Evaluation of Pneumonia Virus of Mice as a Possible Human Pathogen

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Pneumonia virus of mice (PVM), a relative of human respiratory syncytial virus (RSV), causes respiratory disease in mice. There is serologic evidence suggesting widespread exposure of humans to PVM. To investigate replication in primates, African green monkeys (AGM) and rhesus macaques (n = 4) were inoculated with PVM by the respiratory route. Virus was shed intermittently at low levels by a subset of animals, suggesting poor permissiveness. PVM efficiently replicated in cultured human cells and inhibited the type I interferon (IFN) response in these cells. This suggests that poor replication in nonhuman primates was not due to a general nonpermissiveness of primate cells or poor control of the IFN response. Seroprevalence in humans was examined by screening sera from 30 adults and 17 young children for PVM-neutralizing activity. Sera from a single child (6%) and 40% of adults had low neutralizing activity against PVM, which could be consistent with increasing incidence of exposure following early childhood. There was no cross-reaction of human or AGM sera between RSV and PVM and no cross-protection in the mouse model. In native Western blots, human sera reacted with RSV but not PVM proteins under conditions in which AGM immune sera reacted strongly. Serum reactivity was further evaluated by flow cytometry using unfixed Vero cells infected with PVM or RSV expressing green fluorescent protein (GFP) as a measure of viral gene expression. The reactivity of human sera against RSV-infected cells correlated with GFP expression, whereas reactivity against PVM-infected cells was low and uncorrelated with GFP expression. Thus, PVM specificity was not evident. Our results indicate that the PVM-neutralizing activity of human sera is not due to RSV- or PVM-specific antibodies but may be due to low-affinity, polyreactive natural antibodies of the IgG subclass. The absence of PVM-specific antibodies and restriction in nonhuman primates makes PVM unlikely to be a human pathogen.

Pneumovirus of mice (PVM) is an enveloped nonsegmented negative-strand RNA virus of the genus Pneumovirus, family Paramyxoviridae (17, 23). PVM is a relative of human respiratory syncytial virus (RSV). RSV is the leading viral cause of severe respiratory infections in infants and also causes substantial morbidity and mortality in the elderly and in profoundly immunosuppressed individuals (17, 26, 59). Pneumoviruses have genomes of approximately 15 kb that contain 10 genes encoding 11 or 12 proteins. The gene order and constellation of proteins are conserved within the genus, with the exception that the PVM P gene encodes a second protein of unknown function (3, 41) that does not have a counterpart in RSV. The degree of amino acid sequence identity between PVM and RSV ranges from 10% (M2-2 protein) to 60% (nucleocapsid N protein) (41).

The host range and natural history of PVM are poorly understood. PVM was first discovered in laboratory mice in 1938 in a study to isolate human respiratory viruses from patients with respiratory disease (33). In that study, human nasopharyngeal wash specimens were serially passaged in mice. The inoculated animals developed viral pneumonia; however, the same viral pneumonia was also induced by serial passage of lung suspensions from nonsuppressed control mice. This observation led to the identification of PVM as a causative agent of pneumonia in mice (32, 33). The pathogenesis of PVM in inbred mice varies considerably between strains (2); in the commonly used BALB/c strain, the virus is highly pathogenic, with doses of 120 PFU and even lower being lethal (19, 42). In the past, evidence of PVM infection of laboratory mice was abundant (13, 37, 39, 40, 48, 70), and there has been serologic evidence of infection of a number of other laboratory animals, including other rodent species, rabbits, and nonhuman primates (35). However, the virus has now been largely eliminated from laboratory mice by specific-pathogen-free breeding methods and infection control measures. For animals kept without these measures, such as those in pet shops, evidence of PVM infection has continued to be reported (20). While serologic evidence of PVM infection in wild rodents also has been reported (38), most surveys of wild rodents have failed to find such evidence (4, 22, 49, 52, 61). In addition, morbidity or mortality attributed to PVM and isolation of the virus from wild rodents have not been documented. Thus, the natural host(s) and the natural history of PVM remain unclear. More recently, PVM was isolated from dogs with respiratory tract disease, although whether PVM caused the observed disease and is common in dogs is unclear (57).

The prevalence of PVM in captive animals has raised the suggestion that it might arise from human contact. Indeed, two groups have reported serological data suggesting that PVM, or an antigenically related virus, causes widespread human infection (31, 32, 34, 35, 56). However, these studies are difficult to interpret for various reasons, including lack of a control for cross-reaction between PVM and its highly prevalent RSV relative, the small
number of screened sera, and the lack of experimental details sufficient to allow for clear interpretation of the results. Attempts by one group, reporting in the 1940s (31, 32, 34, 35), to detect PVM in human specimens by the induction of antibodies in laboratory animals was confounded by the likely presence of PVM in some of those animals. A second group, reporting in 1986 (56), noted moderate to high titers of PVM-neutralizing antibodies in more than 75% of adult human sera, but reactivity with PVM proteins was not confirmed, and well-defined negative and positive controls were not used. A report of reciprocal cross-reactivity between the N proteins of RSV and PVM highlighted the possibility of cross-reaction in serologic studies (27, 44). To date, PVM has never been reported to be isolated from humans. To investigate the possibility of PVM or a PVM-like virus as a possible human pathogen, we evaluated PVM replication in nonhuman primates, evaluated cross-reactivity between PVM and RSV, and evaluated in detail the reactivity of human sera with PVM.

MATERIALS AND METHODS

Viruses and cells. Recombinant PVM (rPVM) is based on a consensus sequence for virulent strain 15 (42). rPVM, rPVM expressing green fluorescent protein (rPVM-GFP) (42), and rPVM mutants lacking the genes for nonstructural proteins N1 and N2 (rPVMΔNS1, rPVMΔNS2, and rPVMΔNS12) (9) were propagated on BHK-21 cells (CCL-10; ATCC, Manassas, VA) or on Vero cells (CCL-81; ATCC). Recombinant RSV (rRSV) (18) or GFP-expressing RSV (RSV-GFP) (50) was propagated on Vero cells. BHK-21 cells were maintained in Glasgow minimal essential medium (Glasgow MEM; Life Technologies, Carlsbad, CA) supplemented with 4 mM l-glutamine, 2% MEM amino acids (Life Technologies), and 10% fetal bovine serum (FBS) (HyClone, Logan, UT). Vero cells were maintained in Opti-Pro medium (Life Technologies) supplemented with 4 mM l-glutamine. BSR T7/5 cells were maintained in Glasgow MEM as described before (7). A549 cells (CCL-185; ATCC) were maintained in F-12 medium (Life Technologies) supplemented with 5% FBS and 4 mM l-glutamine.

RSV or PVM titers were determined by plaque assay on Vero cells under a 0.8% methylcellulose overlay. Plaques were visualized by immunostaining with rabbit G-specific polyclonal antibody (PAb) raised against recombinant modified vaccinia virus Ankara (MVA)-PVM G (42) or using a mixture of monoclonal antibodies (MAbs) 1129, 1269, and 1243 against the RSV F protein (5), followed by a horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG secondary antibody (KPL, Gaithersburg, MD). Bound antibodies were detected by incubation with a peroxidase substrate (KPL).

To prepare sucrose gradient-purified virus, clarified medium supernatants from virus-infected Vero cells were subjected to centrifugation on discontinuous sucrose gradients (30 to 60% wt/vol) at 26,700 × g for 2 h. The virus band between the 30 and 60% interface was collected, diluted with Opti-MEM (Life Technologies), and pelleted by centrifugation at 26,700 × g for 1 h at 4°C. Pellets were resuspended in Opti-MEM for use in infectivity assays or in TENV buffer (10 mM Tris [pH 7.4], 1 mM EDTA, and 100 mM NaCl) for Western blot analysis. Virus samples were flash frozen on dry ice. UV inactivated rPVMΔNS2 was prepared by irradiation with 240 kJ using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The absence of infectious virus was confirmed by plaque titration.

