GENOMIC CHARACTERIZATION OF TYPE 1 SABIN-RELATED POLIOVIRUSES ISOLATED IN BRAZIL

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Summary. – Eight strains of P1/Sabin-derived polioviruses isolated in Brazil from paralysis cases were analyzed. The serotypes of the viral isolates were identified by neutralization test with hyperimmune equine sera. The relationship of the isolates to the P1/Sabin strain was demonstrated by molecular hybridization and PCR. The isolates were partially sequenced with the objective of finding mutations at nucleotides (nt) 480 and 525 of the 5'-noncoding region (5'NCR) and at nt 6203 of the 3Dpol coding region (3Dpol), which are important for reversion towards neurovirulence. Four isolates from paralysis cases classified as Guillain Barré Syndrome (GBS; three with sequels) were analyzed; one presented G→A (480) and C→U (6203) mutations, one G→A (480) mutation, one G→A (480) and U→C (525) mutations, and one did not mutate at the analyzed positions. Two isolates from transient facial paralysis cases were analyzed ; one presented U→C (525) mutation and the other G→A (480) mutation. One isolate from a transient paralysis case classified as a neuroviral disease and one isolate from a paralysis case with sequels were analyzed and none mutated at the analyzed positions. Although the isolates may not be the causative agent of the disease, a temporal association between the isolation of the P1/Sabin-derived isolates and the disease was observed. The possibility that GBS and the facial paralysis were caused by these isolates could not be excluded.

Key words: poliovirus; Sabin strains; attenuation; vaccine-associated neurovirulence

Introduction

Polioviruses are the causative agents of poliomyelitis, a paralytic and sometimes fatal disease of humans. As polioviruses are grouped into three serotypes designated 1, 2 and 3, based on the antigenicity of the capsid, the oral poliovirus vaccine consists of three attenuated poliovirus strains (Sabin 1, Sabin 2 and Sabin 3), one for each serotype. The attenuated strains developed by A. Sabin have been effectively used as an oral live vaccine to control the disease in Brazil. Although a rare event, vaccine-associated cases of the disease occur particularly with type 2 and type 3 strains in Brazil and other countries (Kew *et al.*, 1981; Minor *et al.*, 1982; WHO, 1982; Nkowane *et al.*, 1987; Fiore *et al.*, 1987), and mutations increasing the neurovirulence of these isolates were identified (Cann *et al.*, 1984; Evans *et al.*, 1985; Minor *et al.*, 1989; Pollard *et al.*, 1989; Equestre *et al.*, 1991; Muzychenko *et al.*, 1991; Macadam *et al.*, 1989, 1991, 1993). Recent studies confirmed that type 1 vaccine-associated cases also occur and mutations were also observed in these isolates (Otelea *et al.*, 1993; Furione *et al.*, 1993; Guillot *et al.*, 1994; Groom *et al.*, 1994). Knowledge of the molecular basis of attenuation and reversion towards neurovirulence of the Sabin strains (Almond, 1987; Racaniello, 1988; Minor, 1992, 1993; Minor *et al.*, 1993), may allow rational improvement of vaccines (WHO, 1990a; Agol, 1993) and production methods (Chumakov *et al.*, 1994), providing alternative models for vaccine safety tests on transgenic mice (Ren *et al.*, 1990; Koike *et al.*, 1991) and/or molecular approaches (Chumakov *et al.*, 1991), avoiding costly safety testing of vaccine pools in primates.

Polioviruses are members of the *Enterovirus* genus, belonging to the *Picornaviridae* family and consist of an icosahedrical particle composed of 60 copies of each of four capsid proteins (Hogle *et al.*, 1985), VP1 to VP4, surrounding a single-stranded positive-sense RNA genome of approximately 7500 nt (Kitamura *et al.*, 1981). The RNA molecule contains 5'NCR of about 740 nt with a terminally

