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Progressive Interstitial Collagen Deposition in Coxsackievirus B3-induced Murine Myocarditis

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Myocardial fibrosis can be produced in certain inbred strains of mice after coxsackievirus B subtype 3 (CVB3) infection. The mechanism responsible for this interstitial matrix alteration is unknown. The presumption is that fibrosis occurs in areas of myocyte damage and inflammation associated with viral infection, analogous to scar formation after cell injury in other organ systems. To test this hypothesis, we examined the hearts of A/J strain mice infected with three CVB3 variants (Crowell [CVB3-CR], Woodruff [CVB3-WD], and Lerner [CVB3-LR]), each known to cause different degrees of acute myocardial injury. With these three variants, virus was present in the heart until day 28 after inoculation but was absent thereafter. Fourteen days after inoculation, inflammation with myocyte necrosis was seen in discrete foci throughout the myocardium with all three variants. Collapse and disorganization of the usually delicate connective tissue matrix identifiable by silver impregnation was seen in these areas of myocyte injury. Persistent, diffuse lymphocytic infiltration of the myocardium was seen 55 days after inoculation with CVB3-WD and CVB3-LR, but hearts initially infected with CVB3-CR showed only rare interstitial lymphocytes comparable to uninfected control hearts. The focal scars produced by myocyte necrosis 14 days after inoculation were accentuated and heavily collagenized 55 days after inoculation with CVB3-WD and CVB3-LR; however, these foci were indistinct 55 days after inoculation with CVB3-CR. Furthermore, the usually delicate network of interstitial collagen fibers surrounding individual myocytes became thickened throughout the heart 55 days after inoculation with CVB3-WD and CVB3-LR, away from visibly scarred areas produced early after infection with these variants. This diffuse reticulin thickening was not seen after infection with the Crowell variant. Only the virus variants associated with persistent interstitial inflammation at day 55 developed major collagen matrix alterations and interstitial fibrosis. We conclude that this persistent interstitial lymphocytic infiltration reflects altered immune function related to specific virus variants in this animal strain. We postulate that these lymphocytes are part of a delayed immunopathogenic response uncoupled from the original viral injury and inflammatory damage. Potential mechanisms by which this interstitial lymphocytic infiltration results in fibrosis are discussed. (Am J Pathol 1990, 136:683–693)

A direct connection between viral-induced myocarditis and cardiomyopathy has remained elusive in humans despite considerable circumstantial evidence in support of the concept that myocardial fibrosis with heart failure is a potential result of viral myocarditis.[1–10] During the past 30 years, studies in experimental animals have provided direct evidence in support of this causal relationship[8–11] and have brought us progressively closer to understanding some of the mechanisms whereby viral infection in the heart may trigger specific pathologic humoral and cellular immunologic responses,[10,12–14] and how these, in turn, produce myocyte injury and death. Furthermore, these experimental studies have documented the late development of interstitial cardiac fibrosis, analogous to that seen in human dilated cardiomyopathy, at a time when viral organisms are not usually detected in the heart.[8–11] The mechanisms responsible for this collagen remodeling are unknown. The present study was undertaken to define the extent and character of interstitial collagen matrix remodeling that occurs in A/J strain mice after infection with specific variants of coxsackievirus group B, subtype 3 (CVB3).

Three CVB3 variants were inoculated into A/J strain mice. This strain was used in the current study based on

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previous observations documenting a vigorous immunopathogenic response to CVB3 infection,[11] and because a potential autoimmune mechanism of inflammatory injury has been implicated in these animals. The virus variants (Crowell [CVB3-CR], Woodruff [CVB3-WD], and Lerner [CVB3-LR]) were selected for study based on previous data suggesting that each was associated with a different degree and persistence of myocardial injury. These variants were selected to explore associations between the acute and chronic aspects of this disease based on the hypothesis that acute CVB3 myocarditis can be uncoupled from chronic myocarditis and cardiomyopathy, depending on the specific interaction of the virus variant with different cellular receptors for the virus. We believe that this latter interaction may be one of the key initiators of immune tolerance dysfunction and the production of autoimmunity in this disease.

Methods

Mice and Infection Strategy

Eight-week-old A/J strain mice (Jackson Laboratories, Bar Harbor, ME) were inoculated with CVB3-CR, CVB3-WD, or CVB3-LR by intraperitoneal injection using 1 ml of a solution of virus adjusted to a concentration of 1.8×10^5 plaque-forming units (PFU) per milliliter. Stock virus concentration was determined before inoculation by standard virus titration in HeLa cells using the plaque formation assay. Two days after inoculation, serum virus titers were determined in each animal using plasma derived from the retro-orbital venous plexus by capillary-tube puncture. Uninfected age-matched control animals were included for study at each experimental interval.

Tissue Processing

Hearts were processed at three intervals after infection (14, 28, and 55 days). Animals were killed by lethal injection of sodium pentobarbital (14d CVB3-CR, n = 5; 14d CVB3-WD, n = 5; 14d CVB3-LR, n = 6; 28d CVB3-CR, n = 6; 28d CVB3-WD, n = 5; 28d CVB3-LR, n = 3; 55d CVB3-CR, n = 3; 55d CVB3-WD, n = 3; 55d CVB3-LR, n = 4). Hearts were removed and sectioned across the ventricles at 2-mm intervals. A portion, including right and left ventricles, was snap frozen and cryosectioned for the plaque-formation assay. One mid-ventricular section was fixed in 10% neutral buffered formalin before dehydration, clearing, and embedment in paraffin wax. Sections from this latter tissue were cut at 4 μ, mounted on glass slides, and rehydrated before the application of histochemical stains.

