Rapid Identification of Viruses by Indirect Immunofluorescence: Isolation and Identification of Adenovirus Types 4 and 7 and Coxsackievirus Type A21 in Microcultures

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Received for publication 7 May 1971

The indirect fluorescent-antibody test (IFAT), employing treated and standardized antiserum in a single pool, was earlier reported to have been used successfully for the preliminary identification of nine respiratory viruses in second to ninth passage multiplying in cells propagated on microscope slides. This report describes parameters affecting the isolation in first passage and the identification by IFAT of adenovirus types 4 and 7 and coxsackievirus type A21 present in stored clinical specimens inoculated into microcultures of WI-38 cells. Isolation frequency was comparable to that obtained in tube cultures, and identification by IFAT of viruses in microcultures could be accomplished in 3 to 4 hr after recognition of a cytopathogenic effect.

With improved cell culture methods and better isolation techniques, the diagnostic virus laboratory in recent years has been required to identify an increasing number and a greater complexity of viruses. In an effort both to simplify and to speed the identification procedure, we have investigated the identification of viruses by the indirect fluorescent-antibody test (IFAT). In a previous study (6) we demonstrated that respiratory viruses, replicating in cells grown on microscope slides, could be presumptively identified by the IFAT by using a single, standardized, pooled antiserum to the viruses of influenza A and B, adenovirus type 2, herpes simplex, mumps, parainfluenza 1, 2, 3, and respiratory syncytial. The earlier report (6) also described (i) the preparation of guinea pig antiserum to these viruses; (ii) assay by complement fixation (CF) and by IFAT of each antiserum for specific and heterologous antibody, using representative strains of the nine viruses; (iii) preparation of the serum pool; and (iv) assay of the pool by IFAT for sensitivity using prototype viruses, as well as 39 respiratory virus isolates in second to ninth passage representative of viruses used in the preparation of the antiserum pool, and several heterologous enteroviruses. As an extension of the above study, the present report describes the identification by IFAT of unadapted viruses in first passage WI-38 microcultures utilizing known positive adenovirus and coxsackievirus A21 clinical throat and rectal specimens.

MATERIALS AND METHODS

Except as noted, materials and methods are the same as previously described (6).

Viruses. Oropharyngeal swabs (OP) of adenovirus types 4 and 7, held at room temperature in charcoal transport medium (CTM) (3), were obtained from the Sixth Army Medical Laboratory (SAML), Fort Baker, Calif., during an adenovirus surveillance program of U.S. Army recruits. Two swabs were collected per individual; one was retained by the SAML for virus isolation and the other sent to us when a virus was isolated. OP specimens were 1 to 7 weeks old when inoculated into cell cultures in our laboratory. Throat washings and rectal swabs of adenoviruses types 4 and 7 and of coxsackievirus A21 in 0.5% veal infusion broth were obtained from the Naval Medical Field Research Laboratory (NMFR, Camp LeJeune, N.C. Specimens were collected from military personnel ill with respiratory disease 5 to 10 months (adenoviruses) and 10 to 12 months (coxsackieviruses) prior to inoculation into cell cultures in our laboratory.

Stock adenovirus types 2, 4, and 7 described previously (6) and coxsackievirus A21, 24R, isolated in this laboratory, were used to infect control microcultures for the IFAT. Coxsackievirus A21 Kuykendall (5), supplied by E. H. Lenette, California State Department of Public Health, Berkeley, was used to prepare an antiserum.

Cell cultures. WI-38 cells in 27 to 29th passage were
grown in Eagle's minimum essential medium in Hanks balanced salt solution (BSS) and 10% fetal calf serum. They were maintained in Leibovitz's L-15 medium containing 3% glutamine, 2% fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin per ml (L-15M).

Cells (10^6/ml) were planted in tubes and slide microcultures as described previously in detail (6). To summarize, the microculture is a monolayer of cells growing within a glass ring (14 by 14 mm) attached to a microscope slide with a paraffin-Vaseline mixture (Fig. 1). Three rings fit onto one glass slide (3 by 1 inch) and five slides are placed in a petri dish (6 inch). Initially 2 to 4 petri dishes were wrapped in clear plastic sheeting and incubated. Later it was found that the pH of cultures was better maintained if the petri dishes were placed in plastic bags, flushed with 5% CO₂/95% air, and sealed. Tube cultures and microcultures were incubated for 1 to 3 days and washed once with Hanks BSS before use.