linked protein (VPg), preceding a single open reading frame coding for the structural and non-structural proteins, and terminates in a 3'-noncoding region of about 70 nt followed by a poly(A)tract (Kitamura *et al.*, 1981; for a review see Wimmer and Nomoto, 1993). Molecular studies demonstrated that the neurovirulent P1/Mahoney strain (Kitamura *et al.*, 1981; Racaniello and Baltimore, 1981), precursor of the attenuated P1/Sabin strain, differs from the P1/Sabin strain by many mutations (Nomoto *et al.*, 1982; Toyoda *et al.*, 1984), and it was demonstrated that many of these mutations are involved in the attenuation of the P1/Sabin strain (Omata *et al.*, 1986). The subsequent studies demonstrated that G at nt 480 of 5'NCR and C at nt 6203 of 3Dpol are important for attenuation of the P1/Sabin strain (Kawamura *et al.*, 1989; Christodoulou *et al.*, 1990; Martin *et al.*, 1991; Tardy-Panit *et al.*, 1993; Horie *et al.*, 1994). A G→A reverse mutation at nt 480 in the 5'NCR (Otelea *et al.*, 1993; Guillot *et al.*, 1994) was found in all five P1/Sabin-derived isolates from vaccine-associated cases, while a C→U reverse mutation at nt 6203 leading to a His→Tyr substitution at aa 73 of the viral RNA polymerase was observed in just one out of these five isolates (Otelea *et al.*, 1993). The mutation at nt 6203 (3Dpol) was also observed in Sabin-derived recombinant isolates from vaccine-associated cases (Furione *et al.*, 1993). U→C mutation at nt 525 (5'NCR) and C→U mutation at nt 6203 (3Dpol) were found in P1/Sabin neurovirulent revertants selected at high temperature (Christodoulou *et al.*, 1990). Vaccine lots of the P1/Sabin strain with an increased amount of G→A (480) and U→C (525) revertants had a higher neurovirulence (Rezapkin *et al.*, 1994).

In this study P1/Sabin-related viruses isolated from paralysis cases were partially sequenced with the objective of finding mutations at nt 480, 525 and 6203, which are important for reversion towards neurovirulence.

Materials and Methods

Faecal samples from paralysis cases were collected from different Brazillian regions (Table 1) and were processed according to standard procedures (WHO, 1990b).

Cells of the RD line used in this study were made available by CDC, Atlanta, GA, USA.

Virus isolation, titration and identification. Viruses were isolated, grown and titrated in RD cells at 37 °C. Virus serotypes were identified by neutralization test with hyperimmune equine sera.

Molecular hybridization and polymerase chain reaction (PCR). The relationship of the isolates to the P1/Sabin strain was demonstrated by molecular hybridization of the RNA of the isolates with a P1/Sabin-specific probe (Da Silva *et al.*, 1991). Confirmation of the P1/Sabin relationship of the isolates was performed by PCR using a pair of specific primers for P1/Sabin-related isolates (Yang *et al.*, 1991).

Nucleotide sequence analysis. Viral genomic RNA was partially sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977; Zimmern and Kaesberg, 1978) using avian myeloblastosis virus reverse transcriptase and synthetic oligonucleotide primers:

primer a (539-565): 5'-ACACGGACACCCAAAGTAGTCGGTTCC-3'

primer b (6370-6398): 5'-AUAUCUCUCUUCUUCUUUCCCAUUGCUAC-3'.

Numbers in parenthesis indicate genomic intervals (nt) that are complementary to the primers.

The last two virus passages cited in the passage history (Table 1) were done with the objective of increasing the viral titer for posterior sequencing.

Results

Isolation and identification of type 1 (Sabin-related) polioviruses

Type 1 polioviruses were isolated from faecal specimen of patients presenting paralysis. Three cases of paralysis with sequels and one case of transient paralysis were classified as GBS, one case of paralysis with sequels was classified as poliocompatible, one case of transient paralysis was classified as a neuroviral disease, and in other two cases transient facial paralysis was observed. A summary of the clinical and epidemiological data collected is reported in Table 1.

Polioviruses were isolated in RD cells and all were neutralized by type 1 hyperimmune equine sera. RNA of all the isolates hybridized specifically with a P1/Sabin-specific probe and with an enterovirus group-specific probe. The isolates were also analyzed by PCR and the RNA of all the isolates was amplified by a pair of P1/Sabin-specific oligonucleotides and by a pair of oligonucleotides specific for the enterovirus group. These results demonstrated the vaccine origin of the isolates and their relationship to the P1/Sabin strain.

