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Cytopathogenic Effect in Cardiac Myocytes but Not in Cardiac Fibroblasts Is Correlated with Reovirus-Induced Acute Myocarditis

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A panel of reovirus strains was used to compare myocarditic potential with induction of cytopathic effect in primary cardiac myocyte and cardiac fibroblast cultures. The results suggest that viral cytopathogenicity in cardiac myocytes, but not in cardiac fibroblasts, is a determinant of reoviral myocarditis.

Viruses are most likely the predominant cause of human acute myocarditis in the United States and western Europe (10, 15, 23). Our current understanding of the pathogenesis of acute myocarditis is primarily attributable to experimental studies in animal models, because of the difficulty in identifying a specific viral etiology in clinical cases. Many investigators, working with members of the *Picornaviridae* and other viruses, suggest that acute viral myocarditis most likely reflects a direct effect of the virus (1a, 4, 9, 10, 17, 18, 24, 25), although some suggest that even during the acute stage, the host immune response exacerbates tissue injury (10, 11).

Reoviruses can cause acute myocarditis in the neonatal mouse (2, 6, 18, 19–21). Our work has focused on a myocarditic variant, 8B, which is a reassortant virus (i.e., contains genes from two different parent viruses) isolated from a mouse injected with type 1 Lang (T1L) and type 3 Dearing (T3D) viruses (18). When injected into neonatal NIH/Sw mice, 8B is efficiently myocarditic (i.e., induces striking macroscopic external cardiac lesions in approximately all mice), T1L is poorly myocarditic (i.e., induces mild macroscopic lesions in approximately 10% of the mice), and T3D is nonmyocarditic (i.e., induces no macroscopic

