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SHORT REVIEW

Mouse Strain-related Variation as a Factor in the Pathogenesis of Coxsackievirus B3 Murine Myocarditis

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Coxsackievirus B3 (CB3) is a well known cause of acute heart muscle disease in humans and experimental animals. After virus is cleared from the heart, a subacute myocarditis or cardiomyopathy may ensue and these conditions are thought to be due to host immune response. During the acute phase of myocarditis, however, the pathogenesis of myocardial fibre destruction remains uncertain. One theory proposing an immune mechanism is based on the 2 to 3 day delay in pathological changes observed after peak virus titres in the heart and their chronological synchrony with the development of cytotoxic T lymphocyte (CTL) activity in the spleen of infected animals (Khatib *et al.*, 1980; Woodruff & Woodruff, 1974). Moreover, adoptively transferred, Cr-labelled sensitized T cells are re-routed to the heart at about the time when myocardial fibre damage becomes apparent by light microscopy (Reyes *et al.*, 1984). Additionally, this theory is supported by the facts that infection of T cell-deficient mice or pharmacological suppression of T lymphocytes by anti-thymocyte serum results in a milder form of myocarditis (Woodruff & Woodruff, 1974) and that the transfer of sensitized T lymphocytes from immunocompetent animals to T cell-deficient subjects augments histopathological changes (Huber & Job, 1983; Hashimoto *et al.*, 1983; Guthrie *et al.*, 1984; Huber *et al.*, 1984).

Another theory proposes a direct virus-induced tissue destruction as the mechanism of cardiac injury. In support of this theory there are several reported studies which show that the extent and severity of myocardial fibre damage is directly related to the presence of infectious virus in the heart. In these experiments, any measure which augments the intensity of myocarditis, such as exercise, hydrocortisone, non-steroid anti-inflammatory agents or cyclophosphamide (CY) therapy, invariably increases virus titres and the duration of virus presence in the heart (Kilbourne *et al.*, 1956; Gatmaiton *et al.*, 1970; Woodruff, 1979; Rager-Zisman & Allison, 1973). Although these observations do not undermine the role of the immune response in the pathogenesis, they imply that direct viral c.p.e. is the principal cause of myocardial damage.

Effects of cyclophosphamide in CD1 mice

To clarify this discrepancy in the proposed pathogenesis of acute CB3 myocarditis we examined the effects of immunosuppression with CY in CB3-infected 2-week-old CD1 mice. This therapy is known to produce profound immunosuppression and to preclude the development of T cell-mediated tissue damage (Gilden *et al.*, 1972). We chose outbred mice to exclude any genetically controlled pathogenesis unique to inbred strains.

The animals were inoculated intraperitoneally (i.p.) with 10^5 $TCID_{50}$ of CB3. Three days later they were randomized to receive a single i.p. injection of saline or CY at 150 mg/kg of mouse weight. Thirteen or 14 animals from each group were sacrificed randomly on days 6 and 9 after infection. Blood was collected from the axillary vein. Hearts were removed aseptically, weighed and the heart to body weight ratio was calculated. An apical segment from each heart was removed and frozen at $-70\,^{\circ}C$ for virus isolation.

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Table 1. *Effects of immunosuppression with CY on antibody titres, virus replication and heart weight to body weight ratios in CB3-infected CD1 mice*

	Days post-infection	Treatment: Cyclosphosphamide	Treatment: Saline
Antibody titres	6	22 ± 9·9* (n = 10)†	96 ± 68·4 (n = 10)
	9	242 ± 121 (n = 10)	155 ± 104 (n = 10)
Virus titres in the heart‡	6	4·7 ± 4·6§	2·5 ± 2·4
	9	3·7 ± 3·5‖	0·5 ± 0·5
Heart weight/body weight ratios × 10^5	6	722·3 ± 98·2¶	620 ± 58·2
	9	947 ± 184·7¶	613·3 ± 117·7

* Mean ± standard deviation.
† Number of individual serum samples.
‡ $TCID_{50}$ (tissue culture infective dose) expressed as logarithm to the base 10.
§ Significantly higher titres ($P < 0·05$, *t*-test).
‖ Significantly higher titres ($P < 0·001$, *t*-test).
¶ Significantly greater ratios on both days ($P < 0·05$, *t*-test).

