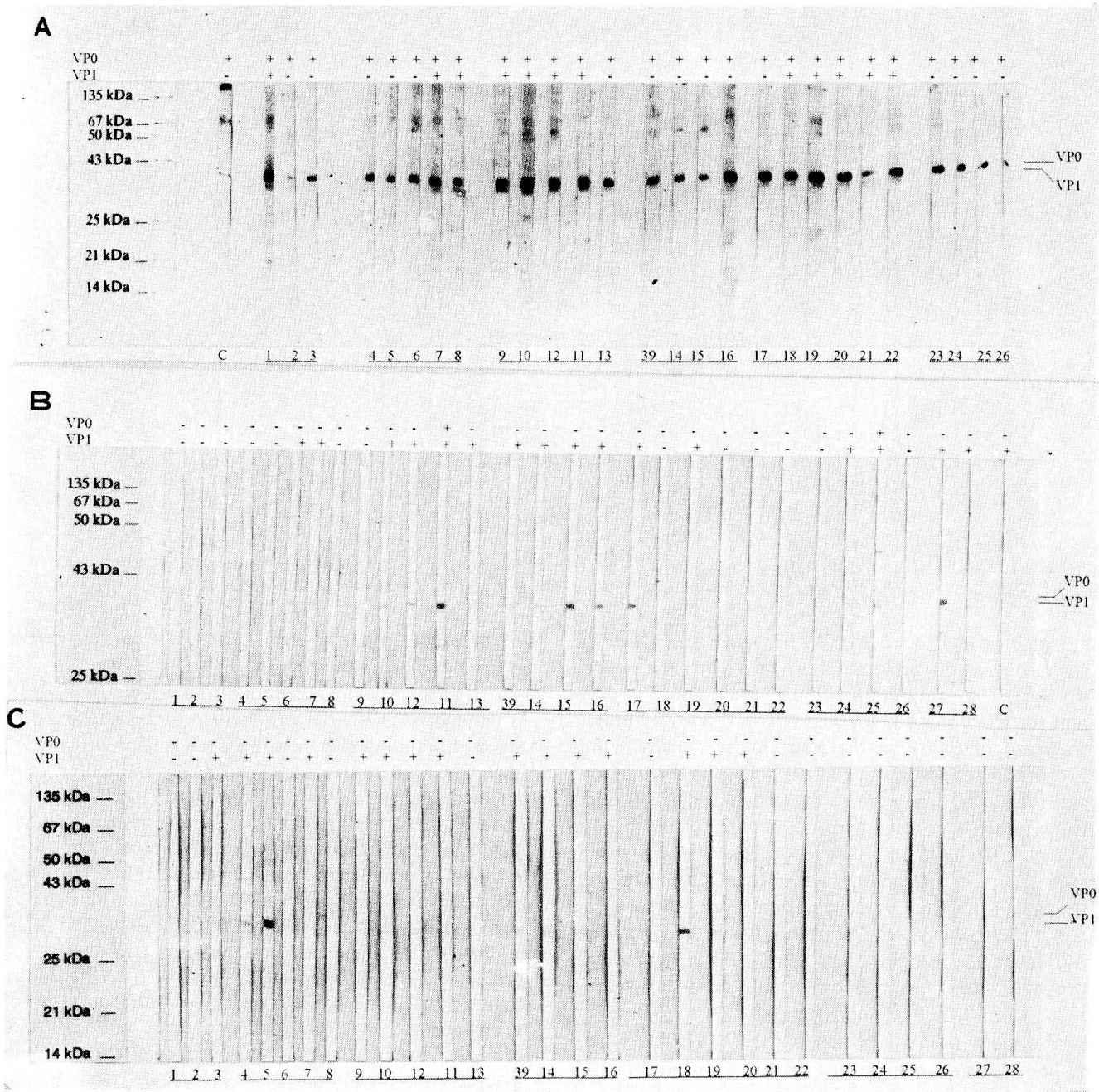

Fig. 1. Western blot analysis of the IgG (A), IgA (B) and IgM (C) response against the C-47 isolate in patients and controls. Numbers are referred to table 1. The reactivity of the sera against the viral proteins VP0 and VP1 is indicated. C, rabbit antiserum against CVB-2.

