Possible Involvement of Infection With Human Coronavirus 229E, but not NL63, in Kawasaki Disease

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Although human coronavirus (HCoV)-NL63 was once considered a possible causative agent of Kawasaki disease based on RT-PCR analyses, subsequent studies could not confirm the result. In this study, this possibility was explored using serological tests. To evaluate the role of HCoV infection in patients with Kawasaki disease, immunofluorescence assays and virus neutralizing tests were performed. Paired serum samples were obtained from patients with Kawasaki disease who had not been treated with γ-globulin. HCoV-NL63 and two antigenically different isolates of HCoV-229E (ATCC-VR740 and a new isolate, Sendai-H) were examined as controls. Immunofluorescence assays detected no difference in HCoV-NL63 antibody positivity between the patients with Kawasaki disease and controls, whereas the rate of HCoV-229E antibody positivity was higher in the patients with Kawasaki disease than that in controls. The neutralizing tests revealed no difference in seropositivity between the acute and recovery phases of patients with Kawasaki disease for the two HCoV-229Es. However, the Kawasaki disease specimens obtained from patients in recovery phase displayed significantly higher positivity for Sendai-H, but not for ATCC-VR740, as compared to the controls. The serological test supported no involvement of HCoV-NL63 but suggested the possible involvement of HCoV-229E in the development of Kawasaki disease.

J. Med. Virol. 86:2146–2153, 2014.

INTRODUCTION

Kawasaki disease is an acute febrile eruptive disease characterized by systemic vasculitis, particularly in small- and medium-sized arteries. More than 80% of Kawasaki disease cases occur in children younger than 5 years, and boys are more prone to Kawasaki disease than girls [Kawasaki et al., 1974; Yanagawa et al., 1995a,b,c, 1996, 1998; Tsuchida et al., 1996].

Though the causative agent of Kawasaki disease remains unclear, an infectious agent is thought to cause or trigger Kawasaki disease because it shows seasonal, temporal, and regional patterns [Burns et al., 2005]. Suggested Kawasaki disease pathogens include Staphylococcus [Hall et al., 1999], Streptococcus [Shinomiya et al., 1987; Kikuta et al., 1992; Anderson et al., 1995], adenovirus [Embil et al., 1985], human herpesvirus 6 [Okano et al., 1989], Epstein–Barr virus [Kikuta et al., 1992], parvovirus B19 [Holm et al., 1995], human lymphotropic virus [Okano, 1999], and human bocavirus [Catalano-Pons et al., 2007];

Abbreviations: ATCC, American Tissue Culture Collection; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; GFP, green fluorescent protein; HCoV, human coronavirus; S, spike; TPB, tryptose phosphate broth; VSV, vesicular stomatitis virus

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan
Conflict of interest: none.

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Accepted 18 March 2014
DOI 10.1002/jmv.23950
Published online 24 April 2014 in Wiley Online Library (wileyonlinelibrary.com).
however, none of these pathogens have been confirmed to cause Kawasaki disease. Recent reports have suggested that heat shock proteins and superantigens produced by bacteria are associated with the development of Kawasaki disease [Matsubara and Fukaya, 2007; Nagata et al., 2009]. However, Rowley et al. [2005] reported that synthetic Kawasaki disease antibodies detected intracytoplasmic inclusion bodies, which were consistent with aggregates of viral proteins and nucleic acids. They also reported that the inclusion bodies seen in Kawasaki disease lesions did not have the substructure characteristic of bacterial inclusion bodies. Instead, RNA, not DNA, was present in the inclusion bodies, suggesting that an RNA virus contributes to the development of Kawasaki disease [Rowley et al., 2008; Rowley, 2011].

