Effect of Preexisting Serum and Mucosal Antibody on Experimental Respiratory Syncytial Virus (RSV) Challenge and Infection of Adults

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We studied preexisting respiratory syncytial virus (RSV)–specific serum and nasal antibodies and their correlation with infectivity, viral dynamics, and disease severity in a human experimental infection model. Higher preinoculation serum neutralizing antibody titers and nasal immunoglobulin (Ig) A predicted lower infectivity and lower measures of viral replication. However, once individuals were infected, no significant protective effect of preexisting antibodies was seen. Lack of correlation between serum and mucosal antibodies was observed, implying that they are independent co-correlates of protection against RSV infection. We suggest that protection from RSV infection is a function of a complex interplay between mucosal and serum humoral immune responses.

Keywords. respiratory syncytial virus; vaccines; serum antibody responses; mucosal antibody responses; correlates of protection.

Despite the very large disease burden, there is no vaccine against respiratory syncytial virus (RSV) infection. The determination of correlates of protective immunity to an infectious disease is fundamental to the development of vaccines. Almost all current vaccines likely work largely through induction of antibodies in serum or mucosa. Earlier studies in healthy young adults experimentally challenged with RSV suggested nasal neutralizing antibody as a more important determinant of resistance than serum antibodies [1]. More-recent studies of elderly persons with natural RSV infection found that lower levels of serum neutralizing antibody and nasal RSV-specific immunoglobulin A (IgA) was associated with increased risk of infection, although the relative importance of each in protection has not been definitively assessed [2, 3]. In addition, the relationship between preexposure antibody concentrations and subsequent viral and disease dynamics also remains ambiguous. We therefore studied preexisting serum and mucosal antibody titers and their correlation with RSV infectivity, viral dynamics, and disease severity in a human experimental RSV infection model.

MATERIALS AND METHODS

Subjects and Study Design
Thirty-five healthy adult male and female volunteers 18–45 years of age were intranasally inoculated with a clinical strain of RSV-A (Memphis 37, a low passage virus manufactured from a hospitalized bronchiolitic infant; inoculum range, 3–6.5 log plaque-forming units [PFU]) after obtaining appropriate ethics committee approval and informed consent from all volunteers. To maximize successful infection, volunteers with relatively lower serum RSV microneutralization titers were selected for this study. The safety, reproducibility, genotype, and full sequence of Memphis 37 have been previously established [4, 5]. Volunteers were admitted to the quarantine unit and evaluated over 11 days. The preinoculation RSV antibody level was measured in serum specimens (obtained on day −2) and nasal wash specimens (obtained on day −1). Subjects were defined as being infected if RSV was detected ≥2 successive times between day 2 and day 8 (inclusive). Nasal wash specimens collected twice daily were analyzed for viral load by real-time quantitative polymerase chain reaction (qPCR) and quantitative culture (qCulture). Identical methods were used to measure disease severity in all volunteers: direct physical examination score determined once daily, symptom score determined twice daily, and cumulative weights of daily nasal mucus production. The study design and symptom scoring system have been previously described [4].

Viral Quantification Assays
RSV qCultures in HEP-2 cell plaque assays were performed [6], and the units are reported as log (base 10) PFU per milliliter (log PFU/mL). A qPCR assay amplifying a portion of the N
gene of RSV was used to detect genomic nucleic acids in the nasal wash specimens [7]. Each specimen was run in duplicate in 96-well plates with internal standards of duplicate pairs of six 10-fold dilutions of RSV RNA extracted from parallel aliquots containing a known quantity of RSV-A Long (ATCC VR-26) as defined by and as used in the plaque assay. Results are expressed as means of duplicates in log (base 10) PFU equivalents per milliliter (log PFUe/mL), with the viral load representing the quantity of RSV nucleic acid detected in a single nasal wash specimen.

