Time-Resolved Fluoroimmunoassay Compared with Virus Isolation for Rapid Detection of Respiratory Syncytial Virus in Nasopharyngeal Aspirates

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Two monoclonal antibodies against two distinct conserved epitopes of the respiratory syncytial virus (RSV) nucleocapsid protein were used in a direct time-resolved fluoroimmunoassay (TR-FIA) for the detection of RSV antigens in nasopharyngeal aspirates. The capture antibody was adsorbed to the solid phase of microdilution strip wells, and the indicator antibody was labeled with a europium chelate. Specimens and label were incubated simultaneously for 1 h at 37°C in the coated wells. After the test samples were washed, fluorescence enhancement solution was added, strips were shaken, and the time-resolved fluorescence was measured. The test procedure took only 75 min, and the total time for 20 specimens, with pretreatment by sonication, was 2 to 3 h. We prospectively evaluated the detection of RSV in nasopharyngeal aspirates of pediatric patients by TR-FIA and by virus isolation in human diploid fibroblasts. TR-FIA detected 40 of 42 isolation-positive specimens. Nine additional isolation-negative specimens were positive by TR-FIA; all proved to be true positives by a blocking-type confirmatory assay. The sensitivity, specificity, positive predictive value, and negative predictive value for TR-FIA were 95, 96, 82, and 99%, respectively, of the values obtained by virus isolation and 96, 100, 100, and 99%, respectively, of the values obtained by virus isolation and the confirmatory assay.

Respiratory syncytial virus (RSV) is one of the most important pathogens causing lower respiratory tract infections in infants and young children (5). Rapid diagnosis of the infection can help physicians avoid unnecessary antibiotic treatment and, in patients with severe underlying disease, facilitate the decision regarding antiviral treatment with ribavirin (6, 14). Since standard virus isolation procedures are too slow, detection of RSV-infected epithelial cells in nasal secretions by immunofluorescence microscopy was introduced (4). A result can be obtained within a few hours with either indirect or direct immunofluorescence staining of infected cells, but the method is laborious and demands special transportation and treatment of specimens. The limitations of immunofluorescence microscopy have focused interest on enzyme immunoassay and related techniques (2, 11, 20) that require less demanding handling of specimens and provide automated recording of results. These tests, however, require prolonged incubations, often overnight, to reach the desired sensitivity.

Time-resolved fluorometry of lanthanide chelates (8, 22) has recently shown great potential in the development of very sensitive immunoassays for detection of viral antigens in clinical specimens (7, 10, 21, 23). We describe a rapid and sensitive time-resolved fluoroimmunoassay (TR-FIA), based on monoclonal antibodies (MAbs), for detection of RSV antigens in nasopharyngeal specimens (NPS) and compare this assay with the virus isolation technique.

MATERIALS AND METHODS

Design of the study. Since the beginning of January 1987 all specimens sent to our diagnostic unit for detection of respi-

ratory virus antigen have been routinely tested with the RSV TR-FIA described here and with similar TR-FIAs for influenza A and B viruses (23), parainfluenzavirus types 1, 2, and 3 (P. E. Halonen, S. Nikkari, M. Waris, H. Siitari, C. Örvell, and J. Hierholzer, Abstr. 5th Int. Symp. Rapid Methods Automation Microbiol. Immunol. 1987, S14/3, p. 170), and adenoviruses (10). During an outbreak of RSV in Finland from March to July 1987, detection of RSV antigens was compared with isolation of virus in children with acute respiratory infection.

Patients and specimens. Specimens for the comparative study were collected from two groups of pediatric patients. Group 1 consisted of 159 outpatients between 1 month and 7 years (median, 1.7 years) of age who were seen in a private medical center in Turku. Group 2 consisted of 131 hospitalized patients between 2 weeks and 14 years (median, 1.4 years) of age who were admitted mainly to the Turku University Hospital.

