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Myocardial Inflammation, Cellular Death, and Viral Detection in Sudden Infant Death Caused by SIDS, Suffocation, or Myocarditis

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ABSTRACT: The significance of minor myocardial inflammatory infiltrates and viral detection in SIDS is controversial. We retrospectively compared the demographic profiles, myocardial inflammation, cardiomyocyte necrosis, and myocardial virus detection in infants who died of SIDS in a safe sleep environment, accidental suffocation, or myocarditis. Formalin-fixed, paraffin-embedded myocardial sections were semiquantitatively assessed for CD3 lymphocytes and CD68 macrophages using immunohistochemistry and for cardiomyocyte cell death in H&E-stained sections. Enteroviruses and adenoviruses were searched for using PCR technology. The means of lymphocytes, macrophages, and necrotic cardiomyocytes were not statistically different in SIDS and suffocation cases. Enterovirus, not otherwise specified, was detected in one suffocation case and was the only virus detected in the three groups. Very mild myocardial lymphocyte and macrophage infiltration and scattered necrotic cardiomyocytes in SIDS are not pathologic, but may occur after the developing heart is exposed to environmental pathogens, including viruses. (***Pediatr Res*** **66: 17–21, 2009)**

Establishing the cause of death in cases of sudden infant death is challenging, especially in the presence of minor pathologic findings, and relies on review of the medical history, circumstances of death, and postmortem examination. Even with this information, boundaries between diseases may be often blurred, especially when infection is considered. Confusion can result from lack of clinically verified classification schemes that establish minimum severity of inflammation and cellular destruction in vital organs necessary to cause death. There is a lack of consensus regarding the significance of organisms isolated from normally sterile body sites at autopsy in the absence of inflammation (1–3).

SIDS is still the leading cause of sudden unexplained death in infants in the United States (4), but the diagnosis becomes controversial with minor inflammatory infiltrates in the heart, brain, and/or respiratory system. Given this situation, several mechanisms have been proposed to explain the cause(s) of death in SIDS. The "triple risk" hypothesis has gained widespread acceptance to accommodate the multiple factors now known to be important in SIDS (5). This states that SIDS results from the cataclysmic and lethal intersection of the infant's age with its concomitant unstable pathophysiologic status associated with an underlying vulnerability while exposed to environmental risk factors. Although it seems increasingly clear that abnormalities in the medullary serotonergic system are particularly important (6), other triggers, such as viral infections, continue to receive attention. In this regard, clinically unrecognized myocarditis is an important consideration. Beckwith (7) identified five cases of myocarditis among 500 cases of sudden infant death. DeSa (8) identified 20 cases, 17 of which were infants, with isolated myocarditis among 3086 necropsies. In a study of 1516 autopsies of children between birth and 18 y of age, myocarditis was present in 28 (1.8%); half occurred in infants and represented 2% of infant deaths referred for autopsy (1). Conversely, in a Swedish study, myocarditis was identified in 16.8% of 410 infants who died naturally and in 7.4% of 27 violent deaths (9). An Israeli study identified myocardial inflammation in 20% of 35 infants whose death had been initially attributed to SIDS (10). Mild cardiac inflammatory infiltrates have been identified not only in SIDS cases, but also in controls, which can make interpretation of their presence difficult (11).

More than 20 viruses have been identified in humans with myocarditis (12). Coxsackie virus is the most common, but adenovirus, cytomegalovirus, human immunodeficiency virus, and mumps virus are also important (13–18). PCR technology has supplanted culture as a means to detect viruses in cases of sudden infant death (14,15,17).