Human coronavirus (HCoV) is an enveloped RNA virus with a single, positive-stranded genome of about 30 kb [Wege et al., 1982; Lai and Cavanagh, 1997]. Several HCoVs have been identified as causative agents of the human common cold, including HCoV-229E, HCoV-OC43 (OC43), HCoV-HKU1 [Woo et al., 2005], and HCoV-NL63 [van der Hoek et al., 2004]. In 2005, it was reported that a new strain, HCoV-NL63 [Esper et al., 2005a], was possibly associated with Kawasaki disease as parts of the spike (S) and replicase genes were detected in 8 of 11 (72.7%) respiratory specimens from patients with Kawasaki disease, as compared to 1 of 22 (4.5%) specimens from age- and region-matched controls [Esper et al., 2005a]. However, many subsequent reports failed to confirm the relationship between Kawasaki disease and HCoV-NL63. For example, Dominguez et al. [2006] tried to detect HCoV-NL63 genes by RT-PCR from nasopharyngeal-wash samples, and detected it in 2 of 26 (7.7%) patients with Kawasaki disease and 4 of 52 (7.7%) matched controls. In addition, Shimizu et al. [2005] detected HCoV-NL63 genes in only 1 of 48 (2%) patients with Kawasaki disease. Additionally, other reports failed to show a relationship between HCoV-NL63 and Kawasaki disease [Belay et al., 2005; Ebihara et al., 2005; Chang et al., 2006; Dominguez et al., 2006; Lehmann et al., 2009]. To detect viral genes in specimens, sampling must be performed at a critical time; if the virus infection does not persist and the samples are collected after the infection is over, then the detection of viral genes is quite difficult. Therefore, we performed a serological study to examine the involvement of HCoV-NL63 in Kawasaki disease. HCoV-229E was included as a control for HCoV-NL63. For the serological test, paired serum samples from patients with Kawasaki disease who had not been treated with γ-globulin and matched control sera were examined. The results suggested that HCoV-NL63 is not a causative agent of Kawasaki disease. Instead, a particular strain of HCoV-229E might trigger Kawasaki disease.

**MATERIALS AND METHODS**

**Cells and Viruses**

HeLa (HeLa-229, American Tissue Culture Collection [ATCC] CCL-2.1) and HeLa-ACE2 [Watanabe et al., 2008] cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) containing 5% fetal calf serum (FCS). BHK cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). 293T/17 cells were obtained from the ATCC (CRL-11268). The BHK and 293T/17 cells were maintained in DMEM (D5796; Sigma, St. Louis, MO) containing 5% FCS. The ATCC strain of HCoV-229E (VR740) was used (National Institute of Infectious Disease [NIID] stock, GenBank accession no. AB691763) [Shirato et al., 2012]. The clinical isolate Sendai-H/1121/04 (Sendai-H), which was isolated from the pharyngeal swab of an outpatient using LLC-MK2 cells in 2004 in Japan, was also examined (accession no. AB691764) [Shirato et al., 2012]. Both viruses were propagated and titrated using HeLa cells, as described previously [Kawase et al., 2009; Shirato et al., 2012]. Pseudotyped vesicular stomatitis virus (VSV) expressing green fluorescent protein (GFP) (VSVΔG) was kindly provided by M.A. Whitt (GTx, Memphis, TN) [Takada et al., 1997]. The seed virus stock of VSVΔG-E, which is a VSV G protein harboring VSVΔG, was prepared using BHK cells, and the VSV pseudotyped with HCoV-229E S protein was generated using 293T/17 cells, as described previously [Kawase et al., 2009; Shirato et al., 2012].

**Clinical Specimens**

This study was approved by the Ethics Review Committee for Human Medical Science Studies of our institute (NIID, Tokyo, Japan). Before sample collection, informed consent was obtained from the patients' parents. All clinical specimens were masked so that the investigators were blind to personal information. Fifteen pairs of serum specimens were obtained from patients with Kawasaki disease who had not...
been treated with γ-globulin at the Japanese Red Cross Medical Center (Tokyo, Japan) in the period 2001 to 2002. All Kawasaki disease cases met diagnostic criteria for Kawasaki disease and thus judged as definitive Kawasaki disease patients. There was no suspected case. Two of them showed cardiac complication, and one of them showed coronary artery ectasia in both acute and recovery phases. Other cases did not show coronary lesions. Control serum specimens collected from healthy children from 2001 to 2002 were obtained from the serum bank at the NIID. Since there was insufficient serum to perform all of the neutralizing tests, the control samples were separated randomly into two groups [group 1 (23 specimens) and group 2 (29 specimens)], and one was used for a neutralizing test with VSV-pseudotyped viruses while the other was used for a neutralizing test with infectious HCoV-229E. The details of the specimens are shown in Table I. No statistical difference in age and sex was detected among control serum and specimens from patients with Kawasaki disease. Serum samples from Kawasaki disease patients as well as control sera of healthy children were collected through the whole year.

**Immunofluorescence Assay**

HeLa-ACE2 cells were seeded in 96-well plates and then infected with HCoV-NL63 or HCoV-229E (VR-740). After a 2-day incubation, the cells were fixed and exposed to Kawasaki disease or control serum at a 1:200 dilution. Next, the cells were stained with FITC-conjugated anti-human IgG (Zymed, South San Francisco, CA). Cellular fluorescence was observed using ultraviolet fluorescence microscopy (AxioCam; Carl Zeiss, Oberkochen, Germany).

