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Abstract

In a matched case-control study of the association between coxsackieviruses and cardiac impairment, 24 human immunodeficiency virus (HIV) type 1–infected children with cardiac impairment were compared with 24 HIV-1–infected control subjects. Serologic evidence of coxsackievirus infection was present in all children, with no significant difference in geometric mean antibody titers between case patients and control subjects. Conditional logistic regression to test for an association between coxsackievirus antibody titer and the presence or absence of cardiac impairment, by any indicator, showed an odds ratio of 1.11 (95% confidence interval, 0.58–2.10; P = .75), indicating no association between coxsackievirus infection and cardiac impairment. Coxsackievirus antibody titers correlated positively with total IgG levels in nonrapid progressors but not in rapid progressors. Paired serum samples taken before and after diagnosis of cardiac impairment in 5 patients showed no evidence of intervening coxsackievirus infection. These results do not identify a causal role for coxsackieviruses for cardiomyopathy in HIV-1–infected children.
Coxsackievirus group B infects 10 million US citizens annually, with most infections occurring among children <5 years old. Coxsackievirus serotypes B2, B3, and B4 are endemic in the United States, whereas serotypes B1 and B5 occur in epidemic patterns [1]. Although only 10% of enterovirus infections result in clinical illness, at least 5% of patients may experience cardiac infection, and an unknown proportion will develop myocarditis. The prevalence of myocarditis in the general population at autopsy is 1%–4% [2]. Coxsackieviruses are present in 40%–50% of hearts with myocarditis or dilated cardiomyopathy, with coxsackievirus B3 being the most common [3]. A higher proportion of patients with chronic myocarditis or dilated cardiomyopathy than patients with heart diseases of other infectious etiologies have antibodies to coxsackievirus B [2, 3].

Cardiac impairment with dysrhythmias and hemodynamic abnormalities occurs frequently in children with human immunodeficiency virus (HIV) type 1 infection [4–6]. Children infected with HIV-1 provide a better opportunity to identify a causal role of coxsackieviruses with HIV-1–associated cardiomyopathy than do adults, since there may be fewer confounding factors affecting cardiac function in children. A matched case-control study was done among 24 HIV-1–infected children with cardiac impairment and 24 HIV-1–infected control subjects without cardiac impairment, to identify differences in coxsackieviruses infection rates and associated immune response as possible risk factors for cardiac impairment.

**Patients, Materials, and Methods**

**Patients and serum**

Children born to HIV-1–infected mothers enrolled in the Pediatric Pulmonary and Cardiovascular Complications of HIV-1 Infection Study (P2C2 Study) from May 1990 through January 1994 were followed prospectively through January 1997 to identify HIV-1 infection and cardiac impairment, as determined by serial echocardiography [7]. All children in the P2C2 Study with documented HIV-1 infection and with sufficient serum samples were included as case patients if they had clinical evidence of cardiac impairment, as indicated by congestive heart failure (1 patient); use of cardiac medications, most commonly digoxin and furosemide and, less frequently, spironolactone, enalapril, and captopril (7 patients); low fractional shortening (<25% after 6 months of age; 9 patients); congestive heart failure and use of cardiac medications (3 patients); low fractional shortening and use of medications (2 patients); or all 3 indicators of clinical evidence of congestive heart failure, use of cardiac medications, and low fractional shortening (2 patients) [6]. HIV-1–infected children were further classified as rapid progressors if they were diagnosed with an AIDS-defining condition (other than lymphocytic interstitial pneumonitis) or with severe immunosuppression (CD4 cell count <750 cells/mm³ or <15% of total lymphocytes) in the first year of life [7].

In total, 24 HIV-1–infected children with cardiac impairment were identified, including 5 children with serum samples obtained before and after diagnosis of cardiac impairment. Serum samples from case patients were obtained within 4 months (median, 37 days; interquartile range [IQR], 1–59 days) after diagnosis of cardiac impairment. An additional 24 HIV-1–infected children without clinical or laboratory evidence of cardiac impairment
served as control subjects and were matched with case patients on the basis of the date of available serum samples. This system was used to minimize the possible effect of unrecognized coxsackievirus outbreaks. Control serum samples were obtained within 45 days of the date of diagnosis of cardiac impairment of the matching case patient.

Coxsackieviruses

The coxsackievirus group B serotype strains used were CVB1 (Conn-5), CVB2 (S.R.), CVB3 (Nancy), CVB4 (Edwards), and CVB5 (Faulkner), as described elsewhere [8]. All strains were propagated and plaque-assayed in HeLa cell cultures. Viruses were purified by a standard procedure that included a final step of banding in cesium chloride [9].

