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Limited Persistence of Viral Antigen in Coxsackievirus B3 Induced Heart Disease in Mice[1] (40455)

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Results from several studies (1–6) suggest that cellular immune mechanisms play a major role in the pathogenesis of Coxsackievirus-induced murine myocarditis. In particular, sensitized cytotoxic thymus-derived lymphocytes (T-cells) have been shown to interact with affected target heart cells following disappearance of detectable infectious Coxsackievirus (3, 6). While the origin and the nature of the antigen(s) in affected heart cells to which specific T-cells are directed remains unresolved, Paque *et al.* (5) have demonstrated that hearts from mice infected with Coxsackievirus B3 contained an antigen(s) which could be extracted with hypertonic salt solution. Using *in vitro* migration–inhibition assays, these workers showed that this antigen(s) inhibited migration of Coxsackievirus B3 sensitized mouse peritoneal exudate cells. The absence of a clear relationship between this antigen(s) and viral structural proteins is in contrast to the earlier observations of Burch *et al.* (7) who, by immunofluorescence techniques, detected type-specific viral antigen in biopsies of human hearts in which there was no detectable infectious virus.

The present study was done to explore further the possibility that viral structural antigen persists in Coxsackievirus infected mouse heart tissue despite the absence of infectious virus.

Materials and methods. The origin of Coxsackievirus B3, strain Nancy has been described elsewhere (8) as has the method for virus purification (9). This strain was used throughout this study except where indicated otherwise. A cardiotropic variant of Coxsackievirus B3, strain Nancy was kindly provided by Dr. Jack Woodruff, Cornell University Medical School, New York. This variant was designated W-1. The virus plaque assay for viral infectivity (8) was done in triplicate or quadruplicate using HeLa cell (JJH) monolayers prepared in plastic tissue culture dishes. NIH/Swiss (random bred) mice were purchased originally from FLOW Laboratories (Rockville, MD.) and thereafter propagated in our laboratory. Tissues for histologic examination were fixed in phosphate buffered formalin, dehydrated, embedded in paraffin, sectioned, and stained with Delafields's hematoxylin and eosin. For immunofluorescence studies, tissues were frozen in liquid nitrogen, cut into 10 micron sections and fixed in cold acetone. Thereafter, the tissue sections were stained and examined for specific fluorescence as described by Goldberg and Crowell (10). Rabbit antiserum made against purified Coxsackievirus B3 and used in fluorescence studies had a virus-specific neutralizing antibody titer of 1:8000. NIH/Swiss mice (6–8 weeks old) were immunized by intraperitoneal inoculation of Freund's complete adjuvant (FCA) mixed with virus or with 10% heart suspensions derived from mouse tissues infected for 7, 14, or 21 days, respectively. Subsequently, two booster doses were given at weekly intervals and consisted of the immunogen plus FCA and the immunogen plus incomplete Freund's adjuvant, respectively. Two weeks later, each mouse was bled and the plasma saved for serologic studies. Mice were bled prior to immunization and their plasma assayed for the presence of antibody to Coxsackievirus B3. Virus neutralization assays were done by the plaque reduction method (11). Samples of plasma from experimental and control mice were tested for neu-

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tralizing antibody to purified Coxsackievirus B3 and to stock pools of Coxsackievirus B3 which had been serially passaged in mice up to 20 times. Immunodiffusion assays were done by methods described elsewhere (12).

Results. Studies to measure persistence of infectious Coxsackievirus B3 in mice following intraperitoneal inoculation revealed (Table I) that while virus was present 6 days postinfection (p.i.) in the heart, pancreas, liver and spleen; only the heart and pancreas contained virus by 11 days. On rare occasion, minimal amounts of infectious virus were detected in the heart at day 17. Similar studies with second, third and sixth serial passage of virus recovered from mouse hearts showed that the virus titer in heart tissue was maximal between 5 and 7 days p.i. and declined thereafter.

