Evidence for Other Non-Poliovirus Enteroviruses Multiplies in L20B Cells.

LUIS SARMIENTO®, PEDRO MÁS, ROSA PALOMERA, LUIS MORIER, MAGILÉ FONSECA, AND SONIA RESIK.

Virology Department, PAHO/WHO Collaborating Center for Viral Diseases, “Pedro Kouri” Tropical Medicine Institute, Havana, Cuba

Corresponding author. Mailing address: Virology Department, PAHO/WHO Collaborating Center for Viral Diseases, “Pedro Kouri” Tropical Medicine Institute, Autopista Novia del Mediodía, Km 6, P.O. Box Marianao 13, Havana, Cuba. Phone: (537) 2020450. Fax: (537) 2046051. E-mail: sarmiento@ipk.sld.cu and lsarmientop@yahoo.com
ABSTRACT

Stool specimens collected from 1515 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 non-polio 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 (16).

Since 1999, WHO Global Polio Laboratory Network has been using L20B cell lines for 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). 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 (3, 8).

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 (1, 12, 14). More recently, studies from India reported that the L20B cell line is susceptible to productive infection by group A coxsackieviruses (9).

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 (4, 5). In the present studies we show the results of the use of this cell line in our country.

Stool specimens were collected from 1515 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, UK. Standard procedures described in the Laboratory Manual for WHO Polio Laboratory Network were used for
propagation of cell lines and their use in virus culture (15). 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 (15). Viruses other than poliovirus were tested in reverse transcription-polymerase chain reaction (RT-PCR) assays using pan-enterovirus and pan-poliovirus primers (2, 17). Enterovirus isolates, i.e., pan-enterovirus RT-PCR positive but pan-poliovirus RT-PCR negative, were taken for virus neutralization tests using the Lim-Benyesh-Melnick (LBM) serum pools A-H for the identification of enterovirus (6, 15).

On primary inoculation of RD cells, a substantial proportion (512/1515, 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 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 non-polio enteroviruses (NPEV).

Out of the 12 isolates, 10 of them showed CPE initially only in RD cells and grew in L20B cells on passage from RD to L20B cells. After passage in L20B cells from the initial RD isolates the CPE progressed to involve the whole monolayer after 3 to 4 days incubation. Only 2 viruses, other than poliovirus, produced CPE on primary inoculation of L20B and RD cells. The isolates that initially grew in both cells produced 10-100 fold higher titres in RD cells than L20B cells (data not shown).

The identity of the 12 NPEV isolates could not be determined by using the LBM serum pools A-H. To identify the serotypes of these untypeable enterovirus-like viruses by a method other than the neutralization assay, we determined the partial VP1 sequences of
all 12 strains by use of primer pairs 012 and 011 or 040 and 011 as described by Oberste et al. (10, 11). The nucleotide sequences of all untypeable isolates were 78 to 86 % identical to the sequences of their respective prototype strains as identified by the BLAST search (www.ncbi.nlm.nih.gov/BLAST/). The predicted partial VP1 amino acid sequences of all untypeable isolates were more than 95 % identical to that of the homologous prototype strain (Table 1).

A phylogenetic tree was constructed 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 by the neighbor-joining method (13). The reliability of the neighbor-joining tree was estimated by bootstrap analysis with 1,000 pseudoreplicate datasets. The untypeable strains were classified nearest to coxsackie A virus types 4, 8 and 10 (Figure 1).

According to results of enterovirus phylogenetic analysis, remicroneutralization tests using in house monospecific rabbit antiserum against reference strains of coxsackie A virus, types 4, 8 and 10 were performed to confirm the serotype of the untypeable strains. This method agreed with the results obtained by partial VP1 nucleotide sequences (Table 1).

The results of our studies show that L20B cells were highly sensitive and selective for 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 non-polio strains produced CPE primarily in L20B cells. This may be due to the high initial titre 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 (7). 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 WHO 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 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 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.
TABLE 1. Correspondence between typing by sequence comparison and by neutralization

| Strain   | Type | % nt sequence identity $^a$ | % aa sequence identity $^b$ | Neutralization type |
|----------|------|-----------------------------|-----------------------------|---------------------|
| 2-49*    | CA10 | 78                          | 96                          | CA10                |
| 13-53    | CA8  | 82                          | 96                          | CA8                 |
| 20-3     | CA4  | 86                          | 95                          | CA4                 |
| 23-5     | CA4  | 85                          | 97                          | CA4                 |
| 23-33    | CA4  | 86                          | 97                          | CA4                 |
| 23-57    | CA10 | 79                          | 96                          | CA10                |
| 24-1     | CA8  | 81                          | 96                          | CA8                 |
| B2-38    | CA10 | 78                          | 96                          | CA10                |
| B3-44    | CA10 | 79                          | 96                          | CA10                |
| B5-19    | CA10 | 78                          | 96                          | CA10                |
| B6-4*    | CA10 | 78                          | 96                          | CA10                |
| B6-17    | CA10 | 79                          | 96                          | CA10                |

$^a$ Primary isolates in L20B cells

$^a$ A serotype was identified when sequence similarity of better than 75% was found with a known enterovirus sequence in the GenBank (10).

