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Group B Coxsackieviruses Readily Establish Persistent Infections in Human Lymphoid Cell Lines

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Exposing human lymphoid cell lines to uncloned or recently cloned group B coxsackieviruses results in the frequent establishment of chronically infected cultures. Persistence is maintained by a carrier culture mechanism involving virus spread through the medium and replication among a minority of cells at any given time. These studies provide a model for persistence by highly cytocidal viruses.

The interactions group B coxsackieviruses (CVB) establish with immunocompetent cells are little understood. The problem is of interest because CVB are important human pathogens, and virus-immunocyte interplay can influence many aspects of infection and disease (3). CVB-infected mice are immunosuppressed (4) and present a generalized depletion of lymphoid organs (18). Here, we examined the response of selected human lymphoid cell (LC) lines to infection by the six CVB immunotypes.

CVB were obtained, and virus titers were obtained as described previously (4). LC suspension cultures were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum and 100 μg of gentamicin per ml. They were infected at a multiplicity of 5, washed thoroughly, adjusted to 5×10^5 or 2×10^5 (Ramos) viable cells per ml, and incubated in 5 ml of medium per flask in plastic 25-ml T-flasks (Corning Glass Works, Corning, N.Y.). Viable cell density and viral titer of supernatants were determined at intervals postinfection (p.i.).

TABLE 1. Response of lymphoid cell lines to CVB infection

Infecting virus	Virus yield and cell density[a]									
	MOLT-4		CCRF-CEM		Ramos		Jiyoye		P3HR-1	
	Virus	Cells	Virus	Cells	Virus	Cells	Virus	Cells	Virus	Cells
CVB-1	5.2	115	1.3	96	6.3	37	5.3	115	5.3	6
CVB-2	5.0	88	<0.3	112	3.6	22	1.3	131	3.6	31
CVB-3	5.5	85	2.6	121	6.3	33	5.5	15	4.5	6
CVB-4	3.6	104	2.5	121	6.5	81	1.5	115	1.6	24
CVB-5	3.5	131	<0.3	117	4.8	18	4.3	114	5.5	12
CVB-6	4.0	0	<0.3	96	4.0	0	5.0	0	3.6	0

[a] Virus yields at day 3 (Ramos) or 4 (other lines) p.i., expressed as log 50% tissue culture infective dose per 0.1 ml of supernatant. Viable cell density expressed as percent uninfected cultures. Control values (no. of cells $\times 10^6$ per ml) were: MOLT-4, 2.6; CCRF-CEM, 2.4; Ramos, 2.7; Jiyoye, 2.6; P3HR-1, 1.6.

The response of the five LC lines examined to infection varied. The CCRF-CEM line completely restricted CVB-2, CVB-5, and CVB-6. All the other virus-cell combinations were permissive, but there was considerable variation in viral yields (Table 1), in the time of appearance of progeny virus (it was present at 4 h p.i. only in some combinations), and in the time titers peaked (which ranged between day 1 and 4). Substantial variation was also seen in the effects on culture viability (Table 1). While CVB-6 caused complete destruction of all the permissive lines, the other immunotypes caused partial to minimal damage. Although the number of lines tested is too low to draw general conclusions, T-cell lines (CCRF-CEM, MOLT-4) appeared somewhat more resistant than B-cell lines, and the response of the latter appeared to be independent of the presence (Jiyoye, P3HR-1) or absence (Ramos) of the Epstein-Barr virus genome, which reportedly influences susceptibility to other viruses (7). A further level of variation was observed when LCs were exposed to different viral clones (Table 2).

Several days p.i., many infected cultures showed no changes in viability or proliferation, despite the production of high virus titers. Since these features were compatible with the establishment of persistence, we determined whether such cultures could be propagated while supporting virus growth. The cultures were split at 1 week p.i. and every 3 to 4 days thereafter. Invariably, they continued to grow and to shed virus for prolonged periods of time.

