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COXSACKIEVIRUS B4 HETEROGENEITY: EFFECT OF PASSAGE ON NEUTRALIZATION AND MORTALITY

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Summary. — We have compared two CB4 isolates for virulence, tissue tropism, and antigenic drift using monoclonal antibodies. Both isolates replicated in C57B1/6 and Balb/c mice. The human isolate Edwards, recovered from a fatal case of encephalohepatomyocarditis, produced lethal infection in adult animals. Lethal infections were associated with high viral titers in visceral organs but not with the presence of specific neutralizing epitopes. Virulence seemed stable upon passage, and also the avirulent JVB isolate retained its phenotype. Mock infection and recovery experiments demonstrated the stability of these characteristics. Neither the tissue from which the virus was isolated nor the cell line used in isolation significantly reduced virulence. However, antigenic variation among isolates was abundant. Thus, the set of monoclonal antibodies employed here may not be appropriate positive markers for virulence. This study suggests that CB4 virulence is stable upon extended in vitro passage and limited in vivo passage and that isolation site and method may not select for or against virulence. It is therefore possible that laboratory adapted strains of CB4, although antigenically different from freshly isolated specimens, may still retain these properties responsible for virulence present in low passage isolates and may be, with regard to virulence, very similar to freshly isolated specimens.

Key words: *Coxsackievirus*; *virulence*; *attenuation*; *lethal infection*; *monoclonal antibody*

Introduction

Coxackieviruses have been implicated in both human myocarditis and viral induced diabetes (Craighead, 1975; Reyes and Lerner, 1985). Both diseases are complex with host and viral genetics interacting to yield a wide range of pathologies (Webb *et al.*, 1976; Webb and Madge, 1980; Hartig *et al.*, 1983; Hartig and Webb, 1983; Kuno *et al.*, 1984; Herskowitz *et al.*, 1985), from asymptomatic infections (Komatsu *et al.*, 1983) to death (Webb

et al., 1979*a*). The natural heterogeneity of Coxsackievirus Group B (CB) has been shown to play a significant role in the diseases produced in genetically defined mice (Cao *et al.*, 1984*a*). Acardio virulence markers for Coxsackievirus group B, type 4 (CB4) have been defined (Jimes and Jamison, 1983; Jimes *et al.*, 1984), unfortunately consistent markers for virulence remain elusive.

Recent studies demonstrated that the picornaviruses (e. g., CB4) can mutate at the rate of one serologically significant mutation for every ten thousand virions (Prabhakar *et al.*, 1982). This high mutation rate suggests a mechanism for extensive viral heterogeneity. Rapid simple mutation implies that post isolation passage of CB4 may produce virus capable of inducing specific pathologies (Komatsu *et al.*, 1983), but pathologies vastly different from the original virus and possibly different from that humans are likely to encounter naturally.

Compounding the problem mutations cause in evaluating viral pathology is the selection of virion subpopulation due to tissue tropism. Viral isolates have been adapted to induce specific pathologies in the organs in which they were passaged (Pappenheimer *et al.*, 1951). Thus any picornavirus population studied contains virions arising from rapid mutation and selected for (or against) by tissue tropism. Furthermore, virions produced by different isolation procedures should differ depending on the cell line or animal used for propagation (e. g., tissue tropism) and the number of passages in culture (e. g., opportunities to mutate).

We have previously documented the ability of CB4 (strain Edwards) to produce lethal infections in adult Balb/c and C57B1/6 mice (Webb and Madge, 1980). Cao *et al.* (1984) have presented studies demonstrating that CB4 disease was associated with high titer viral replication in affected tissue (Cao *et al.*, 1984*a*). In this study we evaluate the role of host genetics (2 strains of mice), mutation (measured by antigenic change), and tissue tropism (measured by viral titer) in lethal CB4 infections. Our results document in vitro and in vivo antigenic changes, the effect of isolation site and method of isolation on antigenic drift, as well as, the effect of these changes on virulence.

Materials and Methods

Animals. Six week old male mice of the inbred strain Balb/c and C57B1/6J were obtained from the virus-free colony of Fredrick Cancer Research Center, Fredrick, Fredrick, MD, housed five per bonnetted cage. Mice were allowed water and food *ad libitum*, light (14 : 10), and temperature was maintained at 25 °C.