**Virus Neutralizing Test**

The neutralizing tests were performed as described previously with modifications [Shirato et al., 2012]. Briefly, a monolayer of HeLa cells was grown in 96-well (for VSV-pseudotyped virus) or 24-well (for infectious HCoV-229E) plates. To neutralize the VSV-pseudotyped virus, ca. 500 GFP count viruses were mixed with DMEM containing 5% tryptose phosphate broth (TPB) and serum (diluted 1:100). For infectious HCoV-229E, ca. 100 PFU of virus were mixed with DMEM containing 5% TPB, 1% normal rabbit serum, and the specimen (diluted 1:20). Then, the samples were incubated for 60 min at 4°C and inoculated onto the HeLa cell monolayers. The viruses were allowed to adsorb for 1 hr at 34°C and the cells were washed three times with DMEM. Next, the infected cells were cultured with DMEM containing 5% FCS (for VSV-pseudotyped virus) or 10% TPB and 1.5% carboxymethyl cellulose (for infectious HCoV-229E). For the VSV-pseudotyped virus, the medium was replaced with fresh phosphate-buffered saline after 24 hr and cell images were captured and the numbers of GFP fluorescence-positive cells were counted with VH-H1A5 software (Keyence, Osaka, Japan). To evaluate HCoV-229E infection, cells were fixed with 20% formalin after a 2-day incubation, stained with crystal violet, and the plaques were counted; 1% FCS was used as a negative control and specific anti-VR740 or -Sendai-H rabbit serum was used as a positive control in each neutralizing test. A specimen showing more than 50% neutralization was considered positive.

**Statistical Analysis**

An unpaired t-test and Fisher’s exact test were used to evaluate the significance of differences between the groups. A P-value <0.05 was considered statistically significant.

**RESULTS**

**Immunofluorescence Assays for HCoV-NL63 and HCoV-229E**

Esper et al. [2005a] reported that HCoV-NL63 was possibly associated with Kawasaki disease based on detection of the viral genome in specimens using RTPCR; however, these observations have not been confirmed using the same method. Therefore, we examined the association of HCoV-NL63 with Kawasaki disease by the serological method using Immunofluorescence assays (Fig. 1). A standard strain of HCoV-229E (VR740) was used as a control for HCoV-NL63. The positive and negative age- and region-matched controls for the test were set for each experiment, and positivity was determined by comparison with these controls (Fig. 1a). As shown in Figure 1b, no difference in HCoV-NL63 antibody positivity was detected between the patients with Kawasaki disease and controls, indicating that there was no valid association between HCoV-NL63 and Kawasaki disease. This result supported the findings by several groups that reported no detection of HCoV-NL63 gene in Kawasaki disease specimens (Belay et al., 2005; Ebihara et al., 2005;
Shimizu et al., 2005; Chang et al., 2006; Dominguez et al., 2006; Lehmann et al., 2009]. Surprisingly, HCoV-229E showed higher positivity in the sera from Kawasaki disease patients, and the difference between the recovery-phase and control groups was statistically significant (\(P < 0.05\)). This result suggests that HCoV-229E, but not HCoV-NL63, is related to the development of Kawasaki disease. Therefore, in a subsequent survey, focus was on HCoV-229E as the causative agent of Kawasaki disease.

**Neutralizing Tests With Sera From Patient With Kawasaki Disease**

As described previously [Shirato et al., 2012], HCoV-229E has two different serotypes: the VR740

