A Survey of Human Myocarditis Cases Diagnosed Using Alexa Immunofluorescence Dyes and Confocal Microscopy

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Abstract: Sudden death as a result of viral myocarditis is a rare but distressing event because it usually occurs in young healthy individuals. Myocarditis is a relatively common complication of viral infections. Specifically, enteroviruses, influenza viruses and adenoviruses have been associated with this disease. In many cases the etiology remains unknown when routine methods are used for diagnosis. With the advent of new molecular techniques such as RT-PCR, stronger amplification techniques such as Alexa dyes for immunofluorescence, and confocal microscopy for reduction of background staining, some of these viruses can be identified. The incidence, clinical history, grosses and microscopic pathology were examined in 22 myocarditis cases that died suddenly. Viral identification was performed on sections from all 22 hearts by Alexa immunofluorescence with confocal microscopy. Influenza A, Coxsackie 3, and Coxsackie 5 were evaluated on all 22 hearts. Cell culture controls transfected with the virus were used as positive controls. One of 22 patients was positive for Influenza A. Six cases were positive for Coxsackie B5, two of which were also positive for Coxsackie B3. Fifteen cases were negative for all antibodies examined. [Life Science Journal. 2006;3(4):37-41] (ISSN: 1097-8135).

Keywords: myocarditis; viral; sudden death

1 Introduction

For the past two centuries, myocarditis has been classified as an inflammatory disease of the myocardium (Carthy, 1997; Feldman, 2000; Bergler-Klein, 2001; Calabrese, 2003). The natural history and pathogenesis is largely still unknown (Hyypia, 1993; Feldman, 2000; Noutsias, 2003). The disease is often fatal and diagnosed on post mortem autopsy (Waller, 1992; Lopes, 2001; Mounts, 2001; Weissel, 2001). The common etiologies are idiopathic, autoimmune and infectious (Furukawa, 2001; Hill, 2001; Leonard, 2004; Pauschinger, 2004; Whitton, 2004). Viral agents, especially the Coxsackie B viruses, are commonly associated with fatal myocarditis (Hyypia, 1993; Carthy, 1997; Feldman, 2000). Other viruses less commonly associated with fatal myocarditis have been Parvovirus (Murry, 2001), Influenza A (Nolte, 2000) and B (Engblom, 1983), Hepatitis C (Matsumori, 2001), HIV, Mumps virus, Cytomegalovirus, Adenovirus (Lozinski, 1994), Echovirus, Poliovirus, and Infectious mononucleosis (Fournier, 2001; Weissel, 2001; Leonard, 2004). In most of the reported studies, only about 20% to 37% of the cases have a definitive viral diagnosis.

Myocarditis has been viewed as the acute stage in the progression of disease leading to heart failure and cardiomyopathy (D'Ambrosio, 2001; Furukawa, 2001; Calabrese, 2003; Mason, 2003; Noutsias, 2003; Whitton, 2004). Generally, myocarditis is viewed histologically as sparse, focal, or diffuse inflammation of the heart associated with focal to multifocal cardiac myocyte necrosis. In end-stage idiopathic cardiomyopathy, the histologic pattern is focal to diffuse myocyte hypertrophy and multifocal to diffuse interstitial fibrosis (Vasiljevic, 2001). In one study using PCR techniques, Hepatitis C was found in a few cases of idiopathic cardiomyopathy (Pauschinger, 2004).

In this study, we report the immunofluorescence analysis using Alexa immunofluorescence amplification of 22 individual cases of fatal myocarditis over a period of 1985 to 2001. As with previous studies, we found a definitive viral etiology in 31% of the cases examined.

2 Materials and Methods

2.1 Tissue specimens

All tissue specimens were retrieved from the Tissue Bank of Indiana University Medical Center Department of Forensic Pathology. All heart specimens were collected from autopsy cases obtained during the period extending from 1985 to 2001. Nine of these cases were collected in the winter of 1997-1998. Three normal cases were used as negative control samples. The samples were selected based on the presence of inflammation after a review by two pathologists using Hematoxylin and

Eosin stained slides. For each of the hearts, two to three histological sections of the left ventricle were examined and at least one section involved a left ventricular papillary muscle.