Although the isolates were collected from stool and may not be the etiological agent of the disease, the possibility that the isolates caused the disease could not be excluded. P1/2360 was isolated from a transient paralysis case classified as GBS. The last vaccine dose was given 31 days before the onset of motor deficiency and the isolate was collected 30 days after the onset of motor deficiency. The interval between the last dose and the isolation of P1/2360 was two months suggesting that the virus could have been replicating in the patient during this period.

P1/2938 was isolated from a paralysis case with sequels classified as GBS. Although the time of last vaccine dose of the 14 year-old patient was unknown, the isolate was collected 17 days after the onset of motor deficiency. The age of the patient suggests that he got the last vaccine dose prob-

Table 1. Epidemiological data on children, from which P1/Sabin-related strains were isolated

Patient	Age (years)	Vaccination		Isolated virus	Origin[c]/ year of isolation	Date of onset of illness	Date of onset of motor deficiency	Date of collection of samples	Clinical background	No. of passage
		No. of doses	Last dose							
J.K.O.	4	7	10/06/89	P1/2360	SP/89	02/07/89	11/07/89	10/08/89	Transient paralysis, GBS	5
M.D.	3	-	-	P1/2933	PE/90	08/06/90	08/06/90	11/07/90	Transient paralysis[a]	5
C.O.S.	3	4	25/08/92	P1/10024	PR/92	20/08/92	20/08/92	03/09/92	Paralysis with sequels[b]	4
L.N.L.	5	4	15/08/87	P1/2746	DF/90	10/06/90	11/06/90	22/06/90	Transient facial paralysis	4
A.Q.C.	14	-	-	P1/2938	BA/90	07/07/90	08/07/90	25/07/90	Paralysis with sequels, GBS	4
T.A.S.	1	2	31/08/91	P1/8879	RJ/92	11/05/92	13/05/92	16/05/92	Paralysis with sequels, GBS	4
A.A.S.	2	5	15/06/91	P1/5838	PR/91	17/06/91	17/06/91	22/06/91	Transient facial paralysis	5
M.F.C.	9	4	19/09/84	P1/7404	RJ/91	10/11/91	17/11/91	26/11/91	Paralysis with sequels, GBS	4

(-) Data not available.
[a]The case classified as a neuroviral disease.
[b]The case classified as polio-compatible.
[c]States in Brazil: SP - Sao Paulo, PE - Pernambuco, PR - Parana, DF - Distrito Federal, BA - Bahia, RJ - Rio de Janeiro.

ably as a baby 13 years ago. The results suggest a persistent infection or the transmission of a P1/Sabin-derived strain to the patient.

P1/7404 was isolated from a paralysis case with sequels classified as GBS. The last vaccine dose was given 7 years before the onset of motor deficiency and the isolate was collected 9 days after the onset of motor deficiency. The interval between the last dose and the isolation of P1/7404 was more than 7 years, suggesting a persistent infection or the transmission of a P1/Sabin-derived strain to the patient.

P1/8879 was isolated from a paralysis case with sequels classified as GBS. The last vaccine dose was given almost 9 months before the onset of motor deficiency and the isolate was collected 3 days after the onset of motor deficiency. The interval between the last dose and the isolation of P1/8879 was almost 9 months, also suggesting a persistent infection or the transmission of a P1/Sabin-derived strain to the patient.

P1/2746 was isolated from a transient facial paralysis case. The last vaccine dose was given almost 3 years before the onset of facial paralysis and the isolate was collected 11 days after the onset of facial paralysis. The interval between the last dose and the isolation of P1/2746 was almost 3 years, also suggesting a persistent infection or the transmission of a P1/Sabin-derived strain to the patient.

P1/5838 was isolated from a transient facial paralysis case. The last vaccine dose was given 2 days before the onset of facial paralysis and the isolate was collected 5 days after the onset of facial paralysis. The interval between the last dose and the isolation of P1/5838 was 7 days. As the last vaccine dose was 2 days before the onset of facial paralysis, the isolate may not be the etiological agent of the disease.

P1/2933 was isolated from a transient paralysis case classified as a neuroviral disease. The data referring to vaccination was unknown. The isolate was collected 33 days after the onset of motor deficiency. The possibility that the virus caused the disease could not be totally excluded.

P1/10024 was isolated from a paralysis case with sequels classified as polio-compatible. The last vaccine dose was given 5 days after the onset of motor deficiency, suggesting that the virus was probably not the etiological agent of the

disease. The isolate was collected 13 days after the onset of motor deficiency. The interval between the last dose and the isolation of P1/10024 was 8 days.