NPS were collected by aspiration with a disposable mucus extractor. Each specimen for virus isolation was taken by dipping a cotton stick into the extracted mucus and placing the stick in a vial containing 2 ml of transport medium (0.5% bovine serum albumin, 5% tryptose phosphate broth, and antibiotics in phosphate-buffered saline). The isolation specimens of group 1 were taken immediately after collection and frozen in dry ice for transport to the laboratory, where they were stored at -70°C. Specimens for antigen detection were transported to the laboratory in the mucus extractor at ambient temperature. The isolation specimens of group 2 were taken only after the NPS arrived at the laboratory, if received on the day of collection, and stored refrigerated in the transport medium until inoculated the same or the following work day.

Virus isolation. Human diploid fibroblasts, prepared in this

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laboratory from foreskins, were maintained in Dulbecco modified Eagle medium supplemented with 2% inactivated fetal calf serum, 0.05% glutamine, and antibiotics. Group 1 isolation specimens were thawed, and 200 μ l of specimen was inoculated into each of two human diploid fibroblast culture tubes containing 1 ml of maintenance medium. The tubes were incubated in a roller drum in a CO₂ incubator at 33°C, and the medium was changed the following day and thereafter twice a week. Isolation for the specimens of group 2 was done in human diploid fibroblasts grown on a cover slip in a shell vial containing 1 ml of medium. The cultures were inoculated with 100 μ l of specimen and centrifuged at 4,000 $\times$ g for 1 h; the medium was then replaced. The tubes were incubated in a CO₂ incubator at 36°C, and the medium was changed twice a week. Cultures from both groups were kept for 3 to 4 weeks and microscopically examined for development of typical syncytium formation three times a week. Cultures of group 1 showing no cytopathic effect at the end of the incubation period, as well as all positive cultures, were tested for antigen detection by TR-FIA.

MAbs. Hybridomas producing MAbs (RSV4 and NC4) directed against different antigenic sites of the RSV nucleoprotein were developed as described elsewhere (17). Ascites fluid was generated by injecting hybridoma cells intraperitoneally in pristane-primed BALB/c mice. Immunoglobulin G (IgG) was purified from ascites fluid by anion-exchange chromatography on a Mono Q HR10/10 column (Pharmacia, Uppsala, Sweden). To avoid frequent regeneration of the Mono Q column, ascites fluid was pretreated as follows. To 2 ml of clarified ascites fluid, solid NaCl was added to a final concentration of 0.4 M. Ascites fluid was then passed through a 1-ml bed of Q-Sepharose FF (Pharmacia) in 20 mM triethanolamine hydrochloride–0.35 M NaCl, pH 7.7, packed into a disposable polystyrene column (QS-Y; Isolab, Inc., Akron, Ohio). The applied volume was quantitatively collected by brief low-speed centrifugation of the column in a test tube. Ascites fluid was then desalted to 20 mM triethanolamine hydrochloride, pH 7.7, with a PD-10 column of prepacked Sephadex G-25 medium (Pharmacia). Finally, the sample was injected through Millex-AA (0.8- μ m pore size) and Millex-GV (0.2- μ m pore size) filters (Millipore, S.A., Molsheim, France) immediately before being loaded onto the Mono Q column equilibrated with 20 mM triethanolamine hydrochloride, pH 7.7. Proteins were eluted with a 160-ml linear gradient of 0 to 100% 20 mM triethanolamine hydrochloride–0.35 M NaCl, pH 7.7, at a flow rate of 2 ml/min and monitored at 280 nm. IgG was eluted at ca. 0.15 M NaCl. The concentration of IgG was determined spectrophotometrically by using an extinction coefficient at 280 nm of 1.4 ml/mg per cm.

Labeling of antibodies. Purified IgG was coupled with Eu³⁺-N'-(*p*-isothiocyanatobenzyl)-N¹,N²,N³,N³-diethylenetriamine tetraacetate (Wallac Oy, Turku, Finland) as described elsewhere (8) with minor modifications. The isothiocyanato group of the Eu chelate reacts with primary amino groups of protein, and triethanolamine (containing tertiary amino groups) does not interfere with the reaction. IgG was concentrated to 4 mg/ml with a Centricon 30 centrifugal microconcentrator (Amicon Corp., Danvers, Mass.). The Eu chelate was dissolved in distilled water to a concentration of ca. 6 mM. The exact concentration was determined by comparing the time-resolved fluorescence of the Eu chelate solution with that of a 1.0 μ M Eu³⁺ standard when both were dissolved in Delfia enhancement solution (Wallac Oy). The time-resolved fluorescence was measured with a model 1250 Arcus fluorometer (Wallac Oy). Eu

