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Time-resolved fluoroimmunoassays with monoclonal antibodies for rapid identification of parainfluenza type 4 and mumps viruses

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Summary. Monoclonal antibodies were prepared to the F and M proteins of parainfluenza 4A and 4B and to mumpsvirus to obtain reagents that could be configured into type-specific yet broadly-reactive IFA, EIA, and TR-FIA tests. Several antibodies to parainfluenza 4A also detected subtype 4B, although to a somewhat lower signal, and thus were well suited to generic parainfluenza type 4 tests that were comparable to similar tests previously described for parainfluenza types 1, 2, and 3. Monoclonals to subtype 4B were less able to detect 4A because of high background problems in one or another test. Monoclonals to mumpsvirus F protein were completely type-specific. These antibodies were screened by IFA and EIA for broad reactivity with diverse strains of each virus and were configured into optimized EIA and TR-FIA tests. The all-monoclonal tests were then compared to polyclonal tests in terms of their ability to detect virus in clinical specimens. The all-monoclonal TR-FIA was uniformly the most sensitive, detecting virus in 80% of culture-positive parainfluenza 4A specimens, 67% of parainfluenza 4B specimens, and 90% of mumps specimens, compared to 40–67% for the monoclonal EIA tests and 33–60% for the polyclonal EIA tests. For parainfluenza 4 TR-FIA, mean P/N values were 379 for subtype 4A cell culture fluids (228 for subtype 4B cultures) and 57 for 4A clinical specimens (43 for 4B specimens). For mumpsvirus TR-FIA, mean P/N values were 27 for culture fluids and 32 for clinical specimens. The sensitivities of the TR-FIA were determined with purified virus to be 0.28 ng virus per well for parainfluenza 4A and 0.70 ng virus per well for mumpsvirus.

Introduction

Rapid antigen tests for respiratory viruses are becoming more frequently utilized as high-quality diagnostic products reach commercial availability and the con-

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venience and practicality of rapid testing become appreciated in the clinical laboratory. This is particularly true for adenovirus, respiratory syncytial virus, parainfluenza viruses 1–3, and measles virus [13]. Rapid tests are not presently available for parainfluenza types 4A and 4B, owing both to the difficulties of isolating and identifying parainfluenza 4 [6, 7, 22–24, 27, 29] and the lack of good epidemiologic data on the prevalence and pathogenicity of this virus [3, 6, 31, 41]. Rapid tests for mumpsvirus have also lagged behind, largely because the incidence of mumps outbreaks has declined over recent years [11, 12, 34] and the infection is often diagnosed on clinical grounds alone [39, 42]. However, rapid tests need to be available for both infections because most parainfluenza 4 infections remain undiagnosed from lack of testing, and the parotid gland swelling that is typical of mumps virus can also be caused by other viruses such as parainfluenza 3 [44].

In this report, we describe the production of monoclonal antibodies to parainfluenza 4 and mumps viruses and describe their use in IFA, EIA, and time-resolved fluoroimmunoassay (TR-FIA), with emphasis on the TR-FIA as a highly-sensitive rapid antigen test [8, 9, 14, 30, 38].

Materials and methods

Viruses and cell culture

The prototype strains of parainfluenza 4A (M-25), 4B (CH-19503), and mumps (Enders) were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD) at the MK_{14}, MK_9, and MK_6 passage levels, respectively. Working stock passages of the prototypes and of multiple isolates of each virus type were obtained from our reference virus collection. Older isolates were initially recovered from clinical specimens by passage in primary rhesus monkey kidney (MK) cell cultures and identified by hemagglutination-inhibition (HI) and serum neutralization (SN) tests [2, 9, 18, 19]. More recent isolates of the paramyxoviruses were made in NCI-H292 cells (ATCC #CRL-1848), which are a proven substitute for MK cells [5]. All passages used in this study were made in H292 cells, and all IFA and SN tests also were carried out in H292 cells.

Antisera

Polyclonal rabbit, guinea pig, horse, and ferret antisera used in some EIA formats were prepared against crude virus supernatants by standard procedures [9, 17, 37].

Production of monoclonals

Monoclones to parainfluenza 4A and 4B and mumps viruses were prepared by modifications of our previous procedures [2, 17]. A basic Opti-MEM medium with 0.1% 2-mercaptoethanol (#320-1985, Gibco Laboratories, Grand Island, NY) was used throughout, with glutamine and penicillin-streptomycin added. Balb/C mice were immunized with crude virus supernatants that were clarified at 1000 × **g** for 10 min at 4 °C and concentrated 15-fold by membrane ultrafiltration (Diaflo PM-10 filters, Amicon Div., W. R. Grace & Co., Beverly, MA). The spleen cells from an immunized mouse were fused with Sp 2/0-Ag 14 myeloma cells (ATCC #CRL-1581) in the presence of 50% PEG (2 ml basic Opti-MEM medium at 37 °C plus 2 ml of melted polyethylene glycol-1500 (#29575, BDH Ltd., Poole, England), filter-sterilized), and the hybridoma cells were selectively grown out by standard media.

Wells with clusters of cells were screened for antibody production by the tissue culture EIA procedure [1]. For parainfluenza 4A, H 292 cells at 20,000 cells per well in 100 µl of growth medium were added to all wells of 96-well tissue culture plates (# 3596, Costar, Cambridge, MA). Entire rows were alternately supplemented with 50 µl of growth medium for the cell control wells or infected with a standard dose of virus in 50 µl to give minimal CPE after 4–5 days of incubation at 36.5 °C under CO_2. The plates were then washed 3 × (see EIA methods), fixed with 75 µl of 80% acetone/20% PBS for 20 min at 4 °C, air-dried, and stored in protective plastic at − 20 °C until needed. For testing, 50 µl of PBS/GT diluent (see EIA methods) was added to all wells, 25 µl of supernatant from the hybridoma wells was added into a virus well and a cell control well, and the test incubated for 1 h at 37 °C in a moist chamber. The plates were then washed 3 × and 75 µl of goat anti-mouse peroxidase (# 14-18-06, Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a 1 : 3,000 dilution in PBS-GT was added; the test was incubated for 1 h at 37 °C as above. After a 6 × wash step, 125 µl of TMB (see EIA methods) was added, reacted 15 min at ambient temperature, stopped with 2 M sulfuric acid, and read spectrophotometrically at 450 nm. Monoclones for parainfluenza 4B were screened in the same manner, with 4B virus infecting the cells.

