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Rapid Subtyping of Influenza A Virus Isolates by Membrane Fluorescence

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During the winter of 1977-1978 three influenza A virus serotypes (A/Vic/3/75, A/Texas/1/77 [both H3N2], and A/USSR/90/77 [H1N1]) circulated in Denver, offering us the opportunity to apply fluorescent antibody techniques to the specific identification of these viruses. Surface antigens of infected, unfixed primary monkey kidney cells were stained in suspension by an indirect immunofluorescence technique with anti-H3N2 and anti-H1N1 antisera. In tests of cells infected with known viruses, the members of the H3N2 family could not be distinguished from one another, but were easily distinguished from H1N1 strains. A total of 101 hemadsorption-positive clinical specimens were evaluated over a 6-month period. Forty-five of 48 influenza A H3N2 and 24 of 29 H1N1 specimens confirmed by hemagglutination inhibition were correctly identified by membrane fluorescence of cultured cells, with no misidentifications among influenza strains and with 1 false positive among 24 non-influenza isolates. The average time to identification by this technique was 4 days compared to 7 days by hemagglutination inhibition. Live cell membrane fluorescence is a simple, rapid, and accurate method for identifying and grouping influenza A viruses.

Fluorescent antibody techniques have been applied to the rapid diagnosis of infection with a number of common viruses from cultured cells, from tissues obtained at postmortem examination, and from clinical specimens such as nasal epithelial cells (6). Subtyping of specific isolates either from tissue culture or directly from clinical specimens has been reported for several viruses, including herpes simplex (17) and parainfluenza viruses (5), but not for influenza A serotypes. During the winter of 1977-1978, influenza A virus of both the H3N2 (A/Vic/3/75 and A/Texas/1/77) and the H1N1 (A/USSR/90/77) families was present in our community and provided the opportunity to apply membrane fluorescence techniques to the differential subtyping of these viruses. The results of this attempt form the basis for this report.

MATERIALS AND METHODS

Fluorescent antibody staining of laboratory strains of influenza A virus. Primary monkey kidney cell monolayers (rhesus or cynomolgous monkey kidney obtained from Flow Laboratories, Rockville, Md.) were maintained in CMRL-1969 medium (Connaught Laboratories, Willowdale, Ontario, Canada) containing 100 U of penicillin per ml and 20 μg of gentamicin per ml and infected with ca. 4,000 hemagglutinating units of egg-passaged strains of influenza A/Vic/3/75, A/Texas/1/77, and A/FW/1/50 or A/USSR/90/77 viruses. All virus strains had been supplied by W. Dowdle, Center for Disease Control, Atlanta, Ga. After a 60-min adsorption, tubes were washed, fed with 1 ml of CMRL-1969 medium, and incubated at 37°C. Monolayers were hemadsorbed daily with guinea pig erythrocytes.

Cells were harvested when the monolayers were widely covered with attached erythrocytes in the absence of cytopathic effect. Each tube was washed three times with 1 ml of phosphate-buffered saline (pH 7.2), followed by incubation with 1 ml of tryspin (0.25% in phosphate-buffered saline) at 37°C to produce a single cell suspension. Trypsinization was arrested by the addition of 10 ml of CMRL-1969 containing 10% fetal calf serum. The cells were then pelleted by centrifugation at 1,500 rpm for 5 min in a model PR-6 centrifuge (International Equipment Co. Needham Heights, Mass.), resuspended in 5 ml of CMRL-1969 with 10% fetal calf serum, repelleted, washed with 3 ml of Hanks balanced salt solution containing 2% fetal calf serum (H2FC), resedimented, and finally resuspended in 0.35 ml of H2FC.

Portions (25 μl) of each cell suspension were incubated for 60 min at 20°C with 20 μl of twofold dilutions (starting at 1:50) in H2FC of either anti-A/Vic/3/75, anti-A/Texas/1/77, or anti-A/USSR/90/77 whole virus antisera prepared in chickens. These antisera, supplied from the Center for Disease Control, were those used for strain typing by hemagglutination inhibition (HI). The cells were then washed three times with 0.5 ml of H2FC with sedimentation at 1,500 rpm for a total of 15 min between washings.

Cells were then incubated for 30 min at 20°C with 20 μl of previously titrated fluorescein isothiocyanate-conjugated rabbit anti-chicken immunoglobulin G (Cappel Laboratories, Inc., Cochranville, Pa.) diluted

1:40 in H2FC. Finally the cells were washed three more times with 0.5 ml of H2FC and pelleted.