chelate solution was added to 1 mg of IgG in a 50-fold molar excess, and the pH of the mixture was raised to 9.0 to 9.5 by addition of ca. 5 μ l of 1 M NaHCO₃, pH 10. Coupling was completed during overnight incubation at 4°C. The uncoupled Eu chelate was then separated by gel filtration on a column (10 mm by 50 cm) of Trisacryl GF 2000 (LKB, Bromma, Sweden) equilibrated with 50 mM Tris hydrochloride–0.15 M NaCl–0.05% NaN₃, pH 7.75. The Eu/IgG ratio was determined by measuring the Eu content of the collected IgG peak and assuming IgG recovery of 100%. Diethylenetriaminepentaacetic acid-treated bovine serum albumin (Wallac Oy) was added to a final concentration of 0.1%.

Virus preparations. Plaque-purified RSV (Randall strain) was propagated in Vero cells, grown in roller bottles, and maintained in Dulbecco modified Eagle medium supplemented with 0.2% bovine serum albumin, 0.03% glutamine, nonessential amino acids, and antibiotics. When advanced cytopathic effect was observed, the cells were washed with phosphate-buffered saline and disrupted with a Dounce homogenizer in 10 mM Tris hydrochloride–0.1 mM EDTA–0.05% NaN₃, pH 7.4. The lysate was centrifuged at 10,000 $\times$ g for 30 min, and the supernatant was used as the control antigen. RSV subgroup A strains Long and CH287 and RSV subgroup B strains WV3212 and WV4843 (15) were kindly provided by C. Örvell (Karolinska Institutet, SBL, Stockholm, Sweden). They were propagated as described above, and infected cells were scraped into the medium and stored at –70°C.

TR-FIA. All immunoreagents were stored in aliquots at –70°C. Once thawed, they were kept at 4°C. The optimal concentrations of the capture MAb (RSV4) and the label MAb (NC4) were determined in preliminary experiments. A label preparation with a molar ratio of 12 Eu/IgG was used. Subgroup A and subgroup B strains of RSV were detected by these MAbs with equal sensitivity in TR-FIA. Each well in microdilution strips (Eflab Oy, Helsinki, Finland) was coated overnight at room temperature with 0.25 μ g of purified RSV4 IgG diluted in 200 μ l of 0.1 M NaHCO₃, pH 9.6. The strips were washed twice and postcoated overnight at room temperature with 50 mM Tris hydrochloride–0.15 M NaCl–0.05% NaN₃, pH 7.75 (250 μ l per well) containing 0.1% gelatin, 0.01% Tween 40, and 20 μ M diethylenetriaminepentaacetic acid and stored at 4°C until used. The washing solution in all steps was 5 mM Tris hydrochloride–0.15 M NaCl–0.05% Tween 20, pH 7.75. Specimens were diluted 1:5 with 20% inactivated fetal calf serum–2% Tween 20 in phosphate-buffered saline, pH 7.2, sonicated to disrupt the mucus, and further diluted 1:2 in 50 mM Tris hydrochloride–0.15 M NaCl–0.05% NaN₃, pH 7.75, containing 0.5% gelatin, 0.01% Tween 40, and 20 mM diethylenetriaminepentaacetic acid (assay buffer). Before use, the strips were washed twice and 100 μ l of diluted NPS or the control antigen was pipetted into each well; 100 μ l of label at a concentration of 100 ng of IgG per well was added immediately thereafter. The samples were tested in duplicate. The strips were incubated for 1 h at 37°C and washed six times, and 200 μ l of enhancement solution was added to each well. The strips were shaken for 10 min on a model 1292 Rack Shaker (Wallac Oy), and time-resolved fluorescence was measured. The measurement time was 1 s per sample, and the resulting value was expressed as counts per second.