Silver Impregnation Method for Reticulin Fibers

Experimental and control heart sections from each PI interval studied were reacted with ammoniacal silver using the Gordon and Sweets method for visualizing reticulin fibers.[15] Briefly, sections were oxidized in potassium permanganate, bleached in oxalic acid, and sensitized in 2.5% ferric ammonium sulphate before impregnation with ammoniacal silver (5 ml of 10.2% aqueous silver nitrate, strong ammonia [sp.gr. 0.88] by drop [until precipitate dissolves], and 5 ml of 3.1% sodium hydroxide). Sections were then reduced in 10% aqueous formalin, toned in 0.1% gold chloride, and fixed in 5% sodium thiosulfate. Experimental and age-matched uninfected control hearts were impregnated together to insure consistency.

Morphologic Analysis

The morphologic distribution and extent of myocyte pathologic changes, inflammation, and collagen abnormalities were evaluated in hearts at the cited time intervals after inoculation with the three virus variants. Inflammation was estimated using a 4-grade system (illustrated in Figure 1). Grade 1 corresponded to rare groups of two or three mononuclear cells displacing myocytes, grade 2 corresponded to larger and more frequent mononuclear cell aggregates, without confluence between adjacent foci, grade 3 inflammation was characterized by larger and more frequent mononuclear cell infiltrates with confluent foci present, and grade 4 inflammation was characterized by the presence of many large, geographic foci of inflammatory cells. Also the number of individual lymphocytes in the interstitium was subjectively assessed at day 55 in hearts infected with each variant. Semiquantitative analysis of reticulin fiber silver impregnation in tissue sections of myocardium was performed by morphometric image analysis (Bioquant Meg IV, R&M Biometrics, Nashville, TN) using pixel grey-scale determinations. Mid-ventricular cross sections from age-matched uninfected control animals and virus-infected experimental animals 55 days after inoculation were evaluated. Silver deposition on reticulin fibers was analyzed by first setting a threshold for black silver grains in control hearts. This value was then retained for use in all subsequent measurements in control and experimental hearts. All measurements were performed using a 40X magnification primary objective, resulting in a final image magnification factor of 1530. Ten measurements were taken from each heart section using a graphic window of fixed size, small enough to be repeatedly overlaid on the myocardium. Approximately 90% of

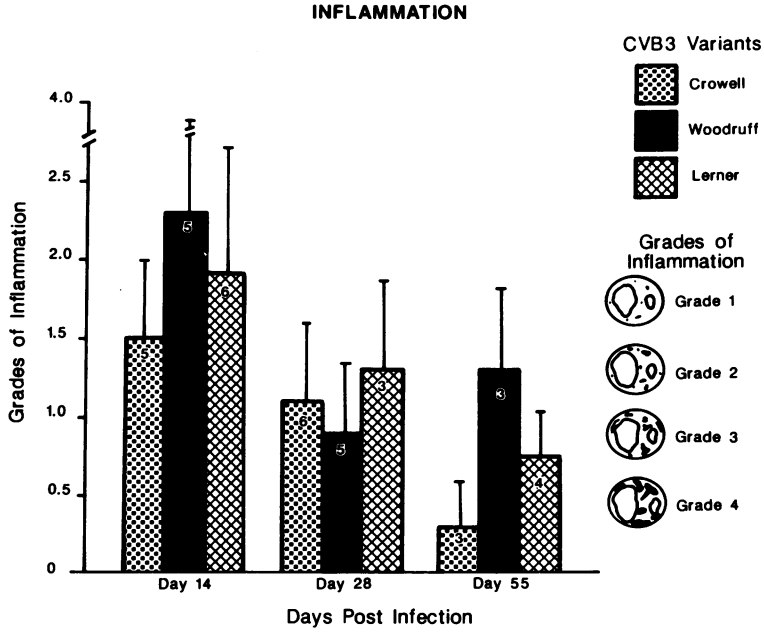

Figure 1. *Myocardial inflammation graded and graphed for the period of study with all three virus variant infections. Grades represent evaluation of* grouped *inflammatory cells only. The amount of inflammation in the hearts infected with CVB3-WD and CBV3-LR appeared to exceed that of hearts infected with CVB3-CR 55 days after infection, but these differences were statistically significant in nonparametric analysis only for the CVB3-WD-infected hearts (P < 0.05).*

each myocardial section was encompassed within the 10 measurements performed.

