Enzyme-Linked Immunosorbent Assay for Detection of Respiratory Syncytial Virus Infection: Development and Description

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An indirect enzyme-linked immunosorbent assay for detection of respiratory syncytial virus (RSV) antigens was developed, using commercially available antisera. Horse anti-RSV and calf antiserum to bovine RSV were used as capture and detector antibodies, respectively. The assay could detect as few as 50 PFU of unpurified RSV per ml in infected cell culture supernatant fluids and as little as 10 ng of affinity-purified RSV antigen per ml. No cross-reactions were observed with heterologous virus types. Freeze-thaw treatment had no effect on RSV enzyme-linked immunosorbent assay titers, but viral transport medium inhibited RSV enzyme-linked immunosorbent assay titers from 10- to 100-fold. The assay can be easily performed in 24 h and is a sensitive and specific method for the detection of RSV antigens.

Rapid methods of virus detection which are not dependent on replication in cell culture have gained popularity as a means of accelerating viral diagnosis (11). Immunofluorescence has been used extensively as a means of direct detection for a variety of viral antigens (5). Application of immunofluorescence to routine viral diagnosis has been limited by the need for specimens with intact cells containing viral antigen. Direct detection of viral enzymes has been reported for influenza A and B (19), but the method is limited to those viruses possessing neuraminidase activity, namely, influenza and, to a lesser extent, parainfluenza viruses.

Solid-phase immunoassays in which radioactive isotopes or, more recently, enzymes are used (12, 16) combine ease of performance with sensitivity. Enzyme-linked immunosorbent assays (ELISA) have been used to detect several respiratory viruses, including adenoviruses (7, 13), influenza A viruses (1, 14), parainfluenza viruses (13, 15), and respiratory syncytial virus (RSV) (3, 8, 13). The antisera used in these studies are not commercially available; thus, widespread application and evaluation of the assays are not possible. We report here a rapid and sensitive indirect ELISA method for detection of RSV antigens, in which only commercially available reagents are used.

MATERIALS AND METHODS

Cells and virus. HEP-2 cells obtained from Flow Laboratories Inc. were grown in 32-oz (ca. 946.2 cm³) prescription bottles with CMRL-1969 medium supple-

mented with 10% fetal bovine or chicken serum. The maintenance medium used for virus production was CMRL-1969 with 2% fetal bovine or chicken serum. RSV (Long strain) was obtained from Robert Chanock (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) and was plaque purified twice in our laboratory.

Propagation of virus. After removal of growth medium, 2 ml of virus suspension containing approximately 5×10^6 PFU of RSV was added to 32-oz prescription bottle cultures of HEP-2 cells. Virus was allowed to adsorb for 1 h at 36°C. Maintenance medium was then added, and the bottles were incubated at 36°C. When a 3+ to 4+ cytopathic effect was seen, the cells were harvested by being scraped into the medium and were disrupted with a probe sonicator for 1 min at 4°C. Virus stocks and mock-infected HEP-2 cells were stored at -70°C.

Plaque titrations. HEP-2 cells grown in 24-mm-diameter wells were inoculated with 0.1-ml dilutions of RSV in Hanks balanced salts plus 0.5% gelatin. After adsorption for 2 h at 37°C, the inoculum was removed, and 2 ml of CMRL-1969 medium supplemented with 5% fetal bovine serum and 0.75% methylcellulose was added per well. Cells were incubated for 5 to 6 days at 37°C and then fixed with 10% Formalin for 1 h and stained with hematoxylin for 30 min to reveal plaques.

Affinity purification of RSV. Antiserum to bovine RSV (lot M-117), raised in gnotobiotic calves (4), was kindly provided by E. J. Stott (Agricultural Research Council, Institute for Research on Animal Diseases, Compton, N. Newbury, Berkshire, United Kingdom). The immunoglobulin fraction was obtained by ammonium sulfate precipitation. Five milligrams of immunoglobulin was coupled to 1 g of cyanogen bromide-activated Sepharose 4-B (Pharmacia Fine Chemicals, Inc.) according to the manufacturer's instructions. RSV-infected cell culture fluid was centrifuged for 10

min at $3,500 \times g$, and 1.5 ml (approximately 5×10^6 PFU) was added to a column (0.7 by 10 cm) which was then washed with phosphate-buffered saline (PBS), pH 7.2, at 4°C . Antigen was eluted with 3 M KSCN in PBS. Fractions were assayed for RSV activity by ELISA, and fractions containing antigen were dialyzed against PBS. Total protein was determined by the method of Bradford (2), according to the manufacturer's (Bio-Rad Laboratories) instructions.