An interface exists between myocarditis and SIDS. A cause of death is easily ascribed to myocarditis in cases with severe myocardial inflammation and necrosis. However, agreement does not exist at the other end of the histologic spectrum: the minimum amount of myocardial inflammation and cardiomyocyte necrosis necessary to cause death is not established, perhaps because of imprecisely applied criteria, limited microscopic sec-

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Abbreviations: HPF, high-power field; **ME,** medical examiner

tioning, and/or inappropriate or absence of controls. In addition, the amount of a viral load necessary to cause sudden infant death is unknown. The current standard for diagnosing myocarditis rests on application of the Dallas criteria (19–21). However, these criteria were not always followed in earlier studies, wherein the diagnosis rested solely on the presence of inflammation without consideration of the presence of myocardial necrosis.

With this in mind, the aims of our study are to: 1) compare the demographic profiles of three groups of infants who died suddenly: SIDS cases who died in a safe sleep environment, infants who accidentally suffocated, and infants who died of myocarditis fulfilling the Dallas criteria, and, in these three groups; 2) compare the severity of myocardial infiltration by CD3-positive lymphocytes and CD68-positive macrophages assessed semiquantitatively; 3) compare the severity of cardiomyocyte necrosis assessed semiquantitatively; and 4) compare incidence of myocardial viruses identified by PCR techniques.

METHODS

Rady Children's Hospital and Health Center institutional review board approved this study. Postneonatal infants (29–365 d of age) accessioned by the San Diego County Medical Examiner's Office (ME) between 1991 and 2005 whose deaths were attributed to SIDS, whereas safe sleeping were selected retrospectively from the SDSRP database, along with a control group of infants who died of accidental suffocation and a reference group of infants with myocarditis fulfilling the Dallas criteria.

Case data were selected from the medical history, death scene, and postmortem information in the investigative and autopsy reports and from two standardized data protocols for the death scene investigation and postmortem examination. Trained, experienced investigators from the ME are charged with collecting this information within 30 h of an infant's death. The data are not complete for every case.

A diagnosis of SIDS was made when criteria of the 2004 San Diego definition were fulfilled (4). Ancillary studies were routinely negative in these cases. Accidental suffocation and myocarditis were established as causes of death after evaluation of the medical histories, death scene reconstructions, and postmortem examinations. A total of 24 SIDS cases, 25 accidental suffocation cases, and three myocarditis cases were included since they had cardiac tissue available.

At least two sections, generally including the left ventricular free wall and interventricular septum, were available in all cases; in some, up to four sections were available. The tissues were initially fixed in 10% buffered formalin for 24 to 72 h and subsequently embedded in paraffin. Myocardial sections were cut at 4 μm from the existent paraffin blocks, batched, and stained for CD3 lymphocytes and CD68 macrophages in a Shandon Varistain Gemini ES automated slide stainer using Dako mouse anti-human antibodies (Carpinteria, CA) reagents at the immunohistochemical laboratory of the University of California, San Diego Medical Center Department of Pathology. The slides stained for CD3 and CD68 were incubated for 60 and 10 min, respectively, at room temperature. Cell membrane staining for CD3 and granular cytoplasmic staining for CD68 were interpreted as positive. Positive and negative controls stained appropriately.

Two independent observers (CF and HM) blinded to each other's results semiquantitatively assessed 20 contiguous 400× high-power fields (HPFs) of the myocardium for interstitial CD3 lymphocytes, CD68 macrophages, and cardiomyocyte death. The latter was ascertained by the presence of nuclear pyknosis or karyorrhexis and cytoplasmic eosinophilic contraction or cell fragmentation. An average number of cells per 20 HPF per case were calculated from the results of each observer. Contiguous fields of the myocardium that seemed to be representative of the pathology of the entire available microscopic area were selected for analysis. That said, the cases assigned to the myocarditis group had areas of severe lymphocytic infiltration and myocardial necrosis easily seen with scanning magnification as opposed to the other two groups in which case interstitial lymphocytes and necrotic cardiomyocytes were difficult to identify without the aid of high magnification.

