Rapid Detection of Respiratory Syncytial Virus and Influenza A Virus in Cell Cultures by Immunoperoxidase Staining with Monoclonal Antibodies

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Peroxidase-labeled monoclonal antibodies against respiratory syncytial virus (RSV) and influenza A virus were used for immunoperoxidase staining (IPS) of cell cultures inoculated with nasopharyngeal aspirates. Cells were grown in 24-well plates, and specimens were inoculated by low-speed centrifugation. Cultures were incubated for 2 days at 37°C and then fixed, stained, and observed by light microscopy. IPS was compared with standard virus isolation by using cultures of human diploid fibroblasts and Vero, HEp-2, and HeLa cell lines for RSV and Madin-Darby canine kidney cells for influenza A virus; these cultures were inoculated with specimens that were previously stored at −70°C. Of 40 known RSV-positive specimens, 30 were found to be positive on reinoculation by both methods, and an additional 5 specimens were found to be positive by IPS only. Of 190 specimens tested for influenza A virus, 14 were positive by IPS and in tubes, and a further 8 specimens were positive by IPS only. IPS was also compared with direct detection of viral antigens in nasopharyngeal aspirates by a time-resolved fluoroimmunoassay (TR-FIA). Fresh nasopharyngeal aspirates were inoculated into human diploid fibroblasts and Madin-Darby canine kidney cells and tested for RSV and influenza A virus, respectively, by IPS. Of 110 specimens tested for RSV, 37 were positive in total, 32 were positive by IPS, and 33 were positive by TR-FIA. Of 150 specimens tested for influenza A virus, 39 were positive in total, 35 were positive by IPS, and 34 were positive by TR-FIA. IPS of cultures inoculated by centrifugation and incubated for 2 days is a sensitive method for the diagnosis of respiratory virus infections, and 24-well plates allow for the easy processing of a large number of specimens.

The availability of specific antiviral therapy for the treatment of infections with respiratory syncytial virus (RSV) and influenza A virus has resulted in an emphasis on the importance of a rapid diagnosis of these pathogens (8, 12, 19). Immunofluorescence microscopy and immunomassay techniques have been developed for direct detection of infected cells or viral antigens in clinical specimens (6, 9, 11, 15, 16, 22, 23). However, virus isolation is still the standard reference method for the evaluation of new alternatives (10). In infected cell cultures, the cytopathic effect (CPE) often develops slowly and is not always conclusive. Hemadsorption and hemagglutination inhibition are commonly used for the identification of influenza viruses, while immunofluorescence staining of infected cells and immunomassays of cell culture material are also suitable for confirmation of RSV isolates. Detection of viral antigens in infected cell cultures makes it possible to detect respiratory viruses before a CPE is recognized (4, 6, 13, 17, 18, 22).

We have recently described a method for the isolation of herpes simplex virus with centrifuged inoculation of cell cultures in 24-well plates and direct immunoperoxidase staining (IPS) with peroxidase-labeled type-specific monoclonal antibodies (Mabs) after overnight incubation (25). In the present study we modified this method for the identification of RSV and influenza A virus. The method was compared with standard isolation and with the direct detection of viral antigens in nasopharyngeal aspirates by a time-resolved fluoroimmunoassay (TR-FIA) for RSV (23) and influenza A virus (15, 22).

MATERIALS AND METHODS

Specimens. Nasopharyngeal aspirates were collected with a disposable mucus extractor (Vycon, Ecouen, France) from children with acute respiratory disease during periods of RSV or influenza A virus outbreaks between December 1987 and January 1989. For virus isolation, a cotton stick was dipped into the freshly collected mucus and then placed in a vial containing 2 ml of viral transport medium (0.5% bovine serum albumin and antibiotics in tryptose phosphate broth), and the rest of the mucus was used for TR-FIA. Both specimens were stored at 4°C until they were further processed. A total of 40 specimens that were originally found to be positive for RSV were used for the evaluation of IPS for RSV after they were stored at −70°C. Another 190 specimens that were previously stored at −70°C were used for the evaluation of IPS for influenza A virus. A total of 260 specimens were tested without prior storage under routine conditions; 110 of those specimens were tested for RSV and 150 were tested for influenza A virus.

Mabs. Mabs against RSV were produced as described earlier (5, 15), using purified virions (20) of the Randall strain as immunizing antigen. An antibody (RS101) that recognizes the fusion protein of both RSV subgroups (1, 14) was selected. The MAb against influenza A virus nucleoprotein (A1) (21) was obtained from the Centers for Disease Control (Atlanta, Ga.). Immunoglobulin G was purified from ascites fluid by fast anion-exchange chromatography on a Mono Q column (Pharmacia, Uppsala, Sweden) (23) and labeled with horseradish peroxidase (24).