Antisera. Horse anti-RSV neutralizing serum was obtained from Flow Laboratories Inc. (lot 42631003). The immunoglobulin fraction was obtained by ammonium sulfate precipitation at one-third saturation, followed by dialysis against PBS. Normal horse serum (Flow), used in the diluent for the ELISA test, was inactivated for 30 min at 56°C . Bovine antiserum to bovine RSV was purchased from Burroughs Wellcome Co. (lots K953 and K9684). This serum had been diluted by the manufacturer so that it was suitable for fluorescence staining of RSV, but it proved possible to dilute it further, as described below, for optimal sensitivity in the ELISA. Swine anti-bovine immunoglobulin conjugated to horseradish peroxidase manufactured by Orion Diagnostics was purchased from MTC Corp.

RSV ELISA procedure. Ninety-six-well flexible round-bottom polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc.) were washed with distilled water before use. Horse anti-RSV immunoglobulin (0.1 ml), diluted in PBS to contain $5 \mu\text{g}$ of protein per ml (1:1,000 dilution of original volume), was added to each well, and the plates were incubated overnight or until needed. Plates were washed three times (for 5

min each time) with PBS containing 0.05% Tween 20 (PBS-T) and shaken to remove excess fluid.

Cell culture supernatant fluids were diluted in PBS containing 0.15% Tween 20 and 0.5% gelatin (PBS-T-G), and 0.1 ml was added to each well, followed by overnight incubation at room temperature (approximately 24°C). After three washes with PBS-T, 0.05 ml of bovine anti-RSV serum (Burroughs Wellcome Co.), diluted 1:50 in PBS-T-G plus 1.5% normal horse serum, was added. After the plates were incubated for 1.5 h at 37°C , they were again washed three times with PBS-T, and 0.1 ml of swine anti-bovine peroxidase conjugate diluted 1:500 in PBS-T-G plus 1.5% normal horse serum was added. The plates were then incubated for 1 h at 37°C . After a final wash with PBS-T, 0.2 ml of *o*-phenylenediamine substrate (0.4 mg of *o*-phenylenediamine per ml in 0.15 M citrate-phosphate buffer [pH 5.5] containing 0.015% H_2O_2) was added per well. Color was allowed to develop in the dark for 1 h at room temperature, and the reaction was terminated by adding 0.05 ml of 3.5 M HCl per well. Absorbance at 492 nm was determined with a Gilford 300-N spectrophotometer equipped with a microsampling cuvette.

Outer wells of the microtiter plate received only substrate, and the mean substrate background absorbance was then subtracted from all values. All of the assays were done in duplicate and reported as mean values corrected for background. A positive result was interpreted as one with a mean absorbance more than 2.1 times that of the corresponding negative control.

RESULTS

The optimal dilutions of antisera were determined by performing a series of checkerboard titrations of each pair of antisera in turn. Initial results indicated that horse serum should be added to the diluent for the swine anti-bovine conjugate to lower background absorbances to acceptable levels.

To determine the assay sensitivity, endpoint serial dilutions were done. Figure 1 compares absorbance with virus infectivity in terms of PFU. Absorbance values for mock-infected cells remained low at all dilutions, and RSV ELISA activity could be detected at a 10^{-6} dilution, a concentration which represented 5 PFU (or 50 PFU/ml) of RSV.

Figure 2 shows RSV ELISA sensitivity when RSV antigens purified by affinity chromatography were titrated in the test. In this test the RSV ELISA was capable of detecting 1 ng of partially purified RSV antigen. To compare the relative sensitivities of a direct (three-layer) and an indirect (four-layer) RSV ELISA, a peroxidase-conjugated bovine antiserum to bovine RSV raised identically to the commercially available indirect reagent was obtained through the courtesy of Colin McLaren of Burroughs Wellcome Co. One-tenth milliliter of bovine anti-RSV peroxidase per well diluted 1:5,000 in PBS-T-G plus 1.5% normal horse serum was determined to be an optimal dilution of the conjugate and was

FIG. 1. Titration of unpurified RSV in cell culture supernatant fluids by ELISA. RSV was grown in HEP-2 cells, sonicated, and clarified. Mock-infected HEP-2 cells were scraped from the glass and treated identically. The mean absorbance for the wells receiving buffer only was 0.016.