2.2 Antibodies

The following antibodies were used for immunohistochemistry:

Coxsackie B5 (Chemicon Temecula, Ca)

Coxsackie B3 (Chemicon Temecula, Ca)

Influenza A monoclonal (Chemicon, Temecula, Ca)

2.3 Tissue preparation

Tissues were fixed overnight in 10% neutral buffered formalin and then transferred to 70% ethanol prior to processing through paraffin. Five-micron sections were microtomed, and the sections were placed on positive charged slides. The slides were then baked overnight at 60 ℃ in an oven.

2.4 Immunostaining

The slides were then deparaffinized in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed by immersing the slides in target retrieval solution (Dako) for 20 minutes at 90 ℃ in a water bath, cooling at room temperature for 10 minutes, washing in water and then proceeding with immunostaining. All subsequent staining steps were performed on the Dako immunostainer. Incubations were done at room temperature. Tris buffered saline plus 0.05% Tween 20, pH 7.4 (TBS - Dako Corp.) was used for all washes and diluents. Thorough washing was performed after each incubation. Slides were blocked with protein blocking solution (Dako) for 45 minutes. After washing, 5 μg/ml of the primary antibodies were added to the slides, and then incubated overnight at room temperature. The secondary antibody, Alexa anti-mouse or anti-rabbit fluorescent dye (Molecular Probes, Eugene OR) was added for 1 hour (Panchuk-Voloshina, 1999). The slides were washed, coverslipped and examined.

2.5 Confocal microscopy

A Bio-Rad MRC-1024ES confocal microscope equipped with a krypton/argon laser and a 60×1.4 numerical aperture objective was used to examine the hearts and obtain images. For each heart, the negative control was used to establish a pixel intensity that eliminated 99% of the background signal. Background fluorescence was then subtracted by applying this threshold to all images, and the percent pixels with the remaining signal and average signal intensity were recorded for each image.

2.6 Slide evaluation

Two investigators using a fluorescence microscope to evaluate the intensity and localization of the staining reviewed the slides. Staining was defined as negative (total absence of staining), 1+ (weak staining), 2+ (moderate), or 3+ (strong, intense staining).

3 Results

Histologic examination showed the inflammatory infiltrate to consist of macrophages and plasma cells without neutrophils. No acute inflammation was observed in any case. The inflammatory lesions were focal in the majority of cases and multifocal to diffuse in a few cases. Some cases were severe and diffuse, going transmural from the endocardium to the epicardial surface such as case number 18 (Figure 1). In this case all multiple histologic sections of the left ventricular myocardium were involved (six sections from different LV areas), and there was severe myodegeneration and myocytolysis. Most cases showed evidence of myofiber degeneration. See Table 1 for histologic scoring of the lesions.

Immunofluorescence revealed strong staining with Coxsackie B5 antibody in 6 of the 22 cases, two of which also stained with the Coxsackie B3 antibody. One case out of the 22 stained with the Influenza A antibody. Table 2 summarizes the immunofluorescence in the myocarditis cases with the three antibodies examined. The cell culture specimens used as positive controls were positive with the specific antibodies and did not cross-react to different antibodies against other viruses. The normal human myocardial cases did not stain with any of the viral antibodies.

The immunolocalization of the Coxsackie antibodies were generally confined to degenerating myocardial cells from sections in the left ventricle (Figure 2). There was no antibody staining with any of the antibodies in normal human heart tissues. Degenerating myocardial cell cytoplasm was distinctly stained with the three antibodies in the myocarditis cases. Occasionally the nucleus was stained with some of the antibodies.

In the one influenza myocarditis case, there was staining of lymphocytes and monocytes in the blood vessels within the myocardium in addition to the degenerating myocytes (Figure 3).

Table 1. Histological assessment of myocardium

Case #	Tissue	Inflammatory infiltrate	Myocyte necrosis	Fibrosis	Diagnosis
1	Heart	2	...	0	Myocarditis
2	Heart	3		1	Acute viral myocarditis
3	Heart	3		0	Viral myocarditis
4	Heart	1		0	Myocarditis
5	Heart	0		2	Myocarditis
6	Heart	1	1	2	Myocarditis
7	Heart	3	2	0	Acute myocarditis
8	Heart	2	1	0	Acute myocarditis
9	Heart	3		1	Viral myocarditis (childe sexual abuse)
10	Heart	2		0	Viral myocarditis
11	Heart	0		1	Viral myocarditis
12	Heart	0		0	Myocarditis
13	Heart	3		2	Myocarditis (lymphocytic, plasmacytic)
14	Heart	1		1	Myocarditis
15	Heart	1		0	Viral myocarditis and viral encephalitis
16	Heart	1		0	Resolving viral myocarditis
17	Heart	1		1.5	Eonsinophilic myocarditis
18	Heart	4	4	0	Myocarditis
19	Heart	1		0	Myocarditis
20	Heart	2	0	0	Acute myocarditis
21	Heart	3 F	0	3 F	Giant cell myocarditis
22	Heart	2		0	Myocarditis

