Prevalence of Antibodies to Four Human Coronaviruses Is Lower in Nasal Secretions than in Serum

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Little is known about the prevalence of mucosal antibodies induced by infection with human coronaviruses (HCoV), including HCoV-229E and -OC43 and recently described strains (HCoV-NL63 and -HKU1). By enzyme-linked immunosorbent assay, we measured anti-HCoV IgG antibodies in serum and IgA antibodies in nasal wash specimens collected at seven U.S. sites from 105 adults aged 50 years and older (mean age, 67 ± 9 years) with chronic obstructive pulmonary disease. Most patients (95 [90%]) had at least one more chronic disease. More patients had serum antibody to each HCoV strain (104 [99%] had antibody to HCoV-229E, 105 [100%] had antibody to HCoV-OC43, 103 [98%] had antibody to HCoV-NL63, and 96 [91%] had antibody to HCoV-HKU1) than had antibody to each HCoV strain in nasal wash specimens (12 [11%] had antibody to HCoV-229E, 22 [22%] had antibody to HCoV-OC43, 8 [8%] had antibody to HCoV-NL63, and 31 [31%] had antibody to HCoV-HKU1), respectively (P < 0.0001). The proportions of subjects with IgA antibodies in nasal wash specimens and the geometric mean IgA antibody titers were statistically higher for HCoV-OC43 and -HKU1 than for HCoV-229E and -NL63. A higher proportion of patients with heart disease than not had IgA antibodies to HCoV-NL63 (6 [16%] versus 2 [3%]; P = 0.014). Correlations were highest for serum antibody titers between group I strains (HCoV-229E and -NL63 [r = 0.443; P < 0.0001]) and between group II strains (HCoV-OC43 and -HKU1 [r = 0.603; P < 0.0001]) and not statistically significant between HCoV-NL63 and -OC43 and between HCoV-NL63 and -HKU1. Patients likely had experienced infections with more than one HCoV strain, and IgG antibodies to these HCoV strains in serum were more likely to be detected than IgA antibodies to these HCoV strains in nasal wash specimens.

Coronaviruses comprise a genus of the family Coronaviridae and are enveloped, single-stranded, positive-sense RNA viruses (30). Four human coronavirus (HCoV) strains have been described, which are associated with a spectrum of disease, from mild, febrile upper respiratory tract illnesses to severe illnesses, including croup, bronchiolitis, and pneumonia, and have a wide geographic distribution (1, 2, 6, 7, 9–14, 16, 20, 25, 26, 31, 32, 35, 39–46). HCoV infection has been a contributor to severe illnesses requiring emergency care and hospitalization of patients with chronic medical conditions (7, 9, 12, 15, 16, 21, 22).

The earliest-described HCoV strains, HCoV-229E and HCoV-OC43, which are group I and group II coronaviruses, respectively, have now been joined by the more recently described group I and II strains HCoV-NL63 and HCoV-HKU1 (13, 30, 42, 45, 46), which were discovered in the search for other pathogenic coronaviruses after the identification of the coronavirus that causes severe acute respiratory syndrome (SARS) (29). HCoV-NL63 may have infected human populations for a long time, since it diverged phylogenetically from HCoV-229E about 1,000 years ago (33), and seroprevalence would likely be high as a result. Cross-sectional and longitudinal seroepidemiological studies have found large proportions of children and healthy adults to have detectable serum antibodies to the four HCoV strains, and seroconversion occurs often in childhood; seroprevalence increases with age, and reinfections may occur (5, 8, 23, 28, 36–38). More information is needed about the seroprevalence of these viruses, the durability of the humoral immune response, correlates of immunity, and mucosal antibody responses to HCoV infection. The present study questioned whether the prevalence of antibodies to the four HCoV strains would be different in nasal secretions than in serum of older adult veterans with underlying chronic obstructive pulmonary disease (COPD) who participated in Department of Veterans Affairs Cooperative Study 448 (18).

MATERIALS AND METHODS

Subjects. A convenience sample of 105 patients who met spirometric criteria for COPD and were enrolled in a larger influenza virus vaccine efficacy trial of patients ≥50 years of age (18) were chosen for analysis in this substudy of the prevalence of antibodies to HCoV, because residual serum and nasal wash specimens collected at the same time for each subject were available for analysis. The 105 subjects were enrolled at seven geographically diverse study sites in the United States, located in the following states: Alabama, Florida, Illinois, Massachusetts, Michigan, Missouri, and Texas. The paired serum and nasal wash specimens were collected at about 3 to 4 weeks following influenza virus vaccination between October 1998 and February 1999 and were not associated with acute respiratory illness at the time of collection. All patients gave written informed consent, and responsible committees on human experimentation approved of the study.

