Differentiation of Respiratory Syncytial Virus Subgroups with cDNA Probes in a Nucleic Acid Hybridization Assay

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A new approach to respiratory syncytial (RS) virus subgroup determination was developed by using a simple nucleic acid filter hybridization technique. By this method, virus-infected cells are bound and fixed in a single step, and the viral RNA in the fixed-cell preparation is characterized directly by its ability to hybridize to cDNA probes specific for either the A or B subgroups of RS virus. The subgroup-specific probes were constructed from cDNA clones that corresponded to a portion of the extracellular domain of the RS virus G protein of either a subgroup B RS virus (8/60) or a subgroup A RS virus (A2). The cDNA probes were labeled with ³²P and used to analyze RS virus isolates collected over a period of three decades. Replicate templates of infected cell preparations were hybridized with either the subgroup A or B probe. The subgroup assignments of 40 viruses tested by nucleic acid hybridization were in agreement with the results of subgroup determinations based on their reactivities with monoclonal antibodies, which previously has been the only method available for determining the subgroup classification of RS virus isolates. The nucleic acid hybridization assay has the advantage of providing broad-based discrimination of the two subgroups on the basis of nucleic acid homology, irrespective of minor antigenic differences that are detected in assays in which monoclonal antibodies are used. The nucleic acid hybridization technique provides a reliable method for RS virus subgroup characterization.

Respiratory syncytial (RS) virus is a major cause of lower respiratory tract infections in infants and young children around the world. No safe and effective vaccine is available, and reinfections occur throughout life (18). RS virus is a member of the *Paramyxoviridae* (genus *Pneumovirus*). It has two major transmembrane surface glycoproteins, the attachment protein G and the fusion protein F (5).

There are two antigenic subgroups of human RS virus which have been differentiated by their patterns of reactivity with monoclonal antibodies (MAbs). The most extensive differences between subgroups reside in the G glycoprotein (3, 20). Analysis of the responses of young children to RS virus infections has shown that a higher neutralizing antibody titer develops against a virus from the homologous infecting subgroup compared with the titer that develops against a virus from the heterologous subgroup (11). Mufson et al. (19) have shown that a first infection with a subgroup A RS virus appears to offer some protection against subsequent subgroup A but not subgroup B RS virus infections. Cotton rats immunized with recombinant vaccinia viruses expressing the glycoprotein G of an RS virus show reduced titers of virus in the lungs after challenge with a virus of the same subgroup as that of the immunizing protein, but show minimal or no protection against heterologous subgroup virus challenge (14, 27).

Thus, the ability to discriminate RS virus subgroups has immediate relevance for analysis of both the epidemiology and the immunology of infections and reinfections with RS viruses and for providing information crucial to vaccine development efforts. In agreement with the evaluation of antigenic differences between subgroups, analysis of the nucleotide sequences of the genes of both subgroup A and subgroup B RS viruses has shown that the greatest degree of sequence divergence between the subgroups occurs in the G

protein gene. The other genes are more conserved between the subgroups (15). In the work reported here, we took advantage of the nucleotide sequence diversity of the G protein gene to develop a nucleic acid hybridization assay capable of establishing the subgroup classification of individual RS virus isolates.

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MATERIALS AND METHODS

Cells and viruses. The RS virus isolates were grown in HEp-2 cells in modified Eagle medium supplemented with 5% fetal bovine serum with penicillin, kanamycin, and streptomycin. Two sets of RS virus isolates were evaluated. One set was previously characterized with MAbs and included representative isolates from subgroups A and B (see Table 1), and one set included recent isolates from New Mexico (provided by Alice Cushing and Leroy McLaren, University of New Mexico Hospital, Albuquerque).

cDNA clones. A cDNA clone of the G protein mRNA of the subgroup A A2 RS virus (6, 28) in a pGEM-1 plasmid vector (Promega) and of the subgroup B 8/60 RS virus (W. M. Sullender, K. Anderson, and G. W. Wertz, *Virology*, in press) in an IBI-76 plasmid vector (International Biotechnologies, Inc.) were constructed for other purposes. For use in this investigation, they were modified as follows. The plasmids containing the cDNA clones were digested with *HincII*, which cleaves a single site in the plasmid vector and a single site in the insert cDNA. The restriction fragments were separated in a low-melting-point agarose gel, and the larger fragment was collected and extracted from the gel piece (17). This larger fragment included the entire plasmid vector and a portion of the G protein cDNA described