Antibody assay

A standard alkaline phosphatase EIA was used, as described elsewhere [10]. Each well was coated with 0.1 mL of purified virus solution in Dulbecco's PBS containing 100 ng of virus/mL (1.1×10^9 pfu ≅ 1 μg of virus). Serum samples were tested at serial 2-fold dilutions until nonreactive or positive at 1:51200. Monkey anti-coxsackievirus serotype reference antisera prepared against CVB1 (Conn-5), CVB2 (Ohio-1), CVB3 (Nancy), CVB4 (JVB), and CVB5 (Faulkner) were obtained from the National Institutes of Health Research Reference Reagent serum bank and were used as positive control serum samples in each EIA performed with each particular purified virus. All dilutions of anti-CVB5 antiserum cross-reacted to the same extent with all 5 purified preparations of coxsackievirus B serotypes. Antibody titers were determined separately against each of the 5 coxsackievirus serotypes. Because of the heterotypic antibody response to coxsackieviruses, the geometric mean titer (GMT) of these 5 serotypes was used to represent a composite coxsackievirus antibody titer for logistic regression.

Western blot

To confirm the specificity of the antibody assay, a representative Western blot was performed, using standard methods, with 2 μg of coxsackievirus B3 antigen and incubated with control and test serum samples with coxsackievirus B3 EIA antibody titers of 1:200–1:51200. In brief, the virus preparation was lysed for 20 min on ice in radioimmunoprecipitation assay lysis buffer, and proteins were separated on a 15% polyacrylamide gel with a 4% stacking gel, blocked in 0.2% 1-block in 0.1% Tween in PBS, incubated overnight with serum diluted 1:50, and detected by incubation with 1:30,000 anti-human IgG alkaline phosphatase in blocking solution.

Statistical analyses

The Wilcoxon matched-pairs signed-rank test was used to compare differences between case patients and control subjects for age and coxsackievirus GMTs. The relationship between cardiac impairment and GMTs was assessed by use of stratified conditional logistic regression analysis, which accounted for the matched nature of the data. The Spearman’s rank correlation coefficient (r) was calculated to determine the association between GMTs and total IgG.
Results

The median age of the 24 case patients was 45 months at diagnosis of cardiac impairment and 46 months at the time of serum sampling. The median age of the 24 control subjects was 59 months at the time of serum sampling. The median difference in age between case patients and their respective matched control subjects at the time of serum sampling was 7.1 months (IQR, −24.5 to 20.9 months; \( P = .87 \), Wilcoxon matched-pairs signed-rank test). The case patients included 7 boys (29%) and 17 girls (71%); the control subjects included 10 boys (42%) and 14 girls (58%) \( (P = .37) \). Of the case patients, 4 (17%) were white, 9 (38%) were black, 10 (42%) were Hispanic, and 1 (4%) was of another ethnic group. Of the control subjects, 4 (17%) were white, 16 (67%) were black, 2 (8%) were Hispanic, and 2 (8%) were of other ethnic groups \( (P = .04) \). Nine (37.5%) of the 24 case patients and 7 (29.2%) of the 24 control subjects were rapid progressors. During the 6-year period of this study, 9 (38%) case patients and 6 (25%) control subjects died \( (P = .35) \).

All children had demonstrable antibodies to coxsackieviruses, and the mode of the antibody titer was 800 for each of the 5 coxsackievirus serotypes tested. The titer of antibody to CVB3 measured by EIA was closely correlated to the signal intensity of virus-specific bands on Western blot, demonstrating the apparent specificity of the EIA test (figure 1). The median coxsackievirus GMT was 988 for case patients (IQR, 606–4536) and 1056 for control subjects (IQR, 651–1838). Paired analysis showed a median GMT difference between case patients and control subjects of 209 (IQR, −2225 to 824; \( P = .96 \), Wilcoxon matched-pairs signed-rank test), with higher titers among control subjects. Antibody titers were not statistically different between girls and boys \( (P = .09) \), but the titers tended to be higher in girls. This trend was consistent for both case patients (median titer, 696 for boys and 1056 for girls) and control subjects (median titer, 919 for boys and 1303 for girls).

High titers were observed in control subjects and all subgroups of children with cardiac impairment, whether indicated by congestive heart failure (median titer, 919; \( n = 1 \)), cardiac medications (median titer, 1056; IQR, 528–4850; \( n = 7 \)), fractional shortening of ≤25% (median titer, 1056; IQR, 606–4222; \( n = 9 \)), congestive heart failure and cardiac medications (median titer, 919; \( n = 3 \)), low fractional shortening and medications (median titer, 2728; \( n = 2 \)), or all 3 indicators of clinical evidence of congestive heart failure, use of cardiac medications, and low fractional shortening (median titer, 5199; \( n = 2 \)). A conditional logistic regression model for matched data to test for an association between coxsackievirus antibody titer and the presence or absence of cardiac impairment, by any indicator, showed an odds ratio of 1.11 (95% confidence interval, 0.58–2.10; \( P = .75 \)), suggesting no association between coxsackievirus antibody titer and cardiac impairment (figure 2A).