**Measurement of Nasal RSV Antibody, RSV-specific IgA**

An enzyme-linked immunosorbent assay (ELISA) was designed to detect and quantify RSV-specific IgA in nasal wash specimens. Plates were coated with purified post-fusion F protein and then blocked with bovine serum albumin (BSA) overnight. Test samples (diluted 1:60) were added in phosphate-buffered saline–BSA (PBS–BSA). After overnight incubation at 4°C, assays were developed with mouse α-human antibody and goat α-human antibody coupled to horseradish peroxidase, followed by addition of tetramethylbenzidine (TMB) ELISA substrate solution and stop solution for optical density (OD) readings at 450 nm and 540 nm (or 570 nm). The OD readings obtained at 540 nm were subtracted from those obtained at 450 nm, to obtain the final OD reading for a particular well. All samples were done in duplicate, and the IgA level assigned to a sample was the average. Each plate had a standard curve.

**Figure 1.** Preinoculation immunity and infectivity. Compared with infected volunteers, the volunteers who remained uninfected despite respiratory syncytial virus (RSV) inoculation had higher preinoculation levels of nasal RSV specific immunoglobulin A (IgA). A, Mean nasal IgA titers (±standard deviation [SD]) in infected and uninfected volunteers, as determined by quantitative polymerase chain reaction (qPCR; uninfected group 1.951 ± 0.2418 log ng/mL [n = 7]; infected group, 1.454 ± 0.09278 log ng/mL [n = 26]; P = .0292). B, Mean levels (±SD) in infected and uninfected volunteers, as determined by quantitative culture (qCulture; uninfected group, 1.814 ± 0.1774 log ng/mL [n = 12]; infected group, 1.414 ± 0.09797 log ng/mL [n = 21]; P = .0391). Each point on the graph in panels A and B is the preinoculation nasal RSV-specific IgA level for a single volunteer. Compared with the infected volunteers, the uninfected volunteers had a higher serum RSV microneutralization titer. C, Mean preinoculation serum microneutralization titers (±SD) in the volunteers who were defined as infected and uninfected by qPCR (uninfected group, 7.853 ± 0.5625 microneutralization units [MU/mL] [n = 8]; infected group, 6.886 ± 0.1387 MU/mL [n = 26]; P = .0022). D, Mean preinoculation serum microneutralization titers (±SD) in the volunteers deemed infected and uninfected by qCulture (uninfected group, 7.598 ± 0.3605 MU/mL [n = 13]; infected group, 6.814 ± 0.1599 MU/mL [n = 21]; P = .0229). Each point on the graph in panels C and D is the preinoculation serum microneutralization titer of a single volunteer expressed in MU. Unpaired t test (2 tailed) was performed to compare the means between the uninfected and infected groups.

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from which OD values of diluted samples were extrapolated and expressed in log nanograms per milliliter (log ng/mL).

**Measurement of Serum RSV-Neutralizing Antibodies**

Serum RSV-neutralizing antibodies were measured by a HEp-2 cell RSV 50% microneutralization assay as previously described [8] but were performed with the Memphis 37 strain. Titers are expressed as the dilution of serum resulting in a 50% reduction of virus growth.

**Statistical Analysis**

Statistical analyses and construction of figures were performed with GraphPad Prism Software v 5.0 (La Jolla, California). Comparisons of preinoculation nasal IgA levels or serum microneutralization titers in uninfected and infected samples were made with t tests. Linear regression was used to compare continuous variables within individual patients. For all analyses, results with 2-sided P values of <.05 are considered significant.

**RESULTS**

**Preinoculation Antibodies and Infectivity**

Of the 35 volunteers included in the analysis, 27 (77%) met the study definition of becoming “infected,” based on results of the sensitive qPCR assay, while 21 of 35 (60%) were deemed “infected” on the basis of qCulture results. The preinoculation nasal IgA level was determined in 33 of 35 volunteers.