Animals were inoculated intraperitoneally (i. p.) with 0.2 ml. of virus suspension. Control animals were inoculated with similar tissue culture fluids or organ suspensions without virus. Mice used in studies of acute infection were sacrificed by cervical dislocation at three days post--inoculation. Their ventral surfaces were washed with 70% ethanol and organs used for viral recovery were aseptically removed. Organs were washed for five minutes in ten times their volume of cold Hank's Buffered Salt Solution (HBSS), containing 0.1% R.I.A. grade bovine serum albumin (BSA) and 0.01% Gentamycin, bissected and homogenized in sterile normal saline. The last 3 cm of large intestine were removed, washed, and the entire piece of tissue homogenized. Homogenates were freeze-thawed 3 ×, and clarified by centrifugation. Virus-containing supernatants were stored at 10 °C until analysis. Serum was recovered to evaluated seroconversion and confirm infection of surviving animals.

Cell cultures. Buffalo green monkey (BGM) kidney cells characterized by Barrow *et al.*, who derived this continuous cell line from the African Green Monkey, *Cercopithecus aethiops* (Barrow *et al.*, 1970), were obtained in passage 130 from the Environmental Protection Agency, (Cincinnati, OH). Unless stated otherwise, BGM cells were used for virus propagation, assay, and serologic evaluations.

LLC-MK2 cells were derived from the kidneys of the *Macaca mulatta* monkey and obtained from the ATCC (Amnerican Type Culture Collection, Rockville, MD) in passage 286.

Cell cultures were maintained in Eagle's Minimal Essential Medium (EMEM), propagated in medium containing 10% Newborn Bovine Serum (Flow Laboratories, McLean, VA 22102, Lot # 29121076, shown not to interfere with Coxsackievirus B4 replication), Gentamycin (0.01%) and sodium bicarbonate buffer. Cell monolayers were subcultured or harvested by suspension in 0.25% trypsin containing 0.05 mM EDTA. Cultures were maintained at 37 °C in a humidified incubator with a 5% CO_2/air atmosphere.

Virus. Two low passage level human isolates of Coxsackievirus Group B Type 4 (CB4) were used. JVB, the approved prototype strain, was obtained from ATCC (Sickles *et al.*, 1955). Sickles *et al.* isolated this virus from the stool of a child who subsequently recovered. It was deposited at ATCC after serial passage in: nine suckling mice, two LLC-MK2 cell cultures and one passage in primary rhesus monkey kidney cells. Edw. was isolated from the myocardium of a child dying of encephalohepatomyocarditis and inflammation of the pancreas (Kibrick and Benirschke, 1958), passaged serially once in suckling mice, monkey renal cells, HeLa cells, then three times in the pancreas of CD-1 mice and once more in HeLa cells (Webb *et al.*, 1976). In addition to these virus stocks, JVB and Edw. were passaged serially in 8 BGM cell cultures. The first and eighth passages of each virus were saved for analysis and designated, JVBp1, Edwp1, JVBp8, and Edwp8, respectively. One clone of JVB was plaque purified in GBM cells (JVBp1). Edw. 59-3, which lacks epitopes recognized by monoclonal antibody, has been described previously.

Viruses for animal inoculation were produced by infecting cell monolayers. When CPE (cytopathic effect) was present in 95% of the cells, monolayers were freeze-thawed 3 ×, clarified by centrifugation, and assayed to determine virus concentration. Titer of virus was determined in BGM cells by $TCID_{50}$ assay. $TCID_{50}$ end-point titer was calculated according to the method of Reed and Muench (1938).

Antisera. The monoclonal antibodies used in this study were a gift from Drs. B. S. Prabhakar and A. L. Notkins, National Institutes of Dental Research, the National Institutes of Health (NIH), the preparation of which has been already described (Prabhakar *et al.*, 1982). Anti-CB4 rabbit antiserum (CB4-Powers isolate as antigen, lot 3-7238) was purchased from Microbiological Associates, Bethesda, MD. All antisera were diluted in HBSS containing 0.1% R.I.A. grade BSA. Polyclonal antisera and serum from convalescent animals were heated to 56 °C for 30 min. to inactivate complement. Sera from convalescent animals were tested for their ability to neutralize 100 $TCID_{50}$ units of JVB. Reciprocal antisera dilutions of greater than twenty were considered proof of seroconversion and infection.