FIG. 2. Titration of affinity-purified RSV antigen by ELISA.

used in the three-layer direct RSV ELISA. Antigen titrations of four separate affinity-purified RSV preparations were performed simultaneously by direct and indirect RSV ELISA. The indirect RSV ELISA was at least 10-fold more sensitive than the corresponding direct assay.

A panel of heterologous virus-infected cell culture supernatant fluids (or, for influenza, allantoic fluids) was assayed by RSV ELISA to determine the extent of cross-reactivity with other viral pathogens. The monolayer-grown virus stocks were harvested when the cytopathic effect reached 4+, frozen at -20 or -70°C , and used undiluted. Infectivity titers of these pools were not determined, but hemagglutination titers of some of the viruses are indicated below. The viruses tested were: influenza A/Victoria/3/75 (H3N2) (1:640); influenza A/USSR/90/77 (H1N1) (1:640); influenza B/Hong Kong/5/72 (1:640); parainfluenza virus types 1 (1:80), 2 (1:320), and 3 (1:320); measles; mumps; rubella; herpes simplex virus types 1 and 2; varicella-zoster virus; cytomegalovirus; poliovirus type 3; coxsackieviruses A-9 and B-2; echovirus type 7; reovirus type 3; SA-11; adenovirus types 2 and 5; and an untyped rhinovirus. All net absorbances (mean of two wells minus mean of substrate controls) were 0.056 absorbance units or less, and all positive-to-negative ratios were 1.6

or less. The only positive-to-negative ratios of >1.0 were found with parainfluenza virus types 2 (1.5) and 3 (1.6) and cytomegalovirus (1.2).

The virus-infected materials examined originated from allantoic fluid or one of several tissue culture types. Initially we encountered nonspecific RSV ELISA activity in several infected cell culture fluids and in the mock-infected controls due to the presence of bovine immunoglobulins in the culture medium. These nonspecific reactions could be eliminated by using virus grown with nonbovine serum, fetal bovine serum, or no serum.

RSV infectivity is highly labile, and freeze-thawing can result in the loss of 1 to 2 logs of infectious titer (6). The effect of freeze-thawing RSV antigens on RSV ELISA is shown in Fig. 3. A single cycle of freeze-thawing did not significantly alter RSV ELISA activity either with RSV-infected supernatant fluid or with mock-infected preparations.

The collection of specimens for viral culture often utilized transport medium consisting of buffered saline, antibiotics, phenol red, and 0.5% gelatin to stabilize viral infectivity. The effect of diluting virus in transport medium on the RSV ELISA is shown in Fig. 3. The use of transport medium inhibited RSV ELISA activity

FIG. 3. Effect of viral transport medium diluent and freeze-thawing on RSV ELISA. Assays were performed on a single microtiter plate. The mean absorbance for the wells receiving buffer only was 0.040.

10 to 100-fold. This was shown not to be due to either the phenol red or the antibiotics, but some component of Hanks salts or to glucose.

N-Acetylcysteine has been reported to reduce nonspecific activity in ELISA (3, 17). To study the effect of *N*-acetylcysteine on RSV ELISA, we added 0.05 ml of virus or mock-infected cell culture supernatant fluid diluted in PBS-T-G to each well, followed by either 0.05 ml of 20% *N*-acetylcysteine (Mucomyst) or PBS-T-G. The results shown in Fig. 4 indicate that *N*-acetylcysteine had no effect on ELISA activity for RSV-infected cells, but slightly decreased ELISA activity in mock-infected negative controls.

DISCUSSION

Detection of RSV antigens by ELISA techniques has been reported by several groups (3, 8, 13). These assays employed antisera produced by immunization with purified RSV nucleocapsids (13), partially purified RSV that required subsequent adsorption of antisera with host cells (3), or antigens extracted from crossed immunoelectrophoresis gels (9). The RSV ELISA described here employs commercially available antisera and should allow widespread applica-

tion and evaluation of the method.