Nasal IgA levels were found to be lower in RSV-infected volunteers, compared with uninfected volunteers, by both qPCR ($P = .0022$; Figure 1C) and qCulture ($P = .0229$; Figure 1D) definitions. There was no significant correlation
between the preinoculation serum and mucosal antibody titers for each individual infected subject (slope of regression line, 0.026 [95% confidence interval (CI), −0.60 to .65]; P = .9312). However, a trend toward statistical significance was seen when all subjects were analyzed (slope of regression line, 0.62 [95% CI, −.61 to 1.30]; P = .073).

**Preinoculation Immunity and Viral Dynamics**

A trend was seen between lower preinoculation RSV mucosal IgA titers and increased viral outcomes (peak viral load and duration of shedding) when all exposed volunteers were studied (Figure 2A and 2C) but not when only infected volunteers were considered (Figure 2B and 2D). Preinoculation serum RSV microneutralization titers were inversely correlated with viral dynamics when all subjects were analyzed (P < .05; Figure 3A and 3C). However, no correlation was noted when only infected subjects were analyzed (Figure 3B and 3D).

**Preinoculation Immunity and Disease Dynamics**

The preinoculation RSV IgA level was found to have no correlation to disease outcome measures (ie, symptom score, physical examination score, and nasal mucus weight; Figure 4A–F). A trend (statistically insignificant) was seen between lower preinoculation RSV microneutralization titers and increased disease outcome (Figure 5A, 5C, and 5E) when all subjects were analyzed. No correlation was noted when only infected subjects were analyzed (Figure 5B, 5D, and 5F).

**DISCUSSION**

Defining the immune correlates of protection is an essential objective criterion for vaccine development against an infectious disease. This has been a major barrier in the development of RSV vaccines. The immune correlates for influenza are well defined, leading to effective vaccination strategies against the disease. Serum hemagglutination titer (mediated by influenza–specific...
immunoglobulin G (IgG) is a well-established immune correlate of protection against influenza disease [9]. Data from efficacy studies of live attenuated influenza virus vaccine highlight the importance of nasal IgA in vaccine-induced immunity [10]. These studies underscore the importance of both serum and mucosal immune responses in respiratory viral infections.

With respect to RSV, low nasal neutralizing titers were shown to be a better correlate of infection than low serum neutralizing titers [1], while a more recent challenge study illustrated that serum neutralization was a modestly accurate predictor of infectivity [11]. Our study demonstrates a role for both serum and mucosal immune responses in protection against RSV infection. Despite the relatively narrow range imposed by the selection criteria for volunteer screening prior to enrollment, preinoculation serum RSV microneutralization titers were found to be lower in subjects who became infected, compared with those who remained uninfected. A definitive seroprotective titer, however, could not be established. This result is consistent with those in other studies [11]. In addition, we found a higher preinoculation anti-RSV IgA level in uninfected volunteers, compared with infected volunteers, a finding also noted in adults with naturally acquired RSV infection [12]. Only 4 of 9 subjects (44%) with a preexisting mucosal IgA titer of >1.8 log ng/mL could be infected, suggesting that this might be a correlate of protection. Thus, under the classification of correlates of protection proposed by Plotkin [13], our observations suggest