Sequence analysis of type 1 poliovirus isolates (P1/Sabin-derived)

The nucleotides at position 481 and 525 of the 5'NCR and at the codon of aa 73 of the 3Dpol coding region of the P1/Sabin strain (Nomoto *et al.*, 1982; Toyoda *et al.*, 1984) are shown and compared with other P1/Sabin-derived isolates from Brazil (Table 2).

Table 2. Nucleotides at positions 480 and 525 in the 5'NCR and in the codon of amino acid 73 of the 3Dpol coding region of the P1/Sabin strain and the P1/Sabin-related isolates

Isolate	5'NCR (nt)		3Dpol	
	480	525	codon	aa 73
P1/Sabin	G	U	CAC	His
P1/2360	G	U	CAC	His
P1/2933	G	U	CAC	His
P1/10024	G	U	CAC	His
P1/2746	G	C	CAC	His
P1/2938	A	U	CAC	His
P1/8879	A/G	U/C	CAC	His
P1/5838	A	U	CAC	His
P1/7404	A	U	TAC	Tyr

The four isolates from cases classified as GBS were analyzed. P1/7404 isolated from a paralysis case with sequels presented G→A mutation at nt 480 of the 5'NCR and C→U mutation at nt 6203 leading to His→Tyr substitution at aa 73 of the viral RNA polymerase. P1/8879 isolated from a paralysis case with sequels presented G and A at nt 480, and C and U at nt 525, and maintained C at nt 6203, indicating that at least two subpopulations were present. P1/2938 isolated from a paralysis case with sequels, presented G→A mutation at nt 480 and maintained U (525) and C (6203). P1/2360 isolated from a transient paralysis case maintained G (480), U (525) and C (6203). As the interval between the last vaccine dose and the isolation of P1/29360 was 2 months, it is very interesting that the isolate maintained G (480) and U (525).

The two isolates from transient facial paralysis cases were analyzed. P1/2746 presented U→C mutation at nt 525 and P1/5838 G→A mutation at nt 480.

P1/10024 isolated from a paralysis case with sequels classified as polio-compatible, maintained G (480), U (525) and C (6203). P1/2933 isolated from a transient paralysis case classified as a neuroviral disease, also maintained G (480), U (525) and C (6203).

Discussion

Although mutations increasing the replication level and neurovirulence of the Sabin strains are important for the establishment of the disease (Macadam *et al.*, 1989, 1991, 1993; Otelea *et al.*, 1993), genomic recombination could perhaps also increase the neurovirulence of the Sabin strains (Lipskaya *et al.*, 1991; Furione *et al.*, 1993), while mutations in antigenic sites (Fiore *et al.*, 1987; Macadam *et al.*, 1989; Minor *et al.*, 1989) could act as an escape mechanism. The isolation of Sabin-derived isolates also presenting reverse mutations from healthy vaccinees (Macadam *et al.*, 1989, 1991, 1993), has suggested that host factors as immune deficiences (WHO, 1982; Nokwane *et al.*, 1987; Zuckerman *et al.*, 1994; Groom *et al.*, 1994), caused perhaps in certain cases by protein/calorie malnutrition (Arya, 1994), deficiency of vitamin A (Arya, 1994) or HIV infection (Wyatt, 1994; Arya, 1994), could also be involved in the establishment of the disease, although other immunodeficiences and other host factors or pathological conditions could also be involved (Agol, 1991). The mutation at nt 480 (Minor and Dunn, 1988; Dunn *et al.*, 1990; Muzychenko *et al.*, 1991; Ogra *et al.*, 1991; Guillot *et al.*, 1994) and 525 (Muzychenko *et al.*, 1991) were also observed in P1/Sabin-derived isolates after passage through the human gut of healthy vaccinees (see also Agol, 1993), demonstrating that although these reverse mutations occur in healthy vaccinees and vaccine-associated cases (Otelea *et al.*, 1993; Guillot *et al.*, 1994), only in rare cases the disease is established.