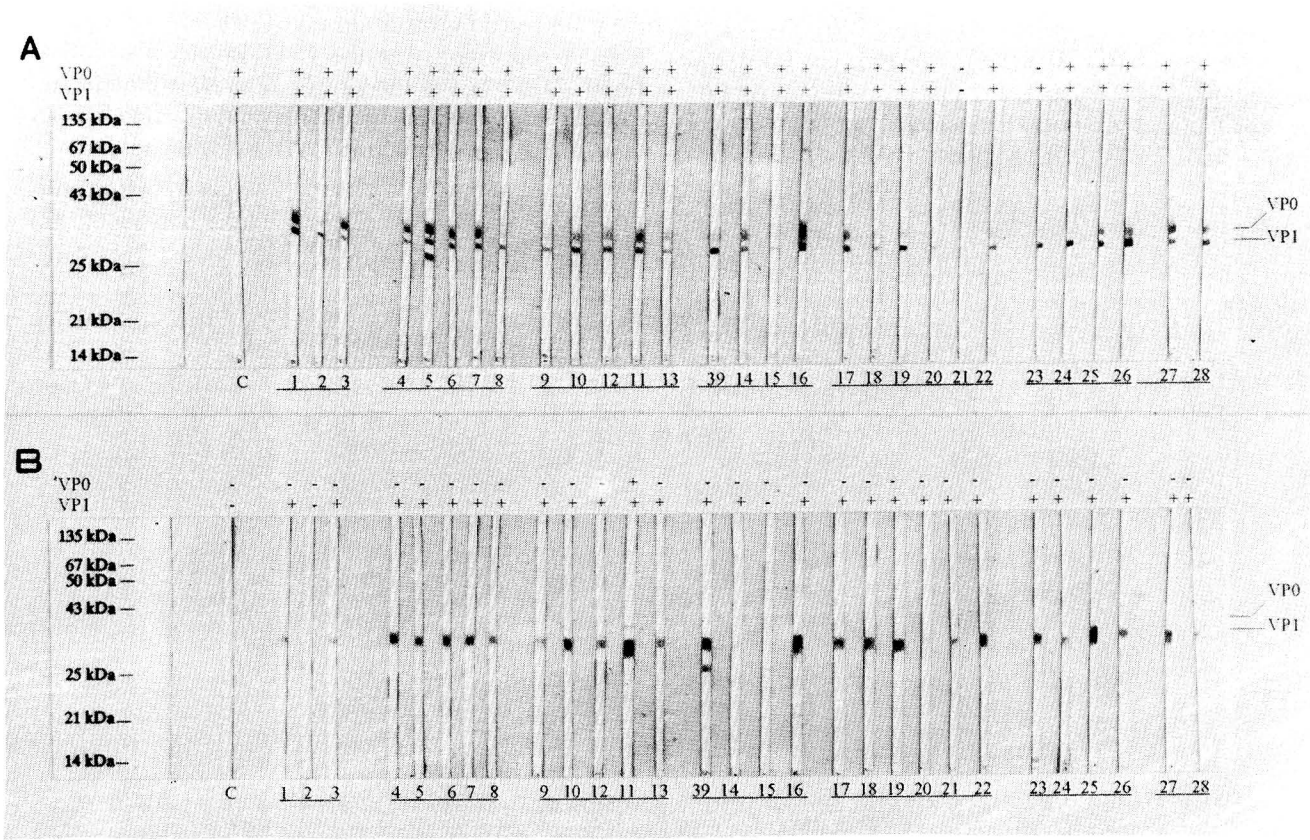

Fig. 2. Western blot analysis of the IgG response against CVB-2 (A) and polio (B) viruses in patients and controls. Numbers are referred to table 1. The reactivity of the sera against the viral proteins VP0 and VP1 is indicated. C, rabbit antiserum against CVB-2.

by some of them (7 out of 30 analyzed sera either did not recognize the protein or recognized it very weakly; fig .2A). The recognition pattern in children was different for C-47 and polio viruses. The C-47 VP1 protein was recognized by all the sick children whereas only two healthy children showed antibodies against it (fig.1 and table 1). Furthermore, these two healthy children were in close contact with adult patients. With polio virus, all the children's sera recognized the VP1 protein (fig. 2B). The VP2 protein was not strongly recognized, thus suggesting that the recognition of VP2 protein from C-47 was not due to crossreactivity with polio virus. Moreover, the recognition of the C-47 VP0 protein was probably due to crossreactivity with the broadly recognized CVB-2 VP0 protein (fig.2A).