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below. This fragment was ligated and used to transform DH5 α F' competent cells (Bethesda Research Laboratories). The resultant plasmids contained the cDNA from bases 386 to 918 (532 nucleotides) of the A2 G protein gene and bases 313 to 914 (602 nucleotides) of the 8/60 G protein gene, and the protein G-specific sequences shared a 67% nucleic acid identity (Sullender et al., in press). This construction resulted in an insert DNA corresponding to most of the proposed extracellular domain of the G protein (30). Plasmid DNA was purified by isopycnic banding in CsCl. To prepare DNA for use as probes, plasmid DNA was digested with *HincII* and *EcoRI* to free the insert DNA from the plasmid vector. The desired fragment was purified by electrophoresis in low-melting-point agarose followed by extraction in phenol. The DNA fragments (0.5 to 1 μ g) were labeled by nick translation with [α - 32 P]dATP (Dupont, NEN Research Products) followed by purification by using a spun column of Sephadex G50 (17).

Glutaraldehyde fixation of infected cells. Templates for hybridization were prepared by the method of Paeratakul et al. (22). HEP-2 cells were split into 12-well plates (Costar) at a dilution which yielded a subconfluent monolayer the following day. The medium was then removed, the viral inoculum was added for an adsorption period of 2 h and then removed, and fresh medium was added. The inoculum was chosen such that an extensive cytopathic effect and syncytium formation would be produced in 2 to 4 days, at which time the cells from one well were scraped into the medium and transferred into a 1.5-ml polypropylene tube on ice. The tube was centrifuged at full speed in an Eppendorf centrifuge (Brinkmann Instruments, Inc.) for 2 min at 4°C. The medium was aspirated, and the cell pellet was suspended in 440 μ l of cold phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 4 mM Na₂HPO₄ · 7H₂O, 15 mM KH₂PO₄ [pH 7]). The sample was stored at -70°C if it was not used immediately.

Nitrocellulose paper (pore size, 0.45 μ m; Schleicher & Schuell, Inc.) was wet with phosphate-buffered saline and placed in a 96-well suction apparatus (Minifold; Schleicher & Schuell, Inc.). Portions of 100 μ l of the infected cells in phosphate-buffered saline were added to each well, and suction was applied. When no liquid remained, the nitrocellulose filter was removed and fixed for 1 h at 4°C in 3% NaCl-100 mM NaH₂PO₄-40 mM Na₂HPO₄ (pH 7.4)-1% glutaraldehyde (grade II; Sigma Chemical Co.). The filter was rinsed three times with 50 mM EDTA-0.1 M Tris hydrochloride (pH 8) and then digested in the same solution with 20 μ g of proteinase K per ml for 30 min at 37°C. The filter was air dried and hybridized immediately or stored at -20°C in heat-sealed plastic bags.

Hybridization. Nitrocellulose membranes prepared as described above were placed in plastic bags, and prehybridization solution was added (4 $\times$ SSC, 5 $\times$ Denhardt solution, 50 μ g of calf thymus DNA per ml, and 0.1% sodium dodecyl sulfate [SDS]) (SSC is 0.15 M sodium chloride and 0.015 M sodium citrate dihydrate; Denhardt solution is 1% Ficoll [Pharmacia Fine Chemicals], 1% bovine serum albumin fraction V, and 1% polyvinylpyrrolidone). The sample was placed at 65°C on a rotary platform for 1 to 4 h, and then the solution was removed. 32 P-labeled probes were boiled for 4 min, quick chilled in ice water, and added with hybridization solution (4 $\times$ SSC, 1 $\times$ Denhardt solution, 50 μ g of calf thymus DNA per ml, 0.1% SDS) to the nitrocellulose membrane. Hybridization was carried out overnight at 65°C on a rotary platform. The hybridization solution was removed; and the membranes were washed in 2 $\times$ SSC-0.1% SDS twice for 5 min each time at room temperature, then in 0.1 $\times$

SSC-0.1% SDS at 42°C for 30 min, and then in 0.1 $\times$ SSC-0.1% SDS at 60°C for 5 min. It was necessary to use wash conditions of this stringency because, with less stringent conditions, cross-hybridization between samples of one subgroup and the probe for the opposite subgroup occurred (data not shown). The membranes were allowed to dry and then exposed to Kodak XAR or Fuji film with an intensifying screen at -70°C for 1 to several days (17).