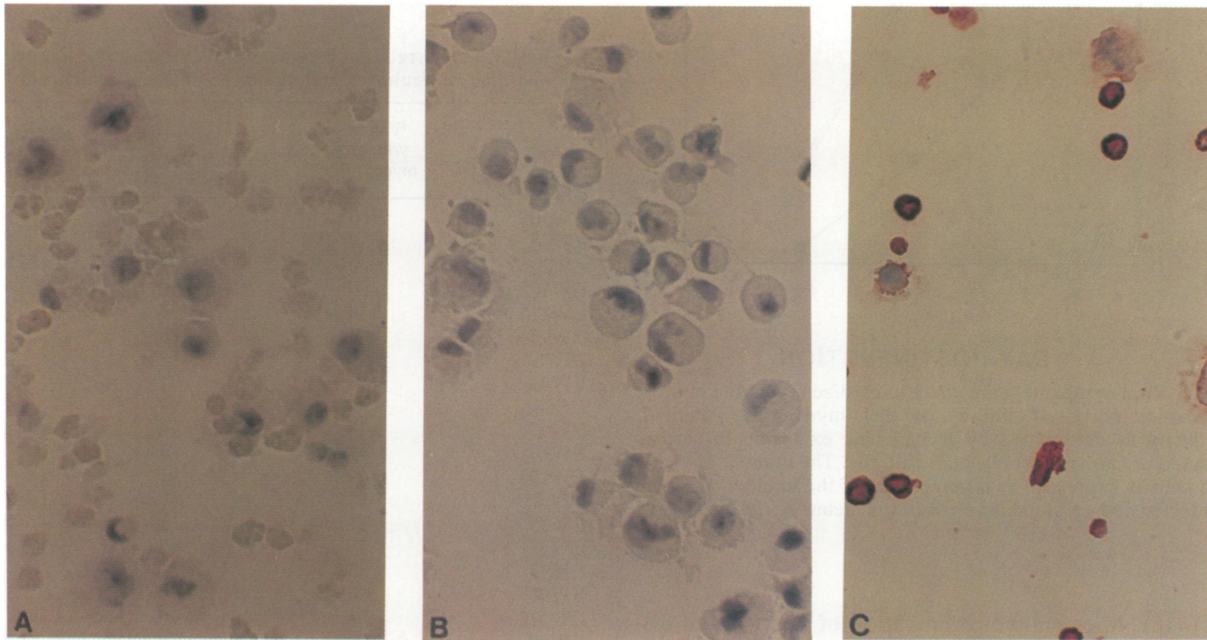

FIG. 1. Cardiac myocytes and fibroblasts are distinguishable by morphology alone. Hematoxylin-counterstained cardiac myocyte cultures (A) and cardiac fibroblast cultures (B). Cardiac myocytes tend to be small and opaque, while fibroblasts are larger and translucent, often with obvious nuclei. (C) Antimyosin (rabbit anti-chicken skeletal muscle myosin antiserum; East-Acres Biologicals, Southbridge, Mass.) immunocytochemistry on cytospin preparations of cardiac myocyte cultures. Magnification, ×400.

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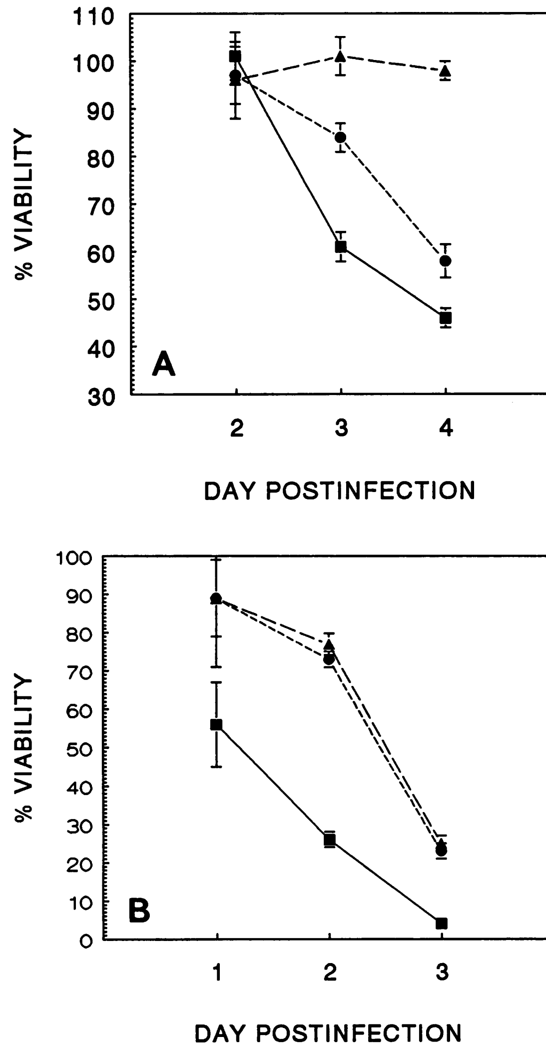

FIG. 2. Viral cytopathogenic effect in cardiac myocyte cultures, but not cardiac fibroblast cultures, parallels myocarditic potential. Cytopathicity as determined by trypan blue exclusion in cardiac myocyte (A) or cardiac fibroblast (B) cultures. The number of viable infected cells is expressed as a percentage of the number of viable mock-infected cells ± standard error of the mean. ▲, T3D; ●, T1L; ■, 8B.

lesions) (18). Microscopic examination of hearts from 8B- and T1L-infected mice reveal necrotic myocytes with mild to moderate inflammatory infiltrate (18). The observations that 8B induces myocarditis in nude (18) and severe combined immunodeficient (17) mice suggest that the cardiac damage is not immune mediated and may be attributable to a direct effect of the virus on cardiac myocytes. Therefore, to investigate the effect of reovirus on cardiac cells, we generated murine primary cardiac myocyte and cardiac fibroblast cultures for infection in vitro.