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**Figure 4.** Mucosal preinoculation immunity and disease measures. Correlation of preinoculation (day −1) nasal respiratory syncytial virus (RSV)-specific immunoglobulin A (IgA) titer expressed in log nanograms/milliliter, with disease measures in all volunteers (A, C, and E) and infected volunteers (B, D, and F). A, Comparison of total symptom scores to nasal RSV-specific IgA titer in all volunteers (mean slope of regression line ±standard deviation [SD]), −4.424 ± 15.06; 95% confidence interval [CI], −35.15 to 26.30; P = .7710). B, Comparison of total symptom scores to nasal RSV-specific IgA titer in infected volunteers (mean slope of regression line ±SD), −30.42 to 54.25; P = .8688). C, Comparison of total directed physical examination score (DPE) to nasal RSV-specific IgA titer in all volunteers (mean slope of regression line ±SD), −11.07 to 1.552; P = .1341). D, Comparison of total DPE score to nasal RSV-specific IgA titer in infected volunteers (mean slope of regression line ±SD), −9.470 to 8.063; P = .8688). E, Comparison of total tissue weight, measured in grams, to nasal RSV-specific IgA titer in all volunteers (mean slope of regression line ±SD), −110.8 to 87.65; P = .8135). F, Comparison of total tissue weight to nasal RSV-specific IgA titer in infected volunteers (mean slope of regression line ±SD), 53.64 ± 64.98; 95% CI, −80.48 to 187.8; P = .4172). Disease severity did not correlate with preinoculation (day −1) RSV-specific IgA titer.
that both serum microneutralization titers and nasal IgA titers are co-correlates of protection against RSV infection. We compared the preinoculation immunity to viral and disease measures. A statistically significant correlation was seen between lower RSV microneutralization titers and increased viral outcomes. Similarly, a trend (statistically insignificant) emerged that suggested a correlation between lower RSV microneutralization titers and increased disease outcomes when all subjects were analyzed. However, once infection was established, we found no significant protective effect of preexisting serum antibody on either viral or disease outcomes. There also was a trend (statistically insignificant) toward increased viral outcomes and lower preinoculation RSV mucosal IgA titers when all subjects were analyzed. However, when infection was established, the titer...
of preexisting nasal antibody did not appear protective. Thus, although both preexisting serum and mucosal immunity protected against RSV infectivity, neither type of preexisting antibodies predicted viral dynamics or disease measures in infected subjects. This is somewhat in contrast to a previous study, in which higher levels of serum neutralizing antibody were associated with a decreased severity of disease among RSV-infected elderly persons or adults with underlying high-risk cardiopulmonary conditions and in whom the titer of nasal IgA to the RSV G protein was inversely correlated with the peak viral titer [3, 12]. The relatively milder upper respiratory tract infection with RSV provoked in healthy adult volunteers may explain why an association with infectivity but not disease severity was seen in our study.

We observed a lack of correlation between virus-specific preexisting mucosal IgA and serum neutralizing antibody titers within individual volunteers, suggesting that they likely offer protection by independent mechanisms. One potential theory, based on animal data, is that plasma IgG is important in protection of the lungs, while mucosal IgA is more important in the protection of the upper respiratory tract [14], the infection of which might be a prerequisite for spread to the lower respiratory tract. In humans, live intranasal vaccines stimulate the production of both nasal mucosal and, to a lesser extent, serum antibodies, while intramuscular or subcutaneously administered vaccines primarily stimulate serum antibodies. Thus, a live vaccine targeting both serum and mucosal antibodies might be expected to be more optimally effective in protection against RSV infection.

Our study has certain limitations. The generalizability of the study to the patient population at risk for severe natural RSV infection is arguable, given that our study is limited to a small number of healthy adult volunteers. Our model used a minimum inoculum of 3 log_{10} PFU of a clinical strain of the most common genotype of RSV. It is possible that we would have seen better correlation between antibody levels and protection if a lower inoculum had been used. The inoculum that mimics natural exposure is unknown. Second, we did not study the role of nasal IgG. We chose to study nasal IgA because of its established defensive role against respiratory viruses by virtue of its prominent presence at the initial point of contact. We have previously shown that RSV-specific IgG is significantly less concentrated in nasal secretions, compared with the lower respiratory tract or the systemic circulation [15]. Last, our model of experimental human infection predominantly involves the upper respiratory tract. Whether protection from clinical manifestations of lower respiratory tract infection would be accomplished by the same relative concentrations of nasal IgA and serum microneutralizing antibodies remains to be determined.

In conclusion, we have shown that both virus-specific nasal and serum antibody responses are independent co-correlates of protection against RSV infection in adults experimentally inoculated with RSV. Hence, an effective RSV vaccine should likely elicit the production of both mucosal IgA and systemic neutralizing antibody. Host factors (age and presence of comorbidities) are also expected to play a vital role in determining the immune response to RSV infection or future RSV vaccines, and thus the immune correlates of protection against RSV in specific vulnerable patient populations need to be better elucidated in future studies.

Notes

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