Antigenic characterization of isolates. The strains were reacted against a panel of previously described MAbs (2, 3) by enzyme immunoassay at a 1:5 dilution in phosphate-buffered saline plus 0.15% Tween 20 and 0.5% gelatin. In this assay, a broadly reacting MAb (131-2g for the G protein and 133-1h for the F protein) was adsorbed to 96-well microdilution plates overnight at 4°C, the plates were washed, and virus was added and incubated overnight at room temperature. The plates were washed, and biotinylated MAbs (see below) were added and incubated for 1 h at 37°C; the plates were washed, peroxidase-conjugated streptavidin was added, and the plates were incubated for 10 min at room temperature; and the plates were washed and 3,3',5,5'-tetramethylbenzidine substrate with 0.005% H₂O₂ was added. The reactions were stopped by the addition of H₂SO₄, and the A₄₅₀ was read (13). In this enzyme immunoassay, subgroup A isolates reacted against MAbs 92-11c and 131-2a but not against MAbs 102-10b, and subgroup B isolates reacted against MAbs 102-10b and 131-2a but not against MAb 92-11c. The subgroup-specific reactivity patterns are not shown. The reactivities of RS viruses against additional MAbs (2) demonstrated the antigenic heterogeneity within the subgroups, and number designations were assigned arbitrarily to illustrate this heterogeneity (see Table 1).

RESULTS

Design of cDNA probes. The G protein-specific cDNAs of A (A2) and B (8/60) subgroup RS viruses (20) were chosen as probes because the greatest sequence differences between the A and B subgroups reside in the G gene (15). In addition, between the G proteins of the two subgroups, the extracellular domain has been shown to have more amino acid differences than the cytoplasmic and transmembrane domains (15). Therefore, we constructed cDNA probes that correlated with the extracellular domain of the G protein of either an A or a B subgroup virus.

Testing of RS virus isolates. The nucleic acid hybridization method of RS virus subgrouping was tested initially against isolates with known subgroup designations (Table 1 and Fig. 1). Replicate templates were prepared, and each template was hybridized with either the subgroup A (probe A) or the subgroup B (probe B) G protein cDNA fragment. The template in Fig. 1a was hybridized with probe A, and all the subgroup A strains in columns 1 and 2 in Fig. 1a had a positive signal. Minimal or no reactivity was seen with mock-infected or subgroup B RS virus-infected samples hybridized with the subgroup A probe.

The template in Fig. 1b was hybridized with probe B, and all seven subgroup B strains in column 3 of Fig. 1b had a positive signal. In contrast, there was no detectable reactivity with mock or subgroup A RS virus-infected samples hybridized with the subgroup B probe. For all the viruses tested (Fig. 1), the subgroup designation was the same by both nucleic acid hybridization and MAb testing.

After establishing the ability of the nucleic acid hybridization assay to effectively differentiate viruses with known

TABLE 1. Date and place of isolation of RS viruses and demonstration of antigenic heterogeneity within subgroups^a

Category	Virus	Isolation		MAb reactivity ^b			
		Yr	Place	143-5a	130-5f	130-9g	130-6d
Subgroup A							
A1	2045-16	1984–1985	Minnesota	+	–	–	+
A1	2045-25	1985–1986	Minnesota	+	–	–	+
A1	2045-33	1985–1986	Minnesota	+	–	–	+
A1	1836a	1985	Georgia	+	–	–	+
A2	159/59	1959	Sweden	–	+	–	+
A2	9324	1977	Massachusetts	–	+	–	+
A2	2045-1	1984–1985	Minnesota	–	+	–	+
A2	2045-32	1985–1986	Minnesota	–	+	–	+
A3	2045-12	1984–1985	Minnesota	–	–	–	+
A3	2012-8	1986	West Virginia	–	–	–	+
A4	1935a	1985	Washington	+	+	–	+
A5	A1	1961	Australia	–	+	+	+
A5	A2	1961	Australia	–	+	+	+
A6	2040-35	1985	Washington	–	–	–	–
A7	2139-4	?	West Virginia	–	–	+	+
Subgroup B							
B1	8/60	1960	Sweden	±	+	–	–
B1	9320	1977	Massachusetts	±	+	–	–
B1	1776	1984	Georgia	±	+	–	–
B1	1842	1985	Georgia	±	+	–	–
B1	2040-23	1985	Washington	±	+	–	–
B2	2045-4	1984–1985	Minnesota	–	+	–	–
B2	2045-5	1984–1985	Minnesota	–	+	–	–
B2	2045-41	1985–1986	Minnesota	–	+	–	–
B2	2040-1	1985	Washington	–	+	–	–
B2	2040-21	1985	Washington	–	+	–	–
B3	18537	1962	District of Columbia	–	±	–	–