Table 2. *Comparative gross and microscopic cardiac lesions in CY- or saline-treated mice infected with CB3*

	Treatment: Cyclophosphamide	Treatment: Saline
No. of animals with grossly evident myocarditis/no. examined (%)	22/27* (82)	13/27 (48)
Score of myocardial fibre necrosis†	3·02 ± 0·92‡	2·26 ± 0·67
Score of inflammation†	2·4 ± 0·3§	2·5 ± 0·3

* Significantly higher incidence ($P < 0·05$, Chi-square test with Yates correction).
† Mean ± standard deviation.
‡ Significantly more extensive necrosis ($P < 0·01$, Student's *t*-test).
§ Inflammation was significantly reduced on day 6 post-infection (see text).

In this experiment CY-treated mice appeared more ill and had a 14·8% mortality (four of 27 compared with none of 27; *P*0·05, Fisher Exact test). Neutralizing antibody titres measured according to a standard procedure (Melnick & Ledinko, 1950) tended to be lower on day 6 post-infection but by day 9 they were comparable to saline-treated animals (Table 1). Virus assay on monolayers of Vero cells in microtitre plates (virus isolates were identified as CB3 by neutralization tests; Melnick *et al.*, 1979) on day 6 post-infection showed that viraemia was absent in all 12 saline-inoculated animals but it was present in five of the 13 CY recipients (*P*0·024, Fisher Exact test). Thereafter, virus was not detectable in the blood of either group (10 and nine animals respectively). Virus was recovered from the heart more frequently from CY recipients on day 6 (13 of 13 compared with seven of 12; *P*0·015, Fisher Exact test) and day 9 (eight of nine compared with one of ten; *P*0·001, Fisher Exact test) post-infection. Moreover, virus titres calculated according to the method of (Reed & Muench, 1938) were remarkably higher in CY recipients (Table 1).

In the pathological examination, cardiomegaly was more prominent in CY-treated animals as shown by heart to body weight ratios (Table 1). Additionally, gross epicardial lesions were present in higher numbers among CY recipients. Microscopically, inflammation (scored as described by Woodruff & Kilbourne, 1970) was diminished on day 6 post-infection in immunosuppressed mice (score of 1·6 ± 0·5 compared to 2·5 ± 0·5; $P < 0·01$, Student's *t* test, Fig. 1). By day 9 post-infection, the extent of inflammation was similar in both experimental groups (score of 2·9 ± 0·3). Myocardial fibre necrosis measured according to a scoring scale from 0 to 4+ (Rezkalla *et al.*, 1986) was significantly more extensive in CY-treated animals (Table 2).