Eighteen of 24 case patients and 19 of 24 control subjects had no history of intravenous immune globulin administration. For these children, there was a statistically significant correlation between the GMT of coxsackievirus antibodies and total IgG \( (r = 0.45; \ P = .005; \ n = 37) \) (figure 2B). The relationship was statistically significant for case patients \( (\rho = 0.55; \ P = .02; \ n = 18) \) but not for all control subjects \( (\rho = 0.29; \ P = .24; \ n = 19) \). However, the correlation among control subjects was similar to that among case patients if the 1 control sample with a log GMT of 11 was excluded \( (\rho = 0.45; \ P = .06) \). For these 37 children, there
was a positive correlation between the GMT of coxsackievirus antibody titers and total IgG for the nonrapid progressors \((r = 0.71; P < .001; n = 24)\), but there was no correlation for the rapid progressors \((r = -0.03; P = .92; n = 13)\). Of these 37 children, 4 of 18 patients and 4 of 19 control subjects died.

Paired serum samples obtained before and after diagnosis of cardiac impairment were available for 5 HIV-infected children. The coxsackievirus GMTs for these 5 paired serum samples were 800 and 919, 1056 and 2111, 528 and 459, 29407 and 12800, and 1213 and 528. The median coxsackievirus GMT of the prediagnosis serum samples was 1056, and the median coxsackievirus GMT of the postdiagnosis serum samples was 919, with a median decrease of 69 \((P = .82)\). The median coxsackievirus GMT of the 5 matched control subjects was 2425.

**Discussion**

These results indicate that coxsackievirus infections are very common among young HIV-1–infected children in the United States. The results of this case-controlled matched study of coxsackievirus antibodies among 24 HIV-1–infected children with cardiac impairment and 24 HIV-1–infected children without cardiac impairment showed no difference in seroprevalence or composite GMT to coxsackieviruses B1–B5. There was no evidence of an inability of HIV-1–infected children to make coxsackievirus-specific antibody, which is similar to findings for other viral infections in HIV-1–infected children [11].

It is of considerable interest that nonrapid progressors maintained concordance between serum coxsackievirus antibody GMTs and total IgG levels, extending a previous report that identified the serum IgG level as a survival factor [12]. Maintenance of specific antibody levels, such as coxsackievirus antibody, implies sufficient specific T cell function to maintain IgG class switching in B cells of nonrapid progressors and is an important prognostic indicator for survival.

Coxsackieviruses may affect cardiac function directly or indirectly as a result of the host response. In challenge experiments in mice with a cardiovirulent coxsackievirus B3 serotype, neutralizing monoclonal antibodies provided protection if they were present prior to challenge but exacerbated myocarditis if they were added 3 days after challenge [13]. Some neutralizing monoclonal antibodies to coxsackievirus B3 recognize epitopes on human cardiac myosin, bind to normal mouse cardiac fibroblasts, and promote complement-mediated lysis or induce cardiopathologic alterations in heart tissues of normal mice [14]. Molecular mimicry between epitopes on CVB3 virions and several molecules from normal cells has been established [15].

Most coxsackievirus infections are asymptomatic but result in an immune response with production of both homotypic and heterotypic antibodies, the latter presumably due to an anamnestic response resulting from previous infection with a different coxsackievirus B serotype [13]. Heterotypic coxsackievirus B antibodies are not neutralizing and recognize a coxsackievirus B group-reactive epitope(s) on the largest surface capsid poly-peptide, VP1 [13]. Although homotypic neutralizing IgM or IgG are found in serum samples of
individuals infected with a single serotype, heterotypic antibodies (even IgM) are detected in many serum samples by EIA [13, 15]. Heterotypic antibody responses to coxsackievirus infections are common in humans, as demonstrated by use of an antigen-capture RIA with purified virus particles [13].

Induction of heterotypic antibodies complicates the serologic diagnosis of coxsackievirus infections; the EIA antibody assay using coxsackievirus B3 also detects some cross-reacting antibodies to other coxsackievirus B serotypes, primarily coxsackie-virus serotypes B2 and B4. Because the EIA used in this study also detected heterotypic antibodies, the EIA antibody titers for coxsackievirus B serotypes 1–5 for each patient were combined to derive a composite GMT representing the total coxsackievirus antibody titer. No correlation was found between this composite EIA antibody titer and the presence or absence of cardiac impairment, implying that coxsackievirus infection was not etiologically associated with either a better or worse cardiac outcome in these HIV-infected children. Nevertheless, the ubiquitous nature of coxsackievirus B infection may provide the background that permits involvement of other factors or modulation by the immune system that ultimately leads to cardiac impairment.