^a Subgroup designations and antigenic categories within the subgroups were determined by enzyme immunoassay, as described in the text. Categories beginning with A are subgroup A viruses, and those beginning with B are subgroup B viruses, with the numbers indicating categories within the main subgroup. The enzyme immunoassay was based on a MAb as a capture antibody, followed by the virus, followed by a second MAb that was biotinylated (2). A positive reaction was one when $P - N_1$ and $P - N_2$ were >0.05 and P/N_1 and P/N_2 were >1.7 , where P is the absorbance of the respective MAb against the virus, N_1 is the absorbance of the respective virus against an adenovirus MAb, and N_2 is the absorbance of the MAb against uninfected HEP-2 cells. The MAbs have been described previously (2), and each reacts with a different epitope on the G protein. MAb 143-5a reacts at epitope G5a, 130-5f reacts at epitope G4, 130-9g reacts at epitope G3b, and 130-6d reacts at epitope G12.

^b +, Positive reaction; -, negative reaction; ±, low-level positive reaction in at least some tests.

subgroup designations, we evaluated clinical samples isolated at the University of New Mexico Hospital during 1989 (Fig. 2). We also tested additional viruses with previously determined subgroup designations in this experiment. Two additional subgroup A RS viruses were tested (Fig. 2a and b, column 1, wells 2 and 3 from the top) and hybridized only with the A probe. Four additional subgroup B RS viruses were tested and only hybridized with the B probe (Fig. 2a and b, column 1, wells 5 through 8 from the top). Of the 14 New Mexico isolates, 13 hybridized with the A probe; only one isolate (Fig. 2b, column 3, well 7 from the top [isolate 89-1355]) hybridized with the subgroup B probe. The viruses from New Mexico were subsequently reacted against the MAbs (data not shown). The subgroup assignments by the MAbs were identical to those of the nucleic acid hybridization assay; 13 viruses were of subgroup A, and 1 virus (89-1355) was of subgroup B.

Demonstration of heterogeneity of the viral population tested. The viruses received from the Centers for Disease Control and tested in the nucleic acid hybridization assay varied in both their dates and places of original isolation (Table 1). MAb reactivity has demonstrated the antigenic heterogeneities of the G proteins within the A and B subgroups (1, 3, 20, 25). To determine whether antigenic heterogeneity existed within the viruses that we tested, we examined several isolates for their reactivities with a group of MAbs (2) and defined antigenic categories within each

subgroup to describe the heterogeneities of the samples we tested. The individual isolates that we tested fell within seven antigenic categories in subgroup A viruses and three antigenic categories in subgroup B viruses (Table 1). Thus, they represented a varied sampling of both subgroup A and B viruses with geographically and temporally disperse origins and demonstrated antigenic heterogeneity. These data show that, even though they were a heterogeneous population of viruses, they segregated clearly with the appropriate subgroup probe in the nucleic acid hybridization assay.

DISCUSSION

We have described a new technique for determining the subgroup designations of individual isolates of RS virus. Infected cells were fixed on nitrocellulose with glutaraldehyde (22), and their RNAs were then hybridized with cDNAs representing a portion of the glycoprotein G gene of either a subgroup A or subgroup B RS virus. A total of 40 isolates were tested; all 28 subgroup A and all 12 subgroup B RS viruses were classified correctly by the nucleic acid hybridization assay.

Previously, the subgroup designation of individual RS virus isolates has been determined based on their reactivities with MAbs by using either immunofluorescence or enzyme-linked immunosorbent assay techniques (3, 20). A potential disadvantage of the use of MAbs arises from the fact that a