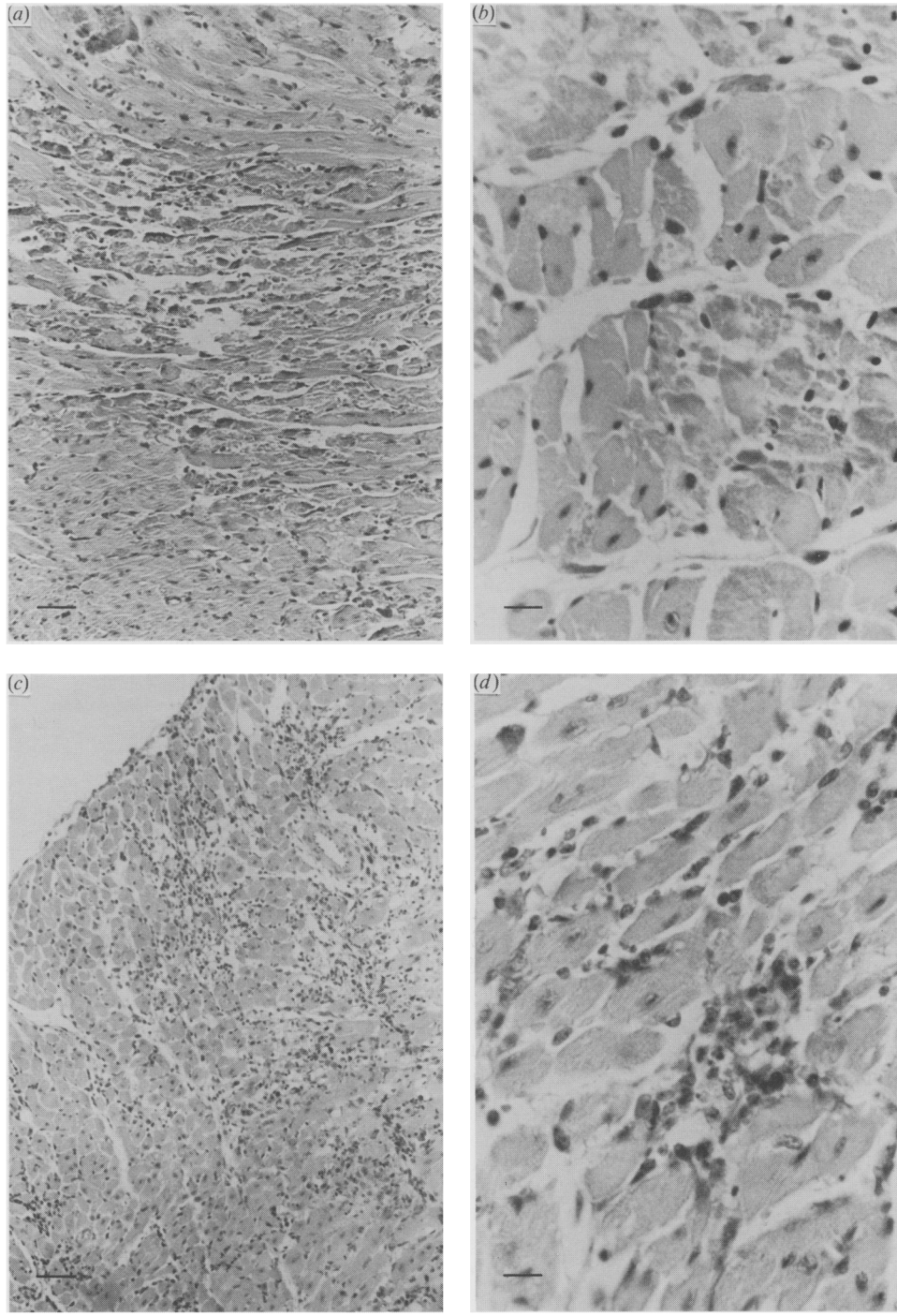

Fig. 1. Microscopic findings of CB3 myocarditis in a 27-day-old CD1 mouse, 6 days after infection. (*a*) Multifocal areas of severe necrosis with minimal mononuclear inflammatory cell infiltration in a CY-treated animal. (*b*) Same specimen showing extensive necrosis without inflammatory cells. (*c*) Multifocal mononuclear inflammatory cell infiltration with limited necrosis in a saline-treated animal. (*d*) Mononuclear inflammatory cell infiltration with mild necrosis in the same specimen. Bar markers represent 100 μm in (*a*) and (*c*); 25 μm in (*b*) and (*d*).

The results of this investigation indicate that virus cytopathogenicity is the principal determinant of acute cardiac injury in CD1 mice. Our conclusion is based on the intensified myocardial damage and cardiomegaly associated with higher virus titres among immunosuppressed mice. Since CY had no direct effect on the heart (data not shown), we believe that these changes are due to enhance virus replication. Although we did not examine the immune response in these animals to verify their immune deficiency, the remarkable reduction of myocardial inflammation in treated mice indicated effective immunosuppression and strongly supported our conclusion. Moreover, the effects of a similar dosage of CY on the host immune status and its modulation of other CTL-mediated diseases are well established (Gilden *et al.*, 1972).

Strain-related variation in pathogenicity

This hypothesis of pathogenesis due to acute myocardial damage may not be applicable to all murine strains. In BALB/c mice, for instance, circumstantial evidence suggests that CTLs play a significant role in mediating histopathological changes, since Woodruff & Woodruff (1974) have shown that infected animals acquire virus-specific CTL responses which selectively damage infected myocardial cells *in vitro*. The peak of CTL activity correlates temporally with maximum inflammation and necrosis in the heart. Additionally, other investigators have demonstrated that depletion of T lymphocytes greatly suppresses pathological changes without affecting virus clearance and transfer of sensitized CTLs to thymectomized, lethally irradiated and bone marrow-reconstituted animals or to genetically T cell-deficient nude mice of BALB/c background restores their susceptibility to severe myocarditis (Woodruff & Woodruff, 1974; Reyes *et al.*, 1984; Huber & Job, 1983; Hashimoto *et al.*, 1983; Guthrie *et al.*, 1984; Huber *et al.*, 1984). These findings are consistent with the proposal that CTLs mediate CB3-induced acute heart muscle damage in BALB/c mice. Among other inbred strains where the pathological spectrum of CB3 myocarditis was noted to be wide (Herskowitz *et al.*, 1985), the mechanisms of early cardiac injury may not be the same. To examine the scope of this diversity, we reviewed the literature for all reports dealing with the pathogenesis of acute CB3 murine myocarditis. We arbitrarily sorted abstracted data into those describing the effects of assessment *in vitro* of the immune response in infected animals, those describing the results of comparing CB3 infection in nude mice with their immunocompetent counterparts and those describing the outcome of immunomodulation of infected but otherwise competent animals.