From our results it seems that the C-47 VP1 protein is poorly immunogenic, but related with the infection. Antibodies against this protein were present in the sera of patients. In patients with optic neuropathy the recognition of the C-47 VP1 protein was of IgG-IgA type, whereas the response in patients with peripheric neuropathy was mainly of the IgA type (fig.1).

Antibodies against the C-47 isolate were present in the CSF of patients with EN

Considering the localization of the viruses in the CSF of patients with EN, it was decided to analyze the presence of antibodies against the C-47 isolate in the CSF. In 9/12 CSFs from patients with EN, antibodies were detected against the C-47 VP0 protein (fig. 3A). In the CSFs from 11 healthy controls, the presence of antibodies against C-47 proteins was not detected (fig. 3B). Although it was impossible to confirm the intra-blood-brain-barrier antibody synthesis in patients with EN, this was considered the most likely hypothesis since the intensity of the signals in western blots was similar for sera and CSF samples. The presence of the virus in the CSF (200-300 particles/mL) without meningeal symptoms and with acellularity (data from the Institute of Neurology in Havana) could be explained assuming that the virus entered the Central Nervous System (CNS) by the hematic route through the choroid plexus, as previously reported for enteroviral infections (Johnson, 1990). The absence of meningeal symptoms could be explained by the fact that the samples were taken after the acute phase of the disease or, alternatively, by the

absence of tropism of these viruses for the CNS. However, in an attempt to explain the role of the virus in the EN, this is one of the most contradictory facts.

The virus circulated within the population with no correlation with the incidence of the disease

A study was conducted in high and low incidence zones after the onset of the epidemic. The analysis of sera from patients with EN from areas with low (fig.4A) and high (fig.4B) incidence of the disease, showed that in both areas all the individuals recognized the C-47 VP1 protein (fig.4), indicating the circulation of the virus within the population.

This evidence supported our initial hypothesis that the causal agent of the disease was not only the virus, although it could play the main role in the neurological damage.

In an attempt to follow the transmission of the virus, a focal study was conducted in families where at least one of the members suffered from the disease (table 2).

In 7 out of 8 focus studied, contacts recognized C-47 VP0, VP1 and VP2 proteins (fig.5), showing the same recognition pattern as the patients. The only focus that did not show this recognition pattern (focus III in table 2) corresponded to a patient whose diagnosis was not confirmed. Focus IV and VI (table 2) were not considered in the analysis because only one patient was included in each. These results are in accordance with those previously showing that the virus circulated within the population and no obvious pattern of transmission can be followed since, as previously stated, the disease was most probably multicausal. However, from 28 contacts included in the study, 10 (35.7%) were recorded as suffering from the disease (table 2). The fact that an obvious pattern of contagion and transmitability was not found, could be explained considering a high number of infected persons with a low frequency of clinical symptoms, as for example in the case of enterovirus infections (Melnick, 1990). Finally, several epidemiological findings found during the SMON epidemic in Japan sug-