Antigen preparation and ELISA. The HCoV antigens used for the antibody enzyme-linked immunosorbent assay (ELISA) were produced as described previously (16). HCoV-229E and HCoV-OC43 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in MRC-5 and HCT-8 cell monolayers (ATCC), respectively. HCoV-NL63 was grown in LLC-MK2 cell monolayers (a gift from Lisa van der Hoeck, University of Amsterdam, Amsterdam, Netherlands). Virus-infected cells were frozen and thawed three times, the supernatant fluid was cleared of cell debris by centrifugation, the virus
was concentrated by overnight centrifugation, and the virus pellet was resuspended in phosphate-buffered saline (PBS). The concentrated virus was inactivated by a psoralen compound (Sigma, St. Louis, MO), followed by irradiation by long-wavelength UV light, as described previously (34). Mock antigen was prepared, in the same way, from uninfected cells.

The His6-tagged recombinant N protein of HCoV-HKU1 was used as an antigen in the ELISA to detect antibody to HCoV-HKU1. Expression vector pET-28b (+) (Novagen, Inc., New Canaan, CT), encoding the N protein of HCoV-HKU1 cloned into the EcoRI and NotI sites in frame and downstream from a series of six-histidine residues, as described previously (45, 46), was a gift from K. Y. Yuen (University of Hong Kong, Hong Kong). The recombinant N protein was expressed by the transformation of BL21(DE3) single competent cells (Novagen, Inc.) by the plasmid and was purified by use of the Ni2+-loaded HiTrap chelating system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions and as described previously (16). In brief, transformed cells were disrupted, and the protein sample was prepared by the isolation of inclusion bodies via sonication and washing. The protein sample was loaded onto the HiTrap chelating HP column prepacked with prechared high-performance Ni2+ Sepharose. The protein was then purified and refolded by serial buffer washes of the column and by liquid chromatography in a fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Piscataway, NJ), with elution of protein, which was collected in fractions. The fractions were analyzed for the presence of the 53-kDa protein by SDS-PAGE. The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and was adjusted to 3 µg/ml for the ELISA. Mock antigen was produced from the same plasmid DNA vector, but without the N protein gene sequence, by the same procedure.

Each viral antigen and its respective control were used to coat flat-bottom 96-well Maxisorp Immunoplates (Nalge-Nune International, Rochester, NY). To measure serum IgG antibodies, the sequence of reagents consisted of serum in a series of eight 2-fold dilutions starting at a 100-fold dilution to generate a broad dose-response curve, mouse anti-human IgG (Fc specific) conjugated with horseradish peroxidase (Accurate Chemical and Scientific, Westbury, NY), and o-phenylenediamine substrate (KPL, Gaithersburg, MD). The optical density (OD) was measured at 405 nm by use of a Tecan (Research Triangle Park, NC) spectrophotometer. The anti-HCoV antibody titer in nasal wash specimens was calculated by use of the standard curve of o-phenylenediamine substrate. The standard curve of known secretory IgA concentrations was determined by use of serial dilutions of human secretory IgA (ICN/Cappel Pharmaceuticals, Inc., Aurora, OH), the nasal wash specimen, goat anti-human IgA (α-chain specific) conjugated with horseradish peroxidase, and o-phenylenediamine substrate. The standard curve of known secretory IgA concentrations was determined by using serial dilutions of human secretory IgA (ICN Biomedical, Inc., Costa Mesa, CA) instead of nasal wash specimens in the ELISA. We repeated the measurement of total IgA concentrations in this study, finding that there was no decay in the total IgA concentration in these specimens that were stored at −70°C for about 9 years after their initial collection. The anti-HCoV IgA antibody titer in nasal wash specimens was mathematically adjusted to an arbitrary total IgA antibody concentration of 100 mg/liter, using the measured total IgA antibody concentration for each specimen.

Statistical methods. Demographic and antibody data were analyzed by using descriptive statistics. Reciprocal antibody titers were logarithmically transformed, and geometric mean titers (GMTs) were compared by using the Wilcoxon signed-rank test. McNemar’s test was used to compare pairwise proportions of subjects with detectable anti-HCoV antibodies. Nonpaired data were compared by using the χ2 test or Fisher’s exact test and the Wilcoxon rank sum test. Logarithmically transformed serum IgG antibody titers against HCoV strains were assessed by linear regression, and Pearson correlations were performed.

### RESULTS

Of the 105 patients studied, 101 (96%) were male and 97 (92%) were white (Table 1). All had spirometrically confirmed obstructive pulmonary disease, 95 (90%) had one or more additional underlying illnesses, and 68 (65%) were 65 years of age or older (Table 1).

