

Association between a Novel Human Coronavirus and Kawasaki Disease

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(See the article by Esper et al., on pages 492–8, and the editorial commentary by McIntosh, on pages 489–91.)

Kawasaki disease is a systemic vasculitis of childhood; its etiology is unknown. We identified evidence of a novel human coronavirus, designated “New Haven coronavirus” (HCoV-NH), in respiratory secretions from a 6-month-old infant with classic Kawasaki disease. To further investigate the possible association between HCoV-NH infection and Kawasaki disease, we conducted a case-control study. Specimens of respiratory secretions from 8 (72.7%) of 11 children with Kawasaki disease and from 1 (4.5%) of 22 control subjects (children without Kawasaki disease matched by age and the time the specimens were obtained) tested positive for HCoV-NH by reverse-transcriptase polymerase chain reaction (Mantel-Haenszel matched odds ratio, 16.0 [95% confidence interval, 3.4–74.4]; $P = .0015$). These data suggest that HCoV-NH infection is associated with Kawasaki disease.

Kawasaki disease is a systemic vasculitis of childhood that may result in aneurysms of the coronary arteries. In the developed world, Kawasaki disease is the most common cause of acquired heart disease in children [1]. The diagnosis of Kawasaki disease is based entirely on clinical features. For classic Kawasaki disease, individuals must have a fever for ≥ 5 days and either meet at least 4 of 5 criteria—(1) bilateral conjunctivitis; (2) erythema of

the mouth or pharynx, strawberry tongue, or stomatitis; (3) polymorphous rash; (4) erythema or edema of the hands or feet; and (5) nonsuppurative cervical lymphadenopathy—or meet at least 3 of these criteria and have evidence of coronary artery abnormalities [2]. Incomplete or atypical Kawasaki disease, in which these criteria are not met, can occur and can also result in aneurysms of the coronary arteries [3]. The etiology of Kawasaki disease is unknown. Laboratory findings are nonspecific, and there are no diagnostic tests for Kawasaki disease.

There is evidence that suggests that Kawasaki disease may be triggered by a response to an infectious agent. Epidemics of Kawasaki disease, with wavelike spread, have been observed in many countries [4]. Epidemics generally occur during winter and spring. Kawasaki disease is rare in infants < 3 months old, which suggests the possibility that they are protected from infection by antibodies that are passively acquired from the mother. Likewise, widespread immunity to a common infectious agent may explain the rarity of Kawasaki disease in adults. A history of an antecedent respiratory illness before the onset of Kawasaki disease has been reported [5]. Although many infectious agents have been proposed as the cause of Kawasaki disease, none have been consistently associated with the disease. By using synthetic antibodies, Rowley et al. recently identified an antigen in respiratory epithelial cells and macrophages from children with Kawasaki disease [6]. The origin of this antigen remains unknown.

Human coronaviruses (HCoVs) have attracted renewed interest recently, in part because of the emergence of a novel HCoV associated with severe acute respiratory syndrome (SARS) [7, 8]. Before the emergence of CoV-SARS, HCoVs were generally thought to cause mild, self-limited infections of the upper respiratory tract. The study of HCoVs has been hampered by the difficulty in propagating these viruses in vitro [9]. To determine whether previously unknown HCoVs are circulating in the human population, we developed genetic probes that target regions of the replicase 1a gene that are conserved among HCoVs, avian CoVs, and mammalian CoVs. The screening of stored specimens of respiratory tract secretions from children < 5 years old revealed evidence of a novel HCoV, designated “New Haven coronavirus” (HCoV-NH) [10]. On the basis of sequence and phylogenetic analysis of the replicase 1a gene, HCoV-NH is closely related to an HCoV recently identified in The Netherlands [11, 12]. One child in our study, a 6-month-old infant who had 2 respiratory specimens test positive for HCoV-NH, was diagnosed with Kawasaki disease. To further investigate the possible association between HCoV-NH infection and Kawasaki disease, we expanded the time frame to the

Received 7 September 2004; accepted 1 December 2004; electronically published 14 January 2005.

Financial support: Patrick and Catherine Weldon Donaghue Medical Research Foundation (to J.S.K.); Friends of Yale Pediatrics (to J.S.K.); Yale Children’s Clinical Research Center (grant M01-RR06022 to J.S.K.); General Clinical Research Centers Program, National Center for Research Resources, National Institutes of Health (NIH); NIH (grants T32 AI07210-20 to F.E. and K24 AI01703 to E.D.S.).