Figure 1 shows how virus production and cell proliferation proceeded in MOLT-4 cells. Several points emerge. First, there were wide fluctuations in virus titers, but these were not regularly cyclical nor temporally related to subculturing, as reported for other persistently infected lines (8, 9, 19). CVB-1 to CVB-4 stabilized at relatively high titers over 1 to 2 months of passaging, while CVB-5 fluctuated for the entire period of persistence. Second, the fluctuations of cellularity observed during the first 4 to 6 weeks were unrelated to the

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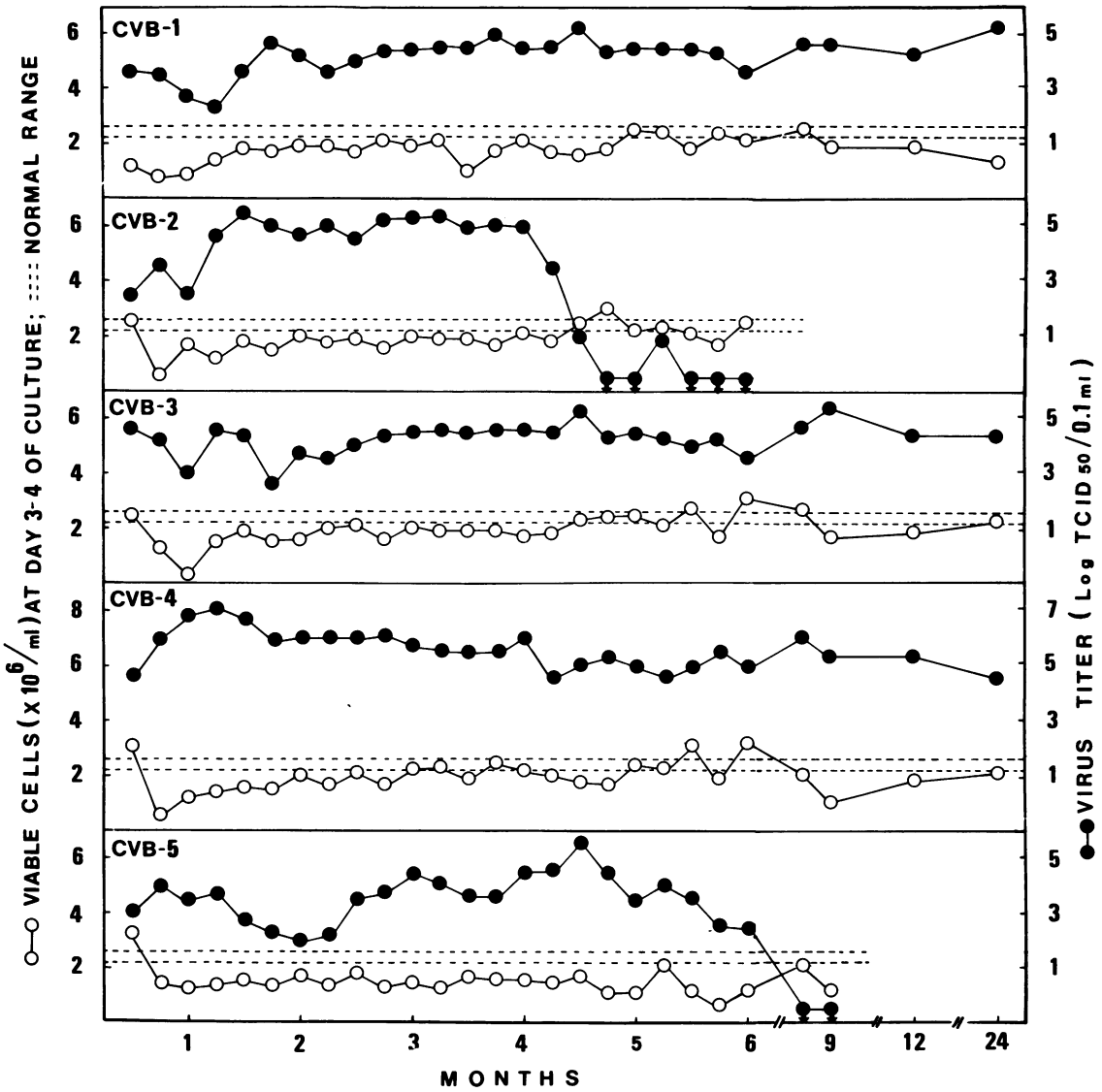