Virus neutralization. The virus suspensions were diluted to 50 $TCID_{50}$/ml. Ten microliter aliquots of this suspension were added to 0.01 ml of monoclonal antibody solution, or polyclonal antiserum and allowed to react for 1 hr at 37 °C under 5% CO_2. One thousand BGM cells in 0.05 ml of EMEM were then added to each sample. Samples were incubated for three days, fixed, stained, and samples containing CPE were judged to contain unneutralized virus. Cell: virus and cell: antiserum containing samples served as controls.

Experimental design. All viruses were screened to determine their ability to be neutralized by the panel of anti-CB4 monoclonal antibodies. This information served as the base for determining variation resulting from manipulations of these viruses, as well as documenting the antigenic nature of JVB and Edw. The epitope recognized by antibody 59-3 present on Edw. but missing on JVB was removed by antigenic manipulation in an attempt to change the biological effect of the virus and thus correlate antigenic differences with virulence.

Five groups of 30 mice, fifteen of the inbred strain C57B1/6 and fifteen of the strain Balb/c were inoculated with 6.5 $TCID_{50}$ (log_{10}) of viruses Edw., JVB, Edw.p8, JVBp8, or Edw. 59-3. At three days post-infection, 5 animals in each group were sacrificed for virus isolation. The remaining 10 animals in each group were monitored and mean survival times were calculated. Animals surviving 21 days were sacrificed and serum saved for analysis. Results from this experiment were used to: evaluate these inbred strains for sensitivity to CB4 infection, document the mortality caused by JVB and Edw. in these strains of mice, and document the virulence of

JVB and Edw. upon passage in vitro. The remainder of the virus recovered from Balb/c animals was pooled according to inoculum and tissue (e. g., JVB Heart), generating virus pools, JVBH, JVBG, Edw.G, Edw.G, Edw.P. Two hundred microliters of each pool was inoculated into 13 Balb/c animals. At 3 days post-infection three animals in each group were sacrificed. Heart and pancreas were saved for viral recovery as was the gut. Ten animals remaining in each group were monitored for 21 days and mean survival times were determined. The data from this experimental group was needed to evaluate the stability of virulence upon passage in vivo, as well as to determine if virion subpopulations with differing tissue tropisms provided different mean survival times.

To determine the combined effect of in vivo and in vitro passage upon virulence and tissue tropism as well as evaluate the effect of isolation procedures on the inadvertant selection of virion subpopulations, one Balb/c mouse was infected with Edw. 59-3 $TCID_{50}$ (log_{10}), and sacrificed at 3 days. Heart, gut, and pancreatic tissues were homogenized and virus recovered. These three tissue isolates were used to inoculate BGM and LLCMK2 cells, which subsequently produced virus stocks hBGM, gBGM, pBGM, hLLC, gLLC, and pLLC, respectively. Antigenic analysis was performed on each stock. Each of these six viruses was used to inoculate thirteen Balb/c animals. Three days after inoculation with 5 $TICD_{50}$ (log_{10}) 3 animals in each group were sacrificed, and virus recovered for the heart, pancreas and gut for antigenic analysis. Animals were monitored for 21 days and mean survival time recorded. This experiment was done to evaluate antigenic changes in the virus and changes in the mortality the virus may produce when it is isolated from different tissue within the same animal and/or, when as part of that isolation, it is passaged in different cell lines.

Statistics. Statistical analysis of all mean survival times demonstrating variation were completed via a 2-way analysis of variance with interaction. As no virus-strain interaction was demonstrated, analysis was effectively one way for virus type. Duncan's multiple range test was used in the Post-hoc separation of means. The probability of generating the F value randomly was 0.0007.

Results

Characterization of JVB and Edw. isolates

Isolates JVB and Edw. produced drastically different outcomes irregardless of the strain of mouse infected. The parental CB4 Edw. isolate and all of its progeny (variants) induced lethal infections in both Balb/c and C57B1/6 mice. Neither the JVB isolate nor its progeny induced lethal infections in either strain of mice (Fig. 1). The isolate specific virulence of CB4 Edw. was passage stable, as was the attenuated nature of JVB (Edw., and Edw. p8 produced fatal infection, neither JVB nor JVBp8 kill even one mouse of either strain). The specific removal of the epitope recognized by monoclonal antibody 59-3 (Edw. 59-3) had no attenuating effect on the virus (Fig. 1). This despite of the fact that 59-3 was one of the few antibodies found consistently on Edw. and missing (or in low frequency) on JVB.