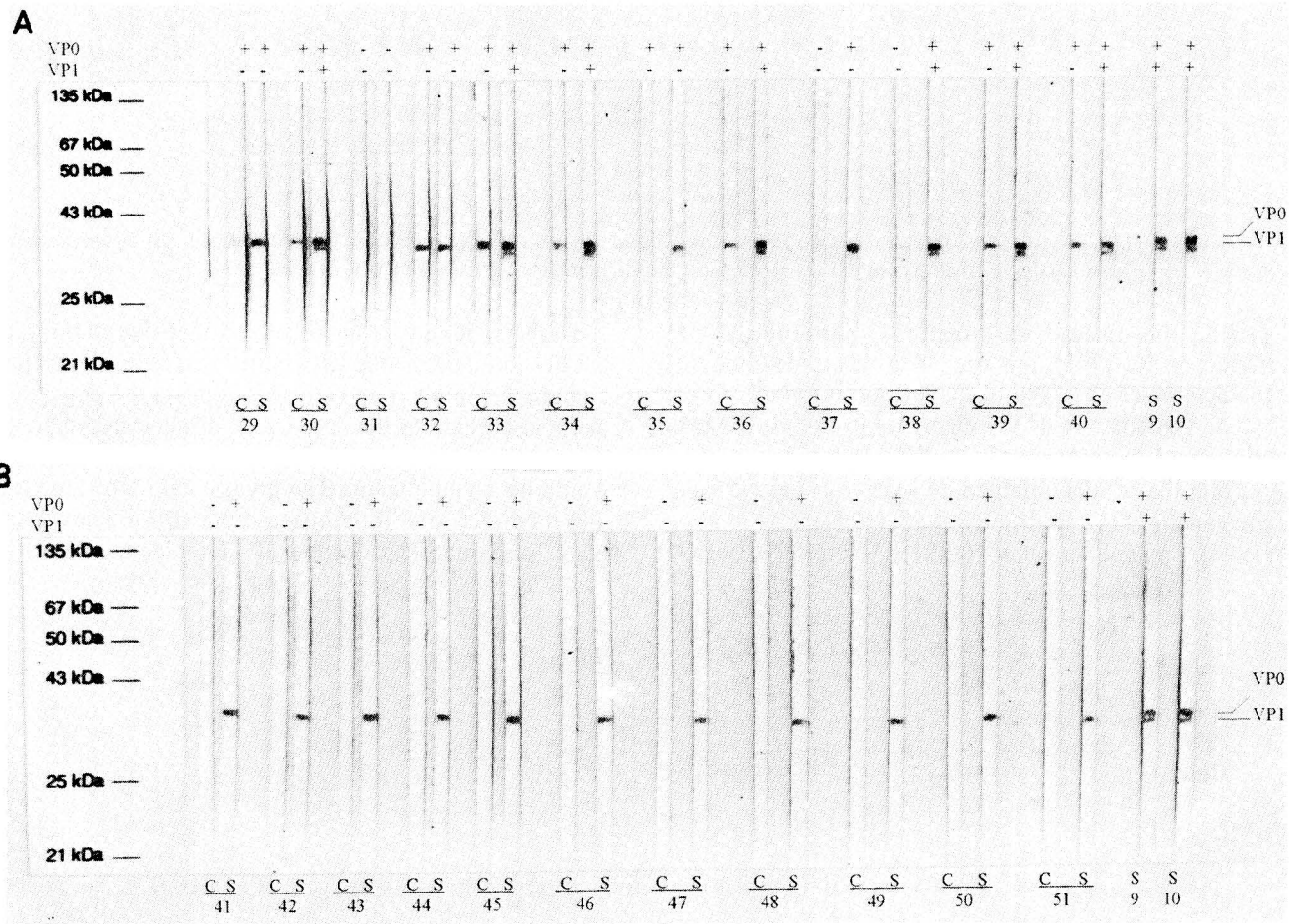

Fig. 3. Western blot analysis of the sera (S) and CSFs (C) of patients (3A) and controls (3B) for the presence of IgG against the C-47 isolate. Numbers are referred to table 1. The reactivity against the viral proteins VP0 and VP1 is indicated.