To interpret the results, the screening mode provided by the fluorometer was used. Briefly, the instrument is programmed to first define an initial cutoff value of two times the mean of negative controls. The samples are then screened with this value, and a new cutoff level is calculated from the

TABLE 1. Comparison of RSV antigen detection by TR-FIA and by RSV isolation using human diploid fibroblasts^a

RSV isolation	No. of TR-FIA specimens					
	Group 1		Group 2		Total	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	22	1	18	1	40	2
Negative	4	132	5	107	9	239

^a For group 1 (outpatients), isolation specimens were frozen and roller cultures were used for virus isolation. For group 2 (hospitalized patients), specimens were taken after transport at ambient temperature and shell vial cultures inoculated by centrifugation were used for virus isolation.

nonreactive sample values (NR) as follows: cutoff = mean of NR + 6 standard deviations of NR. Identical test procedures were followed in the other TR-FIAs, and all tests were performed simultaneously for all specimens.

Confirmatory assay. A procedure similar to that described by Sarkkinen et al. (20) was used for confirmatory assays. Portions (50 μ l) of specimen were simultaneously preincubated for 30 min at room temperature with assay buffer, a 1:100 dilution of guinea pig RSV hyperimmune serum, or a 1:100 dilution of preimmunization serum (50 μ l of each reagent in duplicate wells). The test protocol was then continued as described above. When at least 50% inhibition of a positive reaction was achieved with hyperimmune serum compared with the result obtained with preimmune serum and if the results obtained with preimmune serum and with assay buffer did not differ significantly, the reaction was regarded as specific for RSV.

RESULTS

Of the 290 specimens in the two study groups, 51 (18%) were positive for RSV by one of the methods (49 of these by TR-FIA) and 42 were positive by virus isolation (Table 1). Although the two groups differed with respect to population and isolation procedure, they did not differ with respect to RSV antigen detection or isolation results. All of the nine TR-FIA-positive, isolation-negative specimens produced a specific reaction in the confirmatory assay (Table 2). The two cell culture-positive but TR-FIA-negative specimens were repeatedly negative by TR-FIA. In one of these specimens, weak cytopathic effect was seen only late during incubation. When the cell cultures of these specimens were tested by TR-FIA, however, clearly positive results were obtained.

To compare the TR-FIA counts-per-second values from different test runs, an S/N (specimen counts per second/negative-control counts per second value was calculated; all

positive specimens had an S/N value of 3 or higher (Fig. 1). Positive specimens with an S/N value of less than 10 were retested and gave concordant results. In comparison with results obtained by virus isolation, the sensitivity, specificity, positive predictive value, and negative predictive value of TR-FIA were 95% (40 of 42), 96% (239 of 248), 82% (40 of 49), and 99% (239 of 241), respectively. If specimens producing a positive result in the confirmatory assay were regarded as true positives, these values increased to 96% (49 of 51) for sensitivity, 100% (248 of 248) for specificity, and 100% (49 of 49) for positive predictive value. Also found in these 290 specimens by either antigen detection or virus isolation were adenovirus (10 specimens), cytomegalovirus (2 specimens), influenza A virus (2 specimens), parainfluenza virus 1 (4 specimens), parainfluenza virus 2 (5 specimens), and parainfluenza virus 3 (15 specimens).

The monthly results, including those given above, of RSV antigen detection from the year 1987 are presented in Fig. 2. A total of 3,817 specimens were tested; 756 (20%) of these were positive, 516 (14%) for RSV and 240 (6%) for other respiratory viruses.

DISCUSSION

MAbs have been used as capture antibodies for detection of RSV antigens in indirect assays (9) and in a commercial direct assay with peroxidase-conjugated bovine RSV antiserum as the detector antibody (13). Clayton et al. (3) found a combination of polyclonal serum as a capture antibody and MAbs as detectors superior to MAbs alone in direct-enzyme amplified assays. Only recently, comparable results have been reported by using MAbs both on the solid phase and as the biotinylated indicator antibody (17).

The progress made in time-resolved fluorometry of lanthanide chelates offers the possibility of using high-affinity MAbs as Eu-labeled indicator antibodies in immunoassays, generally without the decrease in immunological activity that may occur during the labeling procedure or because of steric hindrance caused by the relatively large enzyme molecules (M. Waris, S. Nikkari, P. Halonen, I. Kharitonov, and A. Kendal, in A. Turano, ed., *Rapid Methods and Automation in Microbiology and Immunology*, in press). Two additional factors can explain the high sensitivity achieved with the RSV TR-FIA despite the short incubation time: first, the use of two MAbs recognizing two distinct epitopes of a major virus protein (17); and second, the fact that the immunoreaction can take place partially in the liquid phase, since the label is added immediately after the specimen (21). Furthermore, Eu chelate-labeled antibodies are stable and can, because of gentle and efficient labeling, be used at high dilutions.