FIG. 1. Viral yields and viable cell densities in MOLT-4 cells persistently infected with CVB-1 to CVB-5 at various times from infection. The cultures were examined for cell number and viability daily and for virus content in the supernatant before each subculture. Periodically, virus identity was verified by neutralization with reference antisera. $TCID_{50}$, 50% tissue culture infective dose.

viral bursts and showed no predictable regularity. Third, the sublines infected with CVB-2 and CVB-5 abruptly stopped producing virus at 4.5 and 8.5 months, respectively. There were no apparent environmental explanations for such spontaneous cures, since sister cultures infected with the other CVB continued shedding the usual viral yields and are still producing after more than 2 years (256 passages).

In periodical infectious center assays (18), only a small proportion of cells scored as positive (Table 3). Furthermore, the lines were readily cured by the addition of virus-specific antisera. Thus, persistence appeared to be maintained by a carrier culture mechanism involving virus spread through the medium and replication among a minority of cells at any given time.

At least in the MOLT-4 cells, several factors believed to be instrumental for initiation and maintenance of the carrier state (10, 14, 22) do not appear to play a significant role. Despite an exhaustive search (see, for example, Fig. 2C), no viral inhibitors or interferon were detected in uninfected and acutely or chronically infected cultures (but exogenously added interferon rapidly terminated persistence). A significant role of soluble factors has been excluded also in LCs persistently infected by other viruses (6, 15). Involvement of defective interfering particles (12) also seems unlikely because recently cloned CVB initiated persistence effectively and long-term virus killed the original MOLT-4 cells very rapidly.

Consistent with the dynamic nature of persistence in vitro (22), the carrier cells became more resistant to homologous and heterologous CVB, and the virus underwent changes in plaquing, ability to grow at supraoptimal temperatures, virulence for LCs, and animal pathogenicity (data not shown). However, such changes developed gradually, while the conditions leading to persistence were already estab-

TABLE 2. Response of MOLT-4 cells to plaque-purified clones of CVB-3

Viral clone[a]	Virus yield and cell density at incubation temp of[b]:			
	37°C		39.5°C	
	Virus	Cells	Virus	Cells
L-1	6.5	89	5.3	18
L-2	6.0	14	6.3	91
L-3	5.6	64	5.5	64
L-4	6.3	70	5.5	64
L-5	6.3	50	5.3	82
L-6	6.5	30	5.5	20
L-7	6.3	33	5.3	20
L-8	5.5	27	5.5	30
L-9	6.3	89	3.5	103
L-10	5.6	100	3.6	55
S-1	6.0	78	5.0	45
S-2	6.5	93	6.3	109
S-3	5.6	89	0.5	132
S-4	6.3	86	5.5	73
S-5	5.3	43	5.5	134
S-6	6.5	50	4.5	29
S-7	6.5	63	4.6	90
S-8	6.3	66	5.0	29

[a] Clones were isolated by double-plaque purification, grown at a low multiplicity of infection on KB monolayers, and used at passage 2. L, Large plaques on KB cells; S, small plaques.

[b] Virus yield at day 4 p.i., expressed as log 50% tissue culture infective dose per 0.1 ml of supernatant. Viable cell density at day 4 p.i., expressed as percent uninfected cultures. Control value, 2.5×10^6 cells per ml.

lished within a few days p.i. (complete cytolysis in various virus-LC combinations within this time). Thus, selection of cellular and viral mutants may have contributed to the stabilization and continuation of the carrier state but does not seem to be mandatory for the early stages of persistence (1).

Based on available data, CVB persistence in LC seems to be due to the presence and continuous replenishment of a small fraction of susceptible variants among a population of otherwise intrinsically resistant cells. The only finding difficult to reconcile with this conclusion is that eight of nine clones isolated from uninfected MOLT-4 cells were completely lysed by CVB-3 (Fig. 2). The result might, however, be explained by a higher cloning efficiency of susceptible, as compared with resistant, cells. LCs cannot be easily cloned anyway (21), and persistently infected cells proved absolutely refractory to cloning. In any case, the fact that fully susceptible cells could be cloned indicates that permissiveness is genetically determined rather than the expression of a specific moment(s) in the cell cycle.