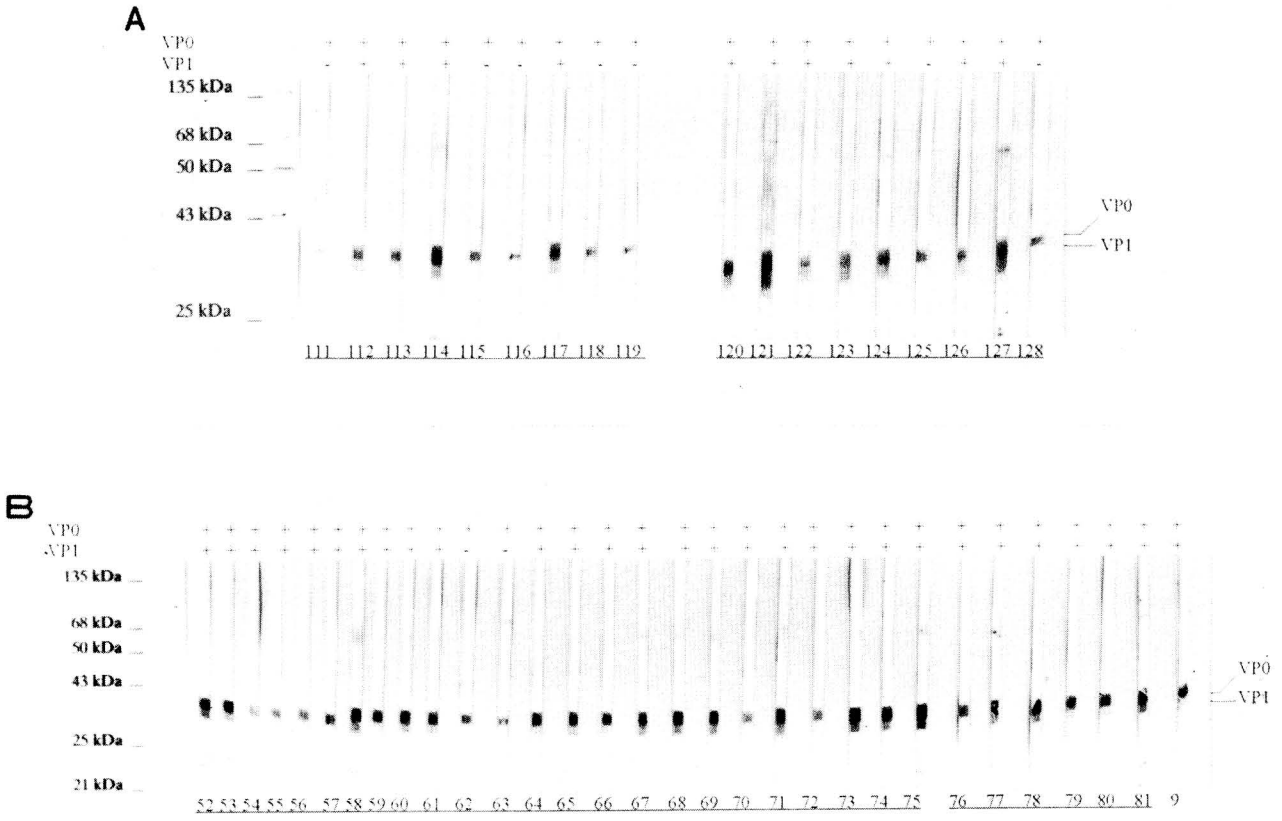

Fig. 4. Western blot analysis of the IgG response against the C-47 isolate in patients and controls from low (A) and high (B) incidence zones. Numbers are referred to table 1. The reactivity of the sera against the viral proteins VP0 and VP1 is indicated.

gesting the disease as infectious, particularly viral (Inoue, Y. K. 1991), are similar to the epidemiological characteristics observed in Cuba (Ramirez *et al.*, 1993).

The virus is one of the elements in the multicausal etiological hypothesis of the EN

From the results obtained by Más *et al.*, (1993); Falcón *et al.*, (1994); Rodríguez *et al.*, (1994); Castro *et al.*, (1994); López-Saura *et al.*, (1994); Alvarez *et al.*, (1995) and others, it can be hypothetized that the etiology of the EN could be related with nutritional deficiencies (eg. vitamin B complex) and stress that made the population more susceptible to the appearance of clinical symptoms because of the infection with a virus that, otherwise, would be asymptomatic. The precise nature of the virus and its direct role in the disease are still unknown and under research. The decline of the epidemic could be