Processing of specimens. OP swabs, after removal from CTM, were agitated on an electrical mixer in 2 to 4 ml of L-15M supplemented by an antibiotic mixture (no. 1), having a final concentration of 250 units of penicillin, 250 μg each of streptomycin and neomycin, 2.5 units of bacitracin, and 5 μg of amphotericin B per ml of L-15M (A. Leibowitz, personal communication). Varying concentrations of penicillin, streptomycin, and amphotericin B were tried with throat and rectal specimens from NMRL. The antibiotic mixture (no. 2) found most useful contained 5,000 units of penicillin, 5,000 μg of streptomycin and 50 μg of amphotericin B per ml; 0.1 ml of this mixture was added to 1.0 to 1.4 ml of specimen. After addition of antibiotics, specimens were incubated for 1 to 2 hr, OP specimens at room temperature and NMRL specimens at 4 C. Rectal specimens were centrifuged in a size one International centrifuge for 15 min at ca. 700 × g; the supernatant fluid was used as inoculum.

Inoculation and processing of microcultures for IFAT. Two of three microcultures per slide and 1 to 3 slides were inoculated per specimen. The central culture served as an uninoculated control. Microcultures were inoculated with 1.0 ml of either undiluted or 1:2 diluted OP specimen, or with 0.2 ml of NMRL specimen followed by 0.8 ml of L-15M added either immediately or after inoculum had adsorbed to the cells for 75 to 90 min at room temperature. In selected experiments, tube cultures were inoculated with similar volumes of specimen. In addition, 15 first-passage adrenovirus isolates (10 obtained from the fluid and cells of tube cultures and 5 from the fluid only of microcultures) were passed a second time in microcultures. Both microcultures and tubes (with loosened caps) were incubated at 34 C in sealed plastic bags with 5% CO₂. All cultures were incubated stationary except on one occasion when comparative microcultures were gently rotated on a laboratory rotator (model G-2; New Brunswick Scientific Co., New Brunswick, N.J.).

Inoculum was removed from microcultures at times varying from 4 hr to 5 days. Thereafter, the medium was changed at 4- to 6-day intervals. Cultures were examined daily for the first 3 days and subsequently at 1- to 3-day intervals.

Microcultures were processed (6) for the IFAT as follows. Fluid was removed and saved. The cells were washed once with phosphate-buffered saline (PBS), pH 7.3, before the glass rings were removed. After air drying, cell monolayers were fixed in acetone at 4 C (adenoviruses for 2.5 hr and coxsackieviruses for 15 min). Adenovirus preparations were stored at -60 C, coxsackievirus at -30 C. Preliminary studies indicated that neither adeno- nor cox-sackievirus A21 replication could regularly be predicted in first-passage microcultures prior to the recognition of infected cells. As few as five to six adenovirus-infected cells per microculture were sufficient to predict a positive test by IFAT. The enterovirus-type CPE of coxsackievirus was more pronounced and easily recognized. For the IFAT, therefore, microcultures were processed when (i) infected cells were seen in both inoculated microcultures on a single slide, or, alternatively, in one culture only; (ii) cultures were degenerated due to toxic or other causes, but no infected cells were seen; and (iii) no infected cells were seen at termination of the run. Cultures inoculated with adenovirus were followed 15 to 24 days, those with coxsackievirus 10 to 12 days. Tube cultures were frozen at -60 C when CPE was 2 to 3 plus or the run was terminated.

Positive control cultures. Microcultures were inoculated with stock adenovirus types 2, 4, and 7 and coxsackievirus A21, 24R, diluted to produce infection of 10 to 50% of cells within 20 to 24 hr after adsorption of 0.1 ml of virus for one hr at room temperature. Medium, incubation, and processing of cultures were the same as described above.

