Rapid Detection and Identification of Respiratory Viruses by Direct Immunofluorescence

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The use of fluorescein-conjugated antiserum against respiratory syncytial (RS) and parainfluenza 1 and 3 viruses was compared with conventional techniques in the rapid detection of virus in tissue cultures inoculated with pharyngeal specimens known to contain these viruses. Twenty-three specimens were tested: 9 RS, 8 parainfluenza 1, and 6 parainfluenza 3. The fluorescein-antibody technique (FA) detected virus in 52% of the tissue cultures in 24 hr, and, by 72 hr, 22 of the 23 cultures were FA-positive whereas only 5 were positive by conventional techniques. Additionally, conjugated antisera were prepared against herpes simplex, influenza A, and adenovirus type 5. All conjugates stained only the homologous virus and were 100- to 10,000-fold more sensitive than conventional techniques in detecting descending dilutions of virus inocula by 24 hr. With the procedures described, several antisera could be conjugated and ready for use within 24 hr. Serum fractionation was by ammonium sulfate precipitation, and with the procedure outlined virtually complete recovery of the globulin fraction and elimination of all of the albumin were accomplished.

Despite the report by Liu (15) in 1956 that influenza infection can be directly demonstrated by immunofluorescence, the fluorescein-antibody technique (FA) has not been widely applied to infections with other respiratory viruses even though it has great potential for rapid and inexpensive diagnosis. One of the most likely reasons for this lack of application is the absence of generally accepted standard procedures for its use. A wide array of methods have been reported for serum fractionation (1, 4, 13), fluorescein isothiocyanate (FITC) labeling (3, 18, 21), and purification of the fluorescein-protein conjugates (8, 24). Many of the techniques described are fairly complex and time consuming and are therefore not readily adaptable to a diagnostic laboratory.

The purpose of the study reported here was to develop a rapid and relatively simple means of producing high-quality fluorescein-labeled antiserum by selecting and modifying, when necessary, those procedures which best fulfilled these criteria and then to test the usefulness of these labeled antisera in the rapid detection of viruses in clinical specimens. A practical ammonium sulfate precipitation technique was developed which yields nearly quantitative recovery of albumin-free globulins and, when combined with the rapid conjugation method of Spendlove (21), permits simultaneous preparation of several sera within 24 hr. The sensitivity of such sera in the detection of inocula containing descending dilutions of each of six common respiratory viruses was compared with conventional techniques. Finally, the rapid FA diagnosis of respiratory syncytial (RS) virus infection previously reported by others (9, 17) was confirmed, and the technique was extended to parainfluenza types 1 and 3.

MATERIALS AND METHODS

Antigen preparation. The antigens against which antiserum was prepared were low passage (no greater than four) strains of RS, parainfluenza types 1 and 3, herpes simplex, and adenovirus type 5. The details of the tissue culture procedures of this laboratory have been described recently (5). Briefly, the tissue cultures (primary rhesus monkey kidney for the parainfluenza viruses, HEP-2 for the others) were grown in 32-oz (ca. 900 ml) prescription bottles by using 50 ml of Eagle's minimal essential medium (MEM) in Earle's salt solution supplemented to 10% with bovine serum. Twenty-four hours before inoculation, the tissue cultures were washed three times with Hank's balanced salt solution (BSS) and maintained with 45 ml of a mixture of 50% MEM and 50% medium 199 in BSS. For the HEP-2 cells, this medium was supplemented to 3% with gamma calf serum. The tissue cultures were inoculated with sufficient virus suspension (3 to 5 ml) to produce, within 7 days, 75 to 100% cell destruction or a hemagglutinin titer of at least 1:128. Then the medium was removed and centrifuged (International centrifuge type SB, size 1) at 2,500 rev/min for 30 min, and the supernatant fluid was used for animal inoculation. To increase the harvest of adenovirus T5, the tissue culture cells were disrupted by
freezing and thawing three times before centrifugation. Influenza A2/Hong Kong/68 was grown in 11-day-old embryonated eggs and harvested at 48 hr.

**Antiserum production.** All antisera were prepared in female goats. With the exception of influenza A2 and herpes simplex viruses, the goats were initially inoculated subcutaneously with 5.0 ml of antigen mixed with 5.0 ml of Freund's complete adjuvant and intravenously with 10.0 ml of undiluted antigen. Intravenous inoculations were continued at weekly intervals until a neutralization antibody titer of at least 1:90 was obtained, usually in 3 to 4 weeks. The herpes simplex antiserum was produced in a similar manner but using 2.0 ml of antigen for the initial subcutaneous injection and 2.0 ml for all intravenous injections. Influenza A2 antisera were prepared by intravenous inoculation only, by using 5 ml of antigen diluted 1:10 in buffered BSS at weekly intervals for 21 days.