Nonhuman primate studies. Four young adult African green monkeys (AGM; Chlorocebus aethiops) and four rhesus macaques (RM; Macaca mulatta) were confirmed to be seronegative for PVM and RSV by plaque reduction assays, which sensitively detect even low levels of activity. The animals were inoculated simultaneously intranasally and intracheally with 1 ml of inoculum per site containing 10^6 PFU of rPVM in L15 medium (Life Technologies). Clinical observations were made on days 0 to 12 postinoculation. Following inoculation, nasopharyngeal (NP) swabs were collected daily for 10 days and on day 12. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, 10, and 12 postinfection. The amount of virus present in NP and TL samples was quantified by plaque titration on Vero cells. All animal experiments were approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases. The studies were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (50a).

Virus-specific control antibodies. Control antibodies specific to RSV included the F-specific mouse MAb 1129 (5), rabbit antiserum raised against sucrose gradient-purified RSV (RSV-specific PAb), and AGM sera collected 35 days following experimental infection with RSV. Control antibodies specific to PVM included rabbit antiserum raised against MVA expressing the PVM F or G protein (PVM F-specific PAb and PVM G-specific PAb, respectively) (42), rabbit antiserum raised against sucrose-gradient-purified PVM (PVM-specific PAb), and post-PVM-infection AGM sera from the experiment whose results are shown in the tables.

Virus neutralization assays. Human sera were obtained from healthy adult donors at the Department of Transfusion Medicine of the National Institutes of Health (NIH, Bethesda, MD) under a protocol approved by the institutional review board of the Clinical Center at the NIH. Written informed consent was obtained from all donors. Sera from healthy infants and children ages 6 to 18 months were provided by Ruth Karron from the Center for Immunization Research (CIR), Johns Hopkins University School of Public Health (Baltimore, MD), under a protocol approved by the Johns Hopkins Institutional Review Board. Written informed consent was obtained from a parent before each child’s enrollment. All human sera were provided without patient identifiers. The 60% plaque reduction neutralizing-antibody titers (PRNT<sub>60</sub>) were determined in a plaque reduction neutralization assay on Vero cells (16) using GFP-expressing rRSV and rPVM. Prior to use, serum samples were incubated for 30 min at 56°C to inactivate complement. In the case of RSV-GFP, 10% of the final volume was guinea pig complement (Lonza, Walkersville, MD), as is typical for RSV neutralization assays (69). Complement was not used with PVM because it inactivates the virus. Plaques were visualized by GFP expression using a Typhoon imager (GE Healthcare, Piscataway, NJ).

qRT-PCR and ELISA. Total RNA was isolated from infected cells using an RNeasy total RNA isolation kit (Qiagen, Valencia, CA) and treated with DNase to remove residual genomic DNA. One microgram of isolated RNA was reverse transcribed using SuperScript II (Life Technologies) and random primers. Two microtiters of the cDNA mix was used for duplex quantitative reverse-transcription PCRs (qRT-PCRs) using a Brilliant qRT-PCR Plus core kit (Life Technologies) for human alpha and beta interferons (IFN-α and -β) (6-carboxy-2′,4′,5′,7′-hexachlorofluorescein [HEX]-labeled probe) with β-actin as the housekeeping gene (6-carboxyfluorescein [FAM]-labeled probe) (62). CXCL10 primers and probes were described previously (5′-TG GACCTCAAGGAGTGCTCCTC-3′ and 5′-CAAAATGTTGCTTGGCAGG AAT-3′) and a HEX-labeled probe (5′-CCGTACCGTGTACCCGACGAGCA-3′) were designed by using the website Primer 3 (http://frodo.wi.mit.edu/primer3/). PVM primers and probes were described previously (9). IRF7, ISG56 (IFIT1), and -18S RNA (ISG56 and IRF7), and expressed as fold change over the values for UV-rPVMΔNS2 controls 24 h postinfection. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentrations of IFN-α and -β (Life Technologies) and of IP-10 and CXCL10 (R&D Systems, Minneapolis, MN) in cell culture supernatants.

Western blots. Sucrose-gradient-purified RSV and PVM were resuspended in TENV buffer (10 mM Tris [pH 7.4], 1 mM EDTA, and 100 mM NaCl). For electrophoresis, viral particles were prepared in 1× NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies), and the highest exposure temperature was 32°C. PVM N and P proteins were identified in cell lysates of BSR T7/5 cells transfected with protein expression plasmids (42) previously prepared for PVM N and P. Cell lysis buffer
containing 1× protease inhibitor (Roche, Indianapolis, IN) and 1× NuPAGE LDS sample buffer was applied to BSR T7/5 monolayers. Cells were scraped and homogenized using a QIAshredder (Qiagen, Valencia, CA). For electrophoresis, cell lysates were diluted in 1× NuPAGE reducing buffer (Life Technologies) and 1× LDS sample buffer and heat denatured at 95°C for 5 min.

One microgram of cell lysates and viral particles was separated on NuPAGE 4-12% bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels with MOPS (morpholinepropanesulfonic acid) electrophoresis buffer (Life Technologies) in parallel with an Odyssey two-color protein molecular weight marker (Li-Cor, Lincoln, Nebraska). Proteins were transferred to polyvinylidene difluoride (PVDF) F membranes (Millipore, Billerica, MA) in 1× NuPAGE buffer. The membranes were blocked with Odyssey blocking buffer (Li-Cor) and incubated with primary antibody in the presence of 0.1% Tween 20. The primary antibodies and the dilutions used were as follows: RSV F-specific mouse MAb 1129, 1:10,000 (5); rabbit RSV-specific PAb, 1:15,000; RSV-specific AGM antiserum, 1:100; rabbit RSV-specific PAb, 1:5,000; RSV F-specific PAb, 1:1,000; and PVM-specific AGM antiserum from the present study, animal W879, 1:300,000. Human sera from donors with known PVM-neutralizing titers were used at a dilution of 1:100. The secondary antibodies (used at a 1:10,000 dilution) were as follows: goat anti-monkey IgG (KPL, Gaithersburg, MD), donkey anti-human IgG IRDye 700DX (Rockland Immunocchemicals, Gilbertsville, PA), goat anti-rabbit IgG IRDye 800 (Li-Cor), and goat anti-mouse IgG IRDye 800 (Li-Cor). The goat anti-mouse IgG antibody was conjugated to IRDye 800CW using the IRDye 800CW Microscale protein-labeling kit (Li-Cor). Membrane strips were scanned on an Odyssey infrared imaging system, and background was corrected using Odyssey software, version 3.0 (Li-Cor).