TABLE 3. Proportion of cells scoring as infectious centers in the MOLT-4 line persistently infected with CVB-3

Time from infection	Infectious centers/10^4 cells	Infectious center size[a]
4 h	270	80% large, 20% small
4 mo	270	100% small
8 mo	45	100% micro
9 mo	40	100% micro
12 mo	10	100% micro

[a] The virus evolved during persistence (manuscript in preparation). One of the changes was in the size of plaques on KB monolayers. Infectious center size varied concomitantly; large, 2 to 4 mm in diameter; small, 0.5 to 1 mm in diameter; micro, foci of 10 to 20 dead cells as observed under the microscope.

This study extends the already long list of viruses that have been shown to persist in LC lines (2, 5, 6, 8, 15). The remarkable propensity of LCs to sustain viral persistence might have important implications. For example, it might be related to the high frequency whereby lymphoid tissues represent reservoirs of virus in chronically infected hosts (3). To date, there are no specific data regarding the capacity of CVB to persist in vivo. However, the lifelong antibody responses observed in CVB-infected humans might be accounted for by repeated antigenic stimulation but also by some kind of latency. On the other hand, in vivo long-term or latent virus infections may be extremely difficult to demonstrate (13), and accumulating evidence indicates that related picornaviruses can give prolonged infections (11, 16, 20, 22). Understanding how CVB-sensitive cells are continuously generated by LCs may be relevant to uncovering in vivo persistence by highly cytocidal viruses.

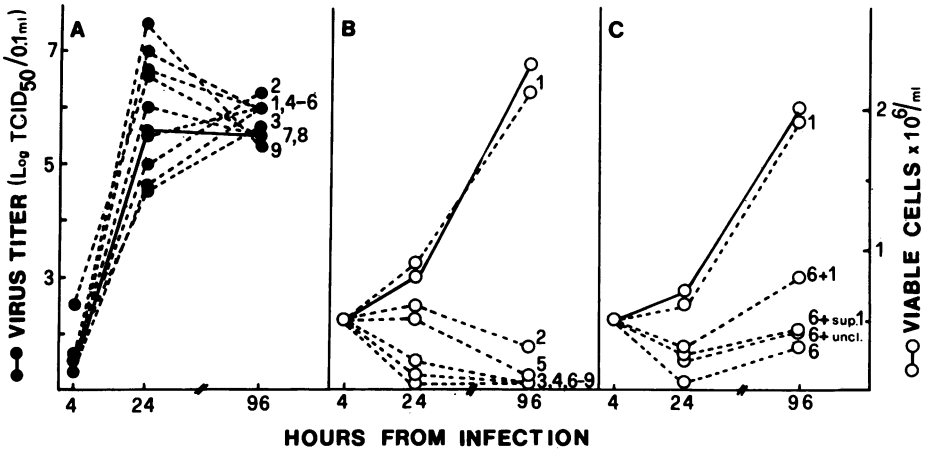

FIG. 2. Viral yields (A) and viable cell densities (B) in clones of MOLT-4 cells infected with CVB-3. The cell clones were obtained by the standard soft-agar technique (17), using conditioned medium to increase efficiency. Each broken line represents a different clone, and the continuous line represents the original uncloned MOLT-4 line. In panel C the susceptibility of clone 6 to CVB-3 was not affected by the addition of 10% cells or supernatant from the resistant clone 1 or from the parental uncloned line. $TCID_{50}$, 50% tissue culture infective dose.