IPS. Cells were grown in 24-well tissue culture clusters (Costar, Cambridge, Mass.). At confluency, growth medium was replaced with 1 ml of maintenance medium and cultures were inoculated with 200 μl of specimen or reference viruses.
and centrifuged in a centrifuge (CRU-5000; International Equipment Co., Div. Damon Corp., Needham Heights, Mass.) at 1,000 × g and ambient temperature for 45 min. The plates were then incubated in a CO2 incubator at 37°C, as there was no difference in sensitivity if IPS plates were incubated at 33 or 37°C. The cells were then washed twice with phosphate-buffered saline (PBS), air dried, fixed with cold 80% acetone in PBS for 10 min, and washed twice with PBS containing 0.05% Tween 20; and 200 μl of peroxidase-labeled MAb diluted 1:1,000 in 5% nonfat dry milk (Valio, Helsinki, Finland)–0.01% antifoaming agent (Cuplaton, Nefarma Oy, Seinajoki, Finland) in PBS was added to each well. After incubation at 37°C for 1 h, the monolayers were washed three times for 5 min with PBS containing 0.05% Tween 20 and once with PBS. Finally, a substrate solution containing 2 mg of 3-amino-9-ethylcarbazole dissolved in 0.5 ml of dimethylformamide and diluted in 9.5 ml of acetate buffer (pH 5.0) and 10 μl of 30% H2O2 was added. The color reaction was developed for 30 min, and the plates were examined with the naked eye and with an inverted microscope at ×100 magnification.

Comparison of IPS with standard isolation for the detection of RSV. Equal volumes of the 40 known positive specimens were simultaneously inoculated into plates and roller culture tubes of human diploid fibroblasts (HDFs) and HEp-2, HeLa (Ohio), and Vero cells and maintained in medium supplemented with fetal bovine serum and glutamine. After a 2-day incubation plates were fixed and stained as described above. Roller tubes were maintained at 33°C, with a rolling speed of 12 rotations per h, and examined microscopically three times a week. Cultures which were negative by CPE after 8 days were subjected to a blind passage and maintained for 10 more days. All negative cultures were fixed and stained by IPS in the tubes.

Comparison of IPS with standard isolation for the detection of influenza A virus. Madine-Darby canine kidney (MDCK) cells, which were maintained in a serumfree medium supplemented with trypsin at a concentration of 2 μg/ml, were used for the isolation of influenza A virus. A 200-μl portion of each of the 190 nasopharyngeal aspirates was inoculated into one well and two tubes of MDCK cells. IPS was done as described above. Tubes were incubated on a roller apparatus at 12 rotations per h at 33°C and observed three times a week for at least 14 days or until an advanced CPE was detected. Negative cultures were then frozen and thawed once and tested for the presence of influenza A virus antigens by TR-FIA as described earlier (15, 22).

Comparison of IPS with TR-FIA for the detection of RSV and influenza A virus. A total of 110 freshly collected nasopharyngeal aspirates were tested for RSV by IPS by using HDF cultures in plates and by direct detection of RSV antigens by TR-FIA (23). Likewise, 150 fresh nasopharyngeal aspirates were tested for influenza A virus by IPS by using MDCK cell plates and by TR-FIA (15, 22). When results obtained by the two methods were in disagreement, TR-FIA results were confirmed by a blocking assay as described earlier (23).

### RESULTS

**RSV.** In preliminary experiments with reference strains and recent isolates of RSV, centrifugation increased the sensitivity of IPS 2- to 40-fold. After staining, infected cells were already detectable by microscopic observation after an overnight incubation, but optimal detection of RSV in clinical specimens was reached after an incubation of approximately 42 h.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Centrifuged plates (IPS)</th>
<th>Roller tube cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>HEp-2</td>
<td>31</td>
<td>23</td>
</tr>
<tr>
<td>HDFs</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>HeLa</td>
<td>26</td>
<td>22</td>
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*Centrifuged plates were incubated for 2 days after inoculation. Roller tube cultures were incubated for 18 days, including one passage. RSV-positive cultures were identified based on a typical CPE and by IPS in the tube at the end of the experiment.*

On inoculation to tubes and plates, RSV was recovered in 35 of the 40 RSV-positive specimens that were stored at −70°C. All 35 specimens were detected by IPS of plate cultures after a 2-day incubation. A total of 20 positive specimens were detected in roller tube cultures, 26 were detected by CPE (9 of which were detected after the passage) and an additional 4 were detected when cultures not showing any CPE were stained with immunoperoxidase after 18 days of incubation. Table 1 shows the results obtained with each cell line. Because of the typical morphology of fibroblast cells (Fig. 1A), single infected and stained cells were more easily recognized in HDFs than in Vero, HEp-2, or HeLa cells.

Of 110 fresh nasopharyngeal aspirates tested for the presence of RSV by IPS using HDF plates and by TR-FIA, 28 were positive by both methods, an additional 4 were positive by IPS only, and 5 were positive by TR-FIA only. The effect of delay in inoculation on the sensitivity of IPS is shown in Table 2.

In three of the four specimens which were positive by IPS but negative by TR-FIA, the virus was also found by standard isolation on reinoculation. Of the five specimens that were positive by TR-FIA but negative by IPS, one was negative and four showed a specific positive reaction in the confirmatory assay.

