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Persistent Infection of Mouse Fibroblasts with Coxsackievirus

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With 1 Figure

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Summary

Infection of fibroblast cell lines initiated from BALB/c or NFR mice with coxsackievirus B3 (CBV-3) or B4 (CBV-4) resulted in infections which persisted for a limited number of subpassages of the infected cells in most cases, but for over a year in one case. In all instances primary acute infections were characterized by cytopathology and release of infectious virus progeny. Viral antigen could be detected during the acute phase of infection, but not in subcultured infected cells. Infectious center assays showed that every cell was infected during the acute phase of infection, but that from the first subcultivation on, the numbers of cells which were able to initiate infection were greatly reduced. The long term persistent CBV-3 infection was characterized by wide fluctuations in titers of virus released into the supernatant fluids. Interferon did not appear to play a role in maintenance of the persistent infection. Information derived from studies on mechanisms of CBV persistence in the *in vitro* model may help to elucidate the role of CBV in chronic human diseases such as myocarditis.

Introduction

Persistent infection of cells in culture has usually been observed with viruses which are not highly cytolytic, although the cell type is also an important factor. In general, enteroviruses are cytopathic in cell cultures which support their replication (15). When persistent cell culture infections

have been established with enteroviruses they have usually required the presence of specific antibody (1—3), addition of 10 percent human serum (19), or daily washing of cells and addition of fresh medium (16). However, a persistent infection of a human fetal diploid cell line was established following infection with a strain of coxsackievirus type B2 (CBV-2) in the absence of specific antibody or human serum and without frequent changes of medium (13).

Persistent infection of L929 cells by Theiler's murine encephalitis virus, a picornavirus, did not require specific antibody (18). In this case interferon was detected in the supernatant fluids of the cultures, and this may have had a role in maintaining the noncytocidal nature of the infection.

These persistent infections could best be described as carrier cultures. That is, a cytocidal virus was kept in check by inhibitory factors in the medium or by the presence in the culture of a minority of susceptible cells and a majority of genetically resistant cells (4).

Persistent infection of cell cultures by coxsackievirus is of interest as a possible *in vitro* model for persistence of coxsackieviruses in chronic diseases such as cardiomyopathies. The possibility that mouse cells might be subject to persistent infection was suggested to us by the observation that primary skin fibroblasts set up for assay of cell mediated immunity were not completely lysed by CBV-3 in 7 days. This report describes certain features of a persistent coxsackievirus infection of mouse fibroblasts which was established without specific antibody or human serum, and without frequent washing or medium changes.

Materials and Methods

Viruses

The myocarditic "M" strain of CBV-3, adapted to the murine heart by Dr. Jack Woodruff, was obtained through the courtesy of Dr. Lilian P. Job, and was passed twice in a continuous line of fetal rhesus monkey kidney cells developed in this laboratory, and once in Buffalo green monkey kidney (BGMK) cells. A CBV-4 field strain was isolated in this laboratory in rhesus monkey kidney (RhMk) cells from the heart of a 10-day-old female with myocarditis, and was passed twice in RhMk cells and twice in BGMK cells. Identity of the virus strains was confirmed by neutralization tests with antisera produced in this laboratory. Vesicular stomatitis virus (Indiana strain) was obtained from the Naval Bioscience Laboratory, Oakland, CA, and was passed once in baby hamster kidney cells.

Infected Cell Cultures

Skin fibroblast cultures were initiated from trypsinized skin of neonatal NFR or BALB/c mice. Cells were subcultured when they reached confluency, usually 2—4 days with a range of 1—14 days. Confluent cultures were infected with CBV-3(M) or CBV-4 at various multiplicities of infection (MOI). Persistently infected cells were established by dispersing cells with a mixture of 0.25 percent trypsin and 0.02 percent

versene (EDTA) 7 days after initiation of infection, and subculturing cells at a 1:2 ratio for subsequent passages. Companion uninfected cultures were passed on the same schedule.

