Use of Immunoperoxidase for the Rapid Identification of Human Myxoviruses and Paramyxoviruses in Tissue Culture

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The immunoperoxidase method, using commercially available antisera, was compared with standard virological methods for the identification and typing of 77 isolates of human myxoviruses and paramyxoviruses. Results of typing using neutralization tests and the immunoperoxidase technique were identical for 76 of the 77 isolates. With the immunoperoxidase method there was one false negative reaction, but no false positive reactions. Cross-reactivity between influenza A (soluble) and A/HK antisera with influenza A isolates was noted, but did not interfere with the interpretation of results. It is concluded that the immunoperoxidase method is ideally suited for the rapid identification and typing of common human respiratory viruses on a routine basis. It offers a number of decided advantages over both immunofluorescence and standard virological methods.

The identification of myxoviruses and paramyxoviruses in tissue culture generally depends on the development of hemadsorption or the typical cytopathic effects (CPE) produced by viruses such as respiratory syncytial virus, mumps, and influenza. Further identification and typing require hemadsorption inhibition or neutralization or both tests. In certain instances, immunofluorescence has been successfully employed to identify some members of this group of viruses, both on tissue culture isolates and directly on clinical specimens (3, 4, 6, 7, 13, 14, 16, 18). Despite its rapidity, immunofluorescence has failed to gain widespread acceptance as a routine diagnostic tool, perhaps due to a number of its inherent difficulties.

Recently, the immunoperoxidase (IP) method has been utilized to identify a wide variety of viral antigens in tissue culture (5, 9, 11, 15, 17, 20). This technique offers a number of distinct advantages over immunofluorescence on a routine basis. The problems of background and fading fluorescence are eliminated, the results are generally easier to interpret, and the slides form a permanent, stable record. Comparative studies between IP and immunofluorescence have demonstrated comparable sensitivity, and the problems of nonspecificity and cross-reactivity are similar in both, being dependent on the quality of the specific antibody preparations (2, 19).

This study was undertaken to adapt the IP method for the routine identification and typing of the common respiratory viruses isolated in a clinical virology laboratory and to compare it with standard virological methods in regard to sensitivity, specificity, and rapidity.

MATERIALS AND METHODS

Cell cultures and virus isolation. Monolayers of diploid cells (rhesus monkey kidney and pigtail monkey kidney) and heteroploid cells grown in culture cells (16 by 25 mm) were routinely employed for isolation. After inoculation, cell cultures were examined daily for CPE and on alternate days for hemadsorption with guinea pig erythrocytes.

Viral identification followed standard methods, utilizing neutralization tests (12).

Specimens. Seventy-seven viruses isolated in the Clinical Diagnostic Virology Laboratory of the University of Washington from clinical specimens were used in this study. Thirty-seven isolates from January 1971 to December 1973 had been stored at -70 C. Two of these had been passaged nine times; the remainder had undergone three or four passages. Forty isolates from 1974 were tested by the IP method on initial isolation and before passage.

Antisera. All antisera were commercially prepared in either guinea pigs or rabbits. All antisera were absorbed with mouse or human liver powder for 1 h at 37 C and 12 h at 4 C. Optimal dilutions for each were found by titration with the IP method. The source of these antisera, animal species, titers, and dilutions used for the IP assay are indicated in Table 1.

Enzyme-antiglobulin conjugates. Enzyme-anti-
globulin conjugates were prepared against guinea pig and rabbit immunoglobulins using the glutaraldehyde coupling method of Avrameas and Ternynck (1) without final chromatography. A 1.0 ml volume of commercially obtained antimmunoglobulin (Antibodies Inc.) was conjugated with 10 mg of horseradish peroxidase, type VI (Sigma Chemical Co.). This usually yielded 1.5 to 2 ml of conjugate. The conjugates were checked for specificity and enzyme activity with passive double gel diffusion followed by washing in phosphate-buffered saline (PBS) and staining the precipitin bands for peroxidase with 3,3-diamino benzidine. Optimal dilutions for the IP assay were found by titration; a 1:200 dilution in PBS (pH 7.3) with 1% bovine serum albumin being most frequently employed. This reagent was stable when stored at 4 C for at least 4 months.