Controls included uninfected primary monkey kidney cells and cells infected with influenza B/Hong Kong/72, parainfluenza 1, 2, and 3, and mumps viruses. Antiserum controls included anti-B/Hong Kong/72 virus (chicken, 1:50), anti-parainfluenza viruses 1, 2, and 3 (rabbit, 1:200), and anti-mumps virus (guinea pig, 1:50), all diluted in H2FC. Fluorescein isothiocyanate-conjugated anti-immunoglobulin G antiserum appropriate to chicken, rabbit (Meloy Laboratories, Springfield, Va.), or guinea pig (Meloy Laboratories) were used in the indirect immunofluorescence assay.

Each portion of pelleted cells was dispensed onto a glass slide, a cover slip was placed on top, and the preparation was sealed with a rim of a mixture of equal parts of petroleum jelly and paraffin wax. Individual cover slips were randomized and examined under code by two different observers (M.F. and K.M.) with a Standard Universal Microscope fitted for excitation epiillumination (Zeiss, West Germany). Fluorescence was assessed by a combination of the intensity of staining of individual cells and by the number of cells stained, and scored from 0 to 4+. Cells were photographed with Kodak Panatonic X film (Kodak, Rochester, N.Y.).

Sources of clinical specimens. Hemadsorption-positive specimens in primary monkey kidney monolayers were examined in an ongoing fashion over a 6-month period (November 1977 through April 1978). First, throat washings were collected from young adult trainees and older permanent staff with febrile respiratory illnesses at Lowry Air Force Base, Denver, Colo. Second, nasopharyngeal and throat cultures (both washings and swabs) were submitted from in- and out-patients to the Viral Diagnostic Laboratory, Colorado General Hospital, Denver. Most of the latter were taken from infants and children.

Fluorescence staining of clinical specimens. After washing and trypsinization, each infected monolayer was divided into two tubes, and staining was performed as described above by using anti-A/Texas/1/77 (diluted 1:150 in H2FC) in one tube and anti-A/USSR/90/77 (diluted 1:400 in H2FC) in the other. Positive and negative controls were from primary monkey kidney cells infected with either influenza A/Texas/1/77 or A/USSR/90/77 and uninfected monkey kidney cells, respectively. Slides were prepared, examined, and scored as described above. Isolates were identified only as H3N2, H1N1, or neither. All specimens were examined by two observers under code.

All hemadsorbing agents were tested for the presence of influenza virus by standard HI techniques employing chicken antisera from the Center for Disease Control directed against A/Vic/3/75, A/Texas/1/77, A/USSR/90/77, and B/Hong Kong/72 (20). Noninfluenza isolates were examined for the presence of parainfluenza viruses 1, 2, and 3 by indirect fluorescent antibody staining of fixed cells (6).

RESULTS

Examination of known viruses with known antisera. The results of the titrations of known influenza A viruses are shown in Table 1. There was complete agreement in all instances between the two observers. A/Vic/3/75-infected cells stained well with both H3N2 antisera, interacting more intensely with antiserum to A/Texas/1/77 than its homologous antiserum. There was no reaction between this virus and antiserum to A/USSR/90/77. A/Texas/1/77-infected cells also stained well with both H3N2 antisera, but were especially strong with the homologous antiserum. Specific fluorescence was similar for all viruses: a bright consistently granular pattern of fluorescence over the cell surface (Fig. 1). There was detectable but faint staining at low dilution with antiserum to A/USSR/90/77 which disappeared at higher dilutions. Both A/FW/1/50- and A/USSR/90/70-infected cells stained intensely with antiserum to A/USSR/90/77 and faintly with low dilutions of H3N2 antisera. Uninfected cells demonstrated no fluorescent staining with any of the antisera tested. Cells infected with the non-influenza A hemadsorbing agents (mumps, influenza B/Hong Kong/72, and parainfluenza viruses 1, 2, and 3) reacted intensely with homologous anti-

TABLE 1. *Live cell membrane fluorescence of H3N2 and H1N1 influenza A viruses*

Virus	Antiserum											
	A/Vic/3/75				A/Texas/1/77				A/USSR/90/77			
	50[a]	100	200	400	50	100	200	400	50	100	200	400
H3N2												
A/Vic/3/75	4[b]	3	1	0	4	4	3	1	NT	0	0	0
A/Texas/1/77	3	2	1	NT	4	4	4	4	NT	1	0	0
H1N1												
A/FW/50	NT[c]	NT	NT	NT	NT	1	1	0	NT	4	4	4
A/USSR/90/77	1	0	0	0	1	0	0	0	NT	4	4	3

[a] Reciprocal serum dilution.