Table 2. Immunofluorescence of viral antigens in heart

Case #	Tissue	Coxsackie B3	Coxsackie B5	Influenza A mono
1	Heart	Negative	2+ myocytes	Negative
2	Heart	Negative	Negative	Negative
3	Heart	Negative	2+ myocytes	Negative
4	Heart	Negative	Negative	Negative
5	Heart	Negative	Negative	Negative
6	Heart	Negative	Negative	endothelial
7	Heart	1+ myocytes	1+ myocytes	endothelial
8	Heart	Negative	Negative	Negative
9	Heart	Negative	3+ myocytes	Negative
10	Heart	1+ myocytes	2+ myocytes	1.5+ lymphocytes
11	Heart	Negative	2+ myocytes	Negative
12	Heart	Negative	Negative	Negative
13	Heart	Negative	Negative	2+ endothelial/macrophage
14	Heart	Negative	Negative	Negative
15	Heart	Negative	Negative	Negative
16	Heart	Negative	Negative	Negative
17	Heart	Negative	Negative	Negative
18	Heart	Negative	Negative	2+ myocytes
19	Heart	Negative	Negative	Negative
20	Heart	Negative	Negative	Negative
21	Heart	Negative	Negative	Negative
22	Heart	Negative	1+ endothelial	1.5+ macrophage