Flow cytometry. Vero cells were infected with rRSV-GFP or rPVM-GFP at a multiplicity of infection (MOI) of 1. Twenty-four hours postinfection, cells were detached using 1 mM EDTA in phosphate-buffered saline (PBS) supplemented with 3% FBS. Three washes were performed after incubation with primary and secondary antibodies. The cells were blocked by incubation in 3% bovine serum albumin (BSA) in PBS supplemented with 0.5 mM EDTA for 20 min on ice. Human and AGM sera with known RSV and PVM PRNT\text{60} were used at dilutions of 1:100, 1:1,000, and 1:5,000. Prior to use, the serum samples were incubated for 30 min at 37°C to inactivate complement. RSV F mouse MAb 1129 (5) and rabbit G-specific PAb were included as positive controls (1:1,000). The secondary antibodies used in these experiments included goat anti-monkey IgG (KPL), goat anti-mouse IgM (KPL), donkey anti-human IgG Alexa 647 (Life Technologies), donkey anti-human IgM Alexa 647 (Life Technologies), goat anti-rabbit IgG (KPL), and goat anti-mouse IgG (KPL). The goat anti-mouse IgG antibody, goat anti-rabbit IgG, and goat anti-mouse IgG were conjugated to Alexa 647 by the NIH NIAID Custom Antibody Conjugation Facility (Rockville, MD). After incubation with secondary antibody, the samples were treated with blue Live/Dead stain (Life Technologies) to monitor cell viability. The samples were then fixed with BD Cytofix (BD Biosciences, San Jose, CA). Flow cytometry analysis was performed on an LSR II flow cytometer (BD Biosciences). Data compensation was performed using mouse CompBeads (BD Biosciences), an ArC amine-reactive compensation kit (Life Technologies), and rRSV-GFP- or rPVM-GFP-infected cells. The data acquired from compensation controls were automatically calculated using BD FACSDiva, version 6.1.3 (BD Biosciences). The data were analyzed using FlowJo software, version 7.6.3 (Tree Star, Ashland, OR). The compensated data were gated using the following pathway: first, a uniform cellular population was identified by using side scatter versus forward scatter; second, the uniform population was gated on forward scatter height versus forward scatter area to eliminate cell clusters and identify single cells; and third, the single cell population was gated on forward scatter versus Live/Dead to eliminate dead cells and identify live cells at the time of staining.

IgG depletion. Serum samples were passed over a protein G Sepharose column (GE Healthcare). To confirm that IgG had been successfully de-

pleated, serum samples were electrophoresed on a 4-to-12% SDS-PAGE gel with MOPS electrophoresis buffer (Life Technologies) under nonreducing and nonnaturating conditions. Gels were stained with Coomassie blue (Life Technologies) to visualize IgG protein bands; human IgG was used as a positive control (Pierce Biotechnology, Rockford, IL). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD). Statistical analysis to compare pre- and post-IgG-depletion neutralization titers was done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

RESULTS

PVM replication is highly restricted in nonhuman primates. Sera from 39 AGM and 20 RM were screened for PVM-neutralizing activity by a plaque reduction neutralization assay (data not shown). PVM-neutralizing activity (which is defined in this study by a cutoff of ≥5.3 reciprocal log\text{2} [≥1:40]) was not detected in any animal, indicating that the 59 animals, which were obtained from breeding colonies at Morgan Island, SC, and St. Kitts in the Caribbean, lacked pre-existing immunity to PVM. Four each of these PVM-seronegative AGM and RM were inoculated intranasally and intratracheally with the high dose of 6.8 log\text{10} PFU of PVM per site (total dose, 7.1 log\text{10} PFU per animal) using low-passage, CDNA-derived PVM (rPVM) that had previously been shown to be highly virulent in mice (9). Nasopharyngeal swabs and tracheal lavage samples were collected over a period of 12 days and analyzed by plaque titration to monitor virus shedding in the upper and lower respiratory tracts, respectively (Tables 1 and 2). From the upper respiratory tract, virus shedding was not detected in any AGM, and very low levels of shedding were detected in 2 of the 4 RM (Table 1). From the lower respiratory tract, low levels of shedding were detected in 3 of the 4 AGM and in 1 of the 4 RM (Table 2). No virus was recovered from either site in 3 animals (AGM W994, RHDA4G, and RHDA43). No clinical symptoms were observed in any animal.

Analysis of sera collected on day 28 postinfection showed that all of the AGM and RM developed remarkably high levels of PVM-neutralizing serum antibodies (Table 3) (mean PRNT\text{60} of 24.2 log\text{2} [1:19,272,000] for AGM and 13.9 log\text{2} [1:15,290] for RM), even in the animals in which replication had not been detected. This suggested that all of the monkeys had been infected with rPVM, even in the absence of detectable shedding. Thus, PVM replicated in the upper and lower respiratory tracts of two species of nonhuman primates, but replication was highly restricted, and shedding was observed in only a subset of animals.

PVM IFN antagonist proteins control type I IFN induction in human cells. As a preliminary investigation of the host range restriction of PVM in primates, we evaluated (i) the ability of PVM to replicate in primate cells, specifically in the human lung epithelial cell line A549, and (ii) the ability of PVM to control the type I interferon (IFN) response in these cells, since controlling the host IFN response is an important factor in the ability of viruses in general to replicate. A549 cells were chosen for this experiment because of their human respiratory epithelial origin and because they are widely used to study IFN responses. PVM had previously been reported to replicate in the primate BSC-1 cell line, but that particular PVM strain had previously been passaged extensively in cell culture, had lost virulence in mice, and had exhibited evidence of widespread mutations (64). In addition to wild-type rPVM, we also evaluated rPVM mutants lacking the NS1 gene (rPVM\text{ΔNS1}), the NS2 gene (rPVM\text{ΔNS2}), or both the NS1 and NS2 genes (rPVM\text{ΔNS1ΔNS2}) (9). The PVM NS2 protein was previously identi-
fied as an inhibitor of murine type I IFN induction, with a minor contribution from NS1 (9, 28).

Monolayer cultures of A549 cells were infected with the various viruses at an MOI of 0.1 PFU/cell in order to evaluate multicycle replication (Fig. 1). rPVM reached a peak titer of approximately 4.2 log10 PFU per ml on day 10 (Fig. 1). This titer is comparable to what we routinely obtain with RSV in A549 cells (62), showing that these human cells are similarly permissive for PVM and RSV. Peak titers of rPVMΔNS1 (4.7 log10 PFU per ml on day 10) were a little higher than those of wild-type rPVM, possibly reflecting a slight replication advantage due to a shorter genome resulting from deletion of NS1. Peak titers for rPVM lacking NS2 (3.5 log10 PFU per ml on day 10) were a little higher than those of wild-type rPVM, possibly reflecting a minor contribution from NS1 (9, 28).

Next, we analyzed the type I IFN response in A549 cells infected with the wild-type and NS deletion rPVM strains using preparations that had been purified on sucrose gradients in order to reduce contamination from small molecules, including cytokines. Replicate cultures of A549 cells were infected with each virus at an MOI of 3 PFU/cell or with UV-inactivated rPVMΔNS2 as a control, and the cultures were incubated and harvested at 24, 44, and 64 h postinfection. Plaque titration of clarified medium supernatants from the 64-h time point showed that the titers of the various viruses were similar (Fig. 2A). Cell-associated viral RNA was quantified by qRT-PCR using primers specific to the PVM N and F genes (Fig. 2B). This showed that, at the earlier time points (24 and 44 h), there was somewhat more N and F RNA in cells infected with rPVM lacking NS2 (rPVMΔNS2 and rPVMΔNS12) than in those infected with wild-type rPVM and rPVMΔNS1. However, by 64 h the levels of RNA were similar for all viruses.

From this same experiment, we evaluated the expression of IFN-α and -β by qRT-PCR of cell-associated RNA and by ELISA of clarified medium supernatants (Fig. 2C). Consistent with our previous observations with mouse embryo fibroblasts (9, 28), rPVM and rPVMΔNS1 induced little or no IFN-α or -β detectable by qRT-PCR or ELISA at any time point, whereas strong IFN-α and -β responses detected by both methods were induced by rPVM lacking NS2 or lacking both NS1 and NS2.