PCR detection of adenoviruses and enteroviruses. From each study subject, DNA and RNA were extracted using the Qiagen kit from 8 to 10 slices of paraffin-embedded tissue that had been initially fixed in 10% buffered formalin for 24 to 72 h using the RNeasy FFPE and QIAamp DNA FFPE kits (Qiagen; Valencia, CA) as per manufacturer's instructions. Reverse transcription was applied to the RNA samples by mixing RNA with random primers (Promega Madison, WI) and M-MLV (Invitrogen; Carlsbad, CA), and cycling at 25°C for 10 min, 42°C for 15 min, and 99°C for 5 min. The presence of nucleic acid in all samples was verified by applying PCR to detect the β-globin gene. Briefly, the FastStart PCR Master-mix (Roche, Indianapolis, IN) was mixed with forward primer (ACACAACTGTGTTCACTAGC), reverse primer (CAACTTCATCCACGTTCACC), probe 1 (CAAACAGACACCATGGTGCACCTGACTCCTGAGGA—FL), and probe 2 (LC705-AAGTCTGCCGTTACTGCCCTGTGGGGCAA—PH), all directed at the β-globin gene. The LightCycler (Roche) was cycled at 95°C for 10 min, followed by 45 cycles of 95°C for 5 s, 62 °C for 15 s, and 72°C for 15 s. Positive samples were those that had a crossing point less than 40 cycles and a single melting peak between 64 and 68°C.

Following the confirmation of adequate nucleic acid, adenovirus PCR was undertaken as previously described. Enterovirus PCR was applied using an in-house clinical assay. In the Enterovirus validation assays, as few as 5.5 copies per reaction could be detected. The low-point sensitivity for the adenovirus assay was not determined. The Roche FastStart master mix was mixed with forward primer (5′-TCCGGCCCCTGAATG-3′), reverse primer (5′-CGGATGGCCAATCCA-3′), probe 1 (5′ AAATGAAACACGGACACCCAAA FL-3′), and probe 2 (5′-LCRED 640 AGTCGGTTCCGCTGCAG PH-3′), using the same settings as the β-globin assay. For all assays, positive and negative controls were included. Samples were deemed positive if they had a crossing point less than 45 cycles, and a single melting peak between 51.7 and 56.7°C.

Data analysis. The SIDS and suffocation cases were compared with each other; myocarditis cases were excluded from analysis because of small size. The groups were compared with one another with respect to perinatal factors, term gestation, gender, ethnicity, and medical history. Categorical variables were analyzed using the χ^2 test or Fisher's exact test. Continuous data were analyzed using ANOVA or t tests and are summarized using means $\pm$ standard deviations. Calculations were performed with SPSS Version 12.0. A p value less than 0.05 was considered significant. In place of a κ test for intraobserver and interobserver reliability, t test was used as a measure of correlation of findings between reviewers.

RESULTS

The cases with myocarditis were older than both the SIDS and suffocation cases (Table 1). The SIDS and suffocation control groups were otherwise statistically similar for all other

Table 1. *Demographics and clinical variables in SIDS, suffocation, and myocarditis*

	SIDS, $n = 24$	Suffocation, $n = 25$	p	Myocarditis, $n = 4$
Age (d)			NS*	
Mean ± SD	79 ± 33	83 ± 39		214 ± 144
Range	34–151	32–153		53–364
Male gender	14 (58%)	12 (50%)	NS	2 (50%)
Prematurity (<37 wk)	$n = 21$	$n = 20$	NS	$n = 4$
	4 (19%)	4 (20%)		1 (25%)
Ethnicity			NS	
White	15 (63%)	10 (40%)		1 (25%)
Hispanic	4 (17%)	6 (24%)		2 (50%)
Black	2 (8%)	4 (16%)		
All others	3 (13%)	5 (20%)		1 (25%)
Prenatal or postnatal exposure to tobacco smoke	$n = 13$ 7 (54%)	$n = 17$ 13 (76%)	NS	NA†
Previous ALTE‡	$n = 21$	$n = 17$	NS	$n = 2$
	3 (14%)	1 (6%)		0
Symptoms of URI within 48 h of death	$n = 17$ 7 (41%)	$n = 23$ 11 (48%)	NS	$n = 4$ 3 (75%)
Any prenatal care	$n = 14$	$n = 12$	NS	$n = 1$
	11 (79%)	12 (100%)		1 (100%)
Ever breastfed	$n = 14$	$n = 12$	NS	$n = 2$
	11 (79%)	9 (75%)		2 (100%)

* Not significant.