$^b$ Amino acid sequence homology of better than 88% was used to identify the serotypes (10).
FIGURE 1. Phylogenetic tree showing genetic relationships between the 12 untypeable strains and 40 prototype human Enterovirus strains available from the GenBank database.

PV: Poliovirus species
HEV-A: Human Enterovirus A Species
HEV-B: Human Enterovirus B Species
HEV-C: Human Enterovirus C Species
HEV-D: Human Enterovirus C Species
REFERENCES

1. Hovi, T., and M. Stenvik. 1994. Selective isolation of poliovirus in recombination murine cell line expressing the human poliovirus receptor gene. J. Clin. Microbiol. 32:1366–1368.

2. Kilpatrick, D.R., B. Nottay, C.F. Yang, S.J. Yang, M.N. Mulders, B.P. Holloway, M.A. Pallansch, and O. Kew. 1996. Group-specific identification of polioviruses by PCR using primers containing mixed-based or deoxyinosine residues at positions of codon degeneracy. J. Clin. Microbiol. 34:2990–2996.

3. Koike, S., H. Itoshi, I. Ise, A. Okitsu, M. Yoshida, N. Iizuka, K. Takeuchi, T. Takegami, and A. Nomoto. 1990. The poliovirus receptor is produced both as membrane-bound and secreted forms. EMBO. J. 9:3217–3224.

4. Más, P., V. Caceres, M. Galindo, H. Gary, M. Valcarcel, J. Barrios, L. Sarmiento, I. Avalos, J. Bravo, R. Palomera, M. Bello, R. Sutter, M.A. Pallansch, and C. de Quadros. 2001. Persistence of vaccine-derived Poliovirus following a mass vaccination campaign in Cuba: Implication for stopping polio vaccination after global eradication. Inter. J. Epidemiol. 30:1029-1034.

5. Más, P., H. Gary, L. Sarmiento, V. Caceres, J. Barrios, R. Palomera, M. Bello, P. Jiménez, M.A. Pallansch, and R. Gonzalez. 2003. Poliovirus detection in wastewater and stools following an immunization campaign in Havana. Inter. J. Epidemiol. 32:772–777.

6. Melnick, J.L, V. Rennick, and B. Hampil. 1973. Lyophilized combination pools of enterovirus equine antisera: Preparation and test procedures for the identification of field strains of 42 enteroviruses. Bull. World. Health. Organization. 48:263–268.
7. Melnick, J.L., H.A. Wenner, and L. Rosen. 1964. The enteroviruses, p. 194–245. *In*: E. Lennette, N. Schmidt, (ed.), Diagnostic procedures for viral and rickettsial diseases. American Public Health Association, New York.

8. Mendelsohn, C., B. Johnson, K.A. Lionetti, S. Nobis, E. Wimmer, and V.R. Racaniello. 1986. Transformation of a human poliovirus receptor gene into mouse cells. Proc. Natl. Acad. Sci. USA. **83**:7845–7849.

9. Nadkarni, S.S., and J.M. Deshpande. 2003. Recombinant Murine L20B Cell Line Supports Multiplication of Group A Coxsackieviruses. J. Med. Virol. **70**: 81-85.

10. Oberste, M.S., K. Maher, D.R. Kilpatrick, M.R. Flemister, B.A. Brown, and M.A. Pallansch. 1999. Typing of Human Enteroviruses by Partial Sequencing of VP1. J. Clin. Microbiol. **37**:1288-1293.

11. Oberste, M.S., K. Maher, and M.A. Pallansch. 1998. Molecular phylogeny of all human enterovirus serotypes based on comparison of sequences at the 59 end of the region encoding VP2. Virus. Res. **58**:35–43.

12. Pipkin, P.A., D.J. Wood, V.R. Racaniello, and P.D. Minor. 1993. Characterization of L cells expressing the human poliovirus receptor for the specific detection of poliovirus. J. Virol. Methods. **41**:333–340.

13. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406-425.

14. Wood, D.J., and B. Hull. 1999. L20B cells simplify culture of polioviruses from clinical samples. J. Med. Virol. **58**:188–192.

15. World Health Organization. 2001. Polio laboratory manual. Department of Vaccines and Biologicals. World Health Organization, Geneva.
16. **World Health Organization.** 2002. Manual for the virologic investigation of poliomyelitis. World Health Organization, Geneva.

17. **Yang, C.F., L. De, B.P. Holloway, M.A. Pallansch, and O. M. Kew.** 1991 Detection and identification of vaccine-related polioviruses by polymerase chain reaction. *Virus Res.* **20**:159–179.
FIGURE 1. Phylogenetic tree showing genetic relationships between the 12 untypeable strains and 40 prototype human Enterovirus strains available from the GenBank database.

PV: Poliovirus species
HEV-A: Human Enterovirus A Species
HEV-B: Human Enterovirus B Species
HEV-C: Human Enterovirus C Species
HEV-D: Human Enterovirus C Species