Antiserum. The group-specific adenovirus type 2 an-

serum and the pooled antiserum to nine respiratory viruses used in this study are the same as described in detail in (6) except as noted below. The same lot of adenovirus antiserum was used singly and in the serum pool. The titer of the adenovirus antiserum was 1:256 by CF and 1:20 by IFAT. With the exception of the coxsackievirus A21 antiserum described below, the other antiseras in the pool had CF titers of 1:64 or greater and IFAT titers of 1:10 or greater (range 1:10 to 1:160). The pool contained 2 to 8 antibody units of each of the nine antisera. In tests versus other viruses used in the preparation of the serum pool both by CF and IFAT, the only heterologous antibody found in any of the sera was a minor (+) reaction between parainfluenza 1 and 3 by IFAT and a similar reaction in the respiratory syncytial antiserum to adenovirus antigen. None of the above antisera stained normal cells or cells infected with several coxsackievirus B5 or rhinovirus isolates. The antiserum pool, exclusive of coxsackievirus antiserum, did not stain coxsackie A21 antigen in the IFAT.

Antiserum to coxsackievirus A21, Kuykendall, was prepared in guinea pigs (600 to 700 g) which were negative when pretested by neutralization at 1:4 dilution of serum to coxsackievirus A21 and by CF to the nine viruses used in the preparation of the antiserum pool. Guinea pigs were immunized by two intranasal instillations, 1 week apart, of 0.3 ml of undiluted 20% (w/v) mouse brain antigen, followed by four intraperitoneal inoculations, 1 to 2 weeks apart, of 1.0 ml of Genetron-treated mouse brain antigen. The titer of the antigen was 10^6 median tissue culture doses (TCD_50) per ml. Animals were exsanguinated 2 weeks after final inoculation. Antisera were assayed by neutralization and the IFAT versus coxsackievirus A21, 24R. Neutralization titers of the three guinea pigs were 1:20, 1:80 and >1:640. The last antiserum was satisfactory for use in the IFAT and had an antibody titer by IFAT of 1:20 versus Coxsackievirus A21, 24R. This serum was negative by CF and IFAT when tested (at a 1:8 and 1:10 dilution, respectively) against other respiratory viruses used to prepare the antiserum pool, including adenovirus type 2. It did not stain normal cells by IFAT. Subsequently, coxsackievirus A21, Kuykendall, was incorporated in the serum pool in place of parainfluenza type 2.

Various lots of fluorescein-conjugated, anti-guinea pig globulin (Antibodies Incorporated, Davis, Calif.) were assayed by (i) capillary precipitin test utilizing serial dilutions of guinea pig serum and 7% gamma globulin (Immunology Incorporated, Glen Ellen, Ill.) as antigen versus undiluted conjugated antibody; and (ii) IFAT utilizing adenovirus- and coxsackievirus A21-infected and control microcultures versus homologous and pooled antisera. Precipitin titers of conjugated antiglobulin varied from 1:10 to 1:80 versus guinea pig serum and 1:10 to 1:40 versus gamma globulin; a reaction end point of two plus was used. IFAT titers giving strong four-plus reactions varied from undiluted to diluted 1:5 or 1:10.

Identification of virus isolates by IFAT. Inoculated and uninoculated control microcultures were stained by the IFAT (usually on the same slide) both with homologous antiserum at 1:10 and with pooled antiserum diluted as previously described (6) with the following exceptions: coxsackievirus A21 Kuykendall antiserum was substituted at 1:5 dilution for parainfluenza type 2, and adenovirus antiserum was decreased to a 1:2.5 dilution instead of 1:5. Details of adsorption of antiseras with chick embryo tissue, the IFAT staining procedure, fluorescence microscopy, and photography are the same as previously described (6) except that microcultures inoculated with coxsackievirus A21 were stained for 1 hr instead of 30 min with antiserum and conjugated antiglobulin.

RESULTS

Factors affecting use of microcultures. WI-38 cells grew readily in microcultures, but cells incubated with 5% CO_2 in plastic bags were healthier and lasted longer than cells without added CO_2. The cell monolayer was 50 to 75% complete within 24 hr, and complete by 72 hr. Tube cell cultures required 4 to 5 days to develop a monolayer.