The Edw. virus consistently replicated to higher titers in both C57B1/6 and Balb/c mice; (JVB 4.9 vs Edw. 7.0 log_{10} virus per gram C57B1/6 pancreas, Table 1). Edw. consistently reached highest titers in the pancreas (Balb/c 7.7/gram) and exceeded the JVB titer regardless of tissue. The antigenic makeup of virus recovered from all organs differed from the inoculum (Table 3, upper). Edw. inoculum possessed epitopes: 204-4, 59-3, 204-3 and 287-2 vs the virus recovered from Balb/c or C57B1/6 pancreas which was not neutralized by any of these antibodies. Conversely, virus recovered from the pancreas of animal 108 acquired epitopes 204-3, 183-2 and 38-1.

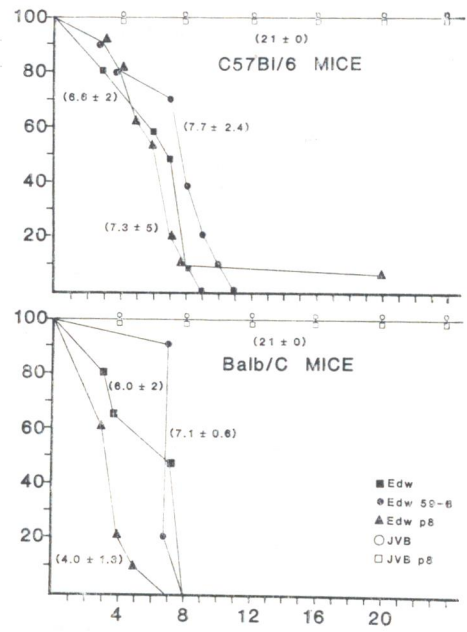

Fig. 1.
Cumulative per cent survival of mice during the 21 day period following infection

At least 10 mice per group received 6.5 $TCID_{50}$ (log_{10}) of virus. Parenthesis contain mean survival times ± standard deviations in days.
Abscissa: days post-infection; ordinate: survival rate (per cent).

Antigenic profiles of virus recovered from the gut and heart yielded similar results (data not shown). The acquisition or loss of epitopes could not be correlated with either passage in vitro, site of isolation, virulence, or animal strain. However, the change does demonstrate viral replication and indicates recovered virus was the product of replication and not residual inoculum. Furthermore, all animals surviving 21 days had high titer CB4 antibody (data not shown). No virus mouse strain interaction could be demonstrated thus, the more docile Balb/c animal was used in further experiments. Likewise, no differences in titer of virus recovered or mortality induced by the original viruses vs their variants could be ascertained. Therefore, subsequent studies employed only the original lowest passaged Edw. or JVB viruses available.

JVB and Edw. were passaged once in vivo and isolated from heart, pancreas and gut yielding virus pools JVBH, JVBP, JVBG, Edw.H, Edw.P, and Edw.G respectively. Appropriate tissue from five individual Balb/c mice made up each pool. Because these in vivo passaged viruses were derived from tissue of more than one animal, inocula were not analyzed for antigenic makeup.

Pooled viruses produced a pattern of infection similar to their parent virus (Table 2, upper). All Edw. derivatives produced lethal infections (mean survival times of 3.5 ± 0.5, 5.7 ± 2.1, 5.7 ± 2.1 days for Edw.H, Edw.P and Edw.G respectively) while JVB in vivo passaged virus failed to kill even one mouse. Curiously, the mean survival time of mice infected with Edw.H was significantly less than all other groups in which fatal infections occurred.