For mumpsvirus, the tissue culture EIA, done both in H 292 cells and in LLC-MK 2 cells, was not as satisfactory as direct antigen-coated plates. Thus, mumpsvirus in NCI-H 292 cells was diluted to 1 : 10 in the pH 9.6 carbonate buffer and added to alternate rows of 96-well plates, at 75 µl/well. H 292 cell control material was diluted in the same fashion and added to wells for the cell control rows. The plates were incubated overnight at 4 °C in a moist chamber, then washed 3 ×. For testing, 50 µl/well of PBS-GT diluent and 25 µl/well of hybridoma culture supernatant were added to parallel virus and control wells, and incubated 1 h at 37 °C moist. The plates were washed 3 ×, 75 µl/well of anti-mouse peroxidase at 1 : 3,000 in PBS-GT was added, and the test was incubated 1 h at 37 °C moist. The plates were then washed and developed with TMB, followed by the acid stop and reading, as in the parainfluenza 4 procedure above.

Positive cultures were cloned by limiting dilutions and again screened for specific antibody. Mouse ascitic fluids were prepared, clarified by centrifugation at 2,850 × **g** for 30 min at 4 °C, pooled, and stored at − 80 °C. These ascitic fluid-derived monoclonal antibodies were tested by the same EIA procedures as the cell culture supernatants, except that they were titrated from 10^{-2} to 10^{-9} dilutions.

Immunoglobulin subclass determination

Monoclone cells were recovered from storage by rapid thaw in a 37 °C water bath, immediate washing with 15 ml of Opti-MEM/6% FCS to remove the DMSO, resuspension in this growth medium, and transfer to an appropriate plate for incubation. Supernatant fluid was then tested in EIA at 1 : 100 and 1 : 500 dilutions against a standard panel of biotinylated subclass antibodies (# 97-6550, Zymed Laboratories, San Francisco, CA) according to the manufacturer's instructions.

Western blots

Parainfluenza 4A, 4B, and mumps viruses were grown in NCI-H 292 cells under RPMI-1640 medium with 1.5 µg/ml trypsin for 3–4 days (when cytopathology just became evident), then the monolayers were washed 1 × with PBS, overlayed with PBS, and incubated 1 additional day. The cultures were harvested by scraping, 2 × freeze-thaws, and light centrifugation. The supernatant fluids containing the viral proteins were solubilized and electrophoresed under both reducing and nonreducing conditions on 10% acrylamide slab gels; molecular weight markers (# MW-SDS-200, Sigma) were biotinylated and included in each gel. The proteins were electrophoretically transferred onto nitrocellulose paper (# 40-21640,

Schleicher & Schuell, Keene, NH), reacted with antibody at 1 : 10–1 : 30 dilutions and goat anti-mouse peroxidase at 1 : 3,000 (or avidin-peroxidase (# A-3151, Sigma) at 1 : 5,000 for the MW markers), and visualized after color development with 3,3′-diaminobenzidine (# D-5637, Sigma) in 3% H_2O_2 [40].

Specimens

Nasopharyngeal aspirates, throat swabs, and nasal swabs positive for parainfluenza 4 or mumps viruses were obtained through our diagnostic service, cultivated for 1–3 passages in H 292 cells, and identified by HA/HI and SN tests. Specimens positive for other respiratory viruses were obtained from this service and from prior studies [8, 9, 15, 16, 37]. All specimens had been stored at − 80 °C, some as a 1 : 5 dilution in PBS containing 20% FCS, 2% Tween-20, and 0.004% merthiolate [9, 37].

Indirect fluorescent antibody test (IFA)

IFA was carried out as previously described [13]. Briefly, 0.5 ml of H 292 cell suspension in growth medium was added to each chamber of 8-chamber sterile slides and incubated under CO_2 at 37 °C for 3 days. The growth medium was decanted and each chamber inoculated with 0.1 MOI of virus. After adsorption for 1 h at RT, 0.45 ml of maintenance medium was added to each chamber and the slides incubated under CO_2 for 2–4 days or until cytopathic effects (CPE) just became evident. The cultures were fixed in cold acetone for 10 min, air dried, and used immediately or stored at − 20 °C sealed in plastic bags. Serial dilutions of monoclonal antibody in PBS were added to appropriate monolayers (30 µl/spot) and incubated 30 min at 37 °C in a humidified chamber. The slides were rinsed with PBS, washed 3 × in PBS with stirring at ambient temperature for 10 min each, and air dried. Finally, 30 µl of 1 : 20 dilution of goat anti-mouse IgG-FITC conjugate (# 02-18-02, Kirkegaard & Perry) was added to each spot and incubated for 30 min at 37 °C in a moist chamber; the slides were washed 3 × as above, air dried, and read at 250 × without oil under incident fluorescence.

Biotinylation

Purified IgG at 1 mg/ml concentration was dialyzed overnight against 0.1 M $NaHCO_3$ at 4 °C and reacted with 0.12 ml of 1 mg/ml biotin (N-biotinyl-ω-aminocaproic acid-N-hydroxysuccinimide ester; # EBP-406, Enzo Biochemical Inc., New York, NY) in DMSO with mild agitation at room temperature for 4 h; the pH was ~ 8.5. The mixture was then dialyzed against PBS (0.01 M phosphate buffer, pH 7.2, with 0.85% NaCl) overnight at 4 °C. The biotinylated IgG was stored undiluted at − 80 °C as a permanent stock or as a 1 : 10 dilution at 4 °C for years barring contamination.