### Table 1: Viral titers of NP swabs from AGM and RM inoculated with rPVM

<table>
<thead>
<tr>
<th>Group</th>
<th>ID</th>
<th>Virus titer (log10 PFU/ml) on day:</th>
<th>Peak titer (log10 PFU/ml)</th>
<th>Days of shedding²</th>
<th>Days of shedding³</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 12</td>
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<td></td>
<td></td>
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<tr>
<td>AGM</td>
<td>W879</td>
<td>— — — — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>W877</td>
<td>— — — — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W872</td>
<td>— — — — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W994</td>
<td>— — — — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>— — — — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
<td></td>
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<tr>
<td>RM</td>
<td>RHCL7C</td>
<td>— — — 1.7 0.7 2.2 1.7 — — — —</td>
<td>2.2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RHCL7E</td>
<td>— — — 1.7 1.4 — — — 1.7 1.0 —</td>
<td>1.7</td>
<td>6</td>
<td></td>
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<tr>
<td></td>
<td>RHDAG</td>
<td>— — — — — — — — — —</td>
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<tr>
<td></td>
<td>RHDA43</td>
<td>— — — — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td>Mean</td>
<td>— — — — — — — — — —</td>
<td>1.3 ± 0.4</td>
<td>2.8 ± 1.6</td>
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* Monkeys were inoculated intranasally and intratracheally with 6.8 log10 PFU of PVM per site (total dose, 7.1 log10 PFU per animal). NP swabs were collected on the indicated days. Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was 0.7 log PFU/ml. —, no detectable virus. The calculation of means used a value of 0.7 for samples with undetectable virus.

¹ Number of days from the day of the first recorded titer to the day of the last recorded titer.

** Table 2: Viral titers of TL samples from AGM and RM inoculated with rPVM

<table>
<thead>
<tr>
<th>Group</th>
<th>ID</th>
<th>Virus titer (log10 PFU/ml) on day:</th>
<th>Peak titer (log10 PFU/ml)</th>
<th>Days of shedding⁴</th>
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<td></td>
<td>2 4 6 8 10 12</td>
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<td></td>
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<tr>
<td>AGM</td>
<td>W879</td>
<td>— 2.9 — — — —</td>
<td>2.9</td>
<td>5</td>
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<tr>
<td></td>
<td>W877</td>
<td>— 0.7 — — — —</td>
<td>1.3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>W872</td>
<td>— — 1.3 — — —</td>
<td>1.3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>W994</td>
<td>— — — 1.3 — —</td>
<td>&lt; 0.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>— — — — 1.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>3.8 ± 1.5</td>
</tr>
<tr>
<td>RM</td>
<td>RHCL7C</td>
<td>— — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RHCL7E</td>
<td>— — — — — 1.0 — 1.9 — 0.7</td>
<td>1.9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>RHDAG</td>
<td>— — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
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<tr>
<td></td>
<td>RHDA43</td>
<td>— — — — — — — —</td>
<td>&lt; 0.7</td>
<td>0</td>
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<tr>
<td></td>
<td>Mean</td>
<td>— — — — — 1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>2.3 ± 2.3</td>
</tr>
</tbody>
</table>

⁴ In the experiment whose results are shown in Table 1, TLs were performed on the indicated days. Virus titers were determined by plaque assay as described for Table 1. —, no detectable virus. The calculation for means used a value of 0.7 for samples with undetectable virus.

⁵ Number of consecutive days starting 1 day before the first recorded titer and ending 1 day after last recorded titer.
TABLE 3 PRNT<sub>60</sub> in serum samples from AGM and RM inoculated with rPVM<sup>a</sup>

<table>
<thead>
<tr>
<th>Group</th>
<th>ID</th>
<th>Day 0</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGM</td>
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<td>3.7</td>
<td>36.6</td>
</tr>
<tr>
<td></td>
<td>W877</td>
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<td></td>
<td>W872</td>
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<tr>
<td></td>
<td>Mean</td>
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<td>24.2 ± 10.1</td>
</tr>
<tr>
<td>RM</td>
<td>RHCL7C</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>Mean</td>
<td>3.7 ± 0.2</td>
<td>13.9 ± 2.1</td>
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<sup>a</sup> From the experiment described in Table 1, sera were collected 28 days postinfection. PVM-neutralizing activity was measured by determining the 60% plaque reduction neutralization antibody titer (PRNT<sub>60</sub>).  

<sup>b</sup> The lower limit of detection of the PRNT<sub>60</sub> assay was 2.5 (reciprocal log<sub>2</sub>). A serum sample was considered positive with a PRNT<sub>60</sub> value of ≥3.3.

NS2 is functional as an efficient inhibitor of type I IFN induction in human cells, just as in mouse cells. IFN-α and -β gene expression was detected starting at 24 h postinfection, was maximal at 44 h, and decreased at 64 h. IFN protein expression lagged behind: it was not detectable at 24 h but reached high levels at 44 h and 64 h.

We also evaluated induction of IFN-stimulated genes by qRT-PCR (ISG56, IRF7, and CXCL10) and by ELISA (CXCL10) (12, 29, 30, 36, 55) (Fig. 2D). We detected strong induction of all three genes in cells infected with rPVMΔNS2 or ΔNS1/2, whereas little or no induction was detected in response to wild-type rPVM or rPVMΔNS1. Peak expression of all three genes was somewhat higher with virus lacking both NS1 and NS2 than with virus lacking only NS2, suggesting the possibility that NS1 may inhibit IFN-inducible genes in human cells to some extent. This will be examined in future studies.

**PVM-neutralizing activity in infant and adult sera.** As the next step in evaluating PVM as a possible human pathogen, we analyzed sera from 30 adult donors and from 17 infants and children, 6 to 18 months of age, for the presence of PVM- and RSV-neutralizing antibodies using plaque reduction neutralization assays (Fig. 3). Serum from each adult human donor had a high titer of RSV-neutralizing antibody, as would be expected for this ubiquitous pathogen, with an average PRNT<sub>60</sub> of 9.1 log<sub>2</sub> (1:549). In addition, 12 (40%) of the adult donor sera also had PVM-neutralizing activity, although these titers were relatively low, with an average PRNT<sub>60</sub> of 6.7 log<sub>2</sub> (1:104) (Fig. 3). There was no evident concordance between high RSV titers and the presence of PVM-neutralizing activity. For example, the four donors with the highest RSV-neutralizing activities (Fig. 3, dots 1 to 4) had no or low PVM-neutralizing activities. This was investigated further for all of the samples using the Wilcoxon signed-rank test, which confirmed a lack of correlation (P < 0.001). This suggested that the observed PVM-neutralizing activity is unlikely to be due to cross-reaction by RSV-specific antibodies.

Seven of 17 (41%) of the sera from infants and young children were seropositive for RSV, and in most cases the titers of RSV-neutralizing antibodies were lower than those in adults (average PRNT<sub>60</sub> of 7.2 log<sub>2</sub>; 1:142). Only one of the pediatric sera had detectable, but low, PVM-neutralizing activity (6.2 log<sub>2</sub>; 1:72), and this particular specimen was RSV seronegative (Fig. 3, dot 5). The apparent increase in PVM seroprevalence from early childhood into adulthood could be consistent with increasing exposure during life to PVM or a PVM-like virus.

**Lack of cross-neutralization between RSV and PVM.** As noted above, the lack of concordance between the titers of RSV- and PVM-neutralizing antibodies suggested that the PVM-neutralizing activity was not due to cross-neutralization by RSV-specific antibodies. To further investigate the possibility of cross-neutralization, we performed neutralization assays with AGM sera collected 28 days following infection with rPVM (from the experiment described above) or with AGM sera collected 35 days following infection with recombinant wild-type RSV, strain A2 (rRSV; P. L. Collins and U. J. Buchholz, unpublished data), thus providing sera specific to either virus without the confounding presence of antibodies to the other virus. The mean PRNT<sub>60</sub> of the RSV-specific and PVM-specific AGM control sera were 8.0 log<sub>2</sub> (1:256) and 24.2 log<sub>2</sub> (1:19,272,000), respectively; it was interesting that much higher titers were induced by PVM even though RSV replicates to higher titers in AGM. When tested individually (Fig. 3), none of the RSV-specific AGM sera had cross-neutralizing activity with rPVM, nor did any of the strongly neutralizing PVM-specific sera have cross-neutralizing activity with RSV. Thus, monospecific AGM sera induced by infection with either rRSV or rPVM do not have detectable neutralizing activity against the heterologous virus.