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The Journal of Infectious Diseases 2005;191:499–502

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0022-1899/2005/19104-0003\$15.00

entire period during which we had obtained specimens, and we conducted a case-control study to assess this association.

Subjects, materials, and methods. Cases of Kawasaki disease were identified from hospital discharge records. As part of an ongoing epidemiological investigation of respiratory viruses, we have archived specimens of respiratory tract secretions that were obtained between November 2001 and May 2004 from children <5 years old and that tested negative for respiratory syncytial virus, influenza viruses A and B, parainfluenza viruses 1–3, and adenovirus by direct fluorescent antibody assay. For each case subject with Kawasaki disease, we identified 2 matched control subjects—the first 2 children (from whom we had a specimen) whose ages did not differ from that of the case subject by >6 months and whose specimens were obtained within 1 week of that of the case subject. Collection of specimens and clinical data was approved by the Yale University Human Investigation Committee.

Specimens were screened for HCoV-NH by reverse-transcriptase (RT) polymerase chain reaction (PCR) with probes specific for the HCoV-NH replicase 1a gene (forward primer, 5'-GCGCTATGAGGGTGGTTGTAAC-3'; reverse primer, 5'-CGCGCAGTAAAAGTCCAGAATTAAC-3' [underscoring indicates G/C clamps]) [10] and the spike gene (forward primer, 5'-CCGCGTAAAGAGTGGTTCACC-3'; reverse primer, 5'-CAGCGGTCATGGCACC-3'; nested forward primer, 5'-CCGCTTGAAGCCACCTGGC-3'; nested reverse primer, 5'-GCGCGGTTGGTTACATGGTGTAC-3') (GenBank accession numbers NC_005831 and AY518894) [11, 12]. The PCR amplification program was as follows: 15 min at 95°C, to activate the HotStar polymerase (Qiagen); 40 cycles of 45 s at 95°C, 60 s at 55°C, and 30 s at 72°C; and a final extension of 10 min at 72°C. Each set of RT-PCR amplifications included appropriate negative controls. PCR products were analyzed by agarose gel electrophoresis. For a specimen to be considered positive for HCoV-NH, it must have tested positive by RT-PCR for both the replicase 1a gene and the spike gene. Amplicons from each positive specimen were sequenced, to confirm that HCoV-NH was in the specimen.

The magnitude of the association (and the associated 95% confidence interval [CI]), as well as the statistical significance of the association, was calculated by use of the Mantel-Haenszel test for matched data, with multiple controls. The software True Epistat was used [13].

Results. From the hospital discharge records, we identified 53 children who had received a diagnosis of Kawasaki disease between October 2001 and April 2004. We had respiratory specimens from 11 (20.8%) of these patients. The mean age of the case subjects was 24.4 months, and the mean age of the control subjects was 23.7 months ($P = .34$). Eight (72.7%) of the 11 case subjects and 1 (4.5%) of the 22 control subjects tested positive for HCoV-NH by RT-PCR (Mantel-Haenszel matched

odds ratio, 16.0 [95% CI, 3.4–74.4]; Mantel-Haenszel χ^2 , 10.1; $P = .0015$). Multiple genetic polymorphisms were observed in the HCoV-NH sequences identified in the isolates from the case subjects (data not shown).

The clinical and laboratory features of the case subjects are shown in table 1. Of the 11 case subjects, 10 met the criteria for classic Kawasaki disease, of whom 8 tested positive for HCoV-NH. The 1 case subject who met 3 criteria (in addition to fever) for Kawasaki disease and had normal echocardiography findings tested negative for HCoV-NH. Nosocomial acquisition of HCoV-NH in children admitted with Kawasaki disease was unlikely, because the respiratory specimens from 10 of the case subjects were obtained either before or on day 1 of hospitalization. The respiratory specimen for 1 case subject, case subject 10, was obtained on day 11 of hospitalization; this child tested negative for HCoV-NH. The median time between the onset of fever and the acquisition of the respiratory specimen that was tested for HCoV-NH was 5 days (range, 4–13 days) (table 1). Seven (63.6%) of the 11 case subjects and 19 (86.4%) of the 22 control subjects had respiratory symptoms that were consistent with an upper respiratory tract infection ($P = .19$). Of the 7 case subjects who had respiratory symptoms, 6 tested positive for HCoV-NH. Specimens from both case subjects and control subjects who did not have respiratory symptoms recorded in the medical records were obtained as part of a diagnostic work-up for fever. All of the subjects in this study presented between November and April.