Assessment in vitro *of the immune response in infected animals*

CTL activity in infected animals has been assessed in BALB/c, CBA, C57B1, C58/WMD, CD1 and C3H/HeJ mice (Huber *et al.*, 1980; Gudvangen *et al.*, 1983; Wong *et al.*, 1977*a*, *b*, *c*; Grun *et al.*, 1984; Huber & Lodge, 1984). The presence of autoreactive or virus-specific CTLs capable of damaging cultured foetal cardiocytes *in vitro* has been demonstrated in all of these strains except C3H/HeJ.

These cells, particularly virus-specific CTLs, are suspected of recognizing a non-virion cardiac neoantigen expressed as a result of infection (Paque *et al.*, 1978, 1979). Their T cell origin is verified by their species restriction and by the absence of cytotoxic activity after treatment with anti-thymocyte serum plus complement. Furthermore, Guthrie *et al.* (1984) showed these cells to be Lyt $1^{-}2^{+}$ (cytolytic/suppressor) subtypes, verifying their cytotoxic nature. Although the activity of these cells *in vitro* is adequately characterized, their role in mediating pathology *in vivo* has not been studied except in BALB/c mice. The results of these studies will be discussed later.

CB3 infection in nude mice

Nude mice, due to their congenital T lymphocyte deficiency, have been used extensively to study T cell dependence in disease expression or control. They have been used by many investigators to study CB3 replication and the pathogenesis of CB3-induced myocardial damage. The results of these investigations seem to depend on the genetic origin of these animals. In nude mice of BALB/c background, clearance of CB3 was shown to be T cell-

Table 3. *Review of CB3 myocarditis in nude mice: mouse background-dependent variability in virus clearance and the severity of myocarditis as compared to T lymphocyte-competent animals*

Reference	Strain of mice	Virus clearance	Myocardial necrosis
Hashimoto *et al.* (1983)	BALB/c	ND*	Diminished
Robinson *et al.* (1981)	BALB/c	Unchanged	Unchanged
Hashimoto & Komatsu (1978)	BALB/c	Unchanged	Diminished
Roesing *et al.* (1979)	ND	ND	Diminished
Schnurr & Schmidt (1984)	NFR	Diminished	Unchanged
Schnurr *et al.* (1984)	N:NIH(S) (II)	Diminished	Delayed

* ND, Not described.

Table 4. *Mouse strain-related variations in the outcome of immunomodulation in CB3 murine myocarditis: review of the literature*

Reference	Mouse strain	Method of immunosuppression	Virus clearance	Myocardial necrosis
Woodruff (1979)	BALB/c	TxBM*	Unchanged	Diminished†
Huber & Job (1983)	BALB/c	TxBM	Unchanged	Diminished†
Guthrie *et al.* (1984)	BALB/c	TxBM	Unchanged	Diminished†
Huber & Job (1983)	BALB/c	TxBM	Diminished	Diminished†
O'Connell *et al.* (1986)	BALB/c	Cyclosporin	Diminished	Increased
Woodruff & Woodruff (1974)	CD1	Cortisone	Diminished	Increased
Kilbourne *et al.* (1956)	CFW	Cortisone	Unchanged	Increased
Woodruff (1979)	CD1	ATS‡	Unchanged	Diminished
Rager-Zisman & Allison (1973)	CBA	Cyclophosphamide	Diminished	Increased
Woodruff & Kilbourne (1970)	MF1	Under-nutrition	Diminished	Increased
Grun *et al.* (1984)	C3H/HeJ	ND§	ND	Unchanged
O'Connell *et al.* (1986)	ICR	Cyclosporin	Diminished	Increased
Khatib *et al.* (this report)	CD1	Cyclophosphamide	Diminished	Increased

* TxBM, Thymectomy, lethal irradiation and bone marrow reconstitution.
† In all four studies myocardial necrosis was intensified after adoptive transfer of T lymphocytes.
‡ ATS, Anti-thymocyte serum.
§ ND, Not described.