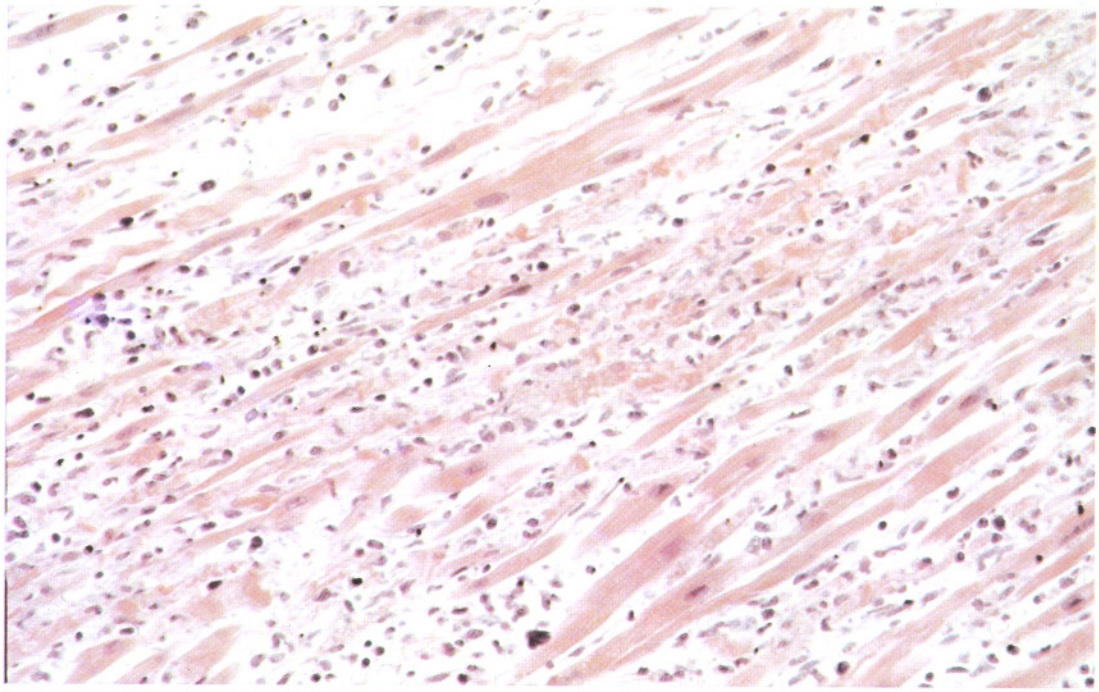

Figure 1. Hematoxylin and Eosin stain on case 18

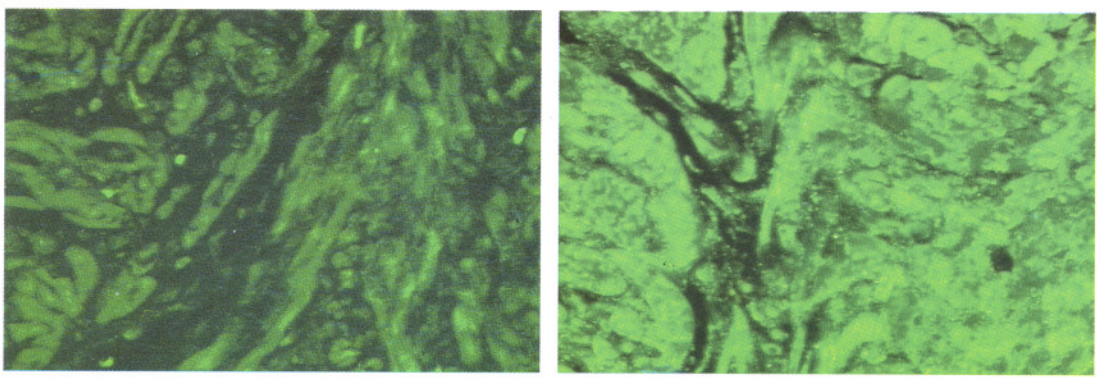

Figure 2. Coxsackie B5 antibody on case 9 (right). Negative control without antibody on case 9 (left).

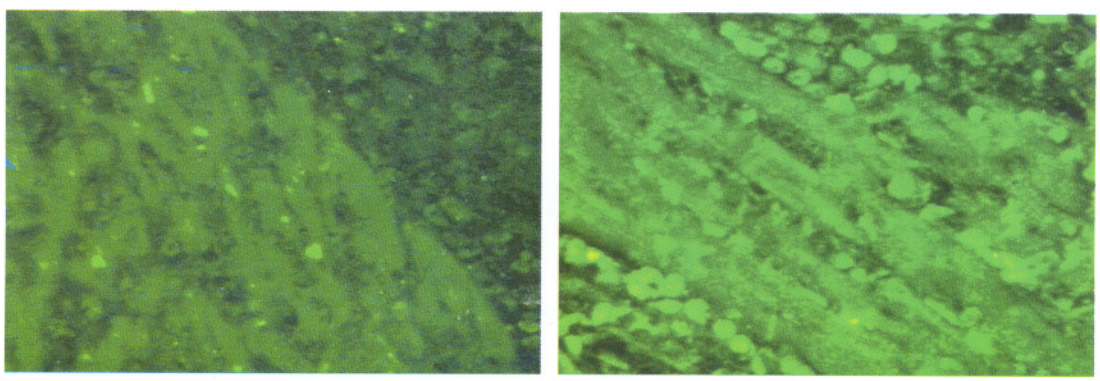

Figure 3. Influenza A antibody on case 18 (right). Negative control without antibody on case 18 (left).

Generally, the surrounding connective tissue stroma, blood vessel smooth muscle, endothelial cells, and associated inflammatory cells in and around the areas of active myocarditis lesions were

not stained with any of the antibodies examined. However, staining was seen in elastic tissue in small arteriole muscular arteries within the myocardium in most cases.

4 Discussion

In this survey we found that about 31% of the cases were positive for viral etiology. This is similar to previous review studies and studies involving collections of human viral myocarditis cases (Hyypia, 1993; Feldman, 2000). Just a few case reports on Influenza A have been reported (Engblom, 1983; Nolte, 2000). This acute case in our survey was very severe, with a severe transmural myocyte degeneration, necrosis, and inflammation.

In this study we used confocal microscopy to evaluate the myocarditis with the new Alexa dyes. These antibodies required long antigen retrieval pretreatment with the Dako antigen retrieval solution in a 90-degree water bath for 25 minutes to obtain reasonable immunofluorescence staining.

The procedure involved long overnight incubation with the Alexa amplification technique to be able to visualize any staining. The confocal microscope allowed us to remove any background scatter fluorescence to see a strong positive signal.

In summary, this survey was similar to previous studies reported in the literatures. This is the first report using confocal microscopy to evaluate human cases of myocarditis.

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