Statistic Analysis

Dunnett's procedure[16] was used for the comparison of experimental and uninfected control heart percentage of reticulin. Inflammatory infiltration was compared between variants at all intervals studied using a nonparametric Kruskal–Wallis test.[17]

Results

Morphologic and Histochemical Observations

Day 14

Well-defined foci of myocardial inflammation were seen in hearts infected with all three variants 14 days after inoculation (Figure 2 A, C, and E); however inflammatory changes were seen as early as day 7 (not shown). Furthermore, myocyte necrosis, evidenced by cytoplasmic hypereosinophilia, granular cytoplasmic disintegration, nuclear pyknosis, and loss of cytoplasmic striations were seen between 7 and 14 days after inoculation associated with these inflammatory foci. There was considerably more myocyte necrosis 7 days after inoculation with CVB3-WD and CVB3-LR than with the CVB3-CR, both in the number of areas involved and the size of the individual inflammatory foci. However, by 14 days after inoculation, all three variants had produced significant necrosis and inflammatory infiltration. The Lerner variant produced more subpericardial lesions than did the other two virus variants. The myocardial lesions produced by CVB3-CR became calcified by day 14. Focal collapse and irregularity of reticulin fibers was present in areas of myocyte necrosis in hearts infected with all three variants (Figure 3A, C, and E).

Day 28

There was subtle, generalized myocyte enlargement and disorganization with all three variants at this stage (not shown). Hearts initially infected with CVB3-WD or CVB3-LR showed persistent myocyte alterations in the form of cytoplasmic vacuolar change and globular inclusions. The reticulin stain showed definite patchy collapse compatible with earlier inflammatory damage and a suggestion of a general increase in reticulin fiber thickness in areas devoid of major architectural changes.

Day 55

The gross appearance of infected hearts is shown in Figure 4. CVB3-CR infection was not associated with myocardial inflammation at this stage (Figure 2B) and there was only minimal, patchy, collagen deposition noted in areas corresponding to scars of earlier inflammatory injury (Figure 3B). Hearts initially infected with CVB3-LR or CVB3-WD, on the other hand, demonstrated microscopic