**Separation and purification of serum globulins.** A 542-g amount of (NH₄)₂SO₄ was dissolved to 1 liter in glass-distilled water and cooled to 4 C, and the pH was adjusted to 6.2 to 6.5 with 1 N NaOH. Just before use, the ammonium sulfate solution was warmed in a 56 C water bath until the precipitated crystals dissolved and then was cooled to room temperature. A volume of ammonium sulfate equal to that of the serum being precipitated was added drop-wise at room temperature under constant slow stirring. The mixture was placed at 4 C, stirred for an additional 2.5 hr, and then sedimented at 10,000 rev/min for 30 min at 4 C in a Spinco centrifuge using a number 30 head. The supernatant was removed, and the precipitate was redissolved in sufficient glass-distilled water (4 C adjusted to pH 7.3 with 1 N NaOH) to bring it to the original serum volume. Two additional precipitations were performed as above, except on the second and third precipitations the ammonium sulfate solution was used at 4 C rather than room temperature and the precipitate was sedimented immediately after the addition of ammonium sulfate. The final precipitate was dissolved in a volume of distilled water approximately one-third the original serum volume. Ten to 15 ml of the globulin solution was placed in a dialysis bag previously boiled for 10 min and dialyzed overnight in 3 liters of 0.85% NaCl (pH 7.3) at 4 C with constant agitation by a magnetic stirrer. Kaufman and Cherry (12) have shown that dialysis for 4 hr, in similar proportions of globulin solution to saline, eliminates interference with biuret determinations or fluorescein labeling by ammonium sulfate contamination. To be certain that removal of (NH₄)₂SO₄ was complete, in several instances the saline was changed after 18 hr and dialysis was continued for an additional 6 hr. At 2 and 6 hr, 0.5 ml of the dialysate was added to a similar volume of a saturated solution of barium chloride. In all instances, this test was negative, indicating the removal of significant (NH₄)₂SO₄ contamination.

**Conjugation with FITC.** With minor modifications, the conjugation procedure followed was that described by Spendlove (21) using FITC (BBL, lot 8021632) dissolved in 0.1 N Na₂HPO₄. Satisfactory labeling was achieved by adding a quantity of FITC solution (1.25 mg of FITC/ml) to produce a ratio of 20 mg of FITC per g of protein as measured by the biuret method. After addition of fluorescein, the pH was immediately raised to 9.5 with 0.04 N NaOH and conjugation was continued at room temperature for 30 min. Unreacted FITC was removed by passage through G-50 medium-grain Sephadex at 4 C. Conjugation and passage through Sephadex usually resulted in a three- to fivefold dilution of the original serum volume. On the whole serum, after fractionation and after conjugation, neutralizing antibody titers (5, 6), total protein concentration, electrophoretic pattern and fluorescein to protein ratio (F:P), in micrograms per milligram, were determined. Electrophoresis was done on a Beckman microscope system (model R101) with the Analystral modified to take microscope cellulose acetate strips. Total protein and F:P of the conjugates were determined by methods described by Wells et al. (23).

**Tissue culture preparation.** HEp-2 and primary monkey kidney cells were grown on cover slips in Leighton tubes. The cover slips were fixed in acetone at room temperature for 10 min after washing twice with 0.01 M phosphate-buffered saline (pH 7.6; PBS) and air drying. After fixation, the cover slips could be stained immediately or stored for several months at -20 C.

**Staining.** For staining, 0.1 ml of conjugate was applied to the coverslip, incubated for 45 min at 37 C in a moist chamber, and then washed in PBS for 10 min. The cover slips were air-dried and mounted on slides with fluid comprised of nine parts glycerol and one part 0.067 M phosphate buffer, pH 8.5. Nonspecific staining (NSS) was eliminated by simultaneously determining the optimal dilution of the conjugate and the concentration of rhodamine (reference 20; FA rhodamine counterstain, Difco) which gave the brightest specific staining without NSS. This was done by making four sets of serial twofold dilutions of conjugate with PBS to which an equal volume of rhodamine, prepared with PBS and 10% fetal calf serum, was added at 2, 4, 6, or 8% concentrations. Each dilution was then tested against known homologous antigen. This procedure did not eliminate NSS sufficiently in the parainfluenza 1 and 3, RS, and herpes simplex conjugates. Absorption with tissue culture cells of the type on which they were to be used, in the ratio of 2 to 4 ml of conjugate to 1 ml of packed cells for 1 hr at 4 C, successfully removed all remaining NSS. The specificity of each conjugate was tested against a number of heterologous antigens and homologous viruses previously identified by conventional techniques. Positive controls and uninfected cover slip tissue cultures of the same batch used for growing the antigen were included in every test.