Table 1. Titer of virus recovered[a]

Mouse	Virus	Heart	Pancreas	Gut
		Virus titer between strains of mice		
C57B1/6	JVB	2.7	4.9	3.3
	Edw.	4.0	7.0[b]	5.5[b]
Balb/c	JVB	2.7	4.8	3.1
	Edw.	3.5	7.7[b]	5.5[b]
		Virus titer after in vivo passage[a]		
Tissue	Virus Name			
Heart	JVBH	N/D	3.0	N/D
	Edw.H	4.0	6.3[c]	4.3
Pancreas	JVBP	N/D	3.7	3.4
	Edw.P	3.9	6.8[c]	4.0
Gut	JVBG	N/D	3.2	N/D
	Edw.G	3.4	6.8[c]	3.9
		Virus titer after in vivo and in vitro passage[c]		
Heart	hBGM	4.6	7.1	5.1
	hLLC	3.9	6.4	3.9
Pancreas	pBGM	4.4	6.7	4.6
	pLLC	4.4	6.4	5.6
Gut	gBGM	3.9	7.1	4.1
	gLLC	3.9	6.7	4.6

(a) Log_{10} of virus per gram. Each value represents the mean of three mice sacrificed 3 days post infection.
(b) Denotes differences of more than 2 logs between viruses within tissues.
(c) One Balb/c mouse was infected with Edw. Three days post infection virus was recovered from heart, pancreas and gut tissue, then passaged once in $LLCMK_2$ or BGM cells.
(N/D) No detected at assay limits.

Virus replication followed a pattern noted earlier (Table 1, middle). Passaged Edw. virus consistently replicated to high titers, regardless of which organ it was passaged in or recovered from. Passage in any particular organ was not reflected in a predilection for that organ as indicated by viral titer. The JVB passaged virus replicated to titer several log lower than comparable Edw. virus (JVBH 3.0 vs Edw.H 6.0 log_{10} recovered from pancreas). In addition, the low viral yields of JVB (JVBH, recovered from heart) were frequently below the limits of the assay system (1000 $TCID_{50}$/ml). This made pooling of recovered virus necessary before antigenic analysis could be accomplished (Table 3, bottom 188—190).

Table 2. Mean survival time[a]

Tissue	Cell Line	Name	
		by in vivo passaged virus[b]	
Heart	N/A	Edw.H	3.5 ± 0.5[c]
Pancreas	N/A	Edw.P	5.7 ± 2.1
Gut	N/A	Edw. G	5.7 ± 2.1
		by in vivo and in vitro passaged virus	
Heart	BGM	hBGM	3.0 ± 0[c]
	$LLCMK_2$	hLLC	3.0 ± 0[c]
Pancreas	BGM	pBGM	3.1 ± 0.3[c]
	$LLCMK_2$	pLLC	3.2 ± 0.4[c]
Gut	BGM	gBGM	3.0 ± 0[c]
	$LLCMK_2$	gLLC	3.0 ± 0[c]

([a]) Mean survival time of 10 Balb/c animals per group.
([b]) All derivatives of Edw. Similar JVB variants failed to produce lethal infections.
([c]) Statistically different from original CB4 Edw. isolate.
(±) SD

Antigenic analysis by monoclonal antibodies

Antigenic analysis of virus recovered from specific tissue after infection with in vivo passaged virus yielded results similar to that of in vitro passaged virus (Table 4, upper). All recovered virus was CB4 and neutralized by monoclonal antibodies 86-3 and 86-6, as well as, polyvalent antisera. There was no pattern of antigenic makeup which correlated with virulence or tissue tropism. Two epitopes (86-3 and 86-6) were highly conserved, all others appeared at random.

The effect of in vivo replication and propagation, in vitro growth, as occurs during isolation, were evaluated. Because of the difficulty in recovering JVB from tissue, indicated by the need for pooling specimens in the previous experiment, only Edw. was used. One Balb/c animal was infected with Edw. and virus was recovered from heart, gut, and pancreas using BGM or LLMCK2 cells for isolation. Viruses were designated hBGM, hLLC, gBGM, gLLC, pBGM and pLLC, respectively. Each represented a different tissue and cell line combination for evaluating this possible synergistic interaction. The six different virus inocula, all obtained by slightly different mock isolation procedures, each produce rapid and fatal infections in Balb/c mice (Table 2 bottom). No animals survived infection and all mice died within 3.2 days. The mean survival time in every group was significantly less than that produced by the original Edw. All of these isolates replicated to high titers

Table 3. Antigenic analysis[a] of virus recovered from different strains of mice and from in vivo passaged inoculum