Enzyme immunoassays (EIA)

The EIA procedures were modifications of those previously described [15, 16]. EIA Format 1 was an all-monoclonal test designed to parallel that used for the TR-FIA as closely as possible. This format required the use of biotinylated IgG, which had been purified as for TR-FIA and tagged with biotin. The EIA was done with 96-well flat-bottom polystyrene microtiter plates (Immulon-2, Dynatech Laboratories, Chantilly, VA) as the solid phase. The specific monoclonal antibody (MAb) (as capture antibody) as purified IgG diluted in carbonate buffer, pH 9.6 was added at 75 µl/well, incubated overnight at 4 °C, and washed 3 ×. The EIA wash buffer used throughout all the EIAs in this study was 0.01 M PBS, pH 7.2, with 0.05% Tween-20 (# P-1379, Sigma). The specimen (NPA, culture supernatant, etc.) at 75 µl/well was added at 1 : 5 dilution in PBS-GT diluent (0.01 M PBS, pH 7.2, with

0.5% gelatin and 0.15% Tween-20) or in the TR-FIA antigen diluent, incubated 1.5 h at 37 °C, and washed 3 ×. Next, the biotinylated detector antibody diluted in PBS-GT was added at 75 µl/well, incubated for 1 h at 37 °C, and the test washed 3 ×. Streptavidin/peroxidase conjugate (#RPN-1231, Amersham International, Amersham, U.K.) diluted in PBS-GT was added at 75 µl/well, and the test was incubated for 10 min at ambient temperature and washed 6 ×. Finally, 125 µl/well of substrate was added: 0.1 mg/ml of 3,3′,5,5′-tetramethyl-benzidine (TMB; #T-2885, Sigma) in 2% DMSO in 0.1 M acetate/citrate buffer, pH 5.5, with 0.005% hydrogen peroxide added when used [10]. The test was incubated for 15 min at ambient temperature, the color reaction stopped with 2 M sulfuric acid, and read at 450 nm in an EIA reader (Dynatech Model MR 5000).

EIA Format 2 for each virus was our optimal polyclonal test derived from hyperimmune antisera. The steps were the same as for Format 1 except that the capture antibody was a polyclonal IgG and the detector antibody was the same IgG after biotinylation. When a different species IgG was tried as detector, followed by the appropriate anti-species IgG-peroxidase conjugate, the diluent for the detector was tried with 2% normal goat serum or 1.5–2% normal species serum, as needed to reduce background signal; the same diluent was then used for the conjugate, with incubation for 1 h at 37 °C followed by a 6 × wash step [14].

Time-resolved fluoroimmunoassay

Purification and labeling of MAbs

A 5 ml sample of mouse ascitic fluid was clarified, dialyzed against 0.05 M Tris-HCl buffer, pH 8.0, and loaded onto a DEAE-Sephacel column (Pharmacia/LKB Biotechnology, Inc., Piscataway, NJ) followed by a salt gradient from 0 to 0.225 M NaCl in the Tris buffer [16]. Eluted proteins were tested by EIA for IgG activity at the 10^{-2} and 10^{-4} dilutions. IgG-positive fractions were pooled, concentrated to 10 ml by ultrafiltration, and precipitated by 50% ammonium sulfate. The protein pellet after centrifugation was resuspended in 2 ml of PBS and dialyzed overnight against PBS. Protein concentration was estimated by absorbance measurements at 280 nm. Alternatively, IgG was purified by affinity chromatography using GammaBind G-Agarose (Genex Corp., Gaithersburg, MD), in which the IgG was bound at neutral pH, eluted with 8 M urea or 0.5 M ammonium acetate, pH 3.0, and then neutralized as appropriate. The purified antibody was divided into 3 parts: some was kept plain to use as capture antibody in both EIA and TR-FIA tests; some was labeled with biotin to use in biotin-avidin EIAs; and some was conjugated with Eu^{3+} to use as detector antibody in TR-FIA.

For europium labeling, the chelate [N′-diethylene triaminopentaacetic acid (DTPA)-europium; Delfia Eu-labeling kit #1244-302, Pharmacia Diagnostics, Fairfield, NJ] was prepared as a 1 mg/150 µl solution in distilled water. It was mixed with the IgG at a molar excess of 50 in 1 M carbonate buffer, pH 9.2. The reaction was incubated overnight at 4 °C, brought to ambient temperature for 2 h, and then stopped by purifying the labeled complex by exclusion chromatography [16]. Fractions of 1 ml were diluted 1 : 5,000 in enhancement solution (Delfia #1244-105, Pharmacia Diagnostics), and counted in a single-photon-counting Model 1232 Arcus fluorometer (Wallac Oy, Turku, Finland). The peak fractions containing IgG (by absorbance at 280 nm) were pooled and BSA was added to 1% concentration for storage at − 80 °C.

One-incubation TR-FIA procedure

Purified monoclonal IgG, diluted to optimal concentration in pH 9.6 carbonate buffer, was added to flat-bottomed wells of 12-well polystyrene strips (Flow Titertek #78-591-99, ICN Biomedicals, Inc., Horsham, PA) in 250 µl volumes and adsorbed overnight at ambient

temperature in a moist chamber. The wells were washed 3 × with aqueous 0.9% NaCl/ 0.05% Tween-20 or with a commercial wash (Delfia #1244-114, Pharmacia Diagnostics) in an automated Platewash (Model 1296-024, Wallac Oy); as in EIA, all wash steps were critical and were performed with care. Wells were then saturated with 250 µl of 0.1% gelatin (Difco, Detroit, MI) in 0.05 M Tris/0.9% NaCl/0.05% NaN_3 buffer, pH 7.75, again with overnight incubation at ambient temperature. After removing the excess volume (leaving ~ 25 µl/well), the strips could be stored up to 1.5 years at 4 °C at this point with no loss of activity.