The microscope used was a binocular Leitz Wetzler equipped with an Osram HBO 200-w light source, dark-field condenser, APO 40 11.00 EL MJ objective, UG 1 1 MM exciter filter, clear barrier filter, and wide field 10X eyepieces. Photomicrographs were made by using Kodachrome II daylight film with a 120-sec exposure.
RESULTS

Each of the six fluorescein-labeled antisera was monitored throughout preparation for the amount and types of protein in each fraction, preservation of antibody activity, and the extent of fluorescein-protein binding (Table 1). The ammonium sulfate precipitation procedure described above resulted in complete recovery of the globulin fraction and elimination of all albumin as determined by electrophoresis. It should be noted that, in the last four conjugates in Table 1, the gamma globulin concentrations and neutralizing antibody titers in the (NH₄)₂SO₄ fraction were slightly higher than those present in the original serum. This resulted from concentration of the globulin solution during the fractionation procedure. The conjugation method used produced quite consistent fluorescein labeling of all antisera with a narrow range of F:P between 11.5 and 14.5 μg/mlg. Although attempts were made to produce sera with high levels of antibody, the optimal staining dilutions in the last line of Table 1 did not correlate well with the height of the neutralizing antibody titers.

Conjugate dilution in conjunction with rhodamine counterstain and, where necessary, tissue culture cell absorption successfully eliminated all nonspecific staining, and no cross-reaction was seen with any of the heterologous antigens tested. However, in evaluating the parainfluenza 3 conjugate, one batch of uninfected monkey kidney controls showed definite specific staining characteristic of a myxovirus. This conjugate was subsequently tested against cells infected with parainfluenza 2 and simian virus 5 and neither antigen was stained. The identity of this contaminating agent was not definitely determined.

The influenza A₂ antiserum was prepared with the Hong Kong strain but contained antibody against the soluble antigen and gave equally bright specific staining with all type A strains used which included A₂/Taiwan/1/64, A₂/Japan/235.

| Table 1. Characterization of fluorescent-antibody conjugates as to protein content and classification, neutralization titer, and fluorescein to protein ratio |
|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Serum fraction | Characteristics analyzed | Influenza A₂/Hong Kong/68 | Parainfluenza 1 | Parainfluenza 3 | Respiratory syncytial | Herpes simplex | Adenovirus type 5 |
| Whole serum | Gamma globulin | 20 | 23 | 31 | 26.6 | 30 | 25 |
| | Albumin | 35 | 28.5 | 28.4 | 33 | 30 | 32 |
| | Neutralization titer (NH₄)₂SO₄ precipitation fraction | 5794/750 | 1024/250 | 1024/100 | 90/500 | 1024/50 | 1024/75 |
| | Gamma globulin | 19 | 23 | 35 | 32 | 33 | 31 |
| | Albumin | 2 | 0 | 0 | 0 | 0 | 0 |
| | Neutralization titer | ND | 1024/250 | 2048/50 | 90/500 | 1280/50 | 2048/75 |
| Final conjugate | Gamma globulin | ND | 25.5 | 35.3 | 31.7 | 32 | 30.8 |
| | Neutralization titer | 5794/750 | 1024/250 | 2048/50 | 90/500 | 1280/50 | 2169/75 |
| | Fluorescein to protein ratio | 11.5 | 14 | 14 | 13.5 | 14.5 | 11.5 |
| | Optimal staining dilution | 1:16 | 1:16 | 1:16 | 1:10 | 1:10 | 1:16 |

a The albumin and globulin concentrations, in milligrams per milliliter, were calculated from total protein determinations by the biuret method and electrophoresis.

b Neutralization titer/TCID₅₀ used in test.

c Not done.

d The globulin concentrations and neutralizing antibody titers are corrected for dilution. The globulin concentrations, in milligrams per milliliter, and fluorescein-protein ratios, in micrograms per milligrams, were determined from a nomograph developed by Wells et al. (23).