[b] Fluorescence score based on the intensity of staining of individual cells and number of cells per preparation stained.

[c] NT, Not tested.

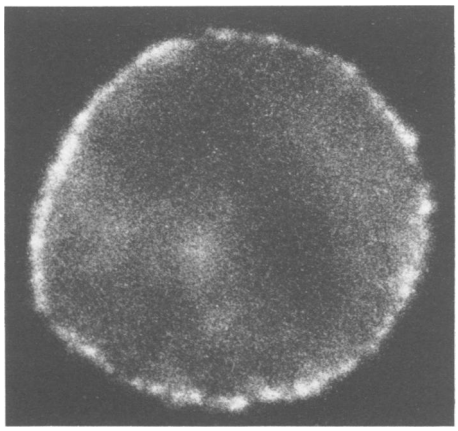

FIG. 1. *Live cell membrane flurorescence. Rhesus monkey kidney cells infected with influenza A/Texas/1/77 and stained with chicken anti-influenza A/Texas/1/77 (1:100) and FITC-conjugated rabbit anti-chicken immunoglobulin G (1:40). × 850.*

sera, but not at all with any of the anti-influenza A antisera at the dilutions tested.

Evaluation of clinical isolates. A total of 101 hemadsorption-positive clinical isolates taken during the period 1 November 1977 to 30 April 1978 were examined (Table 2). There were 77 isolates of influenza A, confirmed by HI, 48 of which were H3N2 (21 A/Texas/1/77 and 27 A/Vic/3/75) and 29 were H1N1 (all A/USSR/90/77). There were 24 hemadsorption-positive isolates that were not influenza A: 9 parainfluenza 1, 7 parainfluenza 2, 6 parainfluenza 3, and 2 isolates not identified as either parainfluenza 1, 2, or 3. There were no isolations of influenza B in Denver during the winter of 1977-1978.

Forty-six of the 48 H3N2 isolates were correctly identified by membrane fluorescence. There were two false negatives (both were A/Vic/3/75). There were no false-positive H3N2 identifications by membrane fluorescence. Of the 29 H1N1 isolates, 24 were correctly identified, with 5 false negatives (all Lowry Air Force Base samples). One specimen, first identified as influenza A/USSR/90/77, was negative for influenza virus by HI and was identified as parainfluenza 2. On retesting, this specimen was negative for H1N1 influenza A by membrane fluorescence.

The average time for identification of all influenza A specimens by membrane fluorescence was 4 days from initial inoculation compared to 7 days for HI. For H3N2 isolates, the mean was 2.5 days (range, 2 to 5 days) and for H1N1 isolates 6.5 days (range, 2 to 9 days) for membrane fluorescence identification. For H3N2 isolates identification by HI took an average of 3.5 days (range, 3 to 30 days). Both of the two specimens negative on several occasions by fluorescence were identified by HI in 3 days. For H1N1 isolates, typing by HI for fluorescence-positive specimens took 7.5 days (range, 4 to 24 days), and for fluorescence-negative specimens, typing took 18 days (range, 4 to 39 days).

DISCUSSION

Previous studies on the identification and subtyping of viruses by fluorescent antibody methods directly from clinical materials have employed fixed cell preparations (6, 17). Such techniques for identifying influenza viruses were first reported by Watson and Coons (24) and first applied to tissue culture isolates and clinical specimens by Liu (12, 13). These findings have been subsequently confirmed and extended by a number of investigators (3, 7, 23). However, Liu noted that the fixed cell could not be used to distinguish among the various influenza A viruses (12).

Differences among influenza A viruses detectable by antisera appear to reside principally in two surface antigens—the hemagglutinin and the neuraminidase (4). The principal internal proteins of the virus, matrix protein and ribonucleoprotein, together representing the bulk of the total virus protein, are quite similar for all influenza A viruses (4). Assays for matrix protein by cell-mediated cytotoxicity suggest this protein may also be expressed on the cell surface (2). Fixation with acetone or ethanol permits the penetration of antiserum into the infected cell and brings antibody in contact with unassembled virion proteins, including the internal proteins (20). The binding of antibody to these common internal proteins thus eliminates the

TABLE 2. *Live cell membrane fluorescence of clinical isolates*

Isolate	Positive fluorescence	Negative fluorescence
Influenza A[a]		
H3N2	46	2
H1N1	24	5
Total	70	7
Non-influenza A[b]		
H3N2	0	24
H1N1	1[c]	23
Total	1	24[d]

[a] Positive by HI test.

