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Evidence for nonpoliovirus enterovirus multiplication in L20B cells

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SUMMARY

Stool specimens collected from 1 515 healthy children following a mass vaccination campaign in Cuba were tested for poliovirus excretion using L20B cell lines. In spite of the selectivity of this cell line for polioviruses (117/129; 90.7 %) some other nonpolio enteroviruses (12/129; 9.3 %), such as coxsackie A virus types 4, 8 and 10, can grow in L20B cells.

Key words: Poliovirus, L20B cell line, coxsackievirus, eradication.

Although recent developments in molecular detection technology make it probable that enterovirus diagnosis/surveillance will increasingly be achieved by non-culture-based methods, culture of poliovirus from clinical samples is the gold-standard method for virological surveillance in the worldwide initiative to eradicate wild type poliovirus.[1]

Since 1999, the WHO Global Polio Laboratory Network has been using L20B cell lines for the isolation of poliovirus for acute flaccid paralysis surveillance. L20B is a transgenic mouse cell line that expresses the human poliovirus receptor on the surface (CD155). The expression of the receptor at the cell surface renders L20B cells susceptible to infection with poliovirus. Furthermore, the CD155 receptor is not shared with other enteroviruses, thus making L20B cells highly specific for poliovirus isolation.[2,3]

Previous studies suggested that L20B cells were highly selective for poliovirus detection, reporting that only coxsackievirus B4 and reovirus type 2, among the viruses other than poliovirus, produced late cytopathic effect (CPE) in this cell line.[4-6] More recently, studies from India reported that the L20B cell line is susceptible to productive infection by group A coxsackieviruses.[7]

In 1998, as part of collaborative studies with WHO in order to know the circulation of vaccine poliovirus, the L20B cell line was introduced in Cuba.[8,9] In the present study we show the results of the use of this cell line in Cuba.

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METHODS

Samples and virus isolation

Stool specimens were collected from 1 515 apparently healthy children following a mass vaccination campaign in Havana, Cuba. The samples were tested for poliovirus excretion using RD (passages 230 to 240) and L20B (passages 25 to 35) cells. Both cell lines were obtained from Dr. DJ Wood, NIBSC, Potters Bar, United Kingdom. Standard procedures described in the laboratory manual for the WHO Global Polio Laboratory Network were used for the propagation of cell lines and for virus culture.[10] All virus isolates from RD cells were passaged in L20B cells. For samples showing CPE in L20B cells, poliovirus serotypes were identified by neutralization with poliovirus typing antisera.[10] Viruses other than poliovirus were tested in reverse transcription--polymerase chain reaction (RT-PCR) assays using pan-enterovirus and pan-poliovirus primers.[11,12] Enterovirus isolates, i.e., pan-enterovirus RT-PCR--positive but pan-poliovirus RT-PCR-negative isolates, were taken for virus neutralization tests using Lim-Benyesh-Melnick serum pools A to H for the identification of enterovirus.[10,13]

Molecular typing of enterovirus isolates

To identify the serotypes of the untypeable enterovirus-like viruses by a method other than the neutralization assay, the partial VP1 sequences of the untypeable strains was determined by the use of primer pairs 012 and 011 or 040 and 011 as described by Oberste et al.[14,15] The sequences were BLAST searched for closely similar sequences in the GenBank using online computer program (www.ncbi.nlm.nih.gov/BLAST/). A serotype was identified when sequence similarity of more than 75 % was found with a known enterovirus sequence in the GenBank.[14] Amino acid sequence homology of more than 88 % was used to identify the serotypes.[14]

Phylogenetic analysis

A phylogenetic tree based on the partial VP1 nucleotide sequence was constructed by the neighbor-joining method.[16] The reliability of the neighbor-joining tree was estimated by bootstrap analysis with 1 000 pseudoreplicate data sets.

Remicroneutralization tests

According to results of enterovirus phylogenetic analysis, remicroneutralization tests using in house monospecific rabbit antiserum were performed to confirm the serotype of the untypeable strains.