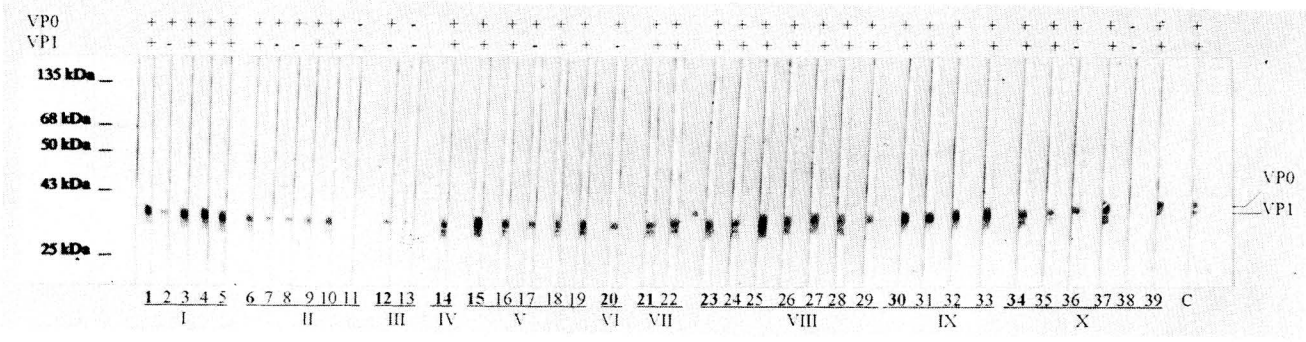

Fig. 5. Western blot analysis of the IgG response against the C-47 isolate in individuals from the families included in the focal study. Numbers are referred to table 2. The reactivity of the sera against the viral proteins VP0 and VP1 is indicated. C, rabbit antiserum against CVB-2.

explained by the elimination of the predisposing element, by the elimination of susceptible persons in the population or by a combination of both factors.

The vitamin administration to the population would prevent the appearance of the clinical symptoms but not spread of the virus. Thus, the seroconvertion in different groups and regions will not be related with the disease and should be essentially the same. The recombinant human interferon-α2b (HeberonR, Heber Biotec S.A., Havana, Cuba) therapy, that resulted useful for the treatment of the peripheral neuritis associated to the EN (López-Saura *et al.*, 1994), will act on the virus without eliminating the predisposing element. Furthermore, in some cases, people that eventually recover from the disease could relapse again with clinical symptoms. However, in a population with a good nutritional balance, the risk of the appearance of the disease should be low.

ACKNOWLEDGEMENTS

We wish to thank Dr. P. Más for providing the C-47/IPK virus isolate. The authors will like to acknowledge the collaboration of the "Frank País" Hospital, the Clinical-Surgical Research Center (CIMEQ), the Pinar del Rio Province Hospital, the Institute of Neurology and the Cuban Ministry of Public Health.