At least 98% of the 105 patients had serum IgG antibodies to HCoV-229E, -NL63, and -OC43, while 96 (91%) had serum IgA antibody to HCoV-HKU1 (Table 2). The proportions of patients with detectable IgG antibodies to HCoV-229E, -NL63, and -OC43 were all greater than the proportion with IgG antibody to HCoV-HKU1 (P < 0.001, Table 2). The proportions of patients with IgA antibodies against HCoV-OC43 and -HKU1 were significantly greater than those with IgA antibodies against HCoV-229E (P = 0.03 and P = 0.0013, respectively) and HCoV-NL63 (P = 0.001 and P < 0.0001, respectively) (Table 2). Anti-HCoV IgA antibody GMTs for nasal wash specimens with detectable antibody were within a 2-fold dilution range when the four viral strains were compared (Table 2). The GMTs were higher and the ranges of individual IgA antibody titers included higher values for HCoV-OC43 and -HKU1 than HCoV-229E (P = 0.003 and P = 0.0006, respectively) and -NL63 (both P < 0.0001). The serum IgG antibody GMT for HCoV-229E was less than that for HCoV-OC43 (P = 0.003), and the serum IgG GMTs for HCoV-229E and -OC43 were each greater than those for HCoV-NL63 and -HKU1 (P < 0.0001). The serum IgG antibody GMTs for HCoV-229E and -OC43 were about 2-fold higher than those for HCoV-NL63 and -HKU1.

The ranges of individual IgG antibody titers extended more than 7-fold higher for HCoV-229E over HCoV-NL63 and 5-fold higher for HCoV-OC43 over HCoV-HKU1 (Table 2), but only 11 sera had an IgG antibody titer against HCoV-229E.
that was greater than the highest titer against HCoV-NL63, and only 2 sera had an IgG antibody titer against HCoV-OC43 that was greater than the highest titer against HCoV-HKU1. Between 13% and 75% of nasal wash specimens with IgA antibody to one HCoV strain had antibody to another viral strain (Table 3). Five nasal wash specimens had titers of IgA antibody to HCoV-OC43 that exceeded the highest titer against HCoV-229E in any specimen, and none of these five specimens had detectable antibody to HCoV-229E. Similarly, seven nasal wash specimens had IgA antibody titers against HCoV-HKU1 that exceeded the highest titer against HCoV-NL63 in any specimen, and only one of these seven specimens had antibody to HCoV-NL63. Hence, the cross-reactive IgA antibodies to more than one viral strain that might be expected for high-titer specimens were often not present for a strain in the other HCoV group.

Since most sera had IgG antibodies to all four HCoV strains, we assessed correlations between serum IgG antibody titers against HCoV strains to characterize possible antigenic relationships between HCoV strains and groups based on seroreactivity. The highest levels of correlation were between strains within each of HCoV group I (Fig. 1A) and group II (Fig. 1B). Comparisons of serum IgG antibody titers against HCoV strains between groups had lower correlation coefficients that were either not statistically significant or at weaker significance levels (Fig. 1C to F) than those observed for the within-group comparisons (Fig. 1A and B). Thus, these data are also consistent with patients having experienced infections with both group I and group II HCoV strains in the past.

Serum IgG and nasal wash specimen IgA antibody GMTs against each HCoV strain were not significantly different when current smokers were compared to nonsmokers and when patients with a history of heart disease were compared to those without a history of heart disease (data not shown). The proportions of patients with nasal wash specimen IgA antibodies to the HCoV strains who were current smokers compared to nonsmokers did not differ significantly (data not shown). The proportion with IgA antibodies to HCoV-NL63 in nasal wash specimens of patients with a history of heart disease was higher than that of patients without heart disease (6 [16%] versus 2 [3%]; \( P = 0.014 \)), but the proportions with IgA antibodies to the other three HCoV strains in nasal wash specimens from patients with and those without heart disease did not differ (data not shown).