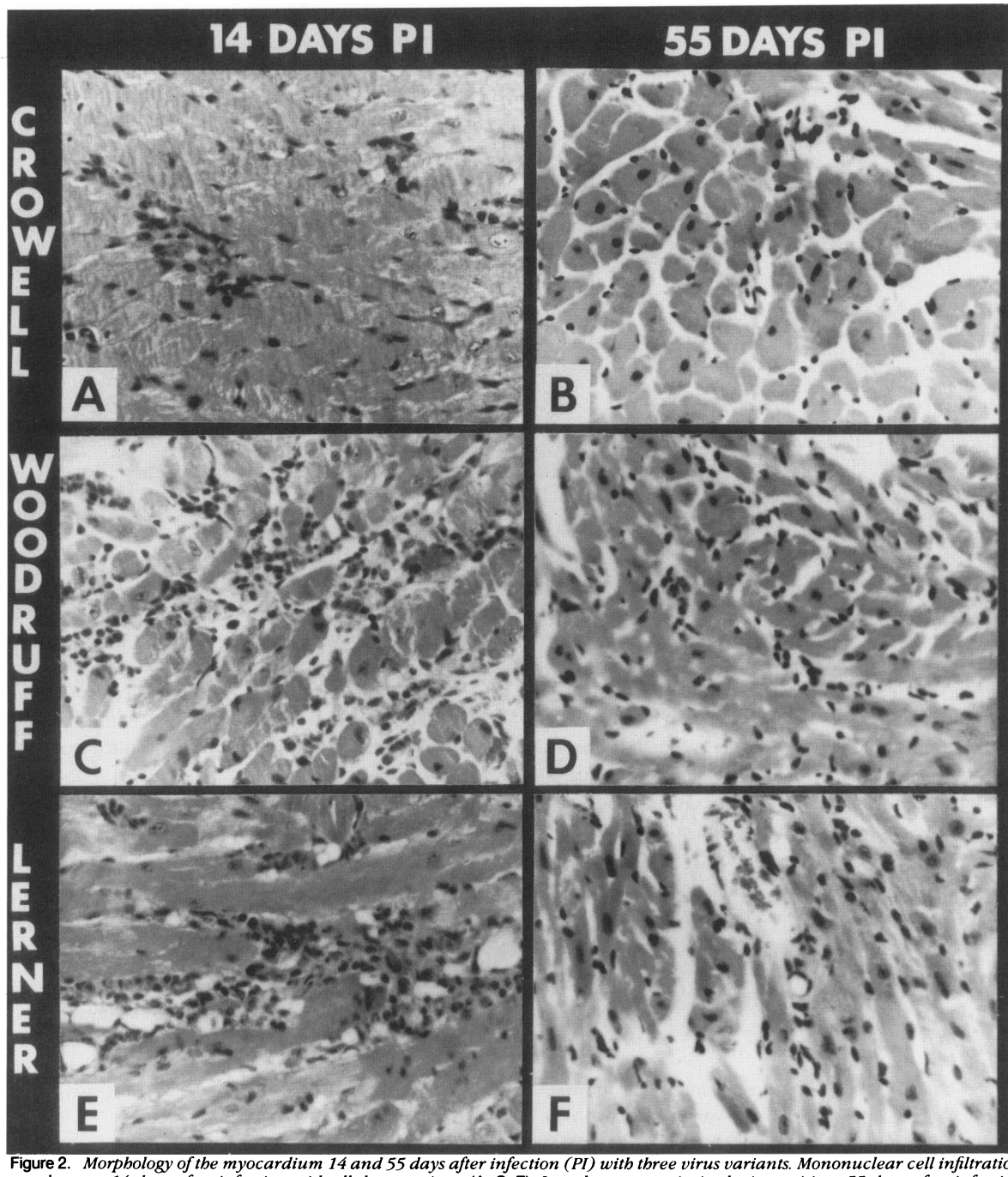

Figure 2. *Morphology of the myocardium 14 and 55 days after infection (PI) with three virus variants. Mononuclear cell infiltration can be seen 14 days after infection with all three variants* **(A, C, E).** *Lymphocytes persist in the interstitium 55 days after infection with CVB3-WD and CVB3-LR. Only rare interstitial lymphocytes are identified 55 days after infection with CVB3-CR (H & E stain, 50× original magnification).*

aggregates of lymphocytes within the interstitium and continued patchy vacuolar alteration of myocytes (Figure 2D and F). It was unclear whether these changes represented early myocyte damage, but no definite necrotic myocytes were seen. The silver stain showed dense reticulin complexity and collapse in areas of pre-existing inflammatory damage (Figure 3B, D, and F) and, in these two variants alone, a generalized increase in reticulin fiber thickness in areas of the heart without architectural alteration (thickening of unscarred reticulin fibers; Figure 5) that exceeded that seen in age-matched uninfected control hearts (Figure 6).

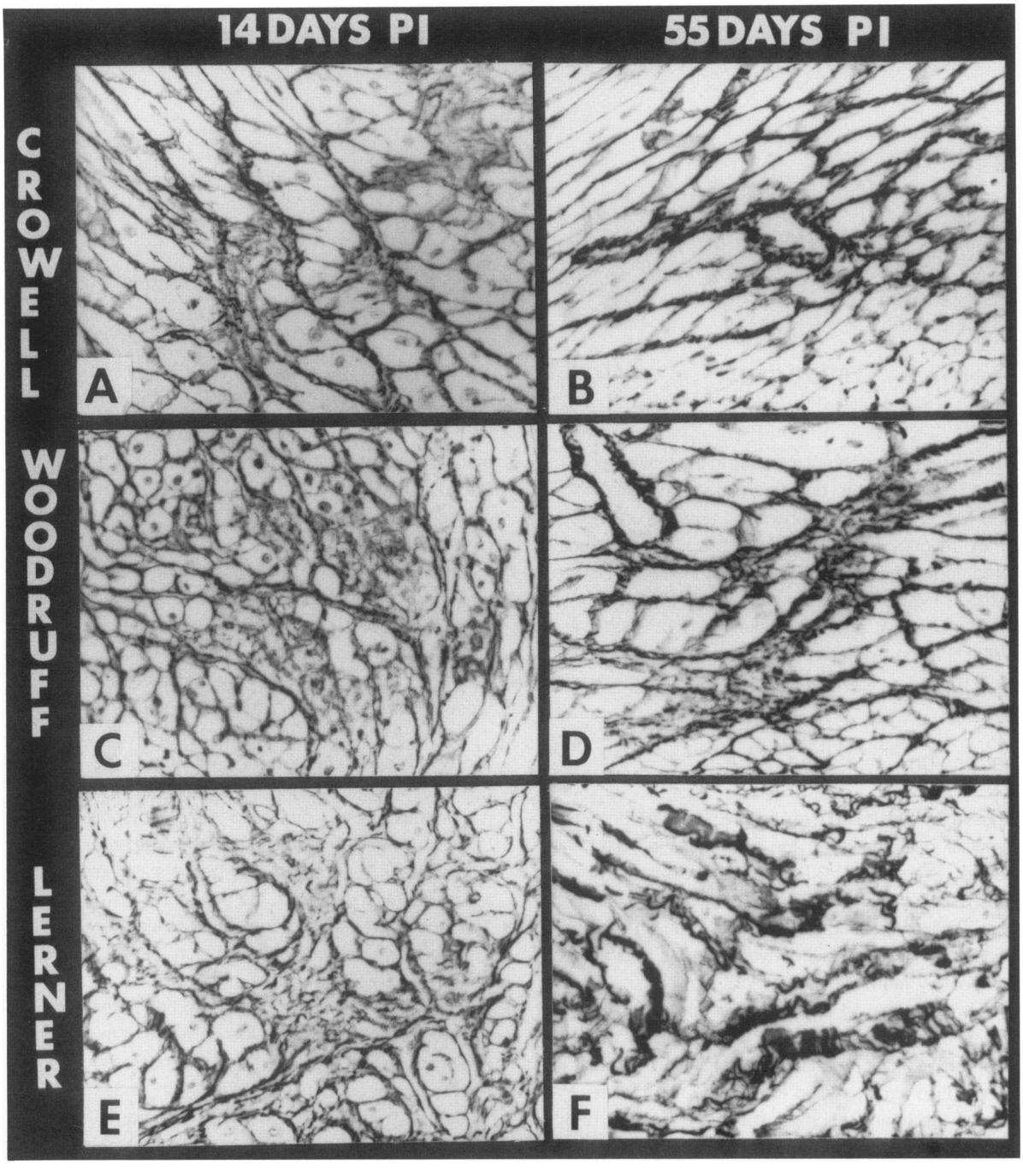

Figure 3. *Reticulin stain of hearts 14 and 55 days after infection (PI) with three virus variants. Note the large pale scars at day 14 with all three variants* **(A, C, E)**. *These scars become progressively collagenized by day 55. Hearts initially infected with CVB3-WD and CVB3-LR show considerably more reduplication of fibers and enlargement of scarred areas when compared to hearts infected with CVB3-CR (Reticulin stain, 50× original magnification).*