Virus[c] inoculum	Mouse strain	Tissue of isolation	Animal number	Antibody[b]														
				CB4	86-3	86-6	287-2	214-5	286-5	9-4	356-4	204-4	59-3	204-3	183-2	93-1	38-1	339-1	204-1
JVB	N/A	N/A	N/A	+	+	+	+	+	+	+	+	+							
	C57B1/6	Pancreas	101	+	+	+													
			104	+	+	+					+							+	
	Balb/c		107	+	+	+						+							
			108	+	+	+	+	+	+	+	+	+		+	+		+		
Edw.	N/A	N/A	N/A	+	+	+	+					+	+	+					
	C57B1/6	Pancreas	114	+	+	+													
			115	+	+	+													
	Balb/c		116	+	+	+													
			117	+	+	+													
Edw. G	Balb/c	Heart	149	+	+	+			+			+	+	+	+				
		Gut	149	+	+	+													
		Pancreas	149	+	+	+						+							
Edw. P		Heart	162	+	+	+			+			+	+	+	+				
		Gut	162	+	+	+													
		Pancreas	162	+	+	+													
Edw. G		Heart	175	+	+	+													
		Gut	175	+	+	+													
		Pancreas	175	+	+	+													
JVBH[d]		Heart	188—190	+	+	+						+							
		Gut	188—190	+	+	+			+				+	+	+	+	+		
		Pancreas	188—190	+	+	+										+			

[a] Table displays representative data from several experiments.
[b] Number designate monoclonal antibody (Prabhakar *et al.*, 1982), CB4 = Rabbit anti-Coxsackievirus B4 antisera.
[c] Inoculum was 5.5 $TCID_{50}$ (log_{10}) of virus, or 0.2 ml of inoculum fluid. Animals were sacrificed at 3 days.
[d] JVBH recovered virus required pooling to recover sufficient virus for antigenic analysis. JVBP, JVBG, also required pooling (data not shown).
(+) Indicates neutralization of 100 $TCID_{50}$ of virus by antibody.

in tissues analyzed (Table 1, bottom). Virus yields were greatest in pancreas with concentrations recovered from gut and heart varying among isolates.

Antigenic analysis of inocula and recovered virus revealed differences in antigenic makeup (Table 4, bottom). These variations, like those of other isolates, failed to correlate with the inocula used to initiate infection, the cell line used to passage the virus, the tissue from which it was subsequently recovered or mortality. All recovered viruses were shown to be CB4 by neutralization with specific antisera. Like all the other isolates these contained the epitopes recognized by monoclonal antibodies 86-3 and 86-6.

Table 4. Antigenic analysis[a] of virus recovered from in vivo and in vitro passaged inoculum

Virus[c] inoculum	Mouse strain	Tissue of isolation	Animal number	Antibody[b]														
				CB4	86-3	86-6	287-2	214-5	286-5	9-4	356-2	204-4	59-3	204-3	183-2	93-1	38-1	339-1	204-1
hBGM	Balb/c	N/A	N/A	+	+	+	+					+							
		Heart	282	+	+	+						+							
		Gut	282	+	+	+													
		Pancreas	281	+	+	+						+							
pBGM		N/A	N/A	+	+	+	+					+							
		Heart	269	+	+	+													
		Gut	269	+	+	+													
		Pancreas	269	+	+	+													
gBGM		N/A	N/A	+	+	+	+					+							
		Heart	295	+	+	+						+							
		Gut	295	+	+	+													
		Pancreas	295	+	+	+					+	+	+	+					
hLLC		N/A	N/A	+	+	+													
		Heart	243	+	+	+													
		Gut	243	+	+	+													
		Pancreas	243	+	+	+													
pLLC		N/A	N/A	+	+	+	+					+							
		Heart	230	+	+	+													
		Gut	230	+	+	+													
		Pancreas	230	+	+	+													
gLLC		N/A	N/A	+	+	+	+					+							
		Heart	256	+	+	+													
		Gut	256	+	+	+													
		Pancreas	256	+	+	+													

For explanations see Legend to Table 3

Discussion

The heterogeneity of CB4 isolates is well documented (Hartig *et al.*, 1983; Hartig and Webb, 1983). The high rate of mutation of CB4 is consistent with other picornaviruses (e. g., Polio) (Prabhakar *et al.*, 1982; Emini *et al.*, 1983). Although the mechanism of mutation remains to be elucidated, similar high mutation rates among two different enteroviruses suggest a common mechanism. If the mechanisms and the significance of mutations in enteroviruses are similar, the data presented here have broad implications.

