Human metapneumovirus (hMPV), first isolated in The Netherlands in 2001, is a member of the genus Metapneumovirus of the subfamily Pneumovirinae of the family Paramyxoviridae (25). This subfamily also includes the genus Pneumovirus, which includes respiratory syncytial virus (RSV). hMPV causes upper respiratory tract infection and flu-like illness (3, 21) but is also associated with lower respiratory tract infections such as bronchitis, bronchiolitis, and pneumonia in very young children, elderly persons, and immunocompromised patients (4, 6, 7, 10, 16). hMPV encodes two major surface glycoproteins, the fusion (F) and attachment (G) proteins (24, 25). The F protein promotes fusion of the viral and cell membranes, allowing penetration of the viral ribonucleoprotein into the cell cytoplasm (27), and the G protein mediates virus binding to the cell receptor (11). In RSV infection, the most immunogenic and protective viral antigen is the F protein, which evokes a strong antibody response and is a target for cytotoxic T cells (14, 17, 26).

The baculovirus expression system provides a method for the production of large quantities of biologically active and antigenic eukaryotic proteins for enzyme immunoassays and for immunoblot and immunofluorescence assays (IFAs) (9, 15, 18, 19, 29). In the present study, we expressed the F protein of hMPV in Trichoplusia ni (Tn5) insect cells by a baculovirus system and demonstrated the utility of the recombinant F protein in an IFA.

**MATERIALS AND METHODS**

*Serum samples.* A total of 200 serum samples were obtained at random from Japanese people (1 month to 41 years old) who visited hospitals. All samples were collected after obtaining informed consent from the children’s parents or the adults.

*Expression of F protein of hMPV in the baculovirus-insect cell system.* A baculovirus expression kit was used to prepare F protein expressed in the baculovirus-insect cell system in accordance with the instructions of the manufacturer (BD PharMingen, San Diego, Calif.). Briefly, the full-length cDNA of F protein from strain JPY88-12 (GenBank accession number AY622381) was amplified by PCR with primers F (5'-GGATCCATGTCTTGGAAAGTGGTGATATTGTTTG-3') and R (5'-GGCGCCGGCTAATTTAGTGATGGAAGCCATTGTGGG-3'). (The restriction sites in the primers used for cloning are underlined.) The PCR product was cloned into the BamHI and NotI sites of the recombinant plasmid pVL1393 baculovirus transfer vector. To generate a recombinant baculovirus, recombinant plasmid pVL1393-F was cotransfected with Baculogold DNA (BD PharMingen) into Sf9 cells. Trichoplusia ni (Tn5) insect cells cultured in Ex-cell 405 medium (JRH Biosciences, Lenexa, Kans.) were infected with the recombinant virus at a multiplicity of infection of 10 virus particles per cell. The cells at 72 h after infection were used as hMPV F protein for IFA and Western blot analysis.

*Western blot analysis.* Cells were lysed with sodium dodecyl sulfate (SDS), and the lysate of 10^6 cell equivalents was subjected to SDS–12% polyacrylamide gel electrophoresis under nonreducing conditions. The separated proteins were electrotransferred onto a nitrocellulose membrane (23). After blocking with 1% bovine serum albumin, hMPV antibody-positive serum (titer of 1:1,280 by hMPV IFA) or hMPV antibody-negative serum (titer of <1:10 by hMPV IFA) at a dilution of 1:200 was allowed to bind to the filter and then to react with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) polyclonal antibody (BioSource International, Camarillo, Calif.), and the proteins were detected by a chemiluminescence assay method (ECL Western Blotting Detection Reagents; Amersham Pharmacia Biotech, Inc., Piscataway, N.J.).
IFA using the baculovirus-insect cell system (Bac-F IFA). Tn5 cells infected with the recombinant virus were spotted onto slides. The cell smears were air dried, fixed in acetone for 10 min, and incubated for 30 min at 37°C with serum samples diluted serially, beginning at 1:10. After incubation, the slides were washed three times in phosphate-buffered saline (PBS) for 10 min each time. They were then incubated for 30 min at 37°C with fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark) at a serum dilution of 1:40. After incubation, they were washed three times in PBS for 10 min each time, air dried, and mounted with PBS-glycerin (1:1). Stained preparations were then examined under a fluorescence microscope. Serum samples that reacted with hMPV at a dilution of more than 1:10 were considered positive for hMPV antibodies. Expression of F protein in Tn5 cells was confirmed by indirect IFA with a guinea pig polyclonal antibody against hMPV, which was a kind gift from Albert D. M. E. Osterhaus, Department of Virology, Erasmus Medical Center. Furthermore, we confirmed that uninfected Tn5 cells did not react with human serum samples.