Microcultures 24, 48, or 72 hr old appeared equally suitable for the isolation of viruses, but cells grown for 24 hr were more sensitive to changes in medium, temperature, inoculum, and antibiotics than cells grown for 48 to 72 hr. The cumulative toxic effect of cells of certain inocula and antibiotics was reduced by eliminating the adsorption period of inoculum to cells and adding maintenance medium immediately after inoculum. This method appeared to be as effective for the isolation of viruses as adsorption, providing the inoculum was left on the microculture for 24 hr. In a limited study with adenovirus type 7, fewer isolations were made when the inoculum was removed at 4 hr compared with 24 hr. Thereafter, inoculum was removed at 24 or 48 hr and replaced with fresh maintenance medium. The concentration of antibiotics initially added to specimens was also decreased to the levels in antibiotic mixture no. 2. This was used successfully for all NMRL specimens but was not extensively evaluated against OP specimens, which frequently contained more yeast and fungus than throat-wash specimens (3).

Duplicate microcultures of adenovirus type 7 specimens incubated stationary or rotated for 72 hr resulted in cell destruction in rotated cultures. There was no evidence by IFAT that virus was isolated in a shorter period of time in rotated cultures compared with cultures held stationary.

On two occasions, cells in suspension in growth medium were mixed with throat or rectal specimens and planted in tubes and microcultures. All such cultures developed slowly and were completely degenerated within 5 to 7 days. This procedure was not repeated.

Isolation of adenoviruses in microculture. Table 1 presents the number and kind of specimens inoculated into microcultures and tube cultures
of WI-38. Tube cultures, when made, were duplicate inoculations of microcultures. Approximately 13% of the microcultures were discarded because of bacterial or fungal contamination; these are not included in Table 1. All remaining microcultures were processed and stained by IFAT, including cultures in which CPE was minimal, negative, or attributed to nonspecific causes. Degenerate cells in cultures 15 to 24 days old were sometimes mistaken for infected cells.

Table 2 correlates the number of adenovirus specimens inoculated into microcultures with the number showing immunofluorescence when stained by group-specific adenovirus type 2 antiserum or by pooled antiserum to nine respiratory viruses. Table 2 shows that of 75 microcultures processed at various times after inoculation with adenovirus type 7, 56 were positive when stained by both group adenovirus and pooled antiserum.

Table 1. Number and kind of specimens inoculated into microcultures and tube cultures of WI-38 cells

<table>
<thead>
<tr>
<th>Virus known to be in specimen</th>
<th>Source of specimen</th>
<th>No. of specimens inoculated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Micro</td>
<td>Tube</td>
</tr>
<tr>
<td>Adenovirus 7</td>
<td>NMRL</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>6AML</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Adenovirus 4</td>
<td>NMRL</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6AML</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Coxsackievirus A21</td>
<td>NMRL</td>
<td>35</td>
<td>20</td>
</tr>
</tbody>
</table>

a NMRL, Naval Medical Research Field Laboratory; 6AML, Sixth Army Medical Laboratory.

Ten additional cultures were positive versus one antiserum but not the other, for a total of 66 adenovirus group identifications. Nine cultures were negative when stained with both antisera, but this group included five cultures from which the inoculum was removed at 4 hr.

Eleven of fifteen adenovirus type 4 microcultures were positive when stained by both group and pooled antiserum; two additional microcultures showed immunofluorescent cells when stained by the pooled, but not the group, antiserum, for a total of 13 positive adenovirus group identifications.

Of the 90 adenovirus specimens inoculated, 88% were recovered as evidenced by IFAT, including six microcultures in which no infected cells were observed or in which CPE was presumed nonspecific. Figures 2 and 3 are photographs of unstained, normal and adenovirus-

Table 2. Cytopathogenic (CPE) and immunofluorescence observed in microcultures inoculated with specimens containing adenovirus types 4 and 7

<table>
<thead>
<tr>
<th>Virus inoculated</th>
<th>No. of specimens</th>
<th>CPE in microculture</th>
<th>IFAT-positive by CPE</th>
<th>IFAT-positive by antiserum used</th>
<th>Total positive IFAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>Adenovirus 7</td>
<td>75</td>
<td>59</td>
<td>55</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Adenovirus 4</td>
<td>15</td>
<td>11</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>70</td>
<td>68</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

a Two microcultures inoculated per slide, left (L) and right (R); Pos, infected cells observed; Neg, no CPE; NS, nonspecific CPE, no infected cells seen but cells degenerated due to toxic or other factors.

b Left microculture stained by indirect fluorescent-antibody test (IFAT) with group-specific adenovirus type 2 (A) antiserum; right with antiserum pool to nine respiratory viruses (P).

c These three positives (A plus P) represent three separate specimens positive by IFAT in one microculture but not the other on a single slide.

d These three positives (A and P) represent three specimens positive by IFAT in both cultures on a single slide.
FIG. 2. WI-38 microculture, uninfected, 6 days after planting cells.
Fig. 3. WI-38 microculture 6 days after inoculation with first-passage adenovirus type 7.
infected microcultures of WI-38 cells. Figures 4–7 show typical adenovirus-infected microcultures stained by IFAT.