**Lack of cross-protection between RSV and PVM in the mouse model.** We further evaluated cross-protection between RSV and PVM in the mouse model. Groups of 15 mice each were infected intranasally with PVM and RSV or mock infected. Since wild-type PVM is lethal for mice even at relatively low doses, we used two attenuated PVM derivatives: (i) rPVM-GFP, which is slightly attenuated due to the presence of the GFP insert (42), was administered at the very low dose of 10 PFU/mouse, and (ii) rPVMΔNS2, which is highly attenuated and apathogenic (9), was administered at a dose of 2,000 PFU per mouse. RSV was administered at 5.7 log<sub>10</sub> PFU, which is a typical dose and well tolerated.
FIG 2 Analysis of the type I IFN response in A549 cells infected with rPVM, rPVMΔNS1, rPVMΔNS2, or rPVMΔNS12 by qRT-PCR and ELISA. Triplicate cultures of A549 cells were mock infected, infected with sucrose gradient-purified rPVM, rPVMΔNS1, rPVMΔNS2, or rPVMΔNS12 at an MOI of 3 PFU/cell, or infected with sucrose gradient-purified UV-inactivated rPVMΔNS2. Cell cultures were harvested at the indicated time points, the medium supernatants were clarified and used for quantification of viral titers and secreted cytokines, and the cell pellets were processed to purify total cell-associated RNA. (A) PVM titers (log_{10} PFU per ml) at 64 h postinfection measured by plaque assay of medium supernatants (9). (B) Levels of cell-associated PVM F and N RNA measured by qRT-PCR. (C) Levels of cell-associated IFN-β and IFN-α RNA measured by qRT-PCR (top) and levels of IFN-β and IFN-α protein in medium supernatants measured by ELISA (bottom). (D) Levels of cell-associated ISG56, IRF7, and CXCL10 RNA measured by qRT-PCR (top) and level of CXCL10 protein in medium supernatants measured by ELISA (bottom). All qRT-PCR results are expressed as difference (fold) relative to values in cells 24 h after inoculation with UV-inactivated rPVMΔNS2.
in mice. Five mice of each inoculated group were sacrificed to evaluate virus replication: sacrifice was at day 4 for the RSV and rPVM/H9004 NS2 groups, which has been shown to be the time of maximum viral replication, and sacrifice was at day 11 for the rPVM-GFP group in order to confirm that the PVM infection was cleared (Fig. 4). We detected moderate levels of replication of RSV and rPVM/H9004 NS2, as expected, and confirmed that rPVM-GFP had been cleared from the animals.

Four weeks after immunization, 5 mice of each group were challenged intranasally with 2,000 PFU of rPVM (Fig. 4), a dose that would be highly lethal for nonimmune mice, and 5 animals in each group were challenged with 5.7 log10 PFU of RSV (Fig. 4). All of the mice that had been immunized with rPVM-GFP or rPVM/H9004 NS2 were completely protected against PVM challenge, whereas the level of replication of PVM in mice that had been immunized with RSV was indistinguishable from that of the mock-infected group (Fig. 4). Thus, immunization with RSV provided no detectable restriction of PVM replication. Conversely, mice that had been immunized with RSV were completely protected against RSV challenge, whereas the replication of challenge PVM was not reduced (Fig. 4). Thus, cross-protection did not occur between PVM and RSV in a mouse immunization and cross-challenge study.

**Human sera with PVM-neutralizing activity did not react with PVM proteins in Western blots.** Since the PVM-neutralizing activity in the human sera did not appear to be due to cross-reaction with RSV-specific antibodies, we investigated reactivity of the sera with RSV (Fig. 5A) and PVM (Fig. 5B) proteins by Western blot analysis, using nondenaturing and nonreducing conditions. Control protein preparations and sera were used to identify major RSV and PVM proteins (Fig. 5). The RSV F and N proteins were identified in blots of sucrose gradient-purified rRSV
FIG 5 Western blot analysis of the 12 adult human sera with PVM-neutralizing activity. The presence of RSV- and PVM-specific antibodies in the 12 adult serum samples with significant PVM-neutralizing activity was investigated by Western blot analysis against sucrose-gradient-purified rRSV or rPVM. Other lanes contain control protein preparations and control antibodies to identify viral proteins. Protein samples were subjected to 4-to-12% SDS-PAGE gel electrophoresis under nonreducing and nondenaturing conditions and then transferred to a PVDF membrane. In the case of human test sera and AGM control sera, the serum dilutions used and the RSV or PVM log₂ PRNT₆₀ of the undiluted sera are indicated above each membrane strip. Note that the RSV- and PVM-seropositive AGM control sera were used at dilutions designed to have PRNT₆₀ similar to that of the human test sera. Data are from one of three independent experiments with similar results. (A) Reactivity with RSV proteins. The left and center panels (lanes a to g) show controls to identify viral protein species. Lanes a to c, rabbit polyclonal antibody raised against gradient-purified RSV (RSV-specific PAb) reacted with mock-infected cell lysate (a) and gradient-purified rRSV (b) and rPVM (c). Lane d, RSV F MAb 1129 (5) reacted with gradient-purified rRSV. Lanes e to g, RSV-specific AGM serum reacted with mock-infected cell lysate (e) and gradient-purified rPVM (f) and rRSV (g). The right panel shows results for the 12 human test sera reacted with blots prepared from gradient-purified rRSV. (B) Reactivity with PVM proteins. The left and center panels (lanes a to i) show controls to identify viral protein species. Lanes a to c, rabbit polyclonal antibody raised against gradient purified PVM (PVM-specific PAb) reacted with lysates of cells that had been mock-transfected (a) or transfected with plasmid expressing the PVM N (b) or P protein (c). Lanes d and e, same PVM-specific PAb reacted with mock-infected cell lysate (d) and gradient-purified rPVM (e). Lane f, rabbit polyclonal antiserum raised against a recombinant vaccinia virus expressing the PVM F protein (PVM F-specific PAb) reacted with gradient-purified rPVM. Lanes g to i, PVM-specific AGM serum reacted with mock-infected cell lysate (g) and gradient-purified rPVM (h) and rRSV (i). The right panel shows results for the 12 human test sera reacted with gradient-purified rPVM.
preparations using polyclonal rabbit serum raised against gradient-purified RSV (RSV-specific PAb) (Fig. 5A, lane b) and the RSV F-specific mouse monoclonal antibody (MAB) 1129 (5) (lane d). This showed that the RSV F protein migrated as an apparent trimer, consistent with a nondenatured, nondenatured state. A largely native conformation for F also is supported by its reactivity with the F-specific MAb, which does not bind efficiently to denatured protein. The rabbit RSV-specific PAb also showed slight cross-reactivity with the PVM N protein in blots of gradient-purified RSV (Fig. 5A, lane c). This is consistent with the previous reports of reciprocal cross-reactivity between the N proteins of RSV and PVM (27, 44).

In the case of PVM, we used a rabbit antiserum against purified PVM (PVM-specific PAb) to identify the PVM N (43 kDa) and P (39 kDa) proteins in blots of lysates of cells transfected with plasmids expressing the PVM N or P protein (Fig. 5B, lanes b and c). In addition, analysis of blots of gradient-purified PVM using the same antiserum detected the PVM N and P proteins as well as a diffuse band of ~55 to 75 kDa corresponding to the G protein (Fig. 5, lane e). There was a slight cross-reactivity of this serum with the RSV N protein in purified rRSV (Fig. 5B, lane d). In addition, the PVM F protein was identified in a blot of gradient-purified PVM using a rabbit serum (PVM F-specific PAb) that had been made using an MVA expressing the PVM F protein (Fig. 5B, lane f). This showed that the PVM F protein migrated entirely as an apparent trimer under these nonreducing, nondenaturing conditions.