Preparation of slides. Slides were prepared by placing drops of glycerin in three rows of nine on standard, alcohol-cleaned microscope slides. Slides were then sprayed with fluorescent (Flurogilde, film bonding grade, Chemplast Inc.), allowed to dry, and rinsed with water. This resulted in 27 wells per slide, each 3 to 4 mm in diameter.

When cultures demonstrated 1 to 3+ CPE or 4+ hemadsorption, the cells were scraped thoroughly with a Pasteur pipette and centrifuged at 800 x g for 5 min; the medium was discarded and the cells and cell debris were suspended in 0.2 ml of PBS. One drop of cell suspension was placed into each well (two rows of nine wells per infected culture). Uninfected cells (heteroploid, pigtail monkey kidney, or rhesus monkey kidney cells) were placed in the third row to serve as negative controls. The slides were air-dried, fixed in acetone for 10 min at 4 C, dried, and stored at room temperature.

IP method. Each cell culture was assayed in duplicate against all eight antisera. To each vertical row of three wells, a specific antiserum was added to completely cover each well. PBS was substituted for an antiserum in the ninth row. The scheme used for applying the specific antiserum was identical to that used for the positive control slide (Table 2). The slides were incubated in a humidified chamber at 37 C for 60 min, rinsed with distilled water, and washed in PBS for 30 min, while being continually agitated. After being washed, slides were gently blotted dry.

The appropriate species antimmunoglobulin-enzyme conjugate was added to each well, and the slides were reincubated for 60 min at 37 C, after which the washing procedure was repeated and the slides were air-dried.

Enzyme activity was demonstrated with the Kaplow stain for peroxidase (10). One drop of the prepared substrate was placed in every well for 60 s. The slide was then rinsed in water, dehydrated through alcohol to xylene, and mounted in Permount.

The presence of peroxidase was evidenced by bright blue granules, whereas the cellular cytoplasm and nuclei stained red. Typical examples of a positive and

| Table 1. Antisera employed to identify myxoviruses and paramyxoviruses |
|---------------------------------------------------------------|
| **Antigen** | **Source** | **Animal** | **Antibody titer** | **Dilution used for IP** |
|------------------|------------------|------------------|--------------------|--------------------------|
| Respiratory syncytial virus | Microbiological Associates | Guinea pig | 1:512 (CF) | 1:60 |
| Parainfluenza 1 | Microbiological Associates | Guinea pig | 1:128 (CF) | 1:20 |
| Parainfluenza 2 | Microbiological Associates | Guinea pig | 1:128 (CF) | 1:40 |
| Parainfluenza 3 | Microbiological Associates | Guinea pig | 1:128 (CF) | 1:40 |
| Mumps | Microbiological Associates | Guinea pig | 1:128 (CF) | 1:40 |
| Influenza A (soluble) | Center for Disease Control | Guinea pig | 1:128 (CF) | 1:20 |
| Influenza B (soluble) | Center for Disease Control | Guinea pig | 1:128 (CF) | 1:40 |
| Influenza A/HK/68 (H1N1) | Flow Laboratories | Rabbit | 1:640 (Neut) | 1:60 |

*Antibody titer listed by supplier. CF, Complement fixation; Neut, neutralizing antibody.

| Table 2. Positive control slide schema* |
|---------------------------------------|
| **Determination** | **Antisera** | **PBS** |
|-------------------|------------------|------------------|------------------|------------------|
| Antigens | RSV | Para 1 | Para 2 | Para 3 | Mumps | Flu A/Eng. | Flu B | Flu A/HK | RSV | Para 1 |
| Antigens | RSV | Para 1 | Para 2 | Para 3 | Mumps | Flu A/Eng. | Flu B/Mass. | Flu A/HK | Para 1 | Para 2 | Para 3 |
| Uninfected cells | HL | PTMK or RMK | PTMK | PTMK | PTMK | PTMK | PTMK | Flu A/Flu B |