For the test, the wells were washed 3 ×, and 100 µl each of antigen and Eu-detector antibody were added to appropriate wells. The antigen (NPA or cell culture harvest) was diluted 1:5 in specimen diluent, consisting of 50 mM Tris, pH 7.75, 0.9% NaCl, 0.01% NaN_3, 0.5% gelatin, 0.01% Tween-40, 20 µM DTPA, and 2% BSA. The Eu-detector antibody was diluted to the appropriate concentration in the same diluent. After the antigen and antibody were added, the strips were incubated for 1 h at 37 °C in a moist chamber. The strips were carefully washed 6 ×, and 200 µl per well of enhancement solution was added. The plates were gently agitated on a shaker (Plateshake Model 1296–002, Wallac Oy) for 10 min at ambient temperature and then placed in the fluorometer for counting. The fluorometer was programmed to take the mean and coefficient of variation (CV) of 12 reagent blanks and to take the mean and CV of the duplicates or triplicates of each specimen minus the reagent blanks. We then further analyzed the printed data by computing the mean and standard deviation (SD) of the negative specimens run in the same test, and used this mean + 3 SDs as the cut-off value for positive specimens [14–16].

Results

Evaluation of monoclonals

Mouse ascitic fluids to all monoclonals which possessed useable homologous titers by IFA and EIA were considered for use in TR-FIA. The protein specificity of these clones was determined by western blots, in which animal hyperimmune antisera were first reacted with the electrophoresed viral proteins to confirm the presence of all proteins in the test lanes, and replicate lanes were then reacted with the individual monoclonals (Fig. 1). Identification of the protein specificity was based on molecular weight and the biological characteristics of the monoclonal (see Tables 1 and 2).

Characterization and labeling data for these antibodies are listed in Table 1. Most had type-specific EIA titers of $> 10^{-4}$ after purification; their Eu: IgG molar ratios varied from 3.3 to 6.5, indicating acceptable labeling of the antibody. Most of the clones with suitable type-specificity but broad reactivity were directed against the fusion (F) protein; one was to the matrix (M) protein. None were directed against the hemagglutinin/neuraminidase (HN), which was the best antibody for similarly-constructed parainfluenza 2 and 3 tests [15].

All antibodies and other reagents (FITC conjugate, streptavidin peroxidase) were titrated to optimal endpoints in checkerboard fashion, so that each test system used all reactions at their optimal dilution to give the highest signal-to-background ratio. Antibody titers were then determined with the prototype virus strains in these tests (Table 2). Heterologous IFA and EIA tests showed that three of the four 4A clones were of sufficiently high titer with both 4A and 4B viruses to act as a "parainfluenza type 4" test; in EIA, all three were

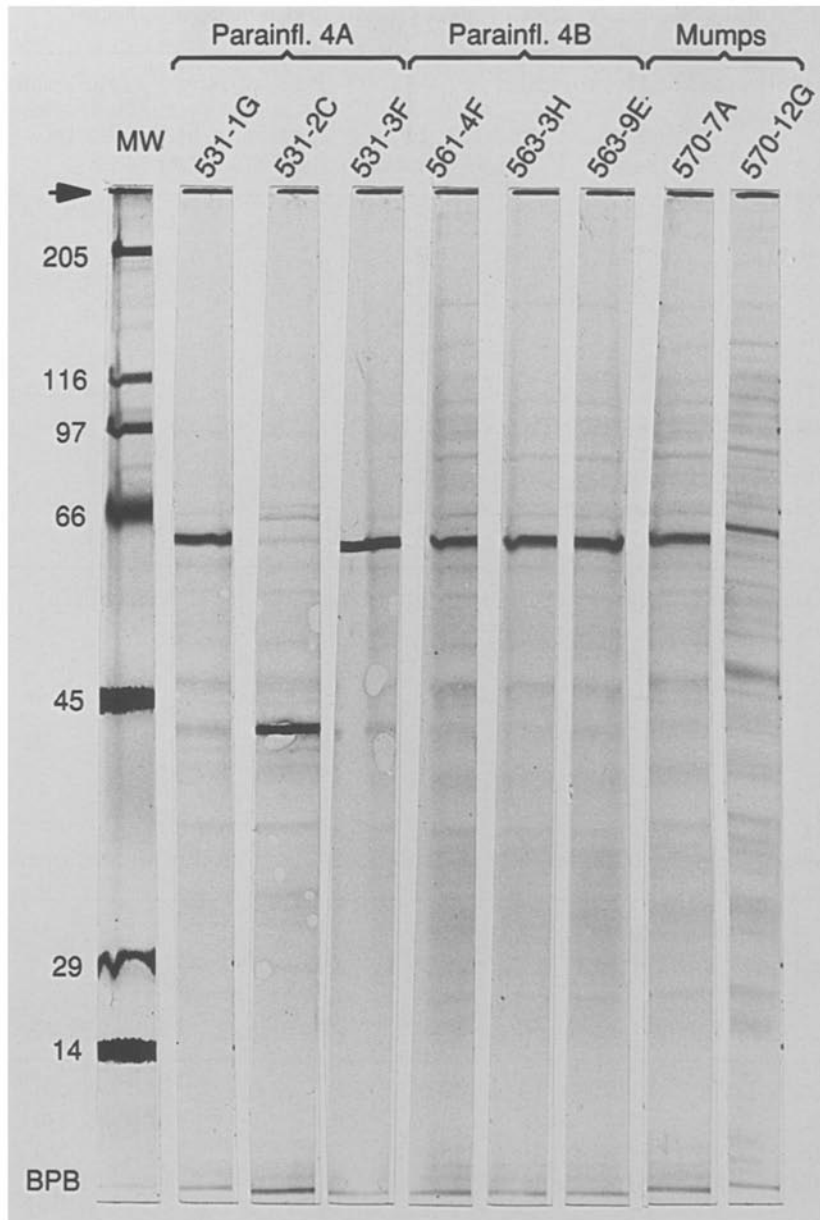

Fig. 1. Western blot of parainfluenza 4 and mumps monoclonal antibodies to identify their protein specificities. Prototype virus strains were grown in H 292 cells, washed in PBS, and electrophoresed in 10% acrylamide gels. The separated proteins were transblotted onto nitrocellulose paper, which was cut into strips, reacted with low dilutions of each antibody, and developed with goat anti-mouse peroxidase and the diaminobenzidine color system. Molecular weight (MW) markers are on the left; the arrow indicates the start position of the proteins during electrophoresis; *BPB* bromphenol blue tracking dye. All the monoclonals were suggested to be anti-fusion (F) clones (MW ~ 61 kDa), except #531-2C (anti-M, MW ~ 40 kDa)