As nt 480 pairs with nt 525 in a predicted secondary structure of the 5'NCR (Pilipenko *et al.*, 1989; Skinner *et al.*, 1989; Hellen *et al.*, 1994), the mutation at nt 480 or 525 could act by restablishing the base paring between these two nucleotides and the secondary structure of the 5'NCR (Kawamura *et al.*, 1989; Christodoulou *et al.*, 1990; Muzychenko *et al.*, 1991; Agol, 1991; Macadam *et al.*, 1992; Minor, 1992, 1993; Tardy-Panit *et al.*, 1993; Minor *et al.*, 1993), favoring its interaction with host factors necessary for translation initiation (Svitkin *et al.*, 1988; Agol, 1991), reestablishing an efficient translation level of the P1/Sabin strain (Muzychenko *et al.*, 1991), increasing its replication level and neurovirulence. The His→Tyr substitution at aa 73 of the viral RNA polymerase could act by increasing the efficiency of the synthesis of viral RNA (Christodoulou *et al.*, 1990; Tardy-Panit *et al.*, 1993).

Although the P1/Sabin-derived isolates may not be the etiological agent of the disease, they were analyzed for the

presence of important reverse mutations able to increase neurovirulence. Although P1/10024, that maintained G (480), U (525) and C (6203), was isolated from a polio-compatible case, the patient received the last dose of the vaccine five days after the onset of the disease, what suggests that it might not be the etiological agent of the disease. P1/2360 isolated from a transient paralysis case classified as GBS, also maintained G (480), U (525) and C (6203). As the patient received the last vaccine dose 31 days before the onset of motor deficiency, the possibility that this isolate caused the disease could not be excluded. P1/2933 that also maintained G (480), U (525) and C (6203), was isolated from a transient paralysis case classified as a neuroviral disease, suggesting that the isolate might be the causative agent of the disease. May not be other mutations occuring in the 5'NCR, able to suppress the attenuating G at nt 480, or certain biochemical characteristics of host factors interacting with this region could select isolates maintaining G at nt 480 in some cases. Different cell lines have demonstrated different selective pressures on these reverse mutations in the 5'NCR (Chumakov *et al.*, 1994; Rezapkin *et al.*, 1994), what strenghtens this suggestion. Another possibility is that mutations in the 2A protease coding region, involved in cap-independent translation, could suppress the effect of the attenuating determinants in the 5'NCR (Minor *et al.*, 1993; Macadam *et al.*, 1994). The observation of isolates not presenting mutations at nt 480 or 525 suggests that isolates maintaining G at nt 480 and U at nt 525 could perhaps also replicate in the central nervous system of primates in some cases, causing a transient paralysis, although in a lower frequency.

P1/7404 presented reverse mutations at nt 480 and 6203, P1/2746 a mutation at nt 525, P1/5838 a mutation at nt 480, P1/2938 a mutation at nt 480, and P1/8879 a mutation at nt 480 and 525 (reflecting the presence of at least two subpopulations); it can be assumed that these isolates display an increased neurovirulence. P1/7404 was isolated from a paralysis case with sequels classified as GBS 7 years after the last dose of the vaccine, and P1/2746 was isolated from a transient facial paralysis case three years after the last dose of the vaccine, suggesting that these isolates were transmitted to the patients. P1/2938 was isolated from a paralysis case with sequels classified as GBS, and although the time of last dose was unknown, the patient was 14 year-old when the disease occured and the virus was isolated, also suggesting a transmission of the P1/Sabin strain to the patient. As P1/8879 was isolated from a paralysis case with sequels classified as GBS 9 months after the last dose of the vaccine, it also suggests a transmission or a persistent infection caused by the P1/Sabin strain. There are studies demonstrating the capacity of P1/Sabin-derived isolates with mutations at nt 525 or elsewhere to cause a persistent infection *in vitro* (Pelletier *et al.*, 1991; Borzakian *et al.*, 1993). The isolation of P1/Sabin-derived isolates from facial paralysis and GBS cases some days or weeks after the onset of the disease demonstrated a temporal association between the disease and the P1/Sabin strain. Although the P1/Sabin-derived isolates from paralysis cases examined in Brazil were collected from the stool and may not be the etiological agent of the disease, the possibility that some of the isolates caused the disease could not be excluded, because a temporal association between the isolation of the P1/Sabin-derived isolates and the disease was observed. As many (71) enteroviruses were isolated from GBS and facial paralysis cases in Brazil (unpublished results), it strengthens the association of enterovirus infections with the appearance of GBS and facial paralysis.

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