IFA using hMPV-infected LLC-MK2 cells (hMPV IFA). hMPV isolate JPS02-76 was inoculated into LLC-MK2 cells. hMPV-infected cells at 2 to 3 weeks after infection were used as hMPV antigen-positive cells for an IFA. The cell smears were air dried, fixed in acetone for 10 min, and incubated for 30 min at 37°C with serially diluted serum samples, beginning at 1:10. After incubation, the slides were washed three times in PBS for 10 min each time. They were then incubated for 30 min at 37°C with fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark) at a serum dilution of 1:40. After incubation, they were washed three times in PBS for 10 min, air dried, and mounted with PBS-glycerin (1:1). Stained preparations were then examined under a fluorescence microscope. Serum samples that reacted with hMPV at a dilution of more than 1:10 were considered positive for hMPV antibodies (5, 6).

Statistical analyses. Correlation between titers obtained by the two different IFAs was evaluated by Pearson’s correlation coefficient. The regression line was calculated using the JSTAT 9.2 software. The degree of concordance between the two assays was assessed by using the kappa statistic, which measures the excess of the observed agreement over that expected purely by chance. Kappa (κ) equals 1.0 for perfect agreement and 0 for no agreement beyond chance; values between 0.4 and 0.8 were considered to represent fair to good agreement beyond chance.

RESULTS

Expression of hMPV F protein and Bac-F IFA. The expected molecular size of the uncleaved and unglycosylated F0 protein of hMPV is 58.5 kDa (8). The observed molecular size of hMPV F protein expressed in the Bac-F-infected Tn5 cells was ~60 kDa, as determined by Western blot analysis with anti-hMPV F antibody-positive human serum (Fig. 1, lane 4, arrowhead). A representative Bac-F IFA result is shown in Fig. 2. hMPV F protein was present on cell surfaces and in the cytoplasm (Fig. 2A). The positive signal was not detected in intact Tn5 cells by IFA with anti-hMPV F antibody-positive human serum (data not shown).

Comparison of the results of the two IFAs. We determined the antibody titers against hMPV F protein by Bac-F IFA, as well as those against whole hMPV protein by hMPV IFA. Results are shown in Fig. 3. The titers obtained by Bac-F IFA and those obtained by hMPV IFA correlated well (correlation coefficient of 0.88). Of the 200 serum samples, all 143 serum samples positive by hMPV IFA were positive by Bac-F IFA. On the other hand, 18 of the 57 serum samples negative by hMPV IFA were positive by Bac-F IFA. The concordance of seroreactivities (titers of more than 10 were regarded as positive, and titers of less than 10 were regarded as negative) between the hMPV and Bac-F IFAs was 91% (κ = 0.76). For 192 of the 200 serum samples, the titers obtained by Bac-F IFA were equal to or higher than those obtained by hMPV IFA. The titers obtained by Bac-F IFA were lower than those obtained by hMPV IFA for only eight serum samples.

DISCUSSION

IFAs using hMPV-infected LLC-MK2 cells or hMPV-infected tertiary monkey kidney cells are widely used and have provided a large volume of serological data (5, 6, 25, 28). However, these conventional IFAs detect antibodies against whole hMPV proteins. In the present study, the specific anti-
bodies against hMPV F protein in human serum were measured by Bac-F IFA. We tentatively used a Bac-F IFA cutoff titer of 1:10 because the hMPV IFA cutoff titer was determined to be 1:10 in our previous study (5, 6). The titers obtained by Bac-F IFA and hMPV IFAs were compared. The values in open circles are the numbers of samples with the indicated titers. The broken lines indicate the cutoffs of the IFAs (serum dilution of 1:10). The solid line is the regression line calculated from the data presented (y = 1.032 × x + 1.44). The calculated r value was 0.88.

The hMPV IFA-positive serum samples reacted weakly with the F protein of hMPV separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, as shown in Fig. 1, suggesting that they were probably directed to conformational-type epitopes of the F protein. The antigenicity and immunogenicity of the F protein of RSV are also highly conserved (2, 8). In our previous study, the hMPV IFA titers of human serum samples tested by LLC-MK2 cells infected with one lineage of hMPV (group 1, JPS03-178) were the same as those of human serum samples tested by cells infected with another lineage (group 2, JPS02-76) (5, 6). Recently, the hMPV F protein has been indicated to be a major antigenic determinant that mediated effective neutralization and protection against both lineages of hMPV in hamsters, chimpanzees, and rhesus macaques (13, 20, 22). Therefore, the Bac-F IFA system made with the recombinant hMPV F protein of one lineage (group 1, JPY88-12) could also detect IgG antibodies against the hMPV F protein of the other lineage (group 2). Detection of a specific antibody against the hMPV F protein by Bac-F IFA should be a useful means for investigating protective immunity.

We conclude that the availability of large quantities of baculovirus-expressed hMPV F protein offers an opportunity to use this recombinant protein as a diagnostic reagent in the IFA and to study antigenic and immunogenic characteristics of the F protein. Further study is needed to determine the false positivity or false negativity rate in the Bac-F IFA by measuring the antibody titers in the acute and convalescent phases after primary hMPV infection.

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