Microcultures infected with adenoviruses and stained by IFAT were easily scanned with the low-power objective of the microscope. In early stages of infection, fluorescing cells were located most often at the periphery of the microculture; infection of the entire cell sheet required several more days. Both adenovirus group-specific and pooled antiserum stained adenovirus-infected microcultures equally well.

Table 3 shows the time after inoculation at which microcultures of adenovirus types 4 and 7 were positive by IFAT. Sixty-two per cent of adenovirus type 7 microcultures were positive between 3 to 10 days, 24% at 11 to 15 days, and 14% at 16 to 24 days. Forty-six per cent of adenovirus type 4 microcultures were positive between 5 to 10 days, 30% at 11 to 15 days, and 23% at 16 to 24 days.

Second-passage microcultures of 15 adenovirus type 7 isolates were all strongly positive when stained by the IFAT utilizing group adenovirus type 2 antiserum 2 to 4 days after inoculation. Tube cultures and microcultures inoculated simultaneously with the same adenovirus type 7 specimens showed no essential differences. Infected cells were noted in microcultures as much as 4 days earlier or later than in tube cultures. Rarely was an adenovirus isolated in one type of culture and not the other.

Throughout the study, control microcultures of adenoviruses types 2, 4, and 7 and normal, uninfected WI-38 microcultures were stained by the IFAT utilizing group adenovirus type 2 antiserum, pooled antiserum to nine respiratory
viruses, and a known negative serum. No un-
toward reactions were seen.

Isolation of coxsackievirus A21 in micro-
cultures. Table 4 presents the results of similar
studies employing known, positive clinical spec-
imens containing coxsackievirus A21. Of 46
specimens inoculated, 26 or 57% produced
within 2 to 5 days typical enterovirus-type CPE
in one or more of four to six microcultures in-
oculated. Cytopathogenesis was variable and
spotty. For example, of nine specimens each
inoculated into six microcultures, only four pro-
duced CPE in all six cultures; two produced CPE
in three cultures; one produced CPE in two cul-
tures; and two produced CPE in one culture.
More coxsackievirus A21 isolations were made
in microcultures than in tube cultures; in a
comparison of 30 specimens, 16 microcultures
but only 10 tube cultures developed CPE. How-
ever, this difference appeared to be a function
of the number of cultures inoculated per speci-
men, since four to six microcultures were used
for each tube culture. Additionally, 15 of 16
coxsackievirus A21 specimens collected during a
1-week period produced CPE when inoculated
into microcultures; by contrast, during a sub-
sequent 3-week period, only 11 of 30 specimens
collected produced CPE in similar microcultures.

The reason for the low recovery rate of coxsackievirus A21 in the latter group of specimens is not
known. Microcultures inoculated with coxsackievirus specimens were not rotated during incuba-
tion, a procedure used successfully by others
(2, 4) for improved isolation of coxsackievirus
A21.

All microcultures inoculated with coxsackievirus specimens were stained by IFAT utilizing
both coxsackievirus A21 and pooled antiserum
regardless of whether an enterovirus-type CPE
was observed. Duplicate CPE-positive micro-
cultures, when available, were stained with both
types of antiserum. However, six cultures showed
CPE in only one microculture; these were stained
with coxsackievirus A21 antiserum. Of 26 CPE-
positive microcultures stained by coxsackievirus
A21 antiserum, 23 were positive by the IFAT;
three were negative or showed questionable
fluorescence (±). In no case was fluorescence
demonstrated in microcultures not showing
CPE. Fluorescence varied in intensity from 1 to 4
plus in different cultures and within the same
culture. Lysis of cells was apparent in areas where
CPE was advanced. Even when the staining time
was increased to 1 hr, first-passage coxsackievirus
A21 in most instances did not stain as well as the
positive control coxsackievirus A21, 24R, which
was in sixth passage. Of 20 CPE-positive micro-
cultures stained with the pooled antiserum, 17
were positive and three were negative. Flu-
orescence was generally weaker with pooled
antiserum but showed the same variation in
intensity as seen with specific antiserum.