To increase the likelihood of acquiring a cardiotropic variant of Coxsackievirus B3 (Nancy), twenty serial passages of infected heart tissue were made in adult mice (13). As shown in Table II, titrations of infected heart tissue taken at each successive passage revealed only relatively small differences in virus titer, *i.e.* less than 50-fold. Furthermore, cardiomyopathic changes induced by the initial mouse passage of B3 virus were not significantly different from those seen in mouse hearts infected with virus which had been passaged 20 times. That is, at passage 1 and at subsequent passages, the myocarditis seen at 7 days p.i. was characterized by discrete localized focal lesions consisting of infiltrates of histocytes, mononuclear cells, and plasma cells which surrounded necrotic myofibers. Slight arteritis and pericarditis also were observed. At 14 and 21 days p.i., similar findings were made except that the size of the lesions and number of foci increased.

Experiments were then performed to determine whether serial passage of heart-adapted virus affected the persistence of infectious virus and/or viral antigens in heart tissue compared to that observed with purified B3 Nancy virus. Accordingly, at selected passage levels between 11 and 19, heart tissue was pooled and homogenates were made at 7, 14 and 21 days, p.i. respectively and assayed for infectious virus. The data (Table III) showed that regardless of passage level, virus titers in heart tissue were either minimal or not measurable by 2 weeks postinfection. Also included in this study was the cardiotropic strain, coxsackievirus B3 (W-1). This strain produced cardiac lesions of greater severity than that produced by the wild-type B3 (Nancy) virus strain used routinely in our laboratory and for this reason was included for comparative purposes. However, the "W-1" strain like that of wild-type B3 virus was not found in significant amounts in infected

TABLE I. RECOVERY OF PURIFIED COXSACKIEVIRUS B3, STRAIN NANCY FROM SELECTED ORGANS OF ADULT N.I.H./SWISS MICE AT INTERVALS POSTINFECTION.

	Days postinfection		
Tissue	6	11	17
Heart	2.5×10^{6}[a]	1.7×10^{6}	1.5×10^{3}
Pancreas	2.1×10^{7}	1.2×10^{4}	<20
Liver	3.4×10^{5}	<20[b]	<20
Spleen	7.3×10^{4}	<20	<20

[a] Titer expressed as PFU/g tissue.
[b] The value of <20 represents the lower limit of the plaque assay for detecting Coxsackievirus in HeLa cells.

TABLE II. COMPARATIVE YIELDS OF COXSACKIEVIRUS B3 FROM HEARTS OF ADULT NIH/SWISS MICE FOLLOWING INFECTION WITH SERIALLY PASSAGED VIRUS DERIVED FROM MOUSE HEARTS.

Serial passage number	Number of mice per passage	Day of harvest	Virus titer[a]
1	4	6	2.5×10^{6}
5	2	5	2.1×10^{5}
10	3	7	4.6×10^{5}
15	3	7	9.5×10^{6}
20	13	7	9.9×10^{5}

[a] Expressed as PFU/g of tissue.

TABLE III. RECOVERY OF SERIALLY PASSAGED COXSACKIEVIRUS B3 FROM THE HEARTS OF ADULT NIH/SWISS MICE AT SELECTED INTERVALS POSTINFECTION.

Virus passage number	Virus titer[a] Days postinfection		
	7	14	21
11	4.4×10^{5}	<20	<20
12	6.7×10^{6}	1.8×10^{2}	<20
17	3.0×10^{6}	<20	<20
19	4.3×10^{6}	3.0×10^{1}	<20
20	9.9×10^{5}	<20	<20
W-1[b]	1.2×10^{6}	<20	<20

[a] Virus titers expressed as PFU/g of tissue.
[b] First NIH/Swiss mouse passage in this laboratory of the cardiotropic variant of Coxsackievirus B3 obtained from Dr. Jack Woodruff (Cornell University Medical School).

heart tissue by 14 days p.i.

To show that the plaque assay in HeLa cells was sufficiently sensitive to detect small amounts of infected virus, numerous samples were assayed in parallel in both HeLa cells and in newborn mice. Those heart tissue suspensions devoid of detectable amounts of virus by the plaque assay also failed to kill newborn mice.