independent whereas pathological changes appeared to require competent T cells (Hashimoto *et al.*, 1983; Robinson *et al.*, 1981; Hashimoto & Komatsu, 1978; Roesing *et al.*, 1979). However, among other strains significant increases in virus titres and duration of virus presence were demonstrable in T cell-deficient animals (Table 3). Moreover, T cell dependence of histopathological changes was not evident or was minimal (Schnurr & Schmidt, 1984; Schnurr *et al.*, 1984). The pertinence of these results to the role of CTLs in CB3 myocarditis is uncertain because of the complexity of differences between these nude mice and their 'normal' counterparts in respect to other aspects of the immune response such as antibody production and natural killer cell activity.

Outcome of immunomodulation of infected animals

In assessing the effect of immunomodulation on the outcome of CB3 infection, strain-related variations were also apparent (Table 4). Immunosuppressive therapy with cortisone, CY, cyclosporin or malnutrition leading to severe atrophy of lymphoid organs increased CB3 titres in CBA, CD1 or MF1 mice and enhanced target organ damage in CBA, CD1, MF1 and CFW mice in all experiments with one exception (Kilbourne *et al.*, 1956; Woodruff, 1979; Rager-Zisman & Allison, 1973; Woodruff & Kilbourne, 1970; O'Connell *et al.*, 1986). Concerning BALB/c mice, however, reports in the literature are conflicting. In some reports T cell depletion (by thymectomy, lethal irradiation and bone marrow reconstitution) and adoptive transfer of lymphocytes implicate CTLs in mediating histopathology (Woodruff & Woodruff, 1974; Huber & Job, 1983; Guthrie *et al.*, 1984; Huber *et al.*, 1984). The authors in these investigations also assumed that the enhanced CB3 cardiopathogenicity observed in males was due to sex-related

differences in CTL activity (Huber *et al.*, 1982; Lyden & Huber, 1984). Other investigators, however, were unable to demonstrate specific CTL activity or to verify their involvement in pathology (Grun *et al.*, 1984). Moreover, in a recently published experiment, immunosuppression of infected BALB/c mice with cyclosporin enhanced virus replication and myocardial necrosis (O'Connell *et al.*, 1986). Although the means of immunomodulation in these investigations differ, they are still considered to be conventional methods of assessing immunopathogenicity and their results should be regarded as equally meaningful.

Concluding remarks

The reasons for these discrepancies in the descriptions of the pathogenesis of CB3 myocarditis in BALB/c mice are not clear. Variable experimental conditions might be responsible for it. However, they might be related to the known genetic variability of this strain (Alter & Bach, 1982), or the tendency of some mice to develop intrinsic cardiomyopathy at an older age (Wilson & Lerner, 1978). It is possible that this form of cardiomyopathy is mediated by myocardiocyte-specific CTLs. During CB3 infection the generation of these cells would be stimulated and amplified at a younger age leading to an early and extensive expression of this 'idiopathic' form of heart muscle disease.

These reports suggest that the severity of histopathology in acute CB3 myocarditis in most murine strains is directly proportional to the presence of infectious virus in the myocardium and is probably related to viral c.p.e. This conclusion conforms with the known results of interactions between T cells and cytolytic or immunopathic viruses *in vivo* (Berger & Blanden, 1981). Depletion of T cells in the host results in reduction of inflammation in target organs for a variety of virus infections. However, the ultimate fate of infected cells is determined by the nature of the pathogen. In dealing with immunopathic viruses such as lymphocytic choriomeningitis virus in adult mice, immunosuppression prevents cell destruction and ameliorates the disease. In contrast, in infections with cytopathic viruses, such as CB3, immunosuppression is expected to increase cell death and the spread of infection.

Although these data question the role of CTLs in acute cardiac injury in CB3-infected mice, we cannot ignore the possibility that these cells are involved in the continuous inflammation and myocardial fibre destruction following virus clearance. In this phase of the disease it is suspected that humoral and cellular autoimmunity mediate pathological changes of cardiomyopathy but the role of these defence mechanisms has not been adequately substantiated (Wolfgram *et al.*, 1985).

In summary, this review of the literature suggests that myocardial damage in the acute phase of CB3 infection is caused by direct virus cytopathogenicity rather than by the host immune response in CD1 mice; this is probably so in all other murine strains with the possible exception of some selectively bred BALB/c mice.

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