**TABLE 2. IgG antibodies to HCoV in serum and IgA antibodies to HCoV in nasal wash specimens**

<table>
<thead>
<tr>
<th>Strain</th>
<th>GMT of all sera</th>
<th>Only sera with detectable antibody</th>
<th>No. of positive samples (%)/total no. of samples tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCoV-229E</td>
<td>994 (^a)</td>
<td>1,022, 1,038</td>
<td>104 (99)/105</td>
</tr>
<tr>
<td>HCoV-NL63</td>
<td>567 (^a)</td>
<td>595, 629</td>
<td>103 (98)/105</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCoV-OC43</td>
<td>1,235 (^a)</td>
<td>1,235, 1,137</td>
<td>105 (100)/105</td>
</tr>
<tr>
<td>HCoV-HKU1</td>
<td>466 (^a)</td>
<td>575, 527</td>
<td>96 (91)/105</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Serum IgG antibodies to all four HCoV strains were present in most study subjects, but IgA antibodies to the four HCoV strains were detected less frequently in nasal wash specimens.
for only 8% to 31% of subjects. Patients represented a broad geographic distribution, from the Midwestern, Southern, and Northeastern regions of the United States. A wide range of IgG and IgA antibody titers against HCoV was observed. The levels of mean IgG antibody to HCoV-229E and -OC43 were higher than levels of mean IgG antibody to HCoV-NL63 and -HKU1, respectively. Nasal wash specimen IgA antibodies to the two group II HCoV strains were more frequently detected than were antibodies to the two group I strains, and the mean antibody titers were higher for the group II strains as well. Since the mean antibody titers against the HCoV strains in serum and also in nasal wash specimens were within about a 2-fold range, it is unclear if the statistical differences in mean titers are important clinically. The concomitant presence of
IgA antibody to more than one HCoV strain was observed for some nasal wash specimens but was universal in the case of serum IgG antibody to HCoV.

The findings may reflect previous infections of patients with more than one viral strain, antibody cross-reactivity between strains due to HCoV group-specific rather than strain-specific antigens, better induction of mucosal IgA antibodies by certain HCoV strains and a shorter period of detectability after infection in secretions than in serum, and a better preservation of antibodies in the nasal wash specimens with antibody to HCoV than in those that had no detectable anti-HCoV antibody despite similar storage conditions. However, the amount of total IgA in the specimens did not decline during storage, so strain-specific IgA antibodies should not have decayed in storage either. Correlation of serum IgG antibody titers within and across HCoV groups showed a more striking association between the levels of antibody to the HCoV strains within each group than across groups. This may reflect antigenic cross-reactivity within HCoV groups but less so between groups. Also, cross-reactive IgA antibodies to HCoV strains between HCoV groups were generally not observed in high-titer nasal wash specimens.

Levels of serum antibody to HCoV-229E and -OC43 in our study in terms of GMT and the frequency of detection were similar to data reported previously by Schmidt et al. (36), who described repeated significant increases in antibody longitudinally over time, suggesting that reinfections occurred with the same virus strain. The prevalence of serum antibodies to HCoV-229E and -OC43 in our study was higher than those reported previously by Hruskova et al. (23) in Czechoslovakia and was higher than the prevalence of serum antibodies to HCoV-229E in children and adolescents in the United States reported previously by Shao et al. (38) but was similar to those reported for HCoV-OC43 in health care workers in France (28). Children in the Netherlands aged 2.5 to 3.5 years had serum antibodies to HCoV-NL63 and HCoV-229E in 75% and 65% of serum samples collected cross-sectionally, respectively (8). Severance et al. (37) previously reported seroprevalence rates of about 90% for HCoV-229E, -NL63, and -OC43 among a largely adult population in Maryland but a seroprevalence rate of only 59% for HCoV-HKU1, which is lower than that reported in the present study. In seroepidemiological studies of families including children and young adults in one community, a high prevalence of antibodies to HCoV-229E and -OC43 was reported, and frequent reinfections occurred despite preexisting serum antibody (27). Thus, there is evidence for infections with these viruses being common in children and adults and occurring over a wide geographical distribution and frequently over time.

We did not identify a difference in antibody titers when current smokers were compared to nonsmokers, unlike what was reported by Severance et al. (37) for IgG antibodies to HCoV-OC43. For unclear reasons, patients with a history of adult smoking were compared to nonsmokers, unlike what was expected. Both smokers and smokers were compared to nonsmokers, unlike what was reported previously by Shao et al. (38) but was similar to those reported for HCoV-OC43 in health care workers in France (28). Children in the Netherlands aged 2.5 to 3.5 years had serum antibodies to HCoV-NL63 and HCoV-229E in 75% and 65% of serum samples collected cross-sectionally, respectively (8). Severance et al. (37) previously reported seroprevalence rates of about 90% for HCoV-229E, -NL63, and -OC43 among a largely adult population in Maryland but a seroprevalence rate of only 59% for HCoV-HKU1, which is lower than that reported in the present study. In seroepidemiological studies of families including children and young adults in one community, a high prevalence of antibodies to HCoV-229E and -OC43 was reported, and frequent reinfections occurred despite preexisting serum antibody (27). Thus, there is evidence for infections with these viruses being common in children and adults and occurring over a wide geographical distribution and frequently over time.

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