Table 1. Characteristics of monoclonals and labeled antibodies[a]

Virus	Monoclone number	MAb properties			Purified Ab		Eu^{3+}-labeled Ab	
		immun. strain	protein specif.	IgG subcl.	protein (mg/ml)	EIA titer	Eu/IgG ratio	protein (mg/ml)
PI-4A	531-1G	M-25	F	2 Ak	15.14	6.3	3.7	0.266
	531-2C	M-25	M	2 Ak	22.39	6.1	3.7	0.259
	531-3F	M-25	F	2 Ak	25.62	7.3	3.3	0.268
	531-5D	M-25	F	2 Ak	2.57	5.6	6.0	0.255
PI-4B	561-4F	CH-19503	F	1 k	8.88	4.5	5.6	0.249
	563-3H	CH-19503	F	2 Bk	20.83	3.0	5.5	0.217
	563-9E	CH-19503	F	1 k	14.75	4.0	5.6	0.232
Mumps	570-7A	Enders	F	1 k	10.25	6.0	6.3	0.232
	570-12G	Enders	F	1 k	10.22	6.0	6.5	0.342
	572-10H	Enders	ND	2 Ak	6.20	2.0	6.1	0.341

[a] MAbs chosen for high reactivity with multiple strains by IFA and EIA
ND Not determined

Table 2. Cross-reactions among parainfluenza 4 and mumps monoclonals[a]

Monoclonal antibody	Virus tested by								
	IFA			EIA			SN		
	PI-4A	PI-4B	mumps	PI-4A	PI-4B	mumps	PI-4A	PI-4B	mumps
4A: 531-1G	5120	80	< 10	10.6	6.8	1.0	300	50	< 10
531-2C	1280	20	< 10	11.0	8.5	1.0	300	30	< 10
531-3F	2560	40	< 10	3.0	2.7	1.0	100	10	< 10
531-5D	640	20	< 10	11.0	7.1	1.0	50	10	< 10
4B: 561-4F	20	160	< 10	4.8	6.2	1.0	30	30	< 10
563-3H	40	40	< 10	3.8	4.8	1.0	100	500	< 10
563-9E	40	40	< 10	4.6	5.8	1.0	100	700	< 10
Mu: 570-7A	< 10	< 10	1280	1.0	1.0	17.6	< 10	< 10	30
570-12G	< 10	< 10	1280	1.0	1.0	13.9	< 10	< 10	10
572-10H	< 10	< 10	1280	1.0	1.0	1.2	< 10	< 10	100

[a] Numbers are endpoint dilutions with prototype virus in each test. For IFA and SN, titers are shown as reciprocals of antibody dilution. For EIA, titers are shown as P/N values for the optimally-diluted homologous antibody format, that is, the same monoclonal antibody used for capture antibody (as purified IgG) was also used for detector antibody after biotinylation

more reactive with parainfluenza 4B virus than any of the 4B monoclonals. The mumps monoclonals were type-specific in both tests. In SN tests with 50 $TCID_{50}$s of virus, read by hemadsorption with guinea pig erythrocytes after 7 days of incubation on a roller drum at 36.5 °C, all of the selected monoclonals had at least some titer, ranging from 1 : 10 in the mumps 570-12 G antibody to 1 : 700 in the parainfluenza 4B 563-9 E antibody. As in IFA and EIA, extensive crossing was observed in both directions between subtypes 4A and 4B, but none with mumpsvirus in either direction. HI tests with the monoclonals versus 4 HA units of each prototype virus showed no HI activity ($< 1:2$) in any of the antibodies.

The selected antibodies were further evaluated by testing against a large panel of homotypic and heterologous virus strains. 17 strains of parainfluenza 4A or 4B and 18 strains of mumpsvirus isolated over a broad range of time and place were correctly identified by the respective monoclonals, with much cross-reaction between 4A and 4B as expected, but no crossing with mumpsvirus in either direction. (These 35 isolates were simultaneously negative for RSV and parainfluenza types 1, 2, and 3 in IFA and EIA tests.) Further, all heterologous IFA and EIA tests with cell culture supernatants were negative: 5 each of parainfluenza 1, 2, and 3 strains, 8 adenoviruses, 10 RSV isolates, 3 measles, 3 influenza A, 2 influenza B, 8 herpes 1 and 2, and 1 each of coxsackie A 9, coxsackie B 2, polio 2, and echo 4 isolates.

Formatting the EIAs

The monoclonal EIA (Format 1) for each virus using the same antibodies as TR-FIA was optimized by checkerboard titration as above. We also biotinylated the other purified antibodies and tested all possible combinations in EIA. The optimal monoclonal EIA formats for antigen detection are given in Table 3. For parainfluenza 4 viruses, several capture/detector combinations were equally sensitive in detecting multiple strains of virus, but the most sensitive combination was not the one chosen as the best TR-FIA format. Antibody 531-5 D as capture with 531-2 C as detector gave OD values ranging from 0.414 to 1.554 with subtype 4A cultures, 0.328 to 1.308 with subtype 4B cultures, and 0.045 to 0.089 with negative cell controls; the mean negative was 0.063, the mean P/N with 4A was 17.8 (range 6.6–24.7), and the mean P/N with 4B was 15.5 (range 5.2–20.8). Thus, this EIA test could detect subtype 4A strains with 13% greater sensitivity than subtype 4B strains. By contrast, the optimal TR-FIA format (531-1 G capture/531-3 F detector) gave a mean negative OD value of 0.080, a mean P/N with 4A of 7.1, and a mean P/N with 4B of 2.4. For mumpsvirus, the optimal Format 1 was monoclonal 570-12 G as capture with 570-7 A as detector; this combination gave OD values ranging from 0.823 to 2.227 with mumps cultures and 0.046 to 0.090 with negative cell controls; the mean negative was 0.067 and the mean P/N with mumps strains was 25.1 (range 12.3–33.2). By contrast, the optimal TR-FIA format (570-12 G capture/570-12 G detector)