At the time of subculture a sample of supernatant fluid from infected cultures was saved for titration of infectious virus. Infectivity titrations were done in serial 10-fold dilutions in BGMK tube cultures, and titers were expressed as 50 percent tissue culture infectious doses ($TCID_{50}$).

Infectious Center Assays

Cells in infected cultures were dispersed with trypsin-versene, washed 3 times in Hanks' balanced salt solution (HBSS), resuspended in 1 ml of HBSS, and 0.1 ml volumes of serial 10-fold dilutions of the suspension were added to monolayers of BGMK cells. The cells were allowed to adsorb to the BGMK monolayers for 30 minutes, and the cultures were overlaid with nutrient medium (Eagle's MEM with 2 percent fetal bovine serum) containing 0.5 percent agarose. Five days later a second agarose overlay containing neutral red to give a final concentration of 1:10,000 was added, and plaques were counted on the following day.

Immunofluorescence Staining

Cell cultures were grown in Lab-Tek slides (Miles Laboratories, Naperville, IL), infected, and stained by the indirect method using immune mouse ascitic fluids as coxsackievirus antisera and fluorescein-labeled goat anti-mouse Ig (Antibodies Inc., Davis, CA).

Radioimmunoassay

Goat anti-mouse Ig was kindly provided by Dr. B. Forghani. The antibody was labeled with ^{125}I by the chloramine-T method (9, 14), and stored at 4° C. Cells for assay were grown in the bottom of 1-dram vials. Infected and uninfected cells were washed in phosphate buffered saline, fixed in acetone for 10 minutes at room temperature, and stored at −20° C. CBV-3 mouse immune ascitic fluid was used as the specific coxsackievirus antibody, and the optimal dilution was determined by titrating 1:500, 1:5000, 1:10,000, 1:50,000, and 1:100,000 dilutions against infected and uninfected BGMK cells. To assay for coxsackievirus antigen in infected cells, the working dilution of the immune ascitic fluid was reacted with the test cells overnight at room temperature, and after washing the cultures, 50,000 CPM of ^{125}I-labeled goat anti-mouse Ig was added to each vial for 70 minutes. After the fluids were removed and the vials were washed 3 times, the bound CPM at each point (tested in triplicate), were counted in a gamma counter. Binding ratios were determined by dividing counts in vials containing infected cells by those in vials with uninfected cells, and ratios ≥ 2.1 were considered to be significant, i.e., to indicate the presence of viral antigen.

Results

Acute Infections

NFR and BALB/c skin fibroblast cells at the 4th and 5th passages respectively were infected with CBV-3(M) or CBV-4 and examined over a 7-day period for release of infectious virus, cytopathic effect, and proportion of cells in which viral antigen was demonstrable by immunofluorescence (IF) staining. Results are shown in Table 1. In both cell types the CBV-3(M)

strain produced more extensive cytopathology, and produced more antigen demonstrable by (IF) staining. However, even after 7 days, only 50 percent of the cells infected with CBV-3(M) showed a cytopathic effect, and only 50—66 percent contained antigen. The CBV-4 strain produced a minimal cytopathic effect (CPE), and very little antigen was demonstrable by IF staining, but moderate levels of infectious virus were released into the cell culture fluids. It seems most likely that the infectious CBV-4 virus in the culture fluids was a result of virus replication rather than residual inoculum, since initial infectivity would have decayed rapidly over the 7-day incubation period.

Table 1. *Acute infection of NFR and BALB/c fibroblasts with CBV-3(M) and CBV-4*

		Cells					
		BALB/c			NFR		
Virus	Days post inoc.	CPE	% cells IF pos.	$TCID_{50}$[a]	CPE	% cells IF pos.	$TCID_{50}$
CBV-3(M)	1	0	0	N.T.[b]	0	8	N.T.
(MOI 100)	2	+	14	>4.5	++	30	>4.5
	3	++	10	>4.5	++	33	>4.5
	5	++	10	>4.5	++	50	>4.5
	7	++	>50	>4.5	++	66	>4.5
CBV-4	1	0	0	N.T.	0	7	N.T.
(MOI 200)	2	±	1	>4.5	++	1—2	4.5
	3	±	0	>4.5	+	1—2	4.5
	5	±	0	>4.5	±	0	4.5
	7	0	0	>3.5	0	0	4.5