A graphic summary of inflammatory changes for all three virus variants is presented in Figure 1. The grades illustrated represent only grouped inflammatory cells and do not include individual interstitial lymphocytes. The amount of inflammation present at day 55 in hearts initially infected with CVB3-WD or CVB3-LR appeared to be greater than that seen in those initially exposed to CVB3-CR. But this difference was statistically significant only for the Woodruff variant-infected hearts ($P < 0.05$). Furthermore, 55 days after inoculation with CVB3-WD and CVB3-

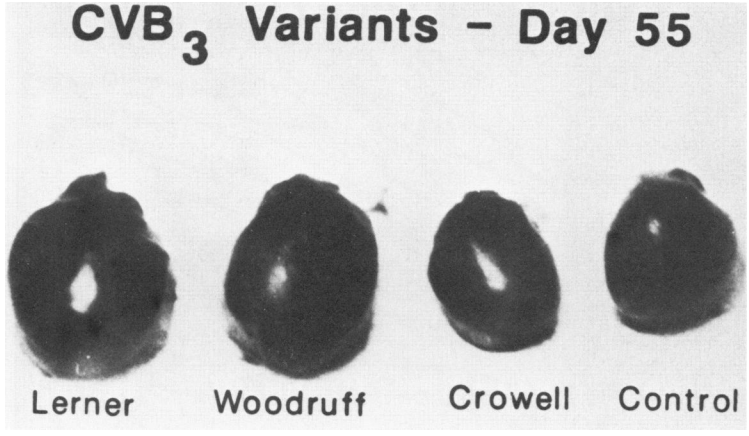

Figure 4. *Gross photograph comparing control and infected hearts 55 days after infection with three virus variants. Note the distinct enlargement of hearts initially infected with CVB3-WD and CVB3-LR compared to uninfected control and CVB3-CR-infected hearts.*

LR, there was an apparent increase in the total number of interstitial lymphocytes, not observed 55 days after inoculation with CVB3-CR. Morphometric analysis of percentage of reticulin in experimental and age-matched uninfected control hearts at 55 days after inoculation is presented in Figure 7. The percentage of reticulin in heart sections 55 days after inoculation with CVB3-WD and CVB3-LR alone exceeded that of age-matched controls ($P < 0.05$).

Discussion

We have demonstrated a relationship between persistent interstitial inflammation in the heart after infection with specific variants of CVB3 and the development of progressive interstitial fibrosis in A/J strain mice. This finding is especially interesting given that all three virus variants used in this study produced myocytolysis and prominent inflammatory mononuclear cell infiltrates throughout the myocardium within 14 days of virus inoculation. This finding was unanticipated in the CVB3-CR–infected animals based on previous reports in which this CVB3 variant (in other mouse strains) failed to produce myocarditis. We are unaware of any reports describing the effects of this virus variant in A/J mice.

The reticulin method used in this study demonstrates the interstitial collagen matrix by reacting with glucosyl and galactosyl (and perhaps other) sugar groups attached to hydroxylysine in collagen, and proteoglycans of the ground substance and basement membranes.[18,19] With this technique, these sugar residues are converted to aldehydes that reduce finely dispersed silver diammine ions in solution to silver metal. Theoretically, four silver ions are initially deposited at the site of each sugar group. These ions are invisible through the light microscope until formaldehyde is added, which causes additional silver metal ions to precipitate at the sites of initial deposition. Because the reticulin demonstrated in this manner includes both collagen and basement membrane proteoglycans, this method is more sensitive than are trichrome methods for demonstrating the interstitial matrix and is, therefore, capable of highlighting changes in total matrix during interstitial remodeling. Our estimations of the amount of reticulin matrix in uninfected control hearts reflect this sensitivity because our measurements exceed those of collagen alone as derived by other methods.

In this regard, two patterns of progressive matrix alteration were seen after infection with the CVB3-WD and CVB3-LR variants. The first pattern was seen in association with focal scars produced in the early phase of the disease. By 14 days after inoculation, large areas of myocyte dropout were replaced by dense stellate scars with tendril-like extensions into the surrounding myocardium. The muscle cells at the edges of these lesions were considerably distorted in their orientation and appeared hypertrophic. By 55 days after inoculation, a considerable percentage of surface area of these hearts reacted with the silver stain for reticulin. We believe that this is a real finding given the amount of myocyte necrosis observed early after inoculation, combined with replacement of these cells by matrix proteins including collagen. The second pattern was manifested by diffuse increase in the thickness of reticulin fibers surrounding individual myocytes in both the endomysial and perimysial connective tissue matrix throughout the heart, even away from areas of focal scar formation. The early scars seen in hearts initially infected with CVB3-CR appeared smaller by 55 days after inoculation, suggesting contraction without much additional matrix deposition at these sites. Alternatively these scars may have been partially remodeled after their initial formation. Furthermore, the diffuse reticulin connective tissue in these hearts was comparable in thickness to age-matched controls, consistent with the