REFERENCES

1. ALVAREZ, M.; V. MUZIO; M. MARRERO; M. BARRO; D. ROSARIO; L .JOMARRON; A. MARTIN; C. SARIOL; M. CANDELARIO; J. MAESTRE; J. DE LA FUENTE; L. HERRERA; M. GUZMAN; P. MAS and G.KOURI (1995). Detection by Polymerase Chain Reaction of enterovirus-like sequences in patients with epidemic neuropathy in Cuba. *BiotecnologIa Aplicada* **12**: 46-51.
2. CASTRO, F. O.; J. BERLANGA; C. ALFONSO; P. RODRIGUEZ; M. HECHEVARRIA; O. HAYES; R. PEREZ; D. PICHARDO; M. PEREZ; I. DORTA; E. LOPEZ; M. C. LOPEZ; L. HERRERA and J. DE LA FUENTE (1994). Evaluation in animal models of the neurovirulence of isolates from patients with epidemic neuropathy. *BiotecnologIa Aplicada* **11**: 138-144.
3. FALCON, V.; J. REYES; O. ANCHETA; P. RODRIGUEZ; R. ALVAREZ; N. BARANOSKY; M. C. DE LA ROSA; P. MAS; G. PADRON; G. KOURI; L. HERRERA and J. DE LA FUENTE (1994). Study by transmission electron microscopy of virus strains isolated from the cerebrospinal fluid of patients with epidemic neuropathy. *BiotecnologIa Aplicada* **11**: 151-159.
4. INOUE, Y. K. (1991). Inoue-Melnick virus and associationed diseases in man: Recent Advances. *Prog. Med. Virol.* **38**: 167-179.
5. JOHNSON, R. T. (1990). Viral Infections of the Nerval System. In: Virology, Ed. by B. N. Fields *et al.* Raven Press, Ltd., New York.
6. LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* **227**: 680- 685.
7. LLANOS, G. C.; D. ASCHER and D. C. BROWN (1993). Epidemic neuropathy in Cuba. *Epidemiological Bulletin PAHO* **14**: 1-4.
8. LOPEZ-SAURA, P.; F. HERNANDEZ; V. LABARTA and the Group for the Study of the Epidemic Neuropathy (1994). Interferon alpha-2b in Epidemic Neuropathy. *J. of Interferon Res.* **14 (Suppl.1)**: S120.
9. MACKINNEY, R. E.; S. L. KATZ and C. M. WILFERT (1987). Chronic enteroviral meningoencephalitis in agammaglobulinemic patients. *Rev. Infect. Dis.* **9**: 334-354.
10. MAS, P.; M. P. RODRIGUEZ; M. G. GUZMAN; M. ALVAREZ; V. MUZIO; O. ANCHETA; A. GOYENECHEA; H. ROCA; L. MORIER; R. ALVAREZ; M. MARRERO; A. MUSACCHIO; A. CASTILLO; V. FALCON; J. L. PELEGRINO; L. LUACES; A. BALMASEDA; M. BARRO; L. RODRIGUEZ; D. GARCIA DEL BARCO; M. SOLER; B. GARCIA; M. A. RIVAS; R. LLEONART; S. RESIK; G. PADRON; L. JOMARRON; M. CANDELARIO; M. MUNE; A. MARTIN; D. ROSARIO; M. G. MORALES; J. LAFERTE; J. REYES; C. SARIOL; R. RICARDO; J. L. MAESTRE; A. RODRIGUEZ; N. BARANOSKY; M. GONZALEZ GRIEGO; P. LOPEZ; E. PENTON; M. LIMONTA; J. DE LA FUENTE; L. HERRERA; A. LLOP and G. KOURI (1993). Resultados preliminares de laboratorio virolOgico en estudios de casos de Neuropatla EpidEmica Cubana. *BoletIn EpidemiolOgico.* NUmero especial **1**: 7-8.
11. MELNICK, J. L. (1990). Enteroviruses: Picornaviruses, Coxsackieviruses, Echoviruses and Newer Enteroviruses. In: *Virology*, Ed. by B. N. Fields *et al.* Raven Press, Ltd., New York.
12. MOORE, M. (1982). Enteroviral Disease in the United States, 1970-1979. *Journal of Infect. Diseases* **146**: 103-108.
13. RAMIREZ, A.; R. RODRIGUEZ; A. MARRERO; G. MESA; M. A. GALINDO and L. IÑIGUEZ (1993). Neropatla EpidEmica Cubana: Breve reseña epidemiolOgica. *BoletIn EpidemiolOgico.* NUmero especial **1**: 1-5.
14. RIEGO, E.; M. MARRERO; E. CALVO; R. BRINGAS; M. ALVAREZ; M. MUNE; H. ROCA; M. GUZMAN and J. DE LA FUENTE (1994). Sequence analysis of coxsackie A9 viruses isolated during 1990-1994 in Cuba from patients with meningitis, myocarditis and epidemic neuropathy. *BiotecnologIa Aplicada* **11**: 145-150.
15. ROCA, H.; M. MARRERO; E. RIEGO; V. FALCON; P. MAS; P. RODRIGUEZ; R. ALVAREZ; V. MUZIO; B. GARCIA; R. LLEONART; G. KOURI; L. HERRERA and J. DE LA FUENTE (1994). Molecular characterization of a virus associated with the outbreak of a polyneurophathy epidemic in Cuba. *Miami Short Reports* **4**: 89.
16. RODRIGUEZ, M. P.; R. ALVAREZ; D. GARCIA DEL BARCO; R. LLEONART; V. FALCON; J. REYES; F. O. CASTRO; J. BERLANGA; A. MUSSACHIO; P. MAS; L. HERRERA and J. DE LA FUENTE (1994). CaracterizaciOn de las cepas virales obtenidas a partir de aislamientos de los lIquidos cefalorraquIdeos de pacientes con cuadros de Neuropatla EpidEmica. *Advances in Modern Biotechnology* **2**: 4.