Since mice infected with Coxsackievirus B3 were shown to undergo seroconversion (unpublished observation), the possibility existed that infectious virus could persist in infected heart tissue as either virus–antibody complexes or cell-associated virus. To examine the likelihood of either phenomenon, representative specimens were assayed for infectious virus before and after treatment of the specimen with glycine buffer at pH 1.5. Such treatment for 1 min. at 0° followed by dilution with BSS-CaS3 to pH 7.0 has been shown to dissociate both virus-antibody complexes and cell-associated virus without reducing the infectivity of the dissociated virus (14). It was found that the specimens assayed before and after low pH treatment yielded the same amount of infectious virus indicating that neither of the postulated phenomena were operative. The finding by Burch and his colleagues (7) of B3 viral antigen in diseased human heart tissue despite the absence of demonstrable infectious virus led us to determine whether similar results could be obtained in our experimental system. Experiments employing both infectivity studies and indirect immunofluorescence assays (IFA) were done on mouse heart tissue obtained at 7 and 21 days following infection by Coxsackievirus B3 at serial mouse passages 9, 12 and 20 respectively. As a positive control, heart tissue infected with the "W-1" strain of B3 virus (see Materials and Methods) was assayed at 7 days p.i. for the presence of infectious virus and for viral antigen by IFA. Negative controls consisted of normal uninfected heart tissue. The results showed that specimens containing as much as 1×10^6 PFU per g of tissue were negative for immunofluorescence. Specimens of mouse pancreas which produced higher yields of virus of about 10^9 PFU/g of tissue, exhibited positive immunofluorescence suggesting that substantial amounts of viral antigen are required in the tissue before positive immunofluorescence could be observed.

In the experiments just described, pathologic changes were found in adult mouse hearts at 14 and 21 days postinfection. However, the absence of demonstrable amounts of infectious virus together with the failure to demonstrate viral antigen by immunofluorescence, precluded the presence of large amounts of viral components in these tissues. These findings did not rule out the possibility, however, that small amounts of viral antigen were present. Since as few as 100 PFU of Coxsackievirus B3 will cause an immunologic response in mice, a series of experiments were done to determine whether virion antigen(s) could be detected in the heart tissue of infected mice by testing these tissues for virus-related immunogenic activity. It was hypothesized that even a small amount of viral antigen, if present, could be related to the mechanisms involved in the observed pathology.

As shown in Table IV, only heart tissues containing infectious virus induced neutralizing antibodies in mice. Conversely, heart tissues devoid of measurable infectious virus did not induce virus neutralizing antibodies in mice. The capacity of these latter mice to respond to antigenic stimulus was demonstrated by their production of virus neutralizing antibodies when subsequently immunized with purified Coxsackievirus B3.

TABLE IV. COMPARATIVE IMMUNOGENICITY IN NIH/SWISS MICE OF COXSACKIEVIRUS B3—INFECTED MURINE HEART TISSUES[a] AT INTERVALS POSTINFECTION.

Days postinfection of heart tissue used as immunogen[b]	Amount of PFU per g of heart tissue	Number of mice immunized	Reciprocal of mean antibody titers (PR_{50}) in plasma against[c]	
			Heart passaged virus	Purified B3 virus
7	4.4×10^6	3	880	840
14	None detected	4	<16	<16
21	None detected	8	<16	<16

[a] Heart tissue suspensions were derived from mice infected with Coxsackievirus serially passaged in mouse hearts.

[b] Heart tissue derived from mice that had been infected with Coxsackievirus B3 and sacrificed 7, 14, or 21 days postinfection, respectively.

[c] A 1:16 dilution of plasma was the lowest dilution tested and the plasma from each mouse was assayed separately for neutralizing antibodies.