To generate primary cardiac cell cultures, term fetuses or 1-day-old neonates were sacrificed and the apical two-thirds of their hearts were minced and successively trypsinized. The cell suspension was plated and incubated for 2 h to allow fibroblasts to adhere (fibroblast cultures), at which time the nonadherent cells were removed to fresh dishes (myocyte cultures). Both cultures were incubated (in Dulbecco modified Eagle medium with 7% fetal calf serum; myocyte cultures with 0.06% thymidine) for 2 days before analysis.

Because myocyte cultures are derived from cardiac tissue, fibroblast contamination is a universal problem (8, 12, 14, 22, 26). It was therefore important to reliably differentiate between myocytes and fibroblasts. When we examined paraformaldehyde-fixed cytospin preparations of cardiac myocyte and cardiac fibroblast cultures, we found that the myocytes tended to be small and opaque while fibroblasts were larger and translucent, often with obvious (hematoxylin-counterstained) nuclei (Fig. 1A and B). Antimyosin staining readily distinguished between fibroblasts and myocytes in the myocyte cultures (Fig. 1C) and confirmed that we could distinguish the two cell types by morphology alone. By these assessments, myocyte cultures were contaminated with 5 to 20% fibroblasts, consistent with levels reported by others who quantitated levels of fibroblast contamination (3, 5, 7, 13). We therefore proposed to conduct parallel experiments in both myocyte and fibroblast cultures, in order to ensure that our results from myocyte cultures were specific to myocytes, not contaminating fibroblasts.

Next, we used 8B (efficiently myocarditic) and its parent viruses T1L (poorly myocarditic) and T3D (nonmyocarditic) to compare induction of cytopathic effect (CPE) in cardiac cell cultures with induction of myocarditis. Myocyte and

TABLE 1. Correlation between viral cytopathogenic effect in cardiac cell cultures and induction of gross myocarditis[a]

Virus	% of mice with gross myocarditis[b]	% Viable cells in[c]:	
		Myocyte cultures	Fibroblast cultures
EW10	0	104	ND
EW38	0	103	ND
EW26	0	102	ND
EW102	0	100	49
EW116	0	95	77
EW90	0	88	37
DB76	0	83	62
EW43	0	72	55
DB68	6	94	74
EW47	38	74	23
EW40	41	82	81
EW100	49	91	16
DB62	57	87	74
EW25	71	75	ND
EW67	75	65	44
EW93	80	81	49
EW96	80	70	54
EW112	83	70	9
EW89	100	79	14
EW60	100	78	44
DB88	100	64	5

[a] The correlation between CPE in cardiac cells and gross myocarditis was determined by the Kruskal-Wallis test. When the fraction of mice with gross myocarditis is assigned to two groups (0 to 6% and 38 to 100%), the P value is 0.003 for myocytes and 0.088 for fibroblasts. When the fraction of mice with gross myocarditis is assigned to four groups (0 to 6%, 38 to 57%, 71 to 83%, and 100%), the P value is 0.009 for myocytes and 0.129 for fibroblasts. The P value for myocytes is still significant if the four viruses which were not tested in the fibroblast cultures are removed and the test is repeated.

[b] Hearts from infected mice were scored for the presence of macroscopic external cardiac lesions (gross myocarditis); a mouse was considered positive if one or more lesions were detected.

[c] Myocyte or fibroblast cultures were infected with the indicated virus at a multiplicity of infection of 5 PFU per cell. Viability was determined by trypan blue exclusion at 3 days postinfection. The number of viable infected cells is expressed as a percentage of the number of viable mock-infected cells. ND, not determined.

fibroblast cultures were mock or virally infected at multiplicity of infection of 5 PFU per cell (inoculum removed and replaced with medium as above after 1 h of attachment). At 1, 2, 3, or 4 days postinfection, cells were harvested and analyzed for viability (trypan blue exclusion). The number of viable infected cells is expressed as a percentage of the number viable mock-infected cells (Fig. 2).