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LITERATURE CITED

1. **Ahmed, R., R. S. Kauffman, and B. N. Fields.** 1983. Genetic variation during persistent reovirus infection: isolation of cold-sensitive and temperature-sensitive mutants from persistently infected L cells. Virology **131**:71–78.
2. **Andiman, W. A., and G. Miller.** 1982. Persistent infection with adenovirus types 5 and 6 in lymphoid cells from humans and woolly monkeys. J. Infect. Dis. **145**:83–88.
3. **Bendinelli, M.** 1984. Immunomodulation in viral infections: virus or infection-induced?, p. 161–195. *In* H. H. Fudenberg, H. D. Whitten, and F. Ambrogi (ed.), Immunopotentiation. New frontiers and advances. Plenum Publishing Corp., New York.
4. **Bendinelli, M., D. Matteucci, A. Toniolo, A. M. Patanè, and M. P. Pistillo.** 1982. Impairment of immunocompetent mouse spleen cell functions by infection with coxsackievirus B3. J. Infect. Dis. **146**:797–805.
5. **Carp, R. I.** 1981. Persistent infection of human lymphoid cells with poliovirus and development of temperature-sensitive mutants. Intervirology **15**:49–56.
6. **Creager, R. S., J. J. Cardamone, and J. S. Youngner.** 1981. Human lymphoblastoid cell lines of B- and T-cell origin: different responses to infection with vesicular stomatitis virus. Virology **111**:211–222.
7. **Creager, R. S., P. Whitaker-Dowling, T. K. Frey, and J. S. Youngner.** 1982. Varied responses of human B-lymphoblastoid cell lines to infection with vesicular stomatis virus. Virology **121**:414–419.
8. **Cummings, P. J., R. J. Lakomy, and C. R. Rinaldo.** 1981. Characterization of herpes simplex virus persistence in a human T lymphoblastoid cell line. Infect. Immun. **34**:817–827.
9. **Damonte, E. B., S. E. Mersich, and C. E. Coto.** 1983. Response of cells persistently infected with arenaviruses to superinfection with homotypic and heterotypic viruses. Virology **129**:474–478.
10. **Friedman, R. M., and J. M. Ramseur.** 1979. Mechanisms of persistent infections by cythopatic viruses in tissue culture. Arch. Virol. **60**:83–103.
11. **Griffith, J. S., S. L. Katz, and M. Moore.** 1977. Persistent enterovirus infections in agammaglobulinemia, p. 488–493. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
12. **Holland, J. J., S. I. T. Kennedy, B. L. Semler, C. L. Jones, L. Roux, and E. A. Grahan.** 1980. Defective interfering RNA viruses and the host-cell response, p. 137–192. *In* H. Frankel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 16. Plenum Publishing Corp., New York.
13. **Holland, J. J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215**:1577–1585.
14. **Joklik, W. K.** 1977. Mechanisms of establishment and maintenance of persistent infections. Summary of mechanisms involved in persistent viral infections, p. 434–438. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
15. **Ju, G., S. Udem, B. Rager-Zisman, and B. R. Bloom.** 1978. Isolation of a heterogenous population of temperature-sensitive mutants of measles virus from persistently infected human lymphoblastoid cell lines. J. Exp. Med. **147**:1637–1652.
16. **Lipton, H.** 1980. Persistent Theiler's murine encephalomyelitis virus infection depends on plaque size. J. Gen. Virol. **46**: 169–177.
17. **MacPherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology **23**:291–294.
18. **Matteucci, D., A. Toniolo, P. G. Conaldi, F. Basolo, and M. Bendinelli.** 1985. Systemic lymphoid atrophy in coxsackievirus B3 infected mice not due to a direct viral effect and exacerbated by immunopotentiating agents. J. Infect. Dis. **151**:1100–1108.
19. **Meinkoth, J., and S. I. T. Kennedy.** 1980. Semliki Forest virus persistence in mouse L929 cells. Virology **100**:141–155.
20. **Miller, J. R.** 1981. Prolonged intracerebral infection with poliovirus in asymptomatic mice. Ann. Neurol. **9**:590–596.
21. **Nillson, K., and G. Klein.** 1982. Phenotypic and cytogenetic characteristics of human B-lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. Adv. Cancer Res. **37**:319–380.
22. **Youngner, J. S., and O. T. Preble.** 1980. Viral persistence: evolution of viral populations, p. 73–135. *In* H. Frankel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 19. Plenum Publishing Corp., New York.