* With 3% rhodamine.
Parainfluenza
Respiratory syncytial
Herpes simplex
Adenovirus
Influenza A2 (HK)

| Fluorescent antiserum employed | Method of detection | CPE | FA | HA | Undiluted | CPE | FA | Undiluted | CPE | FA | Undiluted | CPE | FA | Undiluted | CPE | FA | Undiluted | CPE | FA | Undiluted | CPE | FA | Undiluted |
|-------------------------------|---------------------|-----|----|----|-----------|-----|----|-----------|-----|----|-----------|-----|----|-----------|-----|----|-----------|-----|----|-----------|-----|----|-----------|
| Respiratory syncytial         | CPE                 |     |    |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |
| Parainfluenza 1              | FA                  | 10^-3| 10^-4|   |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |
| Parainfluenza 3              | FA                  | 10^-1| 10^-1|   |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |
| Influenza A2 (HK)            | FA                  | 10^-1| 10^-3|   |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |
| Herpes simplex               | CPE                 |     |    |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |
| Adenovirus T5                | FA                  | 10^-5| 10^-5|   |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |     |    |           |

Day 1 = 10^-10; Day 2 = 10^-6; Day 3 = 10^-2; Day 4 = 10^-2; Day 5 = 10^-2; Day 6 = 10^-2; Day 7 = 10^-2.

Table 2. Comparison of fluorescent-antibody (FA), cytopathic effect (CPE), or hemagglutination (HA) in the detection of laboratory-grown viruses.

* CPE read on the cover slip just before fixation or HA done on the tissue culture fluid at the time of fixation. HA was done with 0.25% guinea pig red cells at room temperature.

* Values represent highest virus dilution positive.

* Not done.

* Except for day 7, after day 2, 10^-4 was the last dilution on which FA staining was done.

** TABLE 3. Comparison of fluorescent-antibody (FA), hemagglutination (HA)*, and hemadsorption (HAd) for detection in rhesus monkey tissue culture (MKTC) of parainfluenza 1 or parainfluenza 3 viruses in throat washings.**

| Time after inoculation | Parainfluenza 1 (8 specimens) | Parainfluenza 3 (6 specimens) |
|------------------------|--------------------------------|--------------------------------|
|                        | FA    | HA | HAd | FA    | HA | HAd |
| 1                      | 1^a   | 1  | ND  | 4     | 0  | ND  |
| 2                      | 4     | 1  | ND  | 6     | 0  | ND  |
| 3                      | 7     | 4  | ND  | 6     | 0  | ND  |
| 4                      | 8     | 6  | ND  | 6     | 0  | ND  |
| 5                      | 8     | 8  | 8   | 6     | 0  | 5   |
| 9                      |       |    |     |       |    | 6   |

* HA was done with 0.25% guinea pig red cells at room temperature.

^ Individual garglings from which either of these two viruses had been previously isolated in MKTC.

^ Number positive.

^ Not done.

Fig. 1. Influenza A2/Hong Kong/68 in monkey kidney tissue culture 48 hr after inoculation with 1 TCID\text{50}.

170/62, and A/FM1/47. The adenovirus T5 conjugate is another preparation which may contain antibody to soluble antigen, but we have not as yet determined its capacity for heterotypic staining.

To compare the sensitivity of the FA technique with the conventional detection methods of reading cytopathic effect or, in the case of the myxoviruses, testing hemagglutination, tissue cultures were infected with 0.1 ml of descending 10-fold dilutions of laboratory-grown virus inocula (Table 2). The FA technique showed markedly increased sensitivity, detecting virus on day 1 at dilutions 100 to 10,000 times higher than conventional methods. By day 3, all viruses had been detected by FA at the highest dilution done. This is illustrated in Fig. 1, which shows influenza A2 (HK) at a 10^-7 dilution 48 hr after infec-
tion. This increased sensitivity was especially evident with RS and adenovirus T5, in which an inoculum of approximately 1 TCID$_{50}$ was detected within 24 hr.

In light of this ability to detect rapidly small virus inocula, the RS and parainfluenza 1 and 3 conjugates were tested by using pharyngeal washings from patients with respiratory infections collected in our earlier epidemiological studies (5). These specimens had previously been shown to contain one of these three viruses. A 0.1-ml amount of each specimen was inoculated into five cover slip tissue cultures and three regular tissue culture tubes. As shown in Table 3, by 48 hr 50% of the parainfluenza 1 and 100% of the parainfluenza 3 were FA-positive (Fig. 2). On day 3, seven of eight parainfluenza 1 specimens were FA positive, whereas only 50% of these specimens showed hemagglutination. Hemadsorption done on the standard tube tissue cultures on day 5 and 9 confirmed that virus was present in all throat specimens done.