Immunofluorescence was not enhanced in
coxsackievirus-infected microcultures when the
concentration of specific antiserum or conjugated
antiglobulin was increased in the IFAT. Best
staining results were obtained when microcultures
were dried 40 to 50 min at room temperature
(instead of 20 min at 37 C), fixed 15 min in acetone
at 4 C, and stained 1 hr at 37 C with both anti-
serum and conjugated antiglobulin.

Figure 8 shows an unstained WI-38 micro-
culture infected with a throat-wash specimen of
coxsackievirus A21. Figures 9 and 10 show micro-
cultures of coxsackievirus A21, 24R, in sixth
passage showing four-plus immunofluorescence
when stained by IFAT. Figure 11, an example of
coxsackievirus A21 in first-passage microculture,
shows variable immunofluorescence from one to
two plus and fragmentation of infected cells.

Fig. 5. WI-38 microculture 4 days after inoculation with a rectal specimen of adenovirus type 7; IFAT versus antirespiratory viral serum pool. 90 sec, 400 X.
DISCUSSION

In a previous study (6), we demonstrated that human diploid, monkey, and rabbit embryonic kidney cells could be grown in microcultures and used for the replication and preliminary identification by IFAT of respiratory viruses in second to ninth passage. These microcultures were maintained for 5 to 6 days; longer periods were not tried. The present study indicates that WI-38 cells can be maintained in microcultures for 21 or more days, providing maintenance medium is changed periodically and cultures are incubated in a 5% CO₂ atmosphere in plastic bags. Such conditions allow many diploid or continuous cell lines to be used under simple laboratory or field conditions.

For this study we used recognition of CPE as a criterion of virus replication and processed slides for the IFAT only when infected cells were seen, since preliminary studies indicated that immunofluorescence could not be predicted in first-passage microcultures of adenoviruses or coxsackieviruses in the absence of CPE. However, it has been our experience that microcultures infected with respiratory viruses in second (or greater) passage could be processed for the IFAT 24 to 48 hr after inoculation, regardless of whether CPE was present, with the reasonable certainty that the IFAT would be positive. That a small amount of virus will give a positive fluorescent antibody test within a relatively short time in the absence of CPE was illustrated by a trial of eight adenoviruses types 4 and 7 in second passage (6). The infectivity titers of these viruses ranged from TCID₅₀ of 10¹⁻₅ to 10⁻³ per 0.1 ml of inoculum; all were positive by IFAT 44 hr after inoculation of microcultures. D'Alessio et al. (1) in a recent report on rapid detection and identification of

FIG. 6. WI-38 microculture 5 days after inoculation with a rectal specimen of adenovirus type 4; IFAT versus antirespiratory viral serum pool. 120 sec, 400 x.
FIG. 7. WI-38 microculture 48 hr after inoculation with adenovirus type 7 in first passage; IFAT versus anti-respiratory viral serum pool. 120 sec, 160 ×.

TABLE 3. Detectability of adenoviruses in microcultures by indirect fluorescent antibody test (IFAT) as a function of time after inoculation

<table>
<thead>
<tr>
<th>Virus inoculated</th>
<th>Total IFAT-positive</th>
<th>IFAT-positive on day after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Per cent</td>
</tr>
<tr>
<td>Adenovirus 7</td>
<td>66</td>
<td>41</td>
</tr>
<tr>
<td>Adenovirus 4</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>47</td>
</tr>
</tbody>
</table>

* No adenovirus type 4 microcultures were processed for the IFAT before 5 days.

TABLE 4. Cytopathogenic effect (CPE) and immunofluorescence observed in microcultures inoculated with specimens containing coxsackievirus A21

<table>
<thead>
<tr>
<th>Virus inoculated</th>
<th>No. of specimens</th>
<th>No. of CPE-positive</th>
<th>No. of IFAT-positive</th>
<th>IFAT-positive (% of inoculated)</th>
<th>IFAT-positive (% of those showing CPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>R</td>
<td>A21</td>
<td>P</td>
<td>A21</td>
</tr>
<tr>
<td>Coxsackievirus A21</td>
<td>46</td>
<td>26</td>
<td>20</td>
<td>23</td>
<td>17</td>
</tr>
</tbody>
</table>

* Two microcultures inoculated per slide, left (L) and right (R). CPE-positive, infected cells observed in one or more of six microcultures (L and R of three slides) inoculated. Nonspecific CPE was not observed with coxsackievirus A21 specimens.