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**Fig. 1.** Immunofluorescence assays of the Kawasaki disease specimens using cells infected with HCoV-NL63 or HCoV-229E (VR-740). a: Positive and negative controls for HCoV-NL-63 (upper panels) and HCoV-229E (lower panels). HeLa-ACE2 and HeLa cells were infected with HCoV-NL63 and HCoV-229E, respectively. At 48–72 hr post-infection, the cells were fixed with methanol-acetone and used as antigens. The positive control of HCoV-NL63 was prepared by staining with anti-feline coronavirus serum, which cross-reacts with HCoV-NL63. HCoV-229E infected cells were stained with anti- HCoV-229E (VR740) rabbit serum. (b) A total of 15 paired serum samples obtained from Kawasaki disease patients and two groups of control sera (23 and 29 specimens) were used for the immunofluorescence assays. The specimens were judged as positive or negative based on a comparison with the positive and negative controls.
and the Japanese clinical isolate, Sendai-H. Therefore, these two viruses were used to estimate the relationship between HCoV-229E and Kawasaki disease development. First, the neutralizing profiles of control specimens were analyzed (Fig. 2). For the neutralizing tests, because the concentrated serum contained a factor that non-specifically blocked infection of the pseudotype virus, the sera were diluted 1:100 for the VSV-pseudotyped virus and 1:20 for infectious HCoV-229E. The control sera showed around 30% positivity in neutralizing tests using the VSV-pseudotype viruses and about 10% positivity using infectious HCoV-229E viruses; no significant difference in positivity was detected between VR-740 and Sendai-H. If HCoV-229E were a causative agent of Kawasaki disease, then seroconversion would be detected in sera collected from the acute and recovery phases of Kawasaki disease. Thus, the levels of HCoV-229E neutralizing antibodies in sera obtained from both phases in patients with Kawasaki disease was examined (Fig. 3). The study using VSV-pseudotype viruses showed that the positivity for VR740 in the acute phase was 13%, while that in the recovery phase was 20%. When examined with infectious viruses, the respective values for VR740 were 13% and 27%. For Sendai-H, the VSV-pseudotyped virus showed that the positivity rates in the acute and recovery phases were 53% and 80%, respectively; however, while using infectious Sendai-H, the respective rates were 20% and 40%. The positivity in the recovery phases seemed to be higher than that in the acute phases; however, no statistically significant difference in seropositivity was detected between them for both viruses. These results suggest that HCoV-229E infection is not a direct trigger of Kawasaki disease. Even in the acute phase of Kawasaki disease patients who had not received any γ-globulin. An neutralizing test was performed for each acute- and recovery-phase sample. More than 50% neutralization was considered positive.

The breakdown of the results

**VR-740**

| VSV-pseudotyped virus | Positive (%) | Negative (%) | Total |
|-----------------------|--------------|--------------|-------|
| Acute phase           | 2 (13)       | 13 (87)      | 15    |
| Recovery phase        | 3 (20)       | 12 (80)      | 15    |
| Infectious virus      |              |              |       |
| Acute phase           | 2 (13)       | 13 (87)      | 15    |
| Recovery phase        | 4 (27)       | 11 (73)      | 15    |

**Sendai-H/1121/04**

| VSV-pseudotyped virus | Positive (%) | Negative (%) | Total |
|-----------------------|--------------|--------------|-------|
| Acute phase           | 8 (53)       | 7 (47)       | 15    |
| Recovery phase        | 12 (80)      | 3 (20)       | 15    |
| Infectious virus      |              |              |       |
| Acute phase           | 3 (20)       | 12 (80)      | 15    |
| Recovery phase        | 6 (40)       | 9 (60)       | 15    |

Fig. 3. Fifteen paired serum specimens were collected from Kawasaki disease patients who had not received any γ-globulin. An neutralizing test was performed for each acute- and recovery-phase sample. More than 50% neutralization was considered positive.

**Fig. 2.** Neutralizing test results for the control specimens. Control sera were collected from healthy children and the specimens were separated into two groups because of the limited volumes. One was used for neutralizing tests with VSV-pseudotyped virus and the other was used for neutralizing tests with infectious HCoV-229Es. Specimens showing more than 50% neutralization were considered positive.
disease, the viral infection had already subsided and antibodies were produced.

The positivity against VR740 was not statistically different in Kawasaki disease specimens from that in the control groups. However, sera from recovery phase patients showed significantly higher positivity of Sendai-H than those from control samples (Fig. 3). This suggests that patients with Kawasaki disease have a greater chance of being infected with Sendai-H type HCoV-229E than healthy control children. In other words, Kawasaki disease could be related to the infection with a particular type of HCoV-229E, which may be prevalent in areas where Kawasaki disease is reported to occur.

**DISCUSSION**

This study examined the involvement of HCoVs, HCoV-NL63, and HCoV-229E, in the development of Kawasaki disease using serological tests. Esper et al. [2005a] reported that HCoV-NL63 was a possible causative agent of Kawasaki disease, but several reports have failed to confirm this [Belay et al., 2005; Ebihara et al., 2005; Shimizu et al., 2005; Chang et al., 2006; Dominguez et al., 2006; Lehmann et al., 2009]. However, most experiments examined genes derived from the virus using RT-PCR. Although RT-PCR has high sensitivity for detecting pathogen genes from clinical specimens, Neutralizing tests are another appropriate way to evaluate the contribution of a viral infection to the development of Kawasaki disease. However, γ-globulin transfers are generally used as a treatment for Kawasaki disease, and this treatment would confuse the serum antibody profile in serological tests. Therefore, the paired serum specimens obtained from Kawasaki disease patients who had not received γ-globulin during the disease was examined.