**Influenza A virus.** Of the 190 isolation specimens stored frozen prior to inoculation, 23 were originally positive for influenza A virus, as determined by TR-FIA. After inoculation to MDCK cells, 22 positive specimens were detected by IPS in plate cultures (Fig. 1B), while in the standard virus isolation procedure only 4 positive specimens were detected by CPE, and an additional 10 specimens were detected by TR-FIA of CPE-negative cultures. One influenza A virus-positive isolation specimen was positive for both influenza A virus and RSV by TR-FIA.

Of the 150 fresh nasopharyngeal aspirates tested by IPS and TR-FIA for influenza A virus, 30 specimens were positive by both methods, 5 were positive by IPS only, and 4 were positive by TR-FIA only. A delay in inoculation had no effect on the sensitivity of influenza A virus IPS when compared with that of TR-FIA.

Three of the five specimens which were positive by IPS but negative by TR-FIA were reinoculated to MDCK cells; influenza A virus was recovered from all of them. Of the remaining two specimens, no material was left for reinoculation. In all four specimens which were positive by TR-FIA but negative by IPS, the positive TR-FIA result was confirmed by the blocking assay.
influenza A virus, the positive IPS result, based on the identification of a single stained cell, was not confirmed by any other method. No false-positive reactions were observed with specimens containing adenovirus, cytomegalovirus, herpes simplex virus, or parainfluenza viruses (data not shown).

The mechanism by which centrifuged inoculation makes cell cultures more susceptible to virus infection is unclear. Enhancement of cytomegalovirus (7) and herpes simplex virus (3) detection has been shown in centrifuged cultures. We used centrifuged inoculation because it increased the sensitivity of IPS with RSV reference strains and clinical isolates. However, our experiments on the effect of centrifugation were not extended to fresh clinical specimens.

Additional RSV-positive specimens were found by IPS of the CPE-negative roller cultures in the tubes. Strong overgrowth of HEp-2 cells in tubes sometimes made it difficult or even impossible to recognize the typical syncytia caused by RSV. After staining, however, infected cells were easily detected by microscopy and, occasionally, even with the naked eye. In contrast to the results of Masters et al. (11), our observations implicate the usefulness of the testing of negative cultures for a maximum yield of RSV isolates. Simultaneous use of several cell lines has been suggested for optimal recovery of RSV in clinical specimens (2). With the exception of HeLa cells, all cell lines were comparatively sensitive for the detection of RSV by IPS 2 days after inoculation. Therefore, one of these cell lines alone, preferably HDFs, because of their characteristic morphology, could be used for clinical purposes.

In our hands, standard isolation of influenza A virus in MDCK cells had a poor sensitivity without additional blind testing of negative cultures, and even then, 8 of 22 IPS-positive specimens remained negative. In a similar study, Swenson and Kaplan (18) tested 70 culture-positive specimens, which were stored at −70°C, by indirect IPS of uncentrifuged rhesus monkey kidney cells 24 h after inoculation. They detected 15 of 19 positive specimens from which influenza A virus was reisolated and 7 positive specimens from which influenza A virus was not reisolated (18). In other studies the sensitivity of immunofluorescence staining of cell cultures by using MABs has been lower (51 to 84%) when compared with that of standard virus isolation (4, 17).

In our earlier study, TR-FIA for RSV was more sensitive than virus isolation (23). In this study, the use of IPS resulted in similar overall sensitivities of the RSV TR-FIA and isolation. However, if specimens were inoculated within 8 h of collection, IPS was more sensitive than TR-FIA. In the detection of influenza A virus, IPS and TR-FIA had nearly equal overall sensitivities. The sensitivity and specificity of TR-FIA were thus comparable to those of virus isolation for the diagnosis of both RSV and influenza A virus. Commercial immunoassays are available only for RSV (10, 11), but they are not yet available for influenza A virus. Direct detection of influenza A virus in specimens by immunofluorescence (18, 19) or enzyme immunoassay (11) has thus far been relatively insensitive with respect to virus isolation. Therefore, IPS, as described here, offers a sensitive and rapid method for the diagnosis of infections caused by influenza A virus.

IPS is a rapid alternative to conventional virus isolation and is applicable for the detection of infectious virus in studies concerning antiviral therapy. It may also prove useful in testing the susceptibilities of virus strains to antiviral drugs. When a sensitive method for the detection of

TABLE 2. Comparison of IPS of infected HDFs with direct detection of antigens by TR-FIA for the detection of RSV in nasopharyngeal aspirates

<table>
<thead>
<tr>
<th>Time (h) of inoculation after specimen collection</th>
<th>No. of specimens</th>
<th>No. of positive specimens</th>
<th>No. of positive specimens by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;8</td>
<td>54</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 to 24</td>
<td>39</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>&gt;24</td>
<td>17</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
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<sup>a</sup> Retesting was negative in two specimens.

<sup>b</sup> Retesting was negative in one specimen.
viral antigens in clinical specimens is available, IPS can be used as a convenient reference method in diagnostic studies.

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LITERATURE CITED