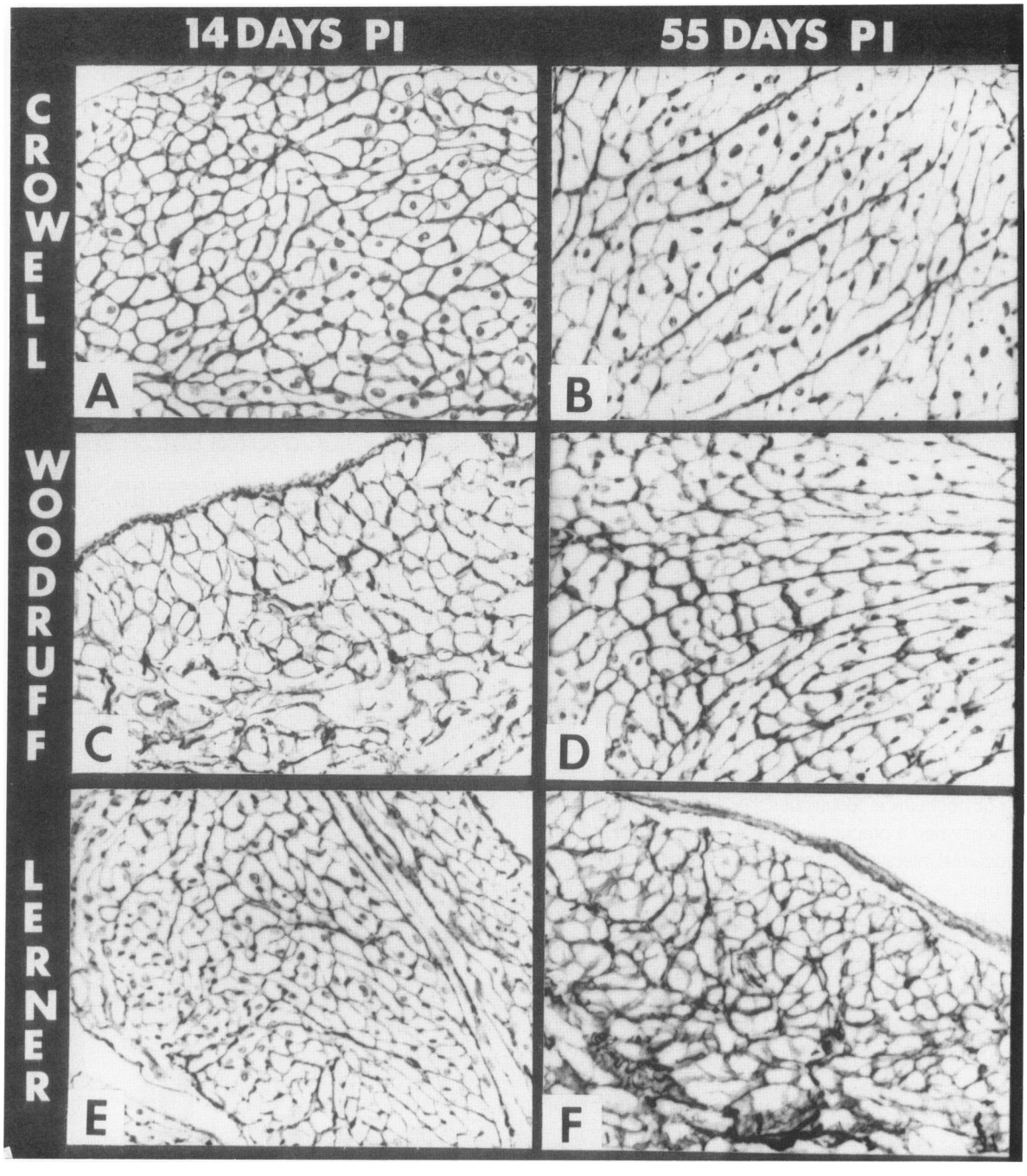

Figure 5. *The reticulin network of the heart 14 and 55 days after infection (PI) is demonstrated after infection with three CVB3 variants. Myocardium away from obvious scars is represented. There is a distinct increase in the reticulin fiber thickness with the Woodruff and Lerner variants between days 14* (A, C, E) *and 55* (B, D, F) *PI. Furthermore, reticulin content away from gross scars 55 days PI appeared to exceed that of age-matched uninfected control hearts (see Figure 5B) (Reticulin stain, 50× original magnification).*

concept that persistent fibrogenic stimuli in the myocardial interstitium may not be present in animals infected with CVB3-CR.