As additional controls, we tested the reactivity of RSV- and PVM-seropositive control AGM sera (Fig. 5A, lanes e to g, and B, lanes g to i). Prior to use, these sera were diluted to levels of virus-neutralizing activity comparable to those of the human test sera (the RSV-specific serum was diluted 100-fold to an RSV PRNT<sub>90</sub> of 9.4 log<sub>2</sub>, and the PVM-specific serum was diluted 300,000-fold to a PVM PRNT<sub>90</sub> of 6.6 log<sub>2</sub>). Under these conditions, the RSV-seropositive AGM serum reacted strongly with RSV F in blots of gradient-purified rRSV (Fig. 5A, lane g) and not with any proteins from gradient-purified rPVM (Fig. 5A, lane f). The PVM-seropositive AGM serum reacted strongly with the PVM G protein in blots of gradient-purified rPVM (Fig. 6B, lane h) and not with any proteins from gradient-purified rRSV (lane i). Sera from the seven other PVM-infected AGM and RM for which data are presented in Tables 1 to 3 also reacted predominantly with the PVM G protein (data not shown). It is interesting that the RSV-seropositive sera reacted mainly with the RSV F protein, while the PVM positive sera reacted with G.

We then used this Western blot assay to investigate whether the 12 adult human sera with PVM-neutralizing activity (Fig. 3) reacted with RSV and PVM proteins. Each of these sera reacted strongly with the RSV F protein (Fig. 5A), which was expected since all had strong RSV-neutralizing activity (Fig. 3). To various degrees, 11 of 12 donors also reacted with the RSV N protein (Fig. 5A). However, none of the 12 sera detected any PVM protein, under conditions in which PVM-specific AGM sera with similar neutralizing activities reacted strongly with PVM G (Fig. 5B).

The PVM-neutralizing activity of human sera is not due to PVM-specific IgG and IgM antibodies. Next, we evaluated the specificity of the human test sera for PVM and RSV by a second assay. Specifically, we evaluated reactivity against Vero cells that had been infected with RSV or PVM, each of which expressed GFP from an added gene. Reactivity was evaluated with intact, native cells in suspension, thus maintaining antigen in native form. The cells were then incubated with a secondary antibody conjugated to Alexa 647. The cells were fixed and analyzed by flow cytometry to compare the level of antibody reactivity with the level of viral protein expression as indicated by GFP expression (Fig. 6).

First, we evaluated the reactivity of virus-specific control antibodies (Fig. 6A). Three different RSV F MAb (results are shown in Fig. 6A for MAb 1129; results with the other two MAb were similar and are not shown) each reacted strongly with RSV-GFP-infected cells, and in each case the intensity of MAb binding correlated positively with the level of GFP expression. In contrast, the RSV F MAb did not react with rPVM-GFP infected cells regardless of the level of GFP expression (Fig. 6A). An isotype control antibody yielded similar negative results (not shown). Rabbit antiserum made against an MVA expressing the PVM G protein (PVM G-specific PAb) reacted strongly with rPVM-GFP-infected cells (Fig. 6A), and the level of antibody staining correlated positively with the level of GFP expression. The rabbit PVM-G-specific PAb did not react significantly with RSV-infected cells regardless of the amount of GFP expression (Fig. 6A). Thus, RSV-specific antibodies did not cross-react with PVM-infected cells, and vice versa. This assay provided a sensitive means to detect virus-specific antibodies by correlating antibody binding with the level of viral antigen expression as indicated by the GFP marker.

We then used this assay to evaluate RSV-positive and PVM-positive AGM sera (serving as additional controls) and, more importantly, a number of selected adult human test sera that were RSV-seropositive and were either PVM seronegative or seropositive. First, binding was assessed with secondary antibodies specific to IgG (Fig. 6B). A preimmune AGM control serum (Fig. 6B) exhibited little or no IgG antibody binding to cells infected with either virus compared to an antibody isotype control, indicating a lack of virus-specific antibodies as expected (Fig. 6). In contrast, when serum from an AGM that had been infected with RSV (RSV<sup>+</sup> PVM<sup>−</sup>; Fig. 6B) was evaluated with RSV-infected cells, it exhibited strong IgG antibody binding, and the level of binding increased concomitantly with increased GFP expression. In contrast, binding to PVM-infected cells (Fig. 6B) was only slightly greater than that of preimmune serum or an isotype antibody control (data not shown), and the level of binding did not increase concomitantly with increased GFP expression. Conversely, serum from an AGM that had been infected with PVM (RSV<sup>−</sup> PVM<sup>+</sup>; Fig. 6B) exhibited the opposite pattern: with PVM-infected cells, IgG antibody binding was strong and increased concomitantly with increased GFP expression, whereas binding to RSV-infected cells was only slightly greater than that of preimmune serum or an isotype antibody control (data not shown), and the level of binding did not increase concomitantly with increased GFP expression. Thus, the postinfection RSV and PVM AGM sera contained IgG antibodies specific to the respective virus, the level of antibody binding increased concomitantly with increased GFP expression, and there was no significant cross-reactivity with the heterologous virus.

Next, representative human sera were evaluated. Serum from an infant that lacked RSV- or PVM-neutralizing activity (Fig. 6B) exhibited a level of binding to either RSV-infected cells or PVM-infected cells that was moderately higher than that of the isotype antibody control (data not shown), but the level of binding did not increase concomitantly with increased GFP expression. An adult donor serum with a high RSV PRNT<sub>90</sub> and low PVM PRNT<sub>90</sub> (RSV<sup>+</sup> PVM<sup>−</sup>; Fig. 6B) exhibited strong binding against
FIG 6 Evaluation of the binding activity of selected human sera to RSV- and PVM-infected cells by flow cytometry. Vero cells were infected at an MOI of 1 with either RSV or PVM, each expressing GFP. Twenty-four hours postinfection, cells were detached with 1.0 mM EDTA. Infected cells were stained with selected human test sera or control antibodies, followed by secondary antibodies conjugated to Alexa 647. The cells were then fixed and analyzed by flow cytometry to compare the level of antibody binding (y axis) to GFP expression as a measure of viral gene expression (x axis). For each human or AGM serum, the reciprocal log_{2} PRNT_{60} for RSV and PVM are noted. Results are representative of three independent experiments. Antibody isotype controls are not shown. (A) Antibody controls. reactivity of RSV F-specific MAb 1169 (left) and PVM G-specific polyclonal antibody (right) with RSV-GFP- and PVM-GFP-infected cells (top and bottom, respectively). (B and C) Analysis of AGM (left) and human (right) sera for IgG (B) or IgM (C) antibodies that react with RSV-GFP- and PVM-GFP-infected cells. One example is shown for each of 6 categories of sera (columns from left to right): (i) preimmune AGM, (ii) RSV+ PVM- AGM, (iii) RSV+ PVM+ AGM, (iv) nonimmune human (from an infant), (v) RSV+ PVM+ adult human, and (vi) RSV+ PVM+ adult human. A total of 4 donors per category were tested, with similar results; the data shown are for one representative individual.
RSV-infected cells, and the level of staining increased concomitantly with GFP expression. Against PVM-infected cells (Fig. 6B), there was a moderate level of antibody staining, but the level of staining did not increase with increased GFP expression. An adult serum with a substantial RSV PRNT<sub>60</sub> and one of the highest observed PVM PRNT<sub>60</sub> (RSV + PVM<sup>+</sup>; Fig. 6B) gave essentially the same results: against RSV-infected cells, there was strong staining that increased concomitantly with GFP expression, whereas against PVM-infected cells, there was a moderate level of staining that did not increase concomitantly with increased GFP expression. These results showed that, in this sensitive assay, the human sera did not contain IgG antibodies that specifically reacted with native PVM antigen, although there was a moderate level of IgG-specific staining that was above that of naïve sera. The same absence of PVM-specific binding was observed with 3 additional sera and with PVM PRNT<sub>60</sub> of 8.6, 7.7, and 7.9 log<sub>2</sub> (data not shown).