TABLE 2. Representative results of the confirmatory TR-FIA

NPS no.	Confirmatory test result (TR-FIA cps) for specimen first incubated with:			Isolation result	Conclusion
	Guinea pig preimmune serum	Guinea pig immune serum	Assay buffer		
Positive control	30,656	7,288	25,392		
139537	382	462	463	Negative	Negative
139538	1,474	603	1,659	Negative	Positive, false-negative by isolation
140796	37,943	4,453	39,309	Negative	Positive, false-negative by isolation
148629	6,977	1,387	8,299	Negative	Positive, false-negative by isolation
148630	2,924	704	3,360	Positive	Positive
150003	542	548	486	Positive	Positive, false-negative by TR-FIA

FIG. 1. Distribution of TR-FIA values for RSV antigen detection compared with virus isolation in the two study groups. The S/N values were calculated by dividing the specimen counts per second by the negative-control counts per second. Values above the line indicate positive by TR-FIA. The mean $\pm$ standard deviation of negative-control values (reagent background) of 70 assay runs was 761 ± 331 cps. See the footnote to Table 1 for descriptions of the groups. -, Negative isolation result; +, positive isolation result.

The sensitivity of RSV antigen detection by enzyme immunoassay has recently been reported to be about 90% of that obtained by virus isolation (12, 13, 19). In our study, 95% of isolation-positive specimens were detected by TR-FIA. In addition, nine isolation-negative specimens were positive by the RSV TR-FIA, and all were confirmed as true positives by the blocking assay. Inadequate treatment of the isolation specimens or a situation in which samples were taken from recovering patients who were still secreting viral antigens but no infectious virus could explain the failure to isolate RSV from these patients. From one patient with bronchitis, a confirmed positive result was obtained only by TR-FIA as late as 30 days after the onset of symptoms. The efficacy of RSV isolation may be enhanced by including

several cell lines (1). On the other hand, the sensitivity of virus isolation was increased in group 1 of our study by immunoassay of cultures that were negative or ambiguous as judged by cytopathic effect. We have found our human diploid fibroblast cell line to be at least as sensitive as the HEp-2, Vero, and HeLa cell lines for isolation of RSV. However, year-to-year variation in host cell sensitivity has been reported (1).

RSV can be divided into two distinct subgroups, A and B, which show variation in reactivity with MAbs (15, 16). The MAbs RSV4 and NC4 have been developed with RSV A2 (subgroup A) nucleocapsid as the immunization antigen (17). However, recent findings indicate that the nucleoprotein of RSV is antigenically more stable than the glycoproteins of

FIG. 2. Monthly results of detection of RSV (■) and other respiratory virus (□) antigens by TR-FIA during 1987. The total number of specimens tested is indicated above each bar.

the viral envelope (18). Thus, the positive reactivity with strains of both subgroups ensures that the MAbs used in our RSV TR-FIA are against conserved epitopes of RSV nucleoprotein, and the sensitivity and specificity of the assay should therefore not be influenced by epidemiological strain variations. As a minor problem associated with the target protein of our assay, we have found that the reactivity of the RSV nucleoprotein decreases during storage in our specimen library at -20°C . This fact can affect the result of a confirmatory assay made afterwards but is partially circumvented if a stored NPS is resonicated after thawing. Some simple chemical treatment of specimens without sonication but with stabilization of the nucleoprotein would improve the assay.

The TR-FIA described here is a sensitive and specific test for detection of RSV antigens in clinical specimens. The short incubation time of only 60 min allows at least 20 specimens to be tested (including pretreatment) in 2 to 3 h and reported on the day they arrive at the laboratory. The simple assay procedure and short incubation time are well suited to the routine of a virological laboratory, particularly if the same specimens are simultaneously tested for the presence of other viral antigens by similar assays.

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