[a] Log_{10} per 0.1 ml culture fluid
[b] Not tested

Experiments were done to determine the number of NFR cells in a culture infected with CBV-3(M) which acquired infectious virus and could produce infectious centers. Cell cultures at the 5th passage were infected with virus at a range of MOIs from 10 to 0.01, and the percentages of cells producing infectious centers were determined on days 1, 2, 4, and 7 post-infection (Table 2). With each inoculum the percentage of cells producing infectious centers reached 100 percent on or before day 7, and before CPE was apparent. These results imply that the cells in the cultures were uniformly susceptible, in that all could initiate a viral infection.

Radioimmunoassay was used as a more sensitive assay than IF staining to study antigen production over a 10-day period in NFR cells acutely infected with CBV-3(M) at an MOI of 50. Binding ratios were insignificant at 0 time, but were significant, indicating the presence of viral antigen, at

days 2 and 5 post-infection. However, at days 7 and 10 binding ratios were no longer significant, possibly as a result of lysis and loss of antigen from infected cells; these results were similar to those obtained by IF staining. Binding ratios for infected NFR cells were lower than those for control infected BGMK cells examined in parallel, suggesting that the latter were more permissive for CBV-3 (M) replication.

Table 2. *Percentage of NFR fibroblasts producing infectious centers after infection with various doses of CBV-3 (M)*

	Percent of cells producing infectious centers at days post infection			
MOI	1	2	4	7
10	100	70	100	100
1	7	11	100	100
0.1	0	0.5	100	100
0.01	0	0	4	100

Table 3. *Persistence of CBV-3 (M) and CBV-4 infections in NFR and BALB/c fibroblasts*

Virus	Cells	Cell passage number	Subpassage number after infection	$TCID_{50}$ in culture fluid[a]
CBV-3 (M) (MOI 0.1)	NFR	7	1	4.5
		8	2	4.0
		9	3	4.0
		10	4	2.5
		11	5	1.5
		12	6	<1.5
CBV-4 (MOI 3.2)	NFR	6	1	7.0
		7	2	6.0
		8	3	4.5
		9	4	4.0
		10	5	1.5
		11	6	<1.5
		12	7	<1.5
CBV-4 (MOI 1)	BALB/c	4	1	6.5
		5	2	6.0
		6	3	6.0
		7	4	4.5
		8	5	3.5
		9	6	1.5
		10	7	<1.0
		11	8	<1.0
		12	9 reinfected	3.5
				(4.5 cont.)

[a] $\log_{10}$ per 0.1 ml

Persistent CBV Infections

NFR and BALB/c fibroblasts at the 4th to 7th passage levels were infected with CBV-3 or CBV-4 and subpassaged at weekly intervals. As shown in Table 3, the titers of infectious virus present in supernatant fluids decreased with subculture of the cells until virus was no longer detectable after the 5th or 6th subpassage. However, it is clear that virus replication and release occurred during the early passages of infected cells, as residual virus from the inocula would be removed by the extensive washing involved in subpassage of the cells.

BALB/c cells which had released infectious virus earlier, but which were no longer doing so, were tested for susceptibility by reinoculating them, and control uninfected cells at the same passage level, with the same inoculum of CBV-4 (1×10^5 $TCID_{50}$). After virus was absorbed for 1 hour, the cultures were washed with HBSS, and maintenance medium was added. As shown in Table 3, the previously infected cells released somewhat less infectious virus than did cells which had not been previously infected, but they were nevertheless susceptible to infection, and the cessation of viral replication could not be explained by a replacement of susceptible cells with insusceptible ones.