If persistent inflammation correlates with progressive collagen deposition in the heart, several important questions arise. What are the potential mechanisms responsible for persistent lymphocytic myocardial inflammation after certain CVB3 variant infections in the absence of identifiable virus in the heart? What is the relationship between chronic inflammation and fibrosis in this disease, and other inflammatory conditions that result in organ fibrosis? What is the implication of diffuse interstitial fibrosis in the

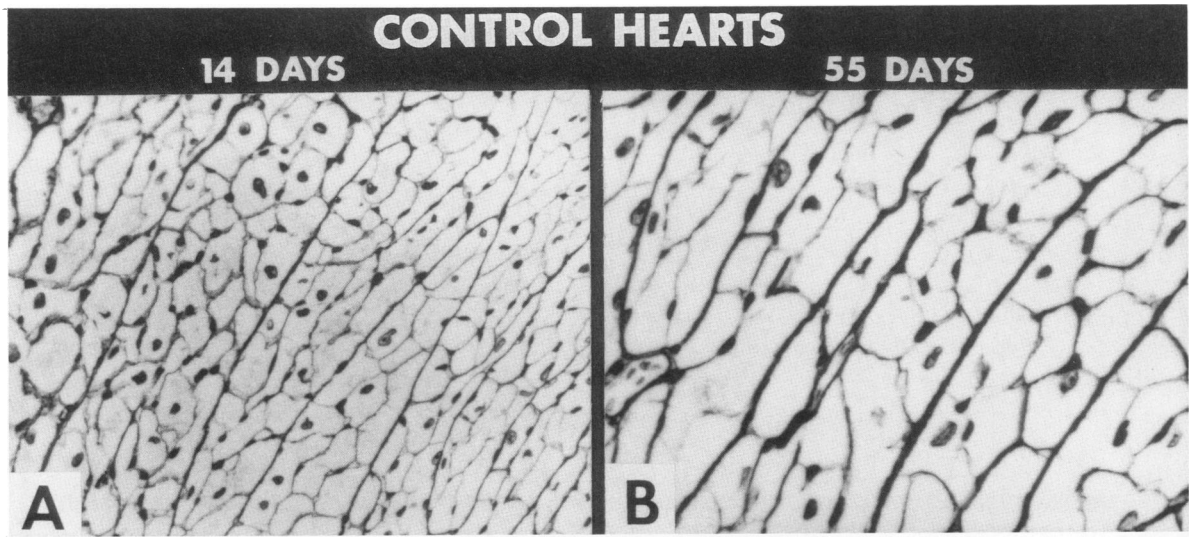

Figure 6. *Age-matched uninfected control hearts stained with the reticulin method. There is a natural increase in the connective tissue matrix of the heart with age. Note that the endomysial and perimysial reticulin fibers are organized into uniform columns or fascicles and there is no focal distortion of fibers as seen in infected hearts (reticulin stain, 50× (A) and 100× (B) original magnification).*

heart relative to myocardial function and organ failure? We will try to address these questions using data derived from human diseases, as well as experimental models of autoimmunity, injury, and repair with fibrosis.

The immunophenotype of the mononuclear inflammatory response in both human myocarditis and CVB3 experimental myocarditis has been characterized using immunocytochemical techniques.[20-25] These studies have demonstrated a preponderance of T lymphocytes in the myocardial interstitial space in association with myocyte necrosis, and diffusely throughout the heart, during active myocarditis. These lymphoid elements reside in the pericapillary space, interposed between the basal laminae of the vascular endothelium and the myocytes. There are several implications of this T-lymphocytic infiltration based on our understanding of the role of these cells in immune surveillance, antigenic stimulation, and cell-mediated immunity. First, the stimulation of antigen-directed T-lymphocytes is dependent on the activation of these cells by antigen coupled to the major histocompatibility complex (MHC) on macrophage cell membranes and other antigen-presenting cells.[26] The nature of the antigen or antigens to which these interstitial T cells respond is unknown. The chronic nature of the inflammatory process, the absence of an identifiable source of nonself antigens in the heart, and the reproducible development of this disease process after specific viral infections has promoted the concept that CVB3-induced chronic myocarditis and its sequelae in experimental animals is an autoimmune disease.[27-30] In humans and some experimental models of chronic virus-induced myocarditis, there is evidence for both cellular and humoral autoimmunity.[10,12-14] The mechanism of this autoimmunity may be related to a variety of cellular events, including alteration of cell-surface membrane proteins resulting from the influence of transient or low-level persistent viral replication within cells of the heart and cross reactivity between viral antigen-sensitized T lymphocytes and specific heart cell membrane components. We propose that the persistent lymphocytic infiltration of the heart seen at day 55 after inoculation with the Woodruff and Lerner variants of CVB3 may represent such a population of antigen-stimulated T lymphocytes.