Table 3. Parameters for optimal EIA tests for antigen detection[a]

Capture antibody		Antigen dilution	Detector antibody		Developing system	
antiserum	dilution		antiserum	dilution	conjugate	dilution
Format 1: Monoclonal sera[b]						
PI-4 531-5D	1:3,000	1:5	531-2C/biotin	1:3,000	streptav.-perox.	1:3,000
Mumps 570-12G	1:3,000	1:5	570-7A/biotin	1:3,000	streptav.-perox.	1:3,000
Format 2: Polyclonal sera						
PI-4A g. pig	1:15,000	1:5	g. pig/biotin	1:1,500	streptav.-perox.	1:3,000
PI-4B ferret	1:1,500	1:5	ferret/biotin	1:1,500	streptav.-perox.	1:3,000
Mumps horse	1:15,000	1:5	horse/biotin	1:1,500	streptav.-perox.	1:3,000

[a] Diluent for capture antibodies was pH 9.6 carbonate buffer, incubated overnight at 4 °C. Antigen was diluted in TR-FIA antigen diluent; incubation was 1 1/2 h, 37 °C. Diluent for both format's detector antibodies was PBS-GT; incubation was 1 h, 37 °C. The developing system for both formats was diluted in PBS-GT and incubated 10 min at ambient temperature

[b] Parainfluenza 4B was not specifically subtyped by any of the parainfluenza 4 monoclones, but was best identified as parainfluenza 4 by the subtype 4A format shown

gave a mean negative OD value of 0.100 and a mean P/N with mumps strains of 15.6.

The polyclonal EIAs (Format 2) also were optimized as shown in Table 3 but were of much less utility because of relatively low signals but high background values in the controls. The parainfluenza 4A test with guinea pig antibody gave OD values ranging from 0.315 to 0.524 with subtype 4A cultures, 0.322 to 0.450 with subtype 4B cultures, and 0.189 to 0.298 with negative cell controls; the mean negative was 0.216, the mean P/N with 4A was 2.4, and the mean P/N with 4B was 2.0. The parainfluenza 4B test with ferret antibody gave similar OD values; the mean P/N ratio with 4A was 2.0 and with 4B was 2.1. For mumpsvirus, the optimal Format 2 was a horse antibody in both capture and detector positions; this combination gave OD values ranging from 0.232 to 0.327 with mumps cultures and 0.056 to 0.088 with negative cell controls; the mean negative was 0.069 and the mean P/N with mumps strains was 4.3 (range 3.4–4.8). For all 3 viruses as cell culture supernatants, the all-monoclonal format was clearly more sensitive than the polyclonal test in antigen detection; limited testing with NPA specimens confirmed both the optimally-sensitive tests described above and the monoclonal tests as superior to polyclonal tests.

Formatting the TR-FIA

The one-incubation TR-FIA was formatted for each virus by testing all combinations of the monoclonals at serial dilutions with tissue culture isolates and NPA or TS specimens. The optimization utilized checkerboard titrations of capture antibodies at 0.25, 0.5, and 1.0 μg/well and detector antibodies at 6.25,

12.5, 25, 50, and 100 ng/well. For each concentration of capture antibody, the optimal concentration of detector antibody was determined by the P/N ratio for representative positive and negative specimens to obtain the greatest sensitivity, as has been shown previously [14]. In the TR-FIA, system background was the mean of 12 reagent (system) controls and was automatically subtracted out when samples were run. Test background was measured as the fluorescence values in known negative specimens that were tested in parallel with positive specimens; thus, a P/N value was the mean of the replicate tests for a positive specimen divided by the mean of all the negative specimens tested (in replicates) in the run.

The P/N values were used to ascertain the best format in TR-FIA (Table 4). Of 10 MAbs evaluated in this study, 6 were found to be useable in either capture or detector position in the TR-FIA. For parainfluenza 4A, clone 531-1 G in capture position with clone 531-3 F in detector position gave a mean P/N value of 379 with subtype 4A cultures and 228 with subtype 4B cultures, and a mean P/N of 57 with 4A clinical specimens and 43 with 4B specimens. As a parainfluenza type 4 test, therefore, this format is 40% more sensitive with 4A cultures than 4B ones and 25% more sensitive with 4A original specimens than 4B ones in terms of actual signal. In 1 : 5-diluted test samples, signals for parainfluenza 4A cultures ranged from 17,336 to 90,628 (mean = 44,978) cps and for 4A clinical specimens were 217–25,146 (mean = 9,737) cps, and for subtype 4B cultures ranged from 7,749 to 41,733 (mean = 27,058) cps and clinical specimens, 297–21,254 (mean = 7,346) cps. The 531-1 G/531-3 F format

Table 4. Comparison of the most sensitive combinations of monoclonals for TR-FIA tests[a]

Virus	Capture antibody	Conc. (μg/well)	Detector antibody	Conc. (ng/100 μl)	Mean P/N of specimens[b]	
					clinical	cultures
PI-4A:	*531-1G*	*0.25*	*531-3F*	25	57(43)	379(228)
	531-3F	0.50	531-3F	50	28(17)	269(79)
	531-2C	0.25	531-3F	25	23(19)	181(64)
	531-5D	0.50	531-2C	50	18(12)	118(59)
PI-4B:	561-4F	0.50	563-3H	50	ND	2(23)
	563-9E	0.25	563-3H	50	ND	2(13)
	561-4F	0.25	563-3H	50	ND	2(12)
Mumps:	*570-*12G	*0.50*	*570-12G*	*25*	32	27
	570-12G	0.50	570-7A	25	19	18
	570-12G	0.50	570-12G	50	3	3