In contrast to the limited persistent infections which could be established in the cell lines shown in Table 3, infection of a second line of NFR

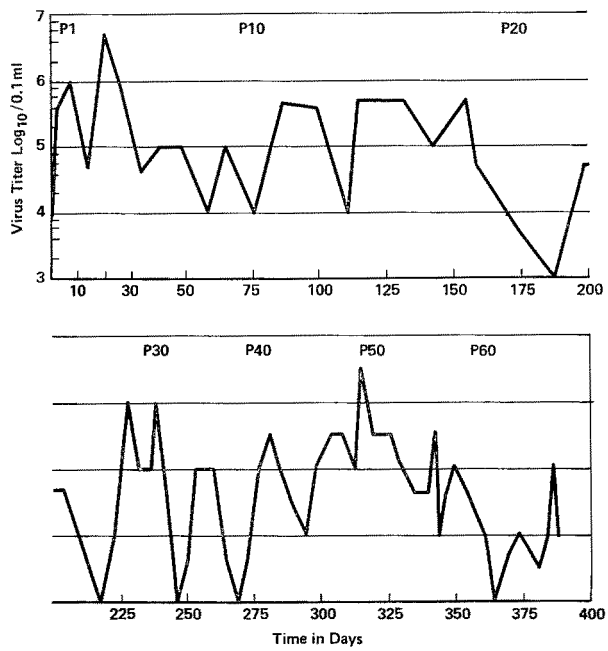

Fig. 1. $TCID_{50}$ of virus in supernatant fluids of persistently infected NFR fibroblasts sampled at the time of subculture. *P1* subculture number 1, etc.

fibroblasts (NFR2) at the 5th passage level resulted in a persistent CBV-3 (M) infection which has been sustained for over 1 year, and uninfected cells passaged in parallel have also been maintained in continuous culture for the same length of time. As shown in Fig. 1, the titers of infectious virus released into the culture fluids fluctuated over a wide range, with no apparent pattern. From passage 19 through 22, the cells were held for relatively long intervals between passages, and at passages 21 the cultures showed foci of overgrown and piled up cells, which differed from the previous appearance of the cultures, suggesting a possible crisis or transformation. From the first subculture through the latest (80th) there was no apparent CPE, but infected cultures grew to lower density and contained approximately 50 percent fewer cells than uninfected control cultures.

Infectious centers were determined at selected passages of the persistently infected cells (Table 4). The percent of positive cells was maximum at passage 1, and greatly reduced at all subsequent points tested. There was no correlation between titers of infectious virus released and numbers of infectious centers in the cultures. Thirty percent of the cells were positive for viral antigen by IF at the first passage, but no positive cells were detected at subsequent samplings.

Infected and control cells from passage numbers 23 and 64 which had been stored in the frozen state in liquid nitrogen were revived up to 4 months after freezing. They showed no loss of viability in culture, and infected cells continued to release virus into the supernatant fluids at the original titers. At passage 83 both infected and uninfected cells had chromosomes with morphology typical of mouse chromosomes.

To test for interferon production by the persistently infected cells, VSV was titrated in infected and uninfected cells. The titer was $1 \times 10^{9.0}$ $TCID_{50}$ in uninfected cells at the 79th passage, and $\geq 1 \times 10^{9.5}$ $TCID_{50}$ in persistently infected cells at the 77th passage. This would suggest that interferon production was not a major factor in the establishment of the persistent CBV-3 infection.

Table 4. *Infectious center and indirect IF assay of NFR cells persistently infected with CBV-3 (M)*

Passage number	% infectious centers	% cells IF positive
1	90	30
2	0.04	—[a]
4	—	0
6	13	—
8	3	—
12	0.7	—
22	5.9	0