Discussion. The cause of Kawasaki disease has been elusive. Epidemiological data suggest that Kawasaki disease may be caused by a common infectious agent. Several pathogens—such as retroviruses [14], Epstein-Barr virus [15], parvovirus B19 [16], and chlamydia [17]—have been suggested by different studies as possibly important in the pathogenesis of Kawasaki disease. However, these studies either lacked control subjects or were poorly controlled, and an association between any of these pathogens and Kawasaki disease has not been established. One of the most promising leads in the investigation of the cause of Kawasaki disease is the detection, recently reported by Rowley et al. [6], of an antigen of unknown origin in respiratory epithelial cells and macrophages from children with acute Kawasaki disease. This finding suggests that a respiratory pathogen may be associated with Kawasaki disease. Our data demonstrate that, in our population during the study period, there was a significant association between HCoV-NH infection and Kawasaki disease.

The clinical spectrum of disease caused by HCoV-NH infection has not been fully characterized, although HCoV-NH [10] and the virus identified in The Netherlands were discovered in children and adults who had symptoms of respiratory tract disease [11, 12]. The 2 common HCoVs, HCoV-229E and HCoV-OC43, are ubiquitous, and infections are common dur-

Table 1. Clinical and laboratory features of children with Kawasaki disease.

Case subject ^a (sex)	Age (month/year of diagnosis), months	Interval, ^b days	Bilateral conjunctivitis	Erythema of the mouth or pharynx	Polymorphous rash	Erythema or edema of the hands or feet	Lymphadenopathy ^c	No. of criteria ^d	Echocardiographic result ^e	HCoVNH by PCR
1 (M) ^f	6 (2/02)	4	+	+	+	+	-	4	CA-D	+
2 (M)	8 (1/04)	6	+	-	+	-	+	3	CA-D	+
3 (M)	12 (4/03)	5	+	+	+	+	-	4	Normal	+
4 (M)	15 (1/04)	4	+	+	+	-	-	3	CA-D	+
5 (F)	21 (3/04)	5	+	+	+	-	+	4	Normal	+
6 (F)	27 (2/04)	10	+	+	+	+	-	4	Normal	+
7 (M)	60 (4/04)	5	+	+	+	-	+	4	Normal ^g	+
8 (M)	67 (3/04)	9	+	+	+	-	-	3	CA-abnl	+
9 (M)	2 (11/02)	5	-	+	+	-	+	3	CA-abnl	-
10 (M)	15 (1/03)	13	+	-	+	+	-	3	Normal	-
11 (M)	34 (12/02)	7	+	+	+	-	+	4	Normal	-

Note. -, negative; +, positive; CA-abnl, abnormal echogenicity of the coronary arteries without evidence of dilation; CA-D, abnormal echogenicity of the coronary arteries with evidence of dilation; F, female; HCoVNH, New Haven coronavirus; M, male; PCR, polymerase chain reaction.

^a All case subjects had fever for >5 days.

^b Between onset of fever and the date the specimen was collected.

^c Cervical lymph node enlargement with at least 1 node >1.5 cm.

^d No. of diagnostic criteria met (in addition to fever).

^e Echocardiograms were obtained at the time of diagnosis of Kawasaki disease.

^f First case identified.

^g Subsequent echocardiogram revealed dilation of the origin of the left coronary artery.

ing winter and spring. If HCoV-NH infection is the cause of Kawasaki disease and is common, it is unclear why the disease occurs in a relatively small number of children. In most individuals, the pathogenesis of Kawasaki disease is likely distinct from the pathogenesis of viral respiratory tract infections. However, Kawasaki disease may result from an abnormal clonal expansion of CD8⁺ T cells [18] in response to an infectious agent. Therefore, predisposition of the host, in addition to the antigenic properties of the virus, may play an important role in the vasculitis of Kawasaki disease.

In the present study, respiratory specimens from case and control subjects were sent to a diagnostic laboratory; therefore, it was not unexpected that 7 (63.6%) of the 11 case subjects and 19 (86.4%) of the 22 control subjects had evidence of respiratory tract disease. The percentage of HCoV-NH–positive control subjects (4.5%) was similar to our previous finding [10]. The selection of control subjects from children with suspected respiratory tract infection would tend to bias the results toward there being no association between HCoV-NH infection and Kawasaki disease—control subjects would more likely be infected with HCoV-NH than would children without symptoms of respiratory tract infection. If this potential bias affected the present study, then the true association would be stronger than we have already shown. The difference in the age distribution between the case subjects and the control subjects was small (mean difference, <1 month), and so it is extremely unlikely that this factor accounted for the difference between the proportions of case and control subjects infected with HCoV-NH. False-positive results that are caused by PCR contamination are a concern in any study that uses genomic amplification techniques. To address this issue, appropriate negative controls were included in each set of RT-PCR amplifications, and we required that 2 regions of the HCoV-NH genome be amplified for the specimen to be considered positive. Moreover, for the observed association to have been caused by contamination, it would have been necessary that the specimens from the case subjects were preferentially contaminated—a highly improbable scenario. Each amplicon was sequenced, to confirm the presence of HCoV-NH. Distinct genetic polymorphisms were observed in the HCoV-NH sequences identified in the isolates from the case subjects. This further reduces the likelihood that PCR contamination explains our findings.