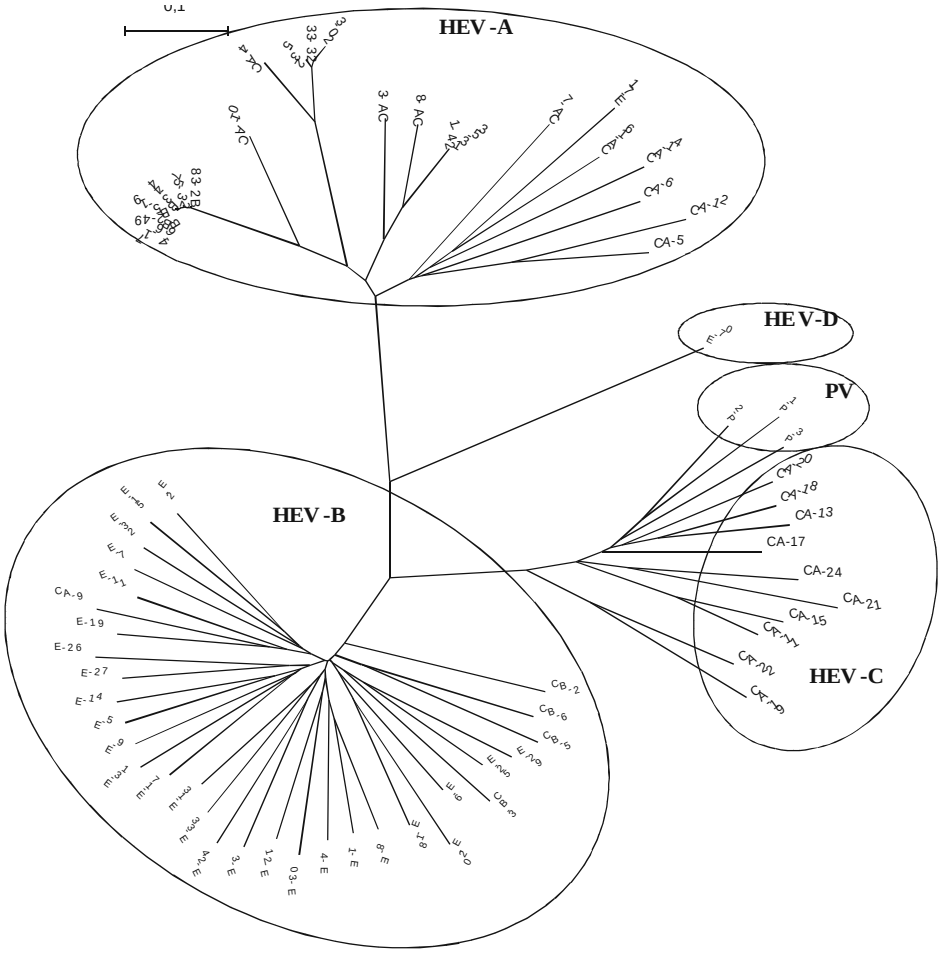
RESULTS

On primary inoculation of RD cells, a substantial proportion (512/1 515; 33.8 %) of isolates was obtained. After the passages onto L20B cells, a total of 129 isolates was obtained. Of these 117/129 (90.7 %) were identified as poliovirus by the neutralization test with poliovirus typing antisera. The remaining 12 isolates were not neutralized by poliovirus antisera. None of the 12 isolates was amplified by using RT-PCR with pan-poliovirus primers. However, all of these isolates gave a positive RT-PCR with pan-enterovirus primers and were therefore grouped as nonpolio enteroviruses (NPEV).

Out of the 12 isolates, 10 of them showed CPE initially only in RD cells and grew in L20B cells after passage from RD to L20B cells. After passage to L20B cells from the initial RD isolates, the CPE progressed to involve the whole monolayer after 3 to 4 days of incubation. Only two viruses other than poliovirus produced CPE after primary inoculation of L20B and RD cells. The isolates that initially grew in both cells produced 10 to 100 fold higher titers in RD cells than L20B cells (data not shown).