*Abbreviations: RSV, respiratory syncytial virus; Para, parainfluenza virus; Flu, influenza virus; PTMK and RMK, pigtail or rhesus monkey kidney cells, respectively; PBS, phosphate-buffered saline; HL, heteroploid; Eng., England; Mass., Massachusetts; Mary., Maryland.
negative reaction are shown in Fig. 1 and 2. The colored product is very stable unless exposed to direct sunlight. In many instances, the results could be read macroscopically, although they were routinely examined microscopically to check for specificity of staining.

Control slides and scheme for identification and typing. One positive control slide was always included when the assays were performed. These slides had been previously prepared using cells infected with known viruses. The antigens and antisera used on the control slide are shown in Table 2.

Two strains of influenza A and B were employed (influenza A/England and influenza A/HK, influenza B/Massachusetts and influenza B/Maryland).

A PBS control was included to check for any nonspecific reaction with the enzyme-antiglobulin conjugate.

RESULTS

Specificity of antisera. Table 3 compares the results of the identification and typing of 77 isolates with the IP method and standard neutralization tests.

With the IP assay there was no cross-reactivity between the antisera and either heterologous viruses or noninfected tissue culture at the dilutions employed. However, influenza A (soluble) and A/HK antisera cross-reacted variably with influenza A isolates. One of the influenza A/England isolates did not react with either antisera, whereas the remainder reacted with one or both.

The antisera were also tested against other antigens (herpes simplex, measles, vaccinia, cytomegalovirus, and simian virus 5) and noninfected cell lines (pigtail monkey kidney, rhesus monkey kidney, heteroploid, HEp-2). No cross-reactivity was observed.

Two hemadsorbing agents that were untypable by standard neutralization tests were assayed. Both demonstrated moderate activity against respiratory syncytial virus and parainfluenza 1 antisera, with one reacting against influenza B antiserum as well, whereas the second reacted with mumps antiserum.

Sensitivity of assay. Three representative viruses were diluted in log₁₀ steps and examined at 48 h of incubation for CPE, hemadsorption

| Virus                        | No. of isolates | Neutralization (no. positive) | Immunoperoxidase (no. positive) |
|------------------------------|-----------------|-------------------------------|--------------------------------|
| Respiratory syncytial virus   | 20              | 20                            | 20                             |
| Parainfluenza 1               | 9               | 3                             | 3                              |
| Parainfluenza 2               | 5               | 5                             | 5                              |
| Parainfluenza 3               | 7               | 7                             | 7                              |
| Mumps                        | 9               | 9                             | 9                              |
| Influenza A*                  | 15              | 15                            | 14                             |
| Influenza B*                  | 16              | 16                            | 16                             |
| Hemadsorbing agents          | 2               | Not able to type (2)          | Not able to type (2)           |

*Influenza A isolates consisted of seven influenza A/England and eight influenza A/HK.

*Influenza B isolates consisted of three B/Massachusetts, three B/Maryland, seven B/Victoria, and three B/Hong Kong.
and the presence of antigen demonstrable by IP (Table 4). IP was considerably more sensitive than the standard methods for virus detection.

Throughout this study it was frequently noted that antigen could be detected by IP prior to the development of CPE or hemadsorption. In one representative experiment (Table 5), respiratory syncytial virus antigen could be detected 12 h after inoculation, whereas the CPE was observed only after 48 h of incubation.

**Rapidity of assay.** The usual time required to type a myxovirus isolated in our laboratory after initial detection ranges between 4 and 43 days. Some of the reasons for the delays are the need for further passage, the problems associated with "breakthrough" in neutralization tests, and batches of monkey kidney cell cultures that sometimes adsorb red cells nonspecifically. The IP method was used on the initial cell cultures in 40 instances, successfully identifying all isolates. The total time required for the assay is between 4 and 5 h.