There was no obvious cell death in any of the infected myocyte cultures at 2 days postinfection (Fig. 2A). At 3 and 4 days postinfection of myocyte cultures, cell death was greatest for 8B infections, present to a lesser extent in T1L infections, and inapparent in T3D infections. Since these cultures contained only 5 to 20% fibroblasts, the cell death observed in 8B- and T1L-infected myocyte cultures reflected predominantly myocyte cell death. Thus, these three viruses exhibited a relative cytopathogenicity to cardiac myocytes that paralleled their potential to induce gross myocarditis in neonatal NIH/Sw mice.

In contrast to the myocyte cultures, there was significant cell death in infected fibroblast cultures at 2 days postinfection (Fig. 2B); indeed, most cells were dead by 3 days postinfection. T3D and T1L were similarly cytopathogenic to fibroblast cultures at 1, 2, and 3 days postinfection, while 8B was more cytopathogenic. The observation that 8B left none of the fibroblasts viable but left 50 to 60% of the myocytes viable is consistent with our immunocytochemical observations that reoviruses can infect essentially all of the fibroblasts but only a maximum of 50% of the myocytes (1).

To test whether, in general, a reovirus strain's myocarditic potential was correlated with induction of CPE in cardiac cells, we compared the two traits using a panel of 8B-derived reassortant viruses (Table 1). These reassortant virus sets (17 or 21 viruses) represent a subset of the panel (31 viruses) previously used to identify the 8B M1 gene as associated with induction of myocarditis (Fig. 1 in reference 16). In that analysis, there were five viruses (EW10, EW38, EW26, EW102, and DB76) which behaved anomalously in that they failed to induce myocarditis even though they had an 8B-derived M1 gene. The inclusion of those viruses here was critical, in order to test whether myocarditic phenotype, regardless of M1 genotype, was correlated with induction of CPE in cardiac cells in vitro. However, their inclusion precluded a genetic analysis of the results, since they represented a large fraction of the viruses tested.

Myocyte and fibroblast cultures were infected at a multiplicity of infection of 5 PFU per cell (Table 1) and were examined for viability at 3 days postinfection (when T1L- and 8B-induced CPEs in myocytes were maximally different; Fig. 2A). As a group, the viruses were more cytopathogenic to fibroblasts than to myocytes (as were 8B, T1L, and T3D; Fig. 2). These same reassortant viruses were injected intramuscularly into neonatal NIH/Sw mice at a high dose (4×10^6 to 4×10^7 PFU), and the hearts were subsequently scored for the presence or absence of gross myocarditis (at death or at 2 weeks postinjection, as in reference 16). These data were analyzed by the Kruskal-Wallis test (Table 1). CPE in infected myocyte cultures, but not in infected fibroblast cultures, was significantly associated with induction of gross myocarditis (*P* values of 0.003 and 0.088, respectively).

Although, in general, the potential to induce CPE in myocytes was correlated with the potential to induce gross myocarditis (statistically significant; Table 1), several viruses displayed behaviors that segregated these two phenotypes. Notably, EW100 was poorly cytopathogenic to myocytes yet induced gross myocarditis, while EW43 was highly cytopathogenic to myocytes yet failed to induce gross myocarditis (Table 1). Induction of CPE in myocytes is therefore not the only determinant of induction of myocarditis.

The data provide two important conclusions. First, it is apparent that cell type (i.e., cardiac myocyte versus fibroblast) determines the cytopathogenicity of a given virus (Fig. 2 and Table 1). Second, there is a significant positive correlation between viral induction of gross myocarditis and cytopathogenicity to cardiac myocyte cultures but not cardiac fibroblast cultures. This provides strong evidence that 8B-induced myocarditis reflects a direct cytopathogenic effect of the virus on the cardiac myocytes. In the future, we will use reovirus-infected cardiac myocyte cultures to identify other parameters critical to reovirus-induced myocarditis.

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