* Left microculture stained by indirect fluorescent-antibody test (IFAT) with coxsackievirus A21 Kuykendall antiserum (A21); right with antiserum pool to nine respiratory viruses (P).
FIG. 8. WI-38 microculture 72 hr after inoculation with a throat-wash specimen of coxsackievirus A21.
respiratory viruses by the direct fluorescent-antibody test also demonstrated that a very small inoculum of virus (approximately 1 TCID₅₀ of either adenovirus type 5 or respiratory syncytial virus) was positive by immunofluorescence at 24 hr.

The present study suggests the feasibility of using WI-38 cells in microcultures for the isolation and prompt identification by IFAT of an unknown virus in first passage, since, from stored throat and rectal specimens known to have contained adenoviruses, 88% of type 7 and 87% of type 4 were reisolated on first passage in WI-38 microcultures and identified by IFAT (Table 2). It should be noted (Table 2) that three of seven adenovirus type 7-inoculated microcultures, processed because of presumed nonspecific degeneration in which no infected cells were seen, were strongly positive by IFAT when stained by both the adenovirus type 2 and pooled antiserum. This indicates that these virus isolations would have been missed if all inoculated microcultures had not been stained by IFAT.

Adenovirus isolations were made during the entire 1 to 7 weeks that OP specimens were stored in CTM at room temperature preceding

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Fig. 9 and 10. WI-38 microculture 24 hr after inoculation with coxsackievirus A21 (24R) in sixth passage; IFAT versus coxsackievirus A21 antiserum, four plus immunofluorescence. 120 sec, 400 X.

Fig. 11. WI-38 microculture 48 hr after inoculation with a throat-wash specimen of coxsackievirus A21; IFAT versus coxsackievirus A21 antiserum, immunofluorescence varies one to four plus. 120 sec, 400 X.
inoculation into microcultures. Isolations were also made throughout the 5 to 10 months that NMRL adenovirus throat and rectal specimens in 0.5% veal infusion broth were stored frozen at -60 C.

Fifteen type 7 adenoviruses, representing both tube cultures and microcultures, required 6 to 19 days for initial isolation and identification by IFAT, but on second passage in microcultures developed CPE and strong immunofluorescence within 48 to 96 hr. This suggests an increase in virus titer over the original inoculum and also demonstrates that the fluid phase of the microcultures is an adequate source of virus for further passage without the necessity of inoculating a parallel tube culture with the original specimen.

The demonstration by IFAT of the presence of coxsackievirus A21 in microcultures of WI-38 was not as clear-cut as for adenoviruses; however, CPE was evident in 26% of the 46 cultures inoculated, and all but three were identified by IFAT utilizing coxsackievirus A21 antiserum. The fluorescence of coxsackieviruses in first passage was variable and of low intensity compared with control coxsackievirus A21, 24R, in sixth passage. This is analogous to the findings of Zalon et al. (8), who also described varying degrees of intensity of fluorescence of group A coxsackieviruses undergoing adaptation in human amnion cells. Accordingly, viruses in fifth passage fluoresced more intensely than those in lower passage or viruses not well adapted to amnion cells. This suggests that the difference in staining properties between first- and sixth-passage virus is antigen-related. Presumably the isolation rate of coxsackievirus A21 in microcultures would have been improved by using unfrozen specimens collected just prior to inoculation or after storage for only a short time (2, 7).

The present study offers additional evidence in support of our view that the slide microculture technique, in conjunction with the IFAT, may be advantageously utilized for rapid identification of a wide spectrum of freshly isolated viruses.

ACKNOWLEDGMENTS

The technical assistance of Robert Chiles and Robert Cochran is gratefully acknowledged. We also thank A. Leibovitz, MSC, USA, formerly of the Sixth Army Medical Laboratory, and W. E. Beam, MSC, USN, Naval Medical Field Research Laboratory, for clinical specimens used in these studies.

LITERATURE CITED