With the nine clinical specimens containing RS virus, FA detection was equally rapid (Table 4). The FA-positive tissue cultures on day 1 were very lightly infected with only several positive cells or nests per monolayer. By 48 hr, however, all specimens were easily read as positive (Fig. 3). Cytopathic effect was apparent on day 3 in only one specimen with the other specimens becoming positive at various times after that, except for specimens 8 and 9 which were still negative at 14 days.

**DISCUSSION**

The techniques described above allow the production of specific, sensitive fluorescein-labeled antiserum within 24 hr, and several sera can be done simultaneously. Rhodamine counterstain and tissue culture cell absorption are quite effective in eliminating NSS, and this latter procedure does not result in the loss of conjugate volume or antibody which occurs with tissue powder absorption (16). The quantitative recovery of serum globulins free from all albumin by ammonium sulfate precipitation was an unexpected but desirable finding which has not been previously reported. In a careful study of serum fractionation procedures, Lewis et al. (14) found that globulin yield could be increased from 60 to 80% by increasing the molarity of (NH$_4$)$_2$SO$_4$.
from 1.56 to 1.95. This increased yield was accompanied, however, by an increase in albumin contamination. The elimination of even small amounts of albumin is desirable, since FITC reacts more rapidly with albumin than with globulin and this can interfere with antibody labeling if low concentrations of FITC are used (14). Labeled albumin also increases problems with NSS (4). Before adopting the procedure outlined, our serum fractions by ammonium sulfate precipitation consistently showed loss of globulin protein as well as 4 to 8% albumin contamination. The changes made in our original procedure consisted of raising the pH of the ammonium sulfate to 6.5 and then increasing the molarity of the solution by dissolving all of the ammonium sulfate at 56 C and using it at room temperature for the first precipitation. In addition, the time of reaction after the first precipitation was increased from 1 to 2.5 hr at 4 C, and the pH of the distilled water used to redissolve the globulin was raised to 7.3. These changes were made simultaneously and have not yet been studied systematically, so that the factor or factors responsible for the improved results are unknown. We have subsequently fractionated rabbit serum by the same technique, with results exactly like those reported here for goat serum.

From our experience, the immunofluorescent technique is immediately applicable to the identification of virus agents previously isolated in tissue culture. Numerous homologous and heterologous viruses, previously identified by conventional techniques, were stained without any false-positive or negative results.

In a small confirmatory study, 25 myxoviruses previously isolated during epidemiological investigations (5) were submitted as unknowns for FA identification. The 10 parainfluenza 1 and 12 parainfluenza 3 strains were correctly identified, and the three influenza B viruses included were negative to both conjugates. Positive results were easily distinguished with no suggestion of cross-reactions. Identification takes only 24 hr, which is considerably faster than neutralization tests and simpler than identification by hemagglutination-inhibition. The only difficulty encountered in identification was, as mentioned above, the staining of an unknown agent in one batch of uninfected monkey kidney controls with the parainfluenza 3 conjugate. The identity of this virus was not determined but it is possible that the agent stained was actually parainfluenza 3, since Hsiung (11) showed that 26% of rhesus monkeys studied manifested seroconversion to parainfluenza 3 during the holding period. This experience again confirms the necessity of including, in every test, uninfected controls of the same batch of tissue culture used to grow the virus.

A more important potential of this technique than mere identification, however, is the rapid detection of viruses from clinical specimens either directly or after inoculation into tissue culture. Of the 23 throat washings inoculated in this study, virally all of the viruses could be detected and identified within 48 hr after culture and half were positive by 24 hr. This confirms other reports (17, 19) with RS virus and adds parainfluenza 1 and 3 as capable of rapid detection. These three viruses account for a large number of the serious lower respiratory tract viral infections in young children (2), and, in many cases of croup, bronchiolitis, and pneumonia, it would be feasible to have an etiologic diagnosis in the same time bacterial cultures take, using only these three conjugates. Gray et al. (9) reported good success in the immediate diagnosis of RS infections by staining smears of pharyngeal mucosal cells much as has been done with influenza (10, 15, 22). However, the specificity of their data has been questioned (7) and more experience with staining of nasopharyngeal smears for RS virus must be accumulated.

In the absence of specific antiviral therapy, there has been much urgency among clinicians for rapid diagnosis of viral infections. The advent of such therapy seems likely in the near future and this will create the same diagnostic needs as with bacterial infections. Additional work with the application of the immunofluorescent technique in the detection and identification of respiratory viruses is necessary to fully define its usefulness. We feel, however, from our own work and from that reported by others that this method is feasible and holds great potential for making diagnostic virology simpler and more rapid.

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