[a] Not tested

Discussion

Persistent viral infections are of interest because of their potential contribution to an understanding of disease processes in viral infections. Persistent infection of mouse fibroblasts by CBV may be useful in understanding coxsackievirus-induced cardiomyopathies in murine and human hosts. In studies from this laboratory which are reported elsewhere CBV-3 (M) has been shown to persist in the hearts of NFR nude mice for up to 1 month (SCHNURR and SCHMIDT, Med. Microbiol. Immunol. in press), and in hearts of NIH II mice for up to 3 months (SCHNURR *et al.*, J. gen. Virol. in press). Other workers have reported persistence of certain myocarditic strains of CBV-3 in the hearts of immunocompetent mice for at least 14 days post-infection (11), and of CBV-4 for 17 days in hearts of mice treated with anti-thymocyte serum (10). Chronic human myocarditis has been epidemiologically linked to prior viral infections (5, 20). Thus an *in vitro* model such as the one described for CBV persistence will be useful for investigating possible mechanisms for *in vivo* viral persistence.

CBV-3 or CBV-4 infection of BALB/c or NFR fibroblasts resulted in infections which persisted for several passages of the infected cells, or for an indefinite period in one instance. In all cases, the primary, acute infection was characterized by a CPE and release of infectious virus progeny. Subculture of surviving cells usually resulted in a restricted infection in the sense that the subcultured cells continued to release infectious virus for only 6 or 7 passages. However, subculture of a second line of NFR fibroblasts infected with CBV-3(M) resulted in a prolonged persistent infection. This suggests that there was a difference between the two strains of cells, and that the host cell type was an important factor in maintaining a persistent viral infection.

Infectious center assays on cells acutely infected with CBV-3 indicated that virtually every cell acquired viral genetic material. However, the cells remained viable and were readily subcultured. These results are similar to those reported on the growth of CBV-3 in neonatal C58 mouse skin fibroblasts (12). The infected C58 cells had cell division rates similar to those in uninfected cultures, and the cells remained 90 to 95 percent viable even when 60 to 70 percent were positive by infectious center assay.

Following the first subculture of our infected NFR or BALB/c cells the percentage of cells positive for infectious centers was greatly reduced, and then fluctuated over a wide range (0.04 to 13 percent) with successive passages. The fluctuation of numbers of infected cells may have been related to the large fluctuation of infectious virus released into supernatant fluids of persistently infected cells, but no correlation was noted between the numbers of infectious centers and $TCID_{50}$ of virus present in the cultures at any given passage.

Viral antigen was detectable by IF or RIA only during the acute phase of infection (first week). IF staining is not a highly sensitive method for detection of CBV antigens, possibly because cells in which viral antigen is produced are lysed rapidly and release antigen. For example, it has been reported that specimens containing as much as 1×10^6 PFU of CBV-3 per gram of tissue were negative by IF (17). In the present studies even RIA was negative for viral antigen by 7 days after infection, suggesting a rapid release of viral antigen from the infected NFR cells.

These results would be consistent with infection of only a few cells in the subpassaged cultures. The continuing presence of infectious virus, together with the ability to reinfect cultures in which virus production has ceased, would indicate that susceptible cells are always present in the cultures.

The mechanism(s) by which persistence of infection was maintained in the mouse fibroblast cultures is not apparent. Interferon was detected in L929 mouse cells persistently infected with Theiler's virus (18), but the fact that growth of VSV was not inhibited in the persistently infected NFR cells would seem to rule out interferon as a factor in this system. Whether infected cells remain viable and can be subcultured is difficult to establish. A useful approach will be to clone persistently infected cultures in the presence of antibody and determine whether any virus-infected clones can be cultured.

In some cases viruses regulate their own expression through mutants such as defective interfering (DI) particles (6, 8), or temperature-sensitive (TS) mutants (21). The wide fluctuation of virus titers in the persistently infected NFR cultures could be indicative of the presence of DI particles (8). Although DI particle enriched virus preparations were not necessary for the initiation of persistent CBV-3(M) infection of NFR cells, DI particles frequently appear during the evolution of a persistent infection in cell culture (7). We intend to examine CBV-3 persistently infected cell cultures for TS mutants, DI particles, and antigenic variants in order to acquire information on the evolution of CBV-3 during persistent infection. However, the limited persistence of infection seen in BALB/c and another line of NFR cells suggests that genetic resistance of most cells in the culture is also an important factor.

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