The same set of AGM and human sera were evaluated by this assay using a secondary antibody specific to IgM (Fig. 6C). In this case, a moderate level of background antibody binding was observed for most of the sera against both RSV- and PVM-infected cells. However, except in a single case, the level of antibody binding did not increase with increased GFP expression. The one exception was with AGM serum from an animal immunized with PVM (RSV<sup>−</sup> PVM<sup>+</sup>; Fig. 6C), for which antibody binding increased, concomitantly with increased GFP expression. This indicated that, 28 days following primary infection with PVM, this AGM contained PVM-specific serum IgM antibodies, which was not completely unexpected. The lack of detectable virus-specific serum IgM in other samples might be indicative of longer periods of time since infection or exposure. In summary, the flow cytometry results showed that the human sera did not contain IgG or IgM antibodies that specifically reacted with PVM-infected cells but that there was some weak IgG- and IgM-based reactivity that could be consistent with the presence of low-affinity (possibly natural) antibodies.

In the experiments described above, the AGM and human sera were tested at a dilution of 1:100. We also evaluated these sera at dilutions of 1:1,000 and 1:5,000 (data not shown). This showed that the magnitude of the positive IgG and IgM fluorescent signals decreased with increased dilution, demonstrating the specificity of the results obtained.

**PVM-neutralizing activity of human sera diminishes after depletion of IgG.** Finally, we investigated whether the observed PVM-neutralizing activity was indeed associated with IgG antibodies (Fig. 7A). IgG was removed using protein G Sepharose columns, which binds to all four subclasses of IgG. To confirm that IgG had indeed been depleted from the serum samples by treatment with protein G, serum samples from before and after treatment were electrophoresed on 4-12% SDS-PAGE gels under nonreducing and nondenaturing conditions, the gels were stained with Coomassie blue, and bands were quantified (Fig. 7A). IgG has a molecular mass range of 150 to 170 kDa; commercially obtained purified human IgG was used as a marker. The representative RSV-positive AGM serum (Fig. 7A, lanes 1) had a reduction of 40%, and the representative PVM-positive AGM serum showed a reduction in IgG band intensity of 66% (lanes 2). For the adult human sera, the reduction in band intensity ranged from 41% (lanes 5) to 79% (lanes 6).

Neutralization titers were compared in samples before and after being processed through the columns. First, we evaluated AGM sera specific for RSV or PVM as monospecific positive controls. This showed that the RSV-neutralizing activity of sera from RSV-infected AGM decreased below background levels following depletion of IgG. The PVM-neutralizing activity of sera from PVM-infected AGM also was reduced by treatment with protein G. The posttreatment titers of PVM-neutralizing antibodies did not fall below background, but this was likely due to the presence of IgM antibodies, as shown in Fig. 6C. Next, the 12 adult serum samples with PVM-neutralizing activity were investigated for RSV- and PVM-neutralizing activity after depletion of IgG. Mean PRNT<sub>60</sub> of RSV-neutralizing antibodies were reduced from 9.2 log<sub>2</sub> to 7.5 log<sub>2</sub>, and mean PRNT<sub>60</sub> of PVM-neutralizing antibodies were reduced from 6.7 log<sub>2</sub> to 3.9 log<sub>2</sub>. RSV and PVM neutralization titers of human sera before and after IgG depletion were compared using a paired t test. The reduction in neutralizing activity was significant in both instances (P < 0.0001). In summary, removal of IgG from adult sera resulted in a concomitant reduction in PVM- and RSV-neutralizing activity.

**DISCUSSION**

New human respiratory pathogens, such as human metapneumovirus (65), human bocavirus (1), and additional human coronaviruses (21, 43, 66, 68), continue to be identified. However, it is estimated that 14 to 23% of viral infections of the lower respiratory tract lack an identified etiologic agent (54). Previous studies indicated that a substantial proportion of human serum samples had PVM-neutralizing activity. This suggested that humans are commonly infected with PVM, or with a virus related to PVM. However, there has never been a report of the isolation or direct detection of PVM in humans. Also, as noted in the introduction, the previous serological surveys had limitations. Therefore, we revisited the question of whether PVM might be a common agent of infection in humans.

Two species of nonhuman primates, namely, AGM and RM, were inoculated via the respiratory tract with a high dose of PVM. Virus was shed sporadically and at very low titers, and some animals failed to shed any detectable virus. This suggested that virus replication was highly restricted, although this is offered with the caveat that we did not sacrifice the animals and measure virus titers directly in lung tissue. Infection of humans by human respiratory viruses, such as RSV, parainfluenza viruses, and influenza virus, results in the shedding of substantial titers of virus in respiratory secretions, and this is a commonly used measure of replication. Infection of nonhuman primates with human respiratory viruses also results in titers of shed virus that are substantial, although lower than those observed with humans. In the case of the severe acute respiratory syndrome (SARS) coronavirus, which infects humans and causes serious disease but is not a natural human pathogen, we and others observed consistent shedding, although at levels that are lower than those observed with human viruses (11, 47). In contrast, the very low and sporadic shedding that we observed in the present study is similar to what we previously observed in AGM and RM with Newcastle disease virus, which is an avian virus (10), or in chimpanzees with bovine RSV (8). In the case of SARS coronavirus and NDV, somewhat higher viral titers were detected when animals were sacrificed and respiratory tract tissue was assayed directly, but the levels of shed virus were predictive. Therefore, the present data suggest that the permissiveness of pri mates for PVM is similar to that for the avian Newcastle.
disease virus and bovine RSV. Thus, PVM appears to have a strong host range restriction in primates. Interestingly, infection of the AGM and RM with PVM in the present study resulted in extremely high titers of PVM-neutralizing serum antibodies. The basis for this is not known. One possibility is that viruses may mutate to lose highly immunogenic B- and T-cell epitopes in response to selective immune pressure during evolution in a natural host but may be more immunogenic in nonnatural hosts in which this selection has not occurred. However, this remains speculative.

The block to PVM infection in nonhuman primates did not appear to be due to an inability to infect and replicate in primate cells. As noted, a number of previous studies employed a variant of PVM that had been adapted to replicate on AGM BSC-1 cells (14, 15, 64); however, this adapted virus had sustained extensive mutations (64) and thus may have an altered host range. For example, a single amino acid change in lymphocytic choriomeningitis virus was sufficient to alter tropism (63). PVM that had been propagated on hamster BHK cells was previously shown to replicate efficiently in AGM Vero cells (6), although these cells lack the ability to produce type 1 IFN and thus do not provide a strict test. In the present study, we found that preparations of PVM that had been grown on BHK cells and confirmed to be virulent in mice

FIG 7 Evaluation of RSV- and PVM-neutralizing activity in human serum samples after partial depletion of IgG antibodies. Human sera previously identified as having PVM-neutralizing activity were passed through protein G Sepharose columns to partially remove IgG antibodies. Sera from RSV- and PVM-infected AGM served as monospecific controls. Some donors are identified by numbers, located below the lanes (A) and above the dots (B), to allow comparison between titers of neutralizing antibodies and level of IgG depletion. (B) PRNT<sub>60</sub> for PVM and RSV were measured before and after IgG depletion. Sera with a PRNT<sub>60</sub> of \( \geq 5.3 \log_2 \) (1:40) were considered seropositive (dashed line). Data are from a single titration experiment; a second experiment provided similar results. (A) Analysis of IgG content by 4-to-12% SDS-PAGE gel electrophoresis under nonreducing and nondenaturing conditions, with commercially obtained purified human IgG as a marker. Gels were stained with Coomassie blue. The reductions in IgG band intensity for the numbered samples were as follows: 1, 80%; 2, 88%; 3, 76%; 4, 45%; 5, 41%; and 6, 79%. ****, \( P < 0.0001 \).
replicated in human airway epithelial A549 cells with an efficiency equivalent to what we typically observe with RSV. While these observations suggest that there is no major block to PVM infection and replication in primate cells, there is the caveat that infection of cell lines can be an unreliable predictor of host range and that for cell lines can be more permissive than the intact host. In some instances, for example, virus entry in cultured cell lines can be mediated by alternative cellular receptors that are irrelevant in vivo (53, 58). In human airway epithelial primary cell cultures, PVM was marginally infectious (Raymond Pickles [University of North Carolina, Chapel Hill, NC], personal communication). However, these primary human airway cultures represent the differentiated cartilaginous respiratory epithelium (71). It will be of interest to further evaluate the permissiveness for PVM infection in primary models of the human distal respiratory tract.