[b] Negative by HI test and/or positive for parainfluenza 1, 2, or 3.

[c] Parainfluenza 2, negative for H1N1 upon repetition.

[d] Includes nine parainfluenza 1, seven parainfluenza 2, six parainfluenza 3, and two non-parainfluenza 1, 2, or 3.

possibility of subtype discrimination.

To circumvent this problem, we have combined two well-described techniques: immunofluorescent staining of infected tissue culture cells and membrane fluorescence of infected living cells. Fluorescence staining of fixed infected tissue culture cells rather than the original specimen has been used to rapidly identify influenza A (19) and influenza B (1) viruses. Membrane fluorescence of living cells, first used to identify murine transplantation (*H-2*) antigens (16), has been applied to the detection of surface antigens expressed by C-type oncornaviruses (10) and Epstein-Barr (11), polyoma (15), and measles (9) viruses. This technique has been employed in an inverse manner to titrate antibodies directed against varicella-zoster (25) and respiratory syncytial (22) viruses. These observations suggested that a fluorescence assay in a living susceptible cell line (primary monkey kidney) could be used to rapidly identify the variable influenza A surface proteins while omitting the obscuring background of the common internal antigens.

By this technique we were able to differentiate between the H3N2 and H1N1 families of influenza A virus, although we could not type viruses within each group. Thus, A/Vic/3/75 and A/Texas/1/77 could easily be distinguished from A/FW/1/50 and A/USSR/90/77, but the members of neither pair could be separated from one another. It is of interest that the two H3N2 viruses examined are temporally closely related (1975, 1977). Older H3N2 viruses (A/Hong Kong/1/68, A/England/42/72) are not easily identified with antiserum directed against A/Texas/1/77 with similar observations among the H2N2 ("Asian") viruses (M. Fishaut, unpublished data). However, the two H1N1 viruses tested appear to be very closely related (18).

This membrane fluorescence technique compared favorably with the standard HI test used to identify influenza viruses in respect to both speed and accuracy of identification. For most samples, identification was faster with the fluorescence technique (mean, 4 days from initial inoculation) than with HI (mean 7 days), perhaps due to the smaller quantity of surface antigen required for detection and perhaps because of detection of unreleased virus. HI identification was delayed when the initial isolate had to be passaged through one or more monkey kidney monolayers to yield enough antigen to perform the test. This occurred often with A/USSR/90/77 strains. Identification of this virus was slower than that of H3N2 viruses in both tests. The time required for identification of positive specimens by membrane fluorescence was longer than that previously reported for both influenza A (19) and influenza B (1) by fixed-cell techniques. This may have been artifactual, as we chose to delay our assay until intense erythrocyte hemadsorption was observed on the infected monolayers. It has also been observed that the expression of influenza viral antigens on cell surfaces is modestly delayed compared to their expression within the infected cell (14, 20).

Membrane fluorescence compared to HI was remarkably accurate. Unlike the Swedish study (19), no comparison was made between either of these tests and seroconversion. Overall sensitivity (the ratio of identified positive specimens to true positive specimens) was 91%, and specificity (the ratio of identified negative specimens to true negative specimens) was 96%. The sensitivity of the test for H3N2 influenza A viruses was 95%, for the H1N1 83%, with the specificity for each at or approaching 100%. The one false positive H1N1 was negative upon repetition. The possibility of a double infection was not completely excluded. The lower sensitivity of membrane fluorescence for the identification of influenza A/USSR/90/77 might be attributed to a number of factors, including low levels of detectable antigen (paralleled by the delays in HI typing of these false negative specimens) and rather weak interactions between certain low-passage H1N1 viruses and antibody (8).

The membrane fluorescence technique allowed us to rapidly examine large numbers of intact cells. Slides could be used for up to 60 min, permitting the re-examination of questionable specimens. End points appeared clean, and distinctions between positive and negative specimens were easily made. The omission of sodium azide from the preparations did not obscure the test. Membrane fluorescence as a means of identifying surface antigens has thus proven to be simple, rapid, and accurate compared to HI for the identification and typing of influenza A viruses from tissue culture. In a laboratory facility equipped to perform fluorescence microscopy, this technique should be useful in the epidemiological investigation of outbreaks of influenza A, particularly in years when viruses of several subtypes are circulating, as well as in the study of the biology and immunology of the membrane antigens of these viruses.

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