[a] Italics denotes optimal test system for direct detection TR-FIA; parainfluenza 4B is best detected by the optimal subtype 4A format

[b] Positive/negative (*P/N*) ratio for parainfluenza 4 formats is listed as the mean ratio with PI-4A specimens followed by the mean ratio with PI-4B specimens (in brackets)

ND Not done

was thus not only useful as a generic type 4 test but was also, on the average, 51% more sensitive than the next best combination (clone 531-3 F in both positions). The best format utilizing subtype 4B monoclonals (561-4 F/563-3 H) was less sensitive than even the bottom-ranked subtype 4A format (531-5 D/531-2 C), and thus the 4B monoclonals were not considered further.

For mumps, the monoclonals were similarly evaluated in capture and detector antibody positions by checkerboard titrations. Two formats gave good results. The best combination was clone 570-12 G in both capture and detector positions, yielding mean P/N values of 27 with culture supernatants and 32 with original specimens. This was 37% greater than the next best combination (clone 570-12 G capture vs. clone 570-7 A detector). In 1 : 5-diluted test samples, signals for mumps cultures ranged from 13,983 to 68,470 (mean = 47,752) cps and for mumps specimens, 1,134–45,622 (mean = 22,447) cps. As in the EIA tests, the optimized TR-FIA formats for parainfluenza 4 and mumpsvirus were type-specific when tested against the same panel of heterologous viruses used in the IFA and EIA tests (see above).

To determine the dose-response curves for the optimized TR-FIA formats, prototype parainfluenza 4A and mumps viruses were grown in H 292 cells, concentrated 10-fold by ultrafiltration, and purified through glycerol/tartrate gradients at 107,000 × g. The visible band for whole virus at the 1.18 density position was harvested, dialyzed, confirmed to be paramyxovirus by electron microscopy, measured for total protein by the Lowry method, and titrated in TR-FIA. The purified parainfluenza 4A sample had a protein content of 325 μg/ml and a fluorescence value at 1 : 10 dilution of 266,000 cps. The mumps sample had a higher protein content (1,100 μg/ml) but somewhat lower fluorescence (221,000 cps) and a much higher background (539 cps), thus the mumps test was less sensitive than the parainfluenza 4 test. The log-log relationship of signal-to-antigen concentration in the optimized tests is shown in Fig. 2. Defining positive as ⩾ background mean + 3 SDs, the all-monoclonal TR-FIA had a threshold of detection of 0.28 ng/well (2.8 ng/ml) of parainfluenza 4A virus and 0.70 ng/well (7.0 ng/ml) of mumpsvirus; these calculate to 1.05×10^6 and 2.62×10^6 virions per well, respectively.

Comparison of TR-FIA with EIAs using clinical specimens

Using the optimum reagents and dilutions just described, the TR-FIA was compared with EIA Formats 1 and 2 in testing original clinical specimens (Table 5). For parainfluenza 4, the comparative evaluation included 5 subtype 4A-positive specimens, 3 subtype 4B-positive specimens, and 12 negative specimens. Eight negative specimens were positive for adenovirus, RSV, parainfluenza types 1 and 3, or CMV and thus were important controls in the study; the remaining four were negative for any recognized virus by culture and TR-FIA. All specimens were tested at 1 : 5 dilution in the specimen diluent. None of the negative specimens were positive by any of the three tests, but several positive specimens failed to be detected by one or more test. One throat swab

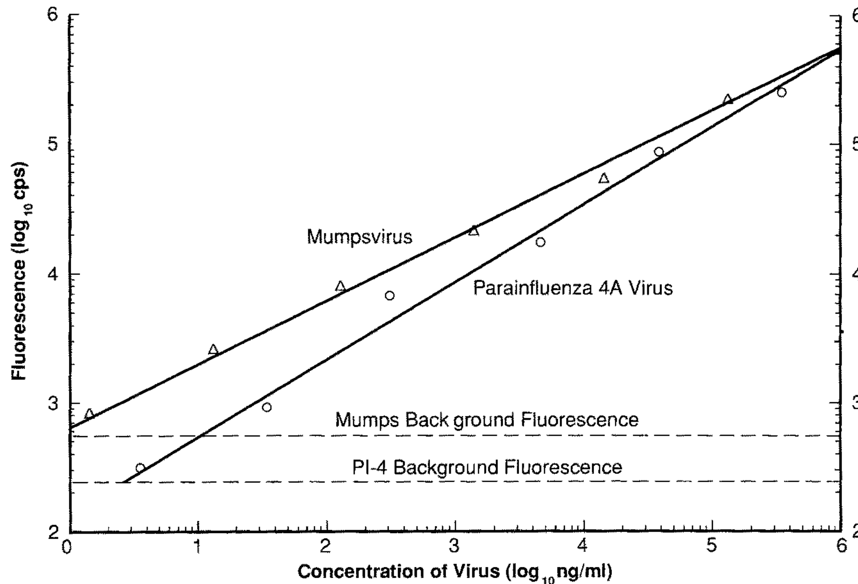

Fig. 2. Dose-response curve for the optimized parainfluenza 4 and mumps TR-FIA tests. Purified virus was titrated to endpoint dilutions in the TR-FIA antigen buffer and quantitated by the optimized tests. The parainfluenza type 4 test can detect 2.8 ng virus per ml and the mumpsvirus test, 7.0 ng virus per ml

Table 5. Comparison of TR-FIA with EIA for detection of antigen in clinical specimens[a]