FIG. 1. Nucleic acid hybridization of infected cell templates by using either RS virus subgroup A (probe A, panel a)- or subgroup B (probe B, panel b)-specific cDNA probes. HEp-2 cells infected with individual RS viruses were prepared for filter hybridization on nitrocellulose membranes by the procedure of Paeratakul et al. (22). Replicate templates were hybridized with [32 P]dATP-labeled cDNA probes that were either subgroup A or B specific. After hybridization and washing as described in the text, membranes were allowed to dry and exposed to X-ray film with an enhancing screen at -70°C . These samples were previously categorized as subgroup A or B viruses by using MABs (3). As indicated in the column heading labeled strains, columns 1 and 2 contain subgroup A viruses and column 3 contains subgroup B viruses. The number designation assigned to each individual isolate, from top to bottom, respectively, is as follows: column 1, A2, 669, 9324, 1936, 2012, 961, 1935, and 2139-4; column 2, 2045-1, 2045-12, 2045-16, 2045-32, 2045-33, mock infected, and two wells empty; column 3, 8/60, 18537, 1842, 2045-5, 2045-41, 2040-1, 2040-23, and mock infected.

single amino acid change in a viral glycoprotein may result in a loss of reactivity with a MAB (16). Garcia-Barreno et al. (10) have shown that RS viruses selected by growth in the presence of an anti-G protein MAB not only lose their reactivities with a group of anti-G protein MABs but also that their G proteins fail to react with polyclonal sera prepared against whole virus. This capacity for antigenic change may explain why investigations in which only a limited number of MABs are used for RS virus isolate analysis are not able to assign a subgroup designation to all RS virus isolates (12, 24). The nucleic acid hybridization assay is based on the extent of genetic homology between the RS virus G protein RNAs and the cDNA probes. The nucleic acid hybridization assay measures the degree of homology over a large part of the G protein RNAs, and the results should be less subject to variation caused by minor mutations and more representative of the degree of relatedness of the entire gene.

The fact that the genetic classification by hybridization reported here corresponds to the antigenic classification is reassuring and supports the appropriateness of the previously described classification of RS virus isolates (3, 20). Since relatively few isolates were studied, additional testing is needed to complete the comparison of the two typing systems. The isolates that we studied, however, represent the known antigenic, temporal, and geographic diversities of RS viruses, including at least seven distinct subgroup A and three distinct subgroup B strains isolated between 1959 and 1989 on several continents. Therefore, it is likely that most, if not all, RS virus isolates tested will give results similar to those presented in this report.

Nucleic acid probes have been used to differentiate anti-

FIG. 2. Nucleic acid hybridization of infected cell templates with either subgroup A (probe A, panel a)- or subgroup B (probe B, panel b)-specific cDNA probes. Template preparation and hybridization were as described in the legend to Fig. 1. The viruses in column 1 are of known subgroup designation. The viruses in columns 2 and 3 are clinical isolates obtained from the University of New Mexico Hospital in 1989 and have not been characterized previously. The first two samples in column 2 are subgroup A (well 1 from the top) and subgroup B (well 2 from the top) controls. The number designation assigned to each individual isolate from top to bottom, respectively, is as follows: column 1, subgroup A—A2, 2040-35, and 2040-25; subgroup B—8/60, 1776, 2045-4, 2040-21, and 9320; column 2, panel a, subgroup A control (A2), subgroup B control (8/60), 89-937, 89-1103, 89-4928, 89-1249, 89-804, and 89-318; column 3, 89-743, 89-1242, 89-1325, 89-1226, 89-384, 89-1051, 89-1355, and 89-1006. CDC, Centers for Disease Control strains; UNM, University of New Mexico Hospital strains.

genically distinct viral strains. Herpes simplex virus types 1 and 2 can be differentiated with cDNA or synthetic oligonucleotide probes (4, 23), rotavirus serotypes can be differentiated with cDNA probes (7, 9, 31), and two closely related bunyaviruses can be discriminated in an assay with synthetic oligonucleotides (21). The nucleic acid hybridization assay described here offered reactivity within each subgroup, even among a diverse population of viruses, and will be of value when the goal is a simple differentiation of RS virus subgroup A or B. When the goal is a more detailed description of the antigenic or molecular diversity within a collection of viruses, testing with a group of MABs (1, 3, 8, 10, 20) or RNA fingerprinting (26) may be useful.

In this study, we demonstrated that nucleic acid hybridization analysis of individual RS virus isolates clearly discriminates between the two subgroups of RS virus and provides the advantage of a broadly reactive assay technique. The adaptation of a non-isotopic-labeling technique would increase the applicability of this technique to a wide variety of situations. In addition to their use as probes of infected cell preparations, these cDNAs might function in assays of clinical materials for the immediate diagnosis of RS virus infections (29). Synthetic oligonucleotides that differentiate between the subgroups of RS virus are also being designed and tested.

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