The possibility was considered that antibodies, other than virus neutralizing antibodies, might be made by mice immunized with heart tissue from which virus had been cleared. Accordingly, plasma from mice immunized with tissue containing infectious virus as well as from mice immunized with heart tissue devoid of infectious virus were tested for virus-specific, precipitating antibody by the Ouchterlony technique. These studies also revealed that plasma from only those animals immunized with heart tissues containing infectious virus possessed antibodies which formed precipitin arcs when reacted against Coxsackievirus B3. Thus, Coxsackievirus B3 virion structural proteins could not be detected in murine heart tissues in the absence of demonstrable infectivity, even though the tissues revealed evidence of typical viral pathology.

Discussion. Postinfectious cardiomyopathy following resolution of acute Coxsackievirus B3 infection has been attributed to involvement of sensitized thymus-derived cytotoxic lymphocytes (1–3, 6). Others, however, have concluded that the observed cytopathology subsequent to resolution of acute viral myocarditis is due to a stage in the healing process of an acute viral myocarditis (15). The pathological characteristics observed in cardiac tissue during the postinfectious period described in the present study is thought to represent both the initial cytolytic effect of Coxsackievirus as well as subsequent immunologic injury. Studies done in our laboratory using athymic "nude" mice support the concept of a cytotoxic T-lymphocyte mediated response since "nude" mice, when infected with Coxsackievirus B3, did not exhibit the same degree of pathology as did similarly infected, immunologically intact mice (unpublished observation).

Peak virus titers were observed in this study to occur at 7 days p.i. In contrast, others (6) have observed maximum virus titers in the heart at 3 days p.i. These differences together with time required for development of severe myocarditis may reflect genetic differences in the strains of mice used as well as differences in the virus variants of Coxsackievirus B3.

While the exact nature of the target antigens to which sensitized T-lymphocytes are directed remains unresolved, several lines of evidence point to either a non-structural viral protein or a cellular neoantigen. In the present report, the inability of postinfection heart tissue suspensions to elicit neutralizing or precipitating antibodies to Coxsackievirus B3 in adult immunocompetent mice suggests that these tissues are deficient in or lack completely the structural Coxsackievirus B3 capsid protein, VP2, which was found to be essential for the induction of neutralizing antibodies (Beatrice, Ph.D. thesis, Hahnemann Medical College, 1976). The absence of precipitating antibodies to B3 virus following immunization of mice with post-infection tissue also indicates the relative or complete absence of the other Coxsackievirus structural proteins. These interpretations, are in accord with evidence obtained by Paque *et al.* (5) who showed that potassium chloride extracts of mouse hearts infected with Coxsackievirus B3 failed to bind Coxsackievirus B3 neutralizing antibodies suggesting that the extracts did not contain viral structural proteins. Further, these KCl extracts in contrast to purified Coxsackievirus B3, were able to inhibit the *in vitro* migration of Coxsackievirus B3-induced immune mouse peritoneal exudate cells.

While Burch (7) detected Coxsackievirus antigen in human heart tissues by immunofluorescence, others (16) have shown that frozen sections of human myocardial tissue may exhibit nonspecific immunofluorescence when stained with fluorescein–conjugated Coxsackievirus antisera. In studies described in the present report, the absence of positive immunofluorescence in frozen sections of infected mouse-hearts may be a reflection of the method employed, since only those tissues containing large amounts of infectious virus were found to be positive by this technique.

Overall, our studies are consistent with the view that Coxsackievirus related, postinfection myocarditis is likely to be due to cytotoxic T-lymphocytes directed against cells containing either non-structural virion proteins or against a neoantigen unmasked or derepressed during Coxsackievirus infection.

Summary. Following acute Coxsackievirus B3 infection of young adult mice, peak virus titers in the heart were found 7 days postinfection, after which virus was cleared. Though postinfection cardiomyopathy was

observed following disappearance of virus, evidence for the continued presence of structural viral antigen in cardiac cells could not be found. Postinfection myocarditis is likely to be due to cytotoxic T-lymphocytes directed against nonstructural viral antigen or, alternatively, to a cellular neoantigen.

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