The identity of the 12 NPEV isolates could not be determined by using Lim-Benyesh-Melnick serum pools A to H. The nucleotide sequences of all untypeable isolates were 78 to 86 % identical to the sequences of their respective prototype strains, as identified by a BLAST search. The predicted partial VP1 amino acid sequences of all untypeable isolates were more than 95 % identical to that of the homologous prototype strain (table).

A phylogenetic tree based on the partial VP1 nucleotide sequences of the 12 enterovirus-like untypeable strains and 40 prototype human enterovirus strains available from the GenBank database was constructed. The untypeable strains were classified nearest to coxsackie A virus types 4, 8 and 10 (fig.).



PV: poliovirus species; HEV-A: human enterovirus A species; HEV-B: human enterovirus B species; HEV-C: human enterovirus species; HEV-D: human enterovirus species.

Fig. Phylogenetic relationships among 12 untypeable strains and 40 prototype human enterovirus strains, based on partial VP1 gene sequences available from the GenBank database.

TABLE. Correspondence between typing by sequence comparison and by neutralization

Strain	Type	Highest-scoring prototype % nt sequence identity	% aa sequence identity	Neutralization type
2-49*	CVA10	78	96	CVA10
13-53	CVA8	82	96	CVA8
20-3	CVA4	86	95	CVA4
23-5	CVA4	85	97	CVA4
23-33	CVA4	86	97	CVA4
23-57	CVA10	79	96	CVA10
24-1	CVA8	81	96	CVA8
B2-38	CVA10	78	96	CVA10
B3-44	CVA10	79	96	CVA10
B5-19	CVA10	78	96	CVA10
B6-4*	CVA10	78	96	CVA10
B6-17	CVA10	79	96	CVA10

* Primary isolate in L20B cells; nt: nucleotide; aa: amino acid.

The results obtained with the remicro-neutralization tests using in-house monospecific rabbit antiserum against reference strains of coxsackie A virus, types 4, 8 and 10 agreed with the results obtained with partial VP1 nucleotide sequences (table).

DISCUSSION

The results of our studies show that L20B cells were highly sensitive and selective for the growth of polioviruses. Of special interest were the isolations of coxsackie A viruses in this cell line. Most of these isolations (10 of 12) were obtained after the passage from RD to L20B cells, indicating that a high multiplicity of infection is required. It is

noteworthy that only two of the nonpolio strains produced CPE primarily in L20B cells. This may be due to the high initial titer of virus in these samples (data not shown).

Newborn mice were the preferred animal hosts for propagation of group A coxsackieviruses from clinical specimens for many years.[17] As L20B cells are of murine origin, the growth of coxsackie A viruses on L20B cells is not surprising because of the ability of these viruses to use receptors on mouse cells.

L20B cells are not absolutely specific for poliovirus; however, the small number of NPEV that grow in L20B cells does not appear to affect the ability of the WHO Global Polio Laboratory Network to detect poliovirus. NPEV that can grow in L20B cells would not be expected to interfere with the detection of poliovirus. This assumption is based on the fact that the majority of coxsackie A virus isolated in L20B cells required a RD cells passage for initial amplification. L20B cells therefore are more sensitive for poliovirus than for coxsackie A virus.

The selectivity of L20B cells for poliovirus was confirmed by the failure of the vast majority of NPEV to grow in L20B cells. In fact, the use of this line simplified the work by reducing the number of isolates to be identified and considerably shortened the time required for reporting the results. These results consistently emphasize the importance of L20B as a powerful tool for the WHO Global Polio Laboratory Network. Nevertheless, it is worth noting that this cell line supports the growth of others NPEV, such as those that belong to coxsackievirus group A.

Evidencia para multiplicación de enterovirus nopoliovirus en células L20B

RESUMEN

Se estudió la excreción de poliovirus en muestras de heces fecales de 1 515 niños saludables, colectados después de la campaña de vacunación en Cuba con el empleo de células L20B. A pesar de la selectividad de esta línea celular para poliovirus (117/129; 90,7 %), algunos enterovirus nopoliovirus (12/129; 9,3 %), como los coxsackievirus A tipos 4, 8 y 10, pueden crecer en células L20B.

Palabras clave: Poliovirus, línea celular L20B, coxsackievirus, erradicación.

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