† Not available.

‡ Acute life-threatening event.

Table 2. *Type of inflammatory and myocardial apoptotic/necrotic cells in SIDS, suffocation, and myocarditis*

Type of cells	SIDS, $n = 24$	Suffocation, $n = 25$	p	Myocarditis, $n = 4$
Lymphocytes, CD3; mean ± SD*	5.5 ± 3.7	5.5 ± 2.7	NS†	36 ± 44.4
Range‡	1.4–14.7	1.5–11.9		3.7–100
Macrophages, CD86; mean ± SD	0.13 ± 0.14	0.18 ± 0.24	NS	1.9 ± 1.6
Range	0–0.425	0–1.1		0.53–4.1
Myocardial apoptotic/necrotic cells; mean ± SD	0.29 ± 0.54	0.13 ± 0.44	NS	0.37 ± 0.27
Range	0–2.2	0–2.16		0.13–0.72

* Mean number of cells per 20 contiguous × 400 fields counted by two observers.

† Not significant.

‡ Range of cells per 20 contiguous × 400 fields counted by two observers.

variables (male gender, term, ethnicity, tobacco exposure, previous acute life-threatening event, symptoms of upper respiratory infection (URI), prenatal care, and ever breastfed).

Statistical results were equivalent among and between groups using each observer's independent assessment scores of CD3 lymphocytes, CD68 macrophages, and cardiomyocyte necrosis, and when their two results were combined and averaged. The combined means are shown in Table 2.

One of the myocarditis cases revealed lymphocytes that were too numerous to count reliably; therefore, a mean of 100 CD3-positive lymphocytes/HPF was arbitrarily assigned.

The means of lymphocytes, macrophages, and necrotic cardiomyocytes for SIDS and suffocation cases were not statistically different (Table 2). The means of lymphocytes and macrophages for the myocarditis cases were higher than the means for lymphocytes and macrophages in the SIDS and/or suffocation groups. The SIDS and suffocation control groups were statistically similar for the mean number of necrotic cardiomyocytes (Table 2).

Adequate nucleic acid was present in all samples, as determined by the PCR assay for human β-globin. Enterovirus, not otherwise specified, was detected by PCR in one of the suffocation cases, but none of the myocarditis or SIDS cases; no adenovirus was detected in any of the myocardial specimens from all three groups.

DISCUSSION

Our study found that the severity of CD3 lymphocyte and CD68 macrophage infiltration into the myocardium as well as the mean number of necrotic cardiomyocytes were not different between SIDS cases found in a safe sleep environment and infants who died of accidental suffocation. In addition, aside from an older mean age for cases dying from myocarditis, the demographic profiles of the three infant groups were not different from each other. Enterovirus, not otherwise specified, was detected in the myocardium of one of the suffocation cases, but none of the SIDS or myocarditis cases; adenovirus was not detected in any of the three groups.

Because the means of the number of lymphocytes and macrophages infiltrating 20 contiguous HPF and the mean number of necrotic cardiomyocytes in a similar area of examination were not different, our study suggests that mild myocardial inflammatory infiltrates, whether or not a causative agent is identified, are not necessarily lethal. Conversely, it suggests that a few scattered inflammatory cells and necrotic cardiomyocytes are not pathologic, but possibly even a "normal" finding in the developing heart that is exposed to new environmental pathogens throughout infancy and early childhood. This conclusion parallels and reinforces that of our earlier study wherein we found essentially no significant differences in rates of clinical URI, severity and type of respiratory system inflammation, and postmortem culture results between SIDS and suffocation cases (22). Previous studies also support our conclusion that inflammatory infiltrates in the cardiac conduction system of infants dying suddenly were present in similar proportions of SIDS cases and control cases or in controls exclusively (23).