Specimen (dilution)	TR-FIA	EIA-1 (mono.)	EIA-2(poly.)
PI-4 (1 : 5)			
Negative (n = 12) range	126–482	0.040–0.117	0.082–0.319
Mean ± SD	219 ± 94	0.076 ± 0.021	0.198 ± 0.048
Mean + 3 SDs	501	0.138	0.342
4A pos. (n = 5) range	237–26822	0.082–0.511	0.284–0.512
Number (%) positive	4 (80)	2 (40)	2 (40)
4B pos. (n = 3) range	331–22902	0.092–0.621	0.316–0.475
Number (%) positive	2 (67)	2 (67)	1 (33)
Mumps (1 : 5)			
Negative (n = 14) range	93–2313	0.037–0.108	0.005–0.061
Mean ± SD	878 ± 513	0.068 ± 0.022	0.038 ± 0.012
Mean + 3 SDs	2417	0.134	0.074
Positive (n = 10) range	934–44547	0.069–1.807	0.055–0.628
Number (%) positive	9 (90)	6 (60)	6 (60)

[a] The original specimens tested here included all the NPA and TS specimens tested for Tables 3 and 4; TR-FIA data are mean counts/s; EIA data are mean absorbance at 450 nm

from which parainfluenza 4A was cultured was above the mean negative value but below the mean + 3 SDs for all three tests; 2 other culture-positive throat swab specimens were positive in TR-FIA but negative in both EIAs. For parainfluenza 4B, two of three culture-positive throat swabs were positive by TR-FIA and EIA-1, and one was positive by EIA-2. Thus, the TR-FIA was the most sensitive of these tests, detecting 80% of subtype 4A and 67% of subtype 4B specimens, compared with 40% and 67% detected by the monoclonal EIA, and 40% and 33%, respectively, detected by the polyclonal EIA. Due to the small number of parainfluenza type 4 specimens available, however, these numbers must be considered provisional.

For mumps, 9 of 10 culture-positive specimens were positive by TR-FIA, 6 were positive by monoclonal EIA, and 6 by polyclonal EIA. The single mumps-positive specimen not detected by TR-FIA was also not detected in either EIA, suggesting that the lower percentage rates in the EIA tests were probably due to low-titered specimens which the less-sensitive tests could not detect.

Discussion

Fluorescent antibody tests, although the first rapid antigen tests developed for respiratory viruses, are still widely used in clinical laboratories and were thus important in this study for evaluating the reactivity and specificity of the monoclonal antibodies we prepared. Enzyme immunoassays were even more important for characterizing the monoclonals, because EIA is more sensitive than IFA and is therefore the critical test with which to compare any new test [7–9, 14, 20, 21, 25, 26, 35–37, 39]. Thus, this study included optimized EIA tests in a direct comparison with optimized TR-FIA tests with monoclonal antibodies.

We have shown previously that the TR-FIA with polyclonal antibodies was more sensitive for detecting respiratory virus antigens than other rapid antigen tests [8, 9]. Subsequent studies with monoclonal antibodies revealed even greater sensitivities for detecting influenzavirus [43], adenovirus [16], respiratory syncytial virus [15], and parainfluenza type 1–3 viruses [15] directly in clinical specimens. In the present study, we describe monoclonal antibodies for parainfluenza 4 and mumpsvirus which are useable in similarly-constructed, all-monoclonal TR-FIA formats. Like the other direct-antigen TR-FIA tests, these tests are also highly useful for identifying viruses isolated in cell culture.

Although many paramyxovirus antigens are known to induce neutralizing or type-specific antibodies and should therefore be useable in diagnostic tests, the protein specificities of the monoclonals are nonetheless relevant to the success of the antibodies in TR-FIA. In RSV tests, monoclonals to the fusion (F) glycoprotein were considerably more sensitive in TR-FIA than antibodies to the nucleocapsid (N) protein, a finding thought to be related to the presence of F as the predominant antigen in nasal secretions during natural RSV infection [15]. The optimized TR-FIA for parainfluenza type 1 also utilized anti-F monoclonals, as did the parainfluenza type 4 and mumpsvirus tests in this report.

The parainfluenza type 2 and 3 tests were configured with anti-HN monoclonals [15]. The activity and type-specificity of these surface glycoproteins in TR-FIA, EIA, and IFA tests on clinical specimens are known to be functions of their role in virus entry and induction of neutralizing antibody [6, 14, 20, 25–29, 33–36, 42], a fact consistent with our findings in this study.

The A and B subtypes of parainfluenza type 4 originally described [4, 24] have remained relatively antigenically stable over the past 30 years [6, 27, 29]. We therefore felt that monoclonals could be found which were directed to epitopes shared by both subtypes and thus allow construction of a "parainfluenza 4" test. This was done, and in fact all of the parainfluenza 4A and 4B monoclonals which passed the IFA and EIA selection testing cross-reacted to at least some degree with the other subtype. The development of a generic parainfluenza type 4 test is fortunate because a rapid test that distinguishes type 4 from types 1, 2, and 3 is all that is required in a clinical laboratory; no epidemiological or pathological distinctions have been noted between the subtypes to warrant further subdivision [23, 29, 32].

For parainfluenzavirus type 4, the most sensitive EIA was a monoclonal assay using clone 531-5D as capture antibody and clone 531-2C as detector, and for mumpsvirus was a monoclonal assay with clone 570-12G as capture and clone 570-7A as detector. These tests detected virus in 40%, 67%, and 60% of parainfluenza 4A-, 4B-, and mumps-containing specimens, respectively. The TR-FIA, on the other hand, detected virus in 80%, 67%, and 90% of parainfluenza 4A-, 4B-, and mumps-containing specimens, respectively. These detection rates may change with additional experience with the formatted tests, because the numbers of original specimens that were known culture-positive and were available for testing were too low for an accurate determination.

The optimized TR-FIA formats described herein should be well-suited to the detection of parainfluenza types 4A and 4B and mumpsvirus directly in clinical specimens, to the identification of virus isolates recovered in cell culture, and to much-needed epidemiologic studies of the role of parainfluenza type 4 in upper and lower respiratory disease.

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