In conclusion, this case-control study suggests that there is an association between HCoV-NH infection and Kawasaki disease. Further studies—such as prospective cohort studies, seroepidemiological investigations, and investigations of inflamed tissue for the presence of virus—are required to determine the precise role played by HCoV-NH in the pathogenesis of Kawasaki disease and to determine whether other infectious agents can also trigger this syndrome.

Acknowledgments

We are indebted to George Miller, John F. Enders Professor of Pediatric Infectious Diseases (Yale University School of Medicine), for his continued support, intellectual and scientific input and exchange, and critical review of the data and manuscript. We thank the staff of the Clinical Virology Laboratory, Yale–New Haven Hospital (CT), for their assistance in this study.

References

1. Rowley AH, Shulman ST. Kawasaki syndrome. *Pediatr Clin North Am* **1999**; 46:313–29.
2. American Academy of Pediatrics. 2000 red book: report of the Committee on Infectious Diseases. 25th ed. Elk Grove Village, IL: American Academy of Pediatrics, **2000**.
3. Rosenfeld EA, Corydon KE, Shulman ST. Kawasaki disease in infants less than one year of age. *J Pediatr* **1995**; 126:524–9.
4. Yanagawa H, Nakamura Y, Kawasaki T, Shigematsu I. Nationwide epidemic of Kawasaki disease in Japan during winter of 1985–86. *Lancet* **1986**; 2:1138–9.
5. Bell DM, Brink EW, Nitzkin JL, et al. Kawasaki syndrome: description of two outbreaks in the United States. *N Engl J Med* **1981**; 304:1568–75.
6. Rowley AH, Baker SC, Shulman ST, et al. Detection of antigen in bronchial epithelium and macrophages in acute Kawasaki disease by use of synthetic antibody. *J Infect Dis* **2004**; 190:856–65.
7. Ksiazek TG, Erdman D, Goldsmith CS, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* **2003**; 348:1953–66.
8. Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* **2003**; 348:1967–76.
9. McIntosh K. Coronaviruses. In: Fields BN, Knipe DM, Howley PM, eds. *Fields Virology*. 3rd ed. Vol 1. Philadelphia: Lippincott-Raven, **1996**: 1095–103.
10. Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS. Evidence of a novel human coronavirus associated with respiratory tract disease in infants and young children. *J Infect Dis* **2004**; 191:492–8 (in this issue).
11. van der Hoek L, Pyrc K, Jebbink MF, et al. Identification of a new human coronavirus. *Nature Med* **2004**; 10:368–73.
12. Fouchier RA, Hartwig NG, Bestebroer TM, et al. A previously undescribed coronavirus associated with respiratory disease in humans. *Proc Natl Acad Sci USA* **2004**; 101:6212–6.
13. Gustafson TL. *True Epistat*. 5th ed. Richardson, TX: Epistat Services, **1994**.
14. Burns JC, Geha RS, Schneeberger EE, et al. Polymerase activity in lymphocyte culture supernatants from patients with Kawasaki disease. *Nature* **1986**; 323:814–6.
15. Kikuta H, Sakiyama Y, Matsumoto S, et al. Detection of Epstein-Barr virus DNA in cardiac and aortic tissues from chronic, active Epstein-Barr virus infection associated with Kawasaki disease–like coronary artery aneurysms. *J Pediatr* **1993**; 123:90–2.
16. Nigro G, Zerbini M, Krzysztofciak A, et al. Active or recent parvovirus B19 infection in children with Kawasaki disease. *Lancet* **1994**; 343: 1260–1.
17. Normann E, Naas J, Gnarp J, Backman H, Gnarp H. Demonstration of *Chlamydia pneumoniae* in cardiovascular tissues from children with Kawasaki disease. *Pediatr Infect Dis J* **1999**; 18:72–3.
18. Choi IH, Chwae YJ, Shim WS, et al. Clonal expansion of CD8⁺ T cells in Kawasaki disease. *J Immunol* **1997**; 159:481–6.