**DISCUSSION**

The results clearly demonstrate that the IP method, using commercially available antisera, is specific enough to be used on a routine basis for the identification of the common human myxoviruses and paramyxoviruses isolated in tissue culture. As Table 3 indicates, typing by this method agreed with standard neutralization tests in all but one instance. One influenza A isolate failed to react with both the influenza A antiserum (prepared from soluble antigen) and the influenza A\textsubscript{2}/HK antiserum. This represents the only false negative reaction. The influenza A isolates could not be identified as to strain with the two antisera used in this study since there was considerable and variable cross-reactivity. However, use of both antisera correctly identified 14 of the 15 isolates as belonging to the group of influenza A viruses. The influenza B antiserum, also prepared against the soluble antigen, reacted equally well with four strains of influenza B (Massachusetts, Maryland, Victoria, and Hong Kong).

Although the IP method was rapid and specific, it was not able to identify the two hemadsorbing agents (presumed myxoviruses) that could not be typed by neutralization tests.

The assay, in addition to its specificity, is adequately sensitive to detect antigen in tissue culture prior to the development of cytopathogenesis and frequently before hemadsorption becomes evident. Similar sensitivity has been demonstrated with immunofluorescence (4).

One important aspect was the finding that commercially produced antisera are adequate for this method. Some immunofluorescent studies have stressed that commercial antisera are frequently inadequate for satisfactory results (8), and most workers have preferred to prepare their own antisera (19).

Another aspect, difficult to quantitate but

### Table 4. Viral titration with immunoperoxidase

| Virus                      | Method of detection | Titrations |
|----------------------------|---------------------|------------|
|                            |                     | 1\textsuperscript{*} | 10\textsuperscript{-1} | 10\textsuperscript{-2} | 10\textsuperscript{-3} | 10\textsuperscript{-4} | 10\textsuperscript{-5} | 10\textsuperscript{-6} |
| Parainfluenza 1            | CPE                 | -          | -          | -          | -          | -          | -          | -          |
|                            | HAD\textsuperscript{*} | +++++      | ++++      | ++++      | ++        | +          | -          | -          |
|                            | IP                  | +          | +          | +          | +          | +          | +          | +          |
| Influenza B/Massachusetts  | CPE                 | +          | -          | -          | -          | -          | -          | -          |
|                            | HAD                 | ++++      | ++++      | ++        | +          | +          | -          | -          |
|                            | IP                  | +          | +          | +          | +          | +          | +          | +          |
| Respiratory syncytial virus| CPE                 | ++++      | ++++      | +          | -          | -          | -          | ND\textsuperscript{*} |
|                            | IP                  | +          | +          | +          | +          | +          | +          | ND         |

\textsuperscript{*} Dilution.
\textsuperscript{*} HAD, Hemadsorption.
\textsuperscript{*} ND, Not done.

### Table 5. Detection of respiratory syncytial virus by immunoperoxidase and by cytopathic effect

| Method                      | Detection |
|-----------------------------|-----------|
|                             | 6 h\textsuperscript{*} | 12 h | 18 h | 24 h | 48 h | 72 h | 96 h |
| IP                          | -         | +    | +    | +    | +    | +    | +    |
| Cytopathic effect           | -         | -    | -    | ±    | +    | +    | +++  |

\textsuperscript{*} Time after inoculation.
especially important, was the ease of interpretation of the results. This is in direct contrast to the expertise and experience required for interpreting results of immunofluorescent staining, especially with scraped and fragmented cells. The disrupted cell morphology and piling up to cells that occurred in many of the wells did not adversely affect the IP assay, but frequently impaired satisfactory immunofluorescent evaluation.

The IP method as outlined in this study is ideally suited for the rapid identification and typing of common respiratory viruses isolated in tissue culture. The technique is simple, rapid, specific, and reproducible. It reduces the need for large amounts of tissue culture and circumvents most of the intrinsic problems associated with neutralization tests. It offers a number of distinct advantages over immunofluorescence including easier interpretation of results and simpler methodology; in addition, a permanent record is available for retrospective evaluation.

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