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Appendix

Pediatric Pulmonary and Cardiovascular Complications of HIV-1 Infection Study Group Members

A complete list of Pediatric Pulmonary and Cardiovascular Complications of HIV-1 Infection study group members can be found in [7]; a partial list follows: National Heart, Lung and Blood Institute: Hannah Peavy (Project Officer), Anthony Kali-ca, Elaine Sloand, George Sopko, and Margaret Wu; Chairman of the Steering Committee, Robert Mellins; Clinical Centers: Baylor College of Medicine, Houston: William Shearer (Principal Investigator [PI]), Gail Demmler, Linda Davis, Debra Moon-eyham; and Teresa Tonsberg (University of Texas); The Children's Hospital, Boston/Harvard Medical School, Boston: Steven Lipshultz (PI), Kenneth McIntosh, Janice Hunter, Ellen Cooper (Boston Medical Center), Suzanne Steinbach (Boston Medical Center), and Karen Lewis (Boston Medical Center); Mount Sinai School of Medicine, New York City: Meyer Kattan (PI), David Hodes, Diane Carp, Stephen Heaton (Beth Israel Medical Center), and Mary Ann Worth (Beth Israel Medical Center); Presbyterian Hospital in the City of New York/Columbia University, New York City: Robert Mellins (PI), Jane Pitt, and Kim Geromanos; University of California, Los Angeles School of Medicine, Los Angeles: Samuel Kaplan (PI), Yvonne Bryson, Helene Cohen, Joseph Church (Children's Hospital Los Angeles), Arnold Platzker (Children's Hospital Los Angeles), Lucy Kunzman (Children's Hospital Los Angeles), Andrea Kovacs (Los Angeles County Hospital/University of Southern California), and Lynn
Fukushima (Los Angeles County Hospital/University of Southern California); Central Laboratory for Epstein-Barr Virus Testing: The University of Texas Health Sciences Center at San Antonio, San Antonio: Hal B. Jenson (PI) and Yasmin Ench; Clinical Coordinating Center: The Cleveland Clinic Foundation, Cleveland: Kirk Easley (PI), Michael Kutner (through December 1999), Mark Schluchter (through April 1998), Richard Martin (Case Western Reserve University), Johanna Goldfarb, Douglas Moodie, Cindy Chen, Victoria Konig, Sunil Rao, Paul Sartori, Susan Sunkle, and Weihong Zhang; and Policy, Data, and Safety Monitoring Board: Henrique Rigatto (Chair), Edward B. Clark, Robert B. Cotton, Vijay V. Joshi, Paul S. Levy, Norman S. Talner, Patricia Taylor, Robert Tepper, Janet Wittes, Robert H. Yolken, and Peter E. Vink.

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Figure 1.
Western blot confirmation of EIA detection and titration of coxsackievirus B3 antibodies. Random serum samples with EIA coxsackievirus B3 antibody titers ranging from 1:200 to 1:51,200 were tested by Western blot, using the purified coxsackievirus B3 preparation that was used for EIA. Positive (+) and negative (−) control serum samples are shown in the 2 left lanes; an additional control (buffer) is shown in the far right lane. The titer of antibody to coxsackie-virus B3 measured by EIA was closely correlated to the signal intensity of virus-specific bands on Western blot. A positive signal on Western blot is faintly detected with serum samples having EIA antibody titers of 1:200 and 1:400, with increasingly stronger signal intensity with serum samples having increasing EIA antibody titers. Coxsackievirus viral protein (VP) 1 is ~34 kDa, VP2 is ~30 kDa, and VP3 is ~26 kDa.
Figure 2.
A. Log geometric mean titer (GMT) to coxsackievirus serotypes B1–B5 for 24 human immunodeficiency virus (HIV) type 1–infected children with cardiac impairment (●) and 24 HIV-1–infected children without cardiac impairment (○). There was no significant difference in GMT between HIV-1–infected children with or without cardiac impairment. B, Log GMT to coxsackieviruses 1–5 for HIV-1–infected children with no history of intravenous immune globulin administration, including 18 of the 24 case patients with cardiac impairment (●) and 19 of the 24 control subjects without cardiac impairment (○). The relationship was statistically significant for case patients ($\rho = 0.55; P = .02; n = 18$) but not for control subjects ($\rho = 0.29; P = .24; n = 19$).