Our immunohistochemical findings do not confirm those of Dettmeyer *et al.* (24) who have reported a significant increase in the number of T lymphocytes in SIDS compared with controls who had died of non-natural causes. The reason for these discordant results is not clear, but may relate to different techniques. Their diagnostic criteria for SIDS are not stated. Their ratio of SIDS to control cases was approximately 6:1 and ours was 1:1. They counted the number of cells in 20 randomized, as opposed to 20 contiguous, 400× high-power fields. Instead of a single observer, we determined our mean values by having two independent observers count cells in contiguous fields at the same magnification. Dettmeyer *et al.* counted CD45-positive cells; in contrast, we counted CD3-postive lymphocytes. Perhaps, most importantly, they created a classification scheme for myocarditis that, to our knowledge, has not been verified to be clinically relevant and was arbitrarily based on their cell counts; in contrast, we did not create an arbitrary classification, but simply reported our observed values for lymphocytes and macrophages.

Coxsackie, adenovirus, Ebstein Barr virus, parvovirus B19, herpes simplex 6, cytomegalovirus, influenza A, and respiratory syncytial virus, either individually or in combination, have been identified in cases with conditions as diverse as myocarditis, arrhythmogenic right ventricular dysplasia, dilated cardiomyopathy, and in cases of sudden infant death diagnosed initially as SIDS (17,25–31). The latter cases deserve comment. In the majority of reported SIDS cases, the myocardial inflammatory infiltrates were described as exceedingly mild by routine light microscopy and were not always different from those in cases who died of known causes (11–13). In other cases of sudden unexpected infant death, immunohistologic techniques were necessary to identify inflammatory infiltrates that had not been seen by routine histology; also, PCR techniques detected viruses in some cases in which inflammatory cells were not detected by either routine or immunohistochemical microscopy (31). Some of the cases clearly had systemic viral disease with the involvement of numerous organs that caused their sudden death as opposed to SIDS (31,32).

In a German study, enteroviruses were detected in 22.5%, adenoviruses in 3.2%, Epstein-Barr viruses in 4.8%, and parvovirus B19 in 11.2% of 62 SIDS cases; evidence of viral infection was not detected in any of the 11 control cases (24). This is in contrast to our cases among which an enterovirus

was detected in the myocardium of one of the suffocation cases, but none of the SIDS or myocarditis cases; adenovirus was not detected in any case of the three groups. Our study did not undertake the range of viral analyses of that performed in the German study, but it is notable that none of our SIDS cases revealed evidence of viruses for which we searched. The failure to identify either enterovirus or adenovirus in the myocarditis cases does not exclude a viral etiology that is suggested by lymphocytic infiltration into the myocardium.