The ability of PVM to infect A549 cells provided the opportunity to investigate its ability to block the human IFN response. This was of interest because, for viruses in general, the ability to block the host IFN response is usually important for efficient replication. Surprisingly, PVM was highly effective in inhibiting IFN production in A549 cells, which is a common model for studying IFN responses. The use of NS deletion PVM mutants showed that the NS2 protein played the major role in inhibiting the human IFN response, as was also the case in murine cells. Thus, the restriction of PVM in nonhuman primates does not appear to be due to an inability of the virus to inhibit the IFN response in these nonrodent hosts. The basis for the host range restriction of a paramyxovirus was previously studied in detail for bovine parainfluenza virus (BPIV3), which is restricted for replication in humans and nonhuman primates such as RM (67). This was analyzed by systematically replacing each gene of HPIV3 with that of BPIV3, followed by evaluation of the chimeras in RM. The results showed that each of the genes made a contribution to the host range restriction (60). This suggested that the restriction does not involve a single major block at any specific step in infection and replication but rather is the aggregate effect of suboptimal functioning of multiple viral components in the nonnative host. The same might be true for PVM.

We evaluated a panel of adult human sera for RSV and PVM neutralization activity. As expected, all of the samples had high RSV-neutralizing activity, indicative of prior exposure to this ubiquitous human pathogen. In addition, approximately 40% had moderate PVM-neutralizing activity, and the rest lacked significant PVM-neutralizing activity. We also analyzed sera from infants and young children and found that 41% were seropositive for RSV, while only one individual was seropositive for PVM. The increased incidence of seropositivity with increased age raised the possibility that exposure to PVM or a PVM-like virus might occur between early childhood and adulthood. However, as noted, the PVM-neutralizing activity was low.

We evaluated the possibility that the observed PVM-neutralizing activity was due to cross-neutralization by RSV-specific antibodies. Three lines of evidence suggested that this was not the case. First, there was no correlation between high titers of RSV-neutralizing antibodies and PVM-neutralizing activity in the human serum samples. Second, no cross-neutralizing activity was observed between monospecific antisera from AGM or RM that had been infected with RSV or PVM. Third, no cross-protection was observed in mice that had a primary infection with RSV or PVM and were cross-challenged with the heterologous virus. Thus, while a low level of cross-reactivity was observed with the N proteins of the two viruses, there was no evidence of cross-neutralization or cross-protection in vitro or in vivo.

We investigated the specificity of the PVM-neutralizing activity by evaluating reactivity with Western blots of PVM and RSV proteins that had been prepared from purified virions under nonreducing and nonneutralizing conditions. The Western blot analysis did not provide evidence of PVM-specific antibodies in human sera. None of the AGM or human sera exhibited any cross-reactivity between RSV and PVM in this Western blot analysis, although, as noted, a low level of cross-reactivity for the N protein was observed with rabbit antisera prepared by repeated immunization with purified virions of either virus. We also investigated the specificity of the PVM-neutralizing sera by reactivity with Vero cells that had been infected with PVM or RSV that expressed GFP. Using the monospecific sera from RSV- and PVM-infected AGM as controls, we showed that the magnitude of binding of IgG correlated with the intensity of GFP expression in cells infected with the homologous GFP-expressing virus, consistent with the expectation that the binding of virus-specific IgG would increase with increased expression of viral antigens. With the adult human sera, all of the samples tested contained IgG antibodies that bound to RSV-GFP-infected cells, and we similarly observed a linear correlation between IgG binding and the level of GFP expression. With PVM-GFP-infected cells, both the PVM-neutralizing and nonneutralizing adult human sera exhibited a low, background level of binding of serum IgG antibodies, and the level of binding did not increase with increasing GFP expression. This was suggestive of low-affinity, non-PVM-specific binding activity. In addition, we detected only a low level of binding of IgM serum antibodies with all of the sera, with the single exception that day 28 sera from PVM-infected AGM exhibited substantial PVM-specific IgM binding. Apart from that single exception, binding did not correlate with GFP expression, indicating an absence of RSV- or PVM-specific IgM.

When we began these studies, we anticipated several possible outcomes: (i) that cells of nonhuman primates would be permissive for PVM and that PVM might be a true human pathogen; (ii) that humans might not be fully permissive for PVM but that PVM might cause incidental infection, similar to the situation with NDV, which can infect and cause generally mild disease in humans with substantial exposure to the virus but which is not a natural human virus; (iii) that a relative of PVM might infect humans; or (iv) that the reported seropositivity might not be PVM specific. The results of the present study support the last conclusion. The idea that PVM might be an authentic human pathogen seems inconsistent with the lack of permissive infection in RM and AGM. The idea that PVM might be a cause of incidental infection in humans is inconsistent with the lack of reactivity with PVM proteins in Western blot or with PVM-infected cells. These same data also speak against the idea that the putative seropositivity might reflect cross-reactivity with a relative of PVM, since that cross-reactivity should have been evident in Western blots.

It is possible that the PVM-neutralizing activity detected in human sera might reflect natural antibodies (46, 51). Natural antibodies are polyreactive, with specificity for multiple different antigens (25, 51). They are produced by B1 cells independent of exogenous antigens, have not undergone affinity maturation, and usually have low affinity for antigen (72). Natural antibodies are of broad specificity. They frequently react with carbohydrate anti-
gens and provide a first line of defense against infections by activating the complement pathway (24). It is true that natural antibodies are often of the IgM subclass, but they can also be of the IgG subclass. Natural polyreactive and autoreactive antibodies are present at birth, representing a contribution of IgG natural antibodies that are of maternal origin as well as a universal congenital IgM profile. Endogenous IgG develops with age, and both the IgG and IgM natural antibody repertoires become more diverse during life (45). This could explain why the low PVM-neutralizing activity of human sera seemed to develop with age.

In summary, we report that the basis of the PVM-neutralizing activity of human sera is not due to high-affinity PVM-specific antibodies or to RSV-specific antibodies cross-reacting with PVM. The PVM-neutralizing activity may be due to low-affinity, polyreactive natural antibodies. It seems very unlikely that PVM is a human pathogen, given its poor replication in nonhuman pri-
mates and the lack of any report of isolation or direct detection in humans. This high level of restriction did not appear to be due to a strong block at the level of infection or IFN antagonism. It also seems unlikely that PVM, or a PVM relative, may incidentally infect humans, given the lack of confirmed PVM-specific serum antibodies. Interestingly, PVM might be useful as a vaccine vector for human immunization via the respiratory tract, since it replicates at low levels and is highly immunogenic. The presence of low levels of PVM-neutralizing serum natural antibodies likely would not be an obstacle to this, since serum antibodies are not efficiently transported to the respiratory lumen and have reduced efficiency in restricting respiratory virus replication.

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