The high levels of nonspecific binding initially observed with the swine anti-bovine peroxidase conjugate were likely due to cross-reactivity between the conjugate and the horse anti-RSV immunoglobulin used as a capture antibody. Adding 1.5% inactivated horse serum to the PBS-T-G diluent reduced background absorbance to acceptable levels. Subsequent experience with other anti-bovine conjugates has indicated that this is not always necessary. The bovine anti-bovine RSV antiserum used as a detector antibody in RSV ELISA was produced by infection of gnotobiotic calves, eliminating the requirement for removal of anti-cell activity seen with most hyperimmune RSV antisera. Because the sensitivity of noncompetitive ELISA is dependent on the positive-to-negative ratio, the low absorbance values obtained with uninfected material are largely responsible for the observed sensitivity of the RSV ELISA.

The high particle/PFU ratio, lability of RSV infectivity, and unknown and possibly variable-free antigen content of virus suspensions make assays of RSV antigen concentration difficult to evaluate. Particle counts by electron microscopy can quantitate only assembled RSV virions, and plaque titration measures the total number of virions capable of undergoing replication. Furthermore, those methods of RSV quantitation are dependent on the efficiency of the cell culture system, infectivity, and the efficiency of purification procedures.

In previous studies of RSV antigen detection, Sarkkinen et al. (13) reported a sensitivity of 10 to 36 ng/ml, using purified RSV nucleocapsid in an indirect radioimmunoassay with anti-RSV nucleocapsid antibody. Chao et al. (3) estimated a sensitivity of 16 PFU (160 PFU/ml) with unpurified RSV-infected cell culture supernatant fluids, using an indirect ELISA assay. Hornsleth et al. (8) reported an assay sensitivity of 2.5×10^3 RSV-infected cells per ml and 10^4 RSV virions per ml when comparing ELISA inhibition with electron microscopy. Figures 1 and 2 show sensitivity limits of 5 PFU (50 PFU/ml) and 1 ng (10 ng/ml) of RSV, respectively. In view of the comments in the previous paragraph, however, it appears impossible to judge the comparative sensitivity of any of these tests.

The relatively higher absorbance values (Fig. 2) with low dilutions of purified RSV are likely due to the fact that partially purified RSV antigens were used, as compared with the assay using unpurified cell suspensions (Fig. 1). HEP-2 cellular antigens could still be detected in the RSV peak fractions obtained after affinity chromatography of RSV antigens. This is consistent with the observation that as much as 90% of RSV activity remains associated with host cells

FIG. 4. Effect of *N*-acetylcysteine on RSV ELISA. RSV or mock-infected HEP-2 cell suspensions were diluted in PBS-T-G with or without 10% *N*-acetylcysteine. The mean absorbance for the wells receiving buffer only was 0.037.

(10) and suggests that Fig. 2 may show an underestimation of the absolute amount of RSV antigens detected. A comparison of an optimized direct RSV ELISA with the indirect method showed at least a 10-fold reduction in sensitivity and is in agreement with a previous study of cytomegalovirus antigen detection by ELISA (18).

RSV ELISA activity was unaffected by freeze-thaw treatments and long-term storage at 4°C, -20°C, or -70°C. This indicates that although RSV infectivity is very labile (6), ELISA activity of RSV antigens is a stable property. This should make RSV ELISA useful for monitoring yields in growth and purification procedures as well as for detecting noninfectious antigens. We have successfully used RSV ELISA to detect RSV antigens in sucrose and cesium chloride gradient fractions and in 1 M MgSO₄ and 3 M KSCN preparations. Several workers have reported that the addition of *N*-acetylcysteine to viral specimens improves ELISA specificity (3, 17). We observed no effect on RSV ELISA sensitivity when 10% *N*-acetylcysteine was added to the antigen step of the assay.

Experiments to improve both the sensitivity and the specificity of RSV ELISA should examine improved solid-phase supports and more sensitive enzyme substrate systems. The horse anti-RSV neutralizing antiserum employed as capture antibody in the assay showed considerable reactivity with HEP-2 cell antigens. The use of monoclonal or affinity-purified antisera should be a further means of improving assay sensitivity and specificity. Results of the present study indicate that RSV ELISA is a sensitive and specific method for detecting RSV antigens. A study describing its usefulness in a pediatric diagnostic virology laboratory is presented in the accompanying paper (10a).

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