Current understanding of the molecular interaction of viruses, inflammatory cells, and cytokines in the myocardium is largely based on experimental models using susceptible (A.BY/SnJ and SWR/J) and resistant (C57BL/6J and DBA/1JJ) mouse strains infected with coxsackievirus virus-B3 and is the subject of a recent, superb review (13). The former develop ongoing myocarditis associated with persistent viral RNA within the myocardium; the latter eliminate the virus after early infection. Although there are acute, subacute, and chronic phases, it is the acute and subacute phases that are most relevant to sudden infant death. During this phase, virus is present in high titer in the myocardium as well as the blood, spleen, and pancreas and undergoes prominent replication in association with cardiomyocyte degeneration and necrosis (33). Cardiomyocytes as well as endothelial cells, fibroblasts, and dendritic cells apparently intrinsic to the myocardium release a host of cytokines, including IL, TNF, and IFN, leading to the subacute phase that is characterized by the release of progeny virus into the interstitium, stimulating natural killer cells and macrophage migration, with an increase in proinflammatory cytokines, and a second wave of inflammatory cells, including CD4+ and CD8+ T lymphocytes. Proinflammatory cytokines lead to marked myocardial infiltration by cytotoxic T lymphocytes that target viral antigens on the surface of infected cardiomyocytes in association with major histocompatibility complex class I (MHC I) antigens. IFN-γ and TNF-α up-regulate the MHC I antigens on the surface of infected cardiomyocytes and up-regulate intracellular adhesion molecule-I on the surface of infected myocytes by providing proper cell-cell contact between T lymphocytes and infected myocytes (34). Also, the MHC class I-restricted peptides generated by the up-regulation of genes processing and presenting viral epitopes increase levels of cardiac immunoproteasomes and alter their proteolytic activity, potentially enabling production of antigenic peptides and subsequent T cell–mediated immune responses (35). Dendritic cells in the mice also become infected and affect levels of IL-10, IL-6, and TNF-α (36). Other studies with animal models have demonstrated that enteroviral protease 2A cleaves dystrophin, thereby disrupting the integrity of the sarcolemmal dystrophin-glycoprotein complex (37–39).

In the final analysis, neither ours nor any of the existing studies succeed in establishing a clinically verifiable or even minimal level of myocardial inflammation and cardiomyocyte necrosis sufficient to cause sudden infant death. Despite the availability of the Dallas criteria (21), pathologists have yet to agree on what constitutes myocarditis (40). Clearly, several variables are at play and potentially include the infant's age, general health, genetic background (13), underlying immune status, exposure to certain risk factors (41), safety of the sleep environment (Hauck FR 1997 Bedsharing: review of epidemiologic data examining links to SIDS. In: Abstracts of the Infant Sleep Environment and SIDS Risk Conference; January 9–10, 1997; Bethesda, MD) (42), site of myocardial inflammation (*e.g.*, conduction system), severity of myocardial necrosis and edema associated with inflammation, and the infant's underlying vulnerability linked to sudden death (*e.g.*, medullary serotonergic system abnormalities (6). Given these aforementioned variables, and surely there are others as well, it is highly unlikely that a clinically reliable quantitative classification scheme will ever be developed and the individual pathologist's judgment will prevail in individual cases.

Our study has limitations. It is retrospective and the number of SIDS and suffocation control cases is relatively small, but the suffocation control group is larger than reported in other similar studies (24,26). The available myocardial sections were not obtained from standardized sites. Had the study been prospective, the use of fresh rather than paraffin-embedded tissues may have resulted in the detection of a larger number of viruses. That said, the paraffin-embedded tissues that were used had not been in 10% buffered formalin for longer that 72 h, most being less than 48 h. The range of myocardial viruses researched was limited, but included those most commonly identified with myocarditis (14,32,43).

Conversely, our study is strengthened by use of SIDS cases that died in a safe sleep environment. Restricting the primary control group to suffocation cases removes natural diseases other than overt myocarditis as a confounding variable in the assignment of the cause of death. The numbers of SIDS and suffocation control cases are similar and closely matched. The myocarditis cases had their diagnosis confirmed using Dallas criteria and their demographic profiles are similar to the other two groups. Other strengths include the use of cases with a confirmed diagnosis of myocarditis, application of the most current SIDS definition, standardized approaches to the postmortem investigation, and quantitative assessment by two investigators with a high degree of agreement in their observations.

In conclusion, we have shown that the severity, *i.e.*, exceedingly minor infiltrates of lymphocytes and macrophages, as well as the small number of necrotic cardiomyocytes in the myocardium in cases of suffocation is not different from that in SIDS. This may indicate exposure of the infant's heart to environmental antigens. Additionally, with the exception of enterovirus being identified in only one suffocation case, viruses were not identified in any of the SIDS or myocarditis cases in this study. Nevertheless, we, like others, have been unable to determine precisely how much inflammation, whether in the heart, lungs, or brain, is sufficient to cause death.

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