We chose to evaluate the change among CB4 variants in three substantially different ways: 1) The complete neutralization of 100 $TCID_{50}$ of virus by monoclonal antibodies. Monoclonal antibodies are the most sensitive and specific tool for antigenic analysis; complete viral neutralization via $TCID_{50}$ is the most sensitive method of evaluating the acquisition of epitopes in a homogenous state (Webb *et al.*, 1986); 2) The titer of virus in specific tissue.

Titer reflects tissue tropism and productive infection in specific tissue. Other studies have suggested viral titer to be a marker for virulence (Cao *et al.*, 1984*b*); 3) Death as an indicator of virulence. Although mortality is the least sensitive method of evaluating differences between variants, it is the most clinically significant outcome of infection.

Two sets of monoclonal antibodies have been produced in attempts to discover antigenic markers for virulence (Prabhakar *et al.*, 1982; Cao *et al.*, 1984*b*). Those used in this study were generated using the JVB (avirulent) isolate of CB4 as the immunogen (Prabhakar *et al.*, 1982). In separate investigations neutralizing and nonneutralizing monoclonal antibodies against CB4 were produced using the myocarditic strain Mil (Cao *et al.*, 1984*b*). As in this study, no site recognized by any of these antibodies positively correlated with any of the pathologies or disease criteria studied. Cao *et al.* (1984*a*, *b*) suggested that the absence of the epitopes recognized by two of their antibodies may indicate virulence. Antigenic marker for virulence may exist, however, we have been unable to demonstrate consistent markers or antigenic changes during in vitro and in vivo passage, or both which are associated with a specific pathologic condition. Perhaps antigenic markers for virulence reside on areas of the virion surface associated with cell attachment and not neutralization. Although these monoclonal antibodies were not markers for virulence, their detection of antigenic changes in recovered virus vs inoculum indicated viral replication in the animals inoculated. In addition, the antigenic changes in recovered virus indicate substantial alterations in virion structure during in vitro and/or in vivo replication, which did not affect virulence.

The most consistent finding in animals with fatal CB4 infection was high virus titers in visceral organs. This is consistent with the findings of Cao *et al.* (1984*a*) who demonstrated higher cardiac viral titers in Balb/c animals infected with amyocarditic low passage field isolates of CB4. Similar animals infected with myocarditic isolates had lower viral titers in the heart. To date, mortality and viral replication in visceral organs of Balb/c animals are the most consistent way to evaluate the potential virulence of an isolate.

This study was designed to evaluate changes in virulence caused by the site of viral isolation (e. g., tissue tropism of viral subpopulations) and changes due to in vitro passage (e. g., mutation and tropism for cultured cells). We have shown virulence to be a stable characteristic. CB4 capable of producing fatal infections remained virulent after eight passages in vitro, this despite monoclonal antibody detected genetic drift which occurred during passage. Virus recovered from animals inoculated with virulent isolates remained virulent despite the site of isolation or the cell line used for isolation. Furthermore, by in vivo and in vitro passages of a virulent isolate accentuation of this phenotype was found (decreased mean survival time). Suggesting current isolation procedure, if they in fact select for specific virus subpopulation, select for those virions which are phenotypically virulent.

Kuno *et al.* (1984) have evaluated several low passaged CB4 isolates for their ability to produce hypoglycaemia and, in contrast to this study, found fecal (i. e., gut) isolates more likely capable of inducing glucose intolerance

in SJL/J mice. All their isolates were recovered on AG-1 cells. Differences in this diabetogenic potential varied among isolates and, in one case, virus recovered the same day from two different sites within one individual varied in their ability to induce glucose intolerance. Because of the short mean survival time in our last experiment (3 days), it is more likely death was due to the cardiotropic properties of our CB4 isolate rather than its diabetogenic potential. Myocarditic properties of CB4 are passage stable and not necessarily tissue specific. Whether the diabetogenic properties of CB4 may be tissue (e. g., isolation site) specific, or passage stable, requires further experimentation.

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