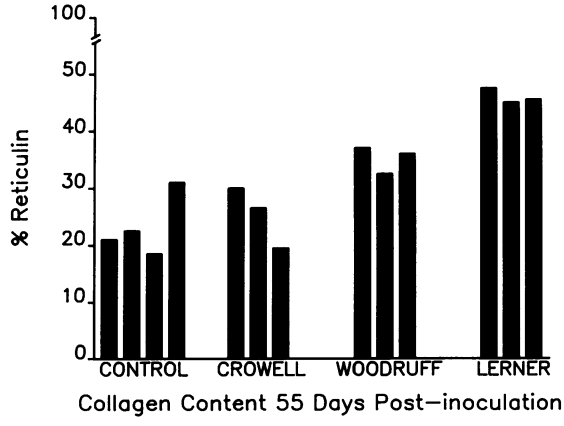

Figure 7. *Collagen content 55 days after infection is represented as the percentage surface area stained with the reticulin method for uninfected controls and hearts infected with three virus variants. The percentage reticulin in the CVB3-WD- and CVB3-LR-infected hearts was statistically different than that of uninfected control hearts using Dunnett's procedure ($P < 0.05$).*

In support of this conclusion, cellular autoimmunity has been demonstrated in this experimental model using lymphocyte-depletion studies conducted on CVB3-WD–infected A/J strain mice in which the combined depletion of LYT2+ and L3T4+ lymphocytes abrogated disease.[11] Still, enlargement of the heart accompanied by interstitial lymphocytic inflammation and prominent myocardial fibrosis occurred in our study only after infection of animals with two of three CVB3 variants. This finding is especially interesting given that all three variants produced equivalent myocyte necrosis and inflammation by 14 days after inoculation. One difference between the variants we used is that the Crowell variant may interact with a different receptor than the other two variants. In support of this concept, Crowell[31] demonstrated disparate receptors for two CVB3 variants *in vitro* in infected HeLa cells, and recently Weller et al[32] have specifically demonstrated different receptors for the Woodruff and Crowell variants of CVB3 *in vivo* in Balb/c CUM mice. Based on these differences, we speculate that the cell membrane receptor(s) for the Woodruff and Lerner CVB3 variants in A/J mice may play an important role in the development of T-cell–related autoimmunity. This phenomenon could occur through several potential mechanisms. First, the interaction of the virus with its receptor might be related to subsequent alteration of the molecular composition of the receptor in connection with MHC presentation in macrophages, analogous to adjuvant-induced autoimmunity. In this scenario, the virus might potentially serve as a hapten for the virus receptor. Alternatively, antibodies directed against the virus may elicit an anti-idiotypic immunoglobulin response that secondarily reacts with the virus receptor on specific heart cells with the eventual production of auto-cytolytic T lymphocytes (ACTL) through MHC coupling in antigen presenting cells.

How these autoimmune cells exert a critical influence on the progressive synthesis and deposition of interstitial collagen is unclear, but there is a considerable volume of experimental data derived from *in vitro* studies on the interactions between stimulated lymphocytes and fibroblasts, as well as circumstantial evidence derived from other organ systems, suggesting a causal relationship between chronic inflammation and interstitial fibrosis.[33–36] In the former studies, secretion of fibrogenic lymphokines such as transforming growth factor beta (TGF-beta) by T lymphocytes has been demonstrated in response to activation. Furthermore, a complex interaction has been suggested between the production of such lymphokines and feedback loops involved in lymphocyte regulation in the microenvironment.[37] Nonetheless, it seems unlikely that autoimmune ACTL serve only as cytokine-producing elements. In fact, their antigen-directed function is more likely aimed at specific antigen-bearing cells within the heart. In this regard, cardiac myocytes have also been shown to elaborate acidic and basic fibroblast growth factors[38] and ACTL directed against myocytes could potentially result in their production and release. Once elaborated, the principal targets of such fibrogenic cytokines are not well defined. The universal presumption has been that the primary responding cell is the cardiac fibroblast, but smooth muscle cells of blood vessels are also capable of producing collagen and may play a role in matrix synthesis in the heart.

The exact relationship between myocardial fibrosis and cardiac failure in dilated cardiomyopathy is unknown. The accumulation of interstitial collagen around individual myocytes might be expected to decrease myocardial compliance and disrupt the synchronous contraction of the ventricles during systole. These changes would be progressive in chronic myocarditis and would effectively increase the workload of the heart leading to myocyte hypertrophy, further decompensation, and finally heart failure with chamber dilatation. The two patterns of myocardial fibrosis identified in our study 55 days after inoculation with CVB3-WD and CVB3-LR clearly represent major alterations in the delicate interfibrillary cross strut configuration that extends between endomysial collagen bundles along the length of the myocyte. This "weave" very likely represents a critical mechanism of passive reconstitution of the ventricular cavities after systole, thereby assisting diastolic filling.[39,40] Thus, at some threshold level, alterations in both macro- and microscopic configurations of this matrix could lead to progressive myocardial decompensation by decreased cardiac output combined with increased intrinsic workload.

In summary, our studies support the hypothesis that acute and chronic viral myocarditis may be distinct pathologic events related to different pathogenetic mechanisms. The absence of significant collagen matrix changes in the Crowell-infected hearts, despite early acute myocarditis, suggests that these two events are associated with dramatically different physiologic and pathologic outcomes. If true, this concept would uncouple these two disease processes and potentially explain how acute myocarditis could be a relatively common pathologic result of viral infection, resolving with little or no morphologic or functional abnormalities analogous to our model of infection with the Crowell variant of CVB3. In the rare instance in which the virus and the host are suitably matched, chronic autoimmune myocarditis would result. Continuous or cyclic infiltration of the myocardial interstitium with ACTL would then result in the stimulation of collagen-producing cells of the interstitial space and the progressive deposition of connective tissue throughout the heart. The specific mechanisms by which this collagen remodeling is achieved remain to be determined.

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