Similar to previous results, it is revealed that HCoV-NL63 was not related to the development of Kawasaki disease using immunofluorescence assays. Instead, HCoV-229E was suggested to be somehow related to Kawasaki disease. Therefore, the relationship between HCoV-229E and Kawasaki disease was studied by using a more virus-specific detection test, the neutralizing test.

As described previously [Shirato et al., 2012], there are at least two serotypes of HCoV-229E: VR740 and Sendai-H. Using these isolates as targets for the neutralizing test, the possible role of HCoV-229E in patients with Kawasaki disease was evaluated. Using VR-740 of HCoV-229E as the antigen, higher positivity was observed by immunofluorescence assays in Kawasaki disease specimens than in controls. In contrast, higher positivity was shown in Kawasaki disease specimens in neutralizing test using Sendai-H, though it was not clearly observed in VR740. Although the neutralizing antigenicity of VR-740 is different from that of Sendai-H [Shirato et al., 2012], VR-740 and Sendai-H showed some cross reactivity by immunofluorescence assays (data not shown). Therefore, it is assumed that greater positivity in Kawasaki disease specimens could be observed by immunofluorescence assays, even using Sendai-H as the antigen, as compared with the results of VR-740.

In the present study, neutralizing tests revealed that HCoV-229E Sendai-H showed higher seropositivity in patients with Kawasaki disease than HCoV-229E VR-740. Sendai-H was isolated recently from a Japanese patient with a common cold and Sendai-H-related viruses are prevalent currently among patients from Japan and other countries [Chibo and Birch, 2006; Shirato et al., 2012]. In contrast, VR-740, an initial strain isolated in the 1960s [Hamre and Procknow, 1966], is not prevalent currently in Australia and Japan [Chibo and Birch, 2006; Shirato et al., 2012]. These facts are in good agreement with the finding that a recent case of Kawasaki disease examined in this study was related to HCoV-229E Sendai-H rather than VR740. Moreover, a mild cold is known to precede the onset of Kawasaki disease and, thus, the agent that causes this respiratory disease could be a good candidate to trigger Kawasaki disease. The present study suggests that HCoV-229E could be one possible candidate. To test this hypothesis, a large-scale serological surveillance of Kawasaki disease, before and after onset, is required.

Some studies reported the activation of peripheral blood B cells and polyclonal B cell activation in acute Kawasaki disease [Leung et al., 1982, 1987; Furukawa et al., 1991, 1992; Nonoyama, 1991]. It is also suggested that new endothelial cell antigens induced by cytokine production, and generation of autoantibodies directed to these antigen caused the endothelial cell injury in Kawasaki disease [Leung, 1991]. However, in this study, there is no increase in antibody for HCoV-NL63 even if polyclonal B cell activation occurred, suggesting that our results would not result from such polyclonal B cell activation.

If HCoV-229E Sendai-H were a causative triggering agent for Kawasaki disease, then a significant increase in seropositivity from acute to recovery phase would be anticipated; however, the results in the present study do not support this theory. One possible explanation for this result is that the virus infection has already subsided when Kawasaki disease is first apparent in patients, and that the antibody against HCoV-229E is already present even in the acute phase of Kawasaki disease. In the recovery phase, a slight increase in positivity may occur, although such an increase was not statistically significant in our study. Alternatively, many cases of Kawasaki disease examined in the present study may not seem serious. Serious cases are normally treated with γ-globulin transfer in about 80% of patients with Kawasaki disease in Japan [Yanagawa et al., 1995d]. Virus infection may not have been intense in patients with Kawasaki disease and, thus, the antibody response was low. Future studies would be facilitated by the availability of sera from normal
individuals before Kawasaki disease onset and before γ-globulin treatment after Kawasaki disease onset. Such cohort studies would be invaluable in determining whether HCoV is the Kawasaki disease triggering agent.

In addition, it is thought that common cold caused by HCoVs generally shows winter seasonality, however, Gaunt et al. [2010] reported that HCoV-229E detected sporadically through the year, though epidemiological surveillance studies for HCoVs are very limited. On the other hand, it is reported that Kawasaki disease cases increase in summer and winter [Burns et al., 2005]. Therefore, it makes sense to postulate that HCoV-229E is a possible causative agent for Kawasaki disease if HCoV-229E infection occurs through the year, though it is very likely that some environmental and genetic factors, in addition to virus infection, considerably influence the development of Kawasaki disease.

ACKNOWLEDGMENTS

We thank Dr. Tomisaku Kawasaki for his invaluable suggestions and many helpful discussions. This work was supported financially by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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