An Immunofluorescent Staining Method for Rapid Identification of Respiratory Syncytial Virus.* (30486)

JACK H. SCHIEBLE, EDWIN H. LENNETTE AND ALICE KASE
Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley

The serological identification of respiratory syncytial (RS) virus is generally effected through use of serum-neutralization tests done in cell culture systems or, less frequently, by determining the capacity of a test isolate to fix complement in the presence of known complement-fixing antibody to RS virus(1). Problems of maintaining continuous passage cell cultures in a suitable condition during neutralization tests for RS virus are often resolved by renewing the maintenance medium after several days incubation(2). Other reports(1,3) indicate that addition to the inoculated cell cultures of neutral red, a vital dye, is useful as an aid in demonstrating syncytia in assay tests for RS virus or RS virus immune serum. The inherent dangers of cross contamination during manipulations required for feeding inoculated cultures are obvious. Also the need for the use of vital dyes points to the difficulties sometimes encountered in detecting a cytopathic effect (CPE) induced by RS virus at, or near, the endpoint. The purpose of this report is to describe a sensitive and reliable immunofluorescence test for the rapid identification of RS virus.

Material and methods. Virus strain. The Balin strain of RS virus was isolated in this laboratory in human fetal diploid cells (HFD) and during this study was used in its 5th to 7th passage in these cells. The Long strain of RS virus used as antigen in the preparation of one lot of immune serum was received from Dr. R. M. Chanock and had undergone 11 passages in KB cells in this laboratory.

Tissue culture. KB cells were grown in Eagle's minimum essential medium (MEM) and 10% fetal bovine serum. Monkey kidney cells (MK) were prepared by a modification of the method of Bodian(4). HFD lung or kidney cells were initiated and subcultured according to the method of Hayflick and Moorhead(5). The outgrowth medium for HFD cells was the same as that used for KB cells. For maintenance of all cells, Leibovitz Medium #15(6) and 2% heat-inactivated (56°C for 30 min) fetal bovine serum was used. All media contained 250 units of penicillin and neomycin, 250 μg of streptomycin, and 1 μg of amphotericin B per ml.

Cover slip cell cultures used for immunofluorescent identification of RS virus were prepared by placing 3 circular cover slips (approximately 15 mm in diameter) in sterile dry petri dishes and seeding each with approximately 0.3 ml of HFD cell suspension at a concentration of 3 to 5 $\times$ 10^5 cells per ml. The cells, suspended in Eagle's MEM without serum, were allowed to attach to the cover slips for 1 hour and then 5 ml of outgrowth medium (with serum) was carefully added to the petri dishes. The cultures were placed in a humidified atmosphere of CO_2 at 36°C. Confluent cell sheets were usually formed in 3 to 4 days.

Preparation of immune serum. Immunizing antigens were prepared by inoculating bottles of KB cell cultures with either the Long or Balin strain of RS virus. For this purpose, the usual 2% fetal bovine serum was replaced by 1% heat-inactivated monkey serum. Infectious tissue culture fluids were harvested when approximately three-fourths of the cell sheet had undergone degeneration. The cellular debris was removed by centrifugation at 1000 rpm for 15 minutes and 6-ml samples of the supernatant fluids were stored at —70°C in flame-sealed glass ampules until needed for immunization. Equal portions of RS virus antigen and complete Freund's adjuvant (Difco Co., Detroit) were homogenized and each of the 2 rhesus monkeys was given 4 injections of 4.0 ml of virus-adjuvant mixture (2.0 ml in each calf muscle) at

* The work on which this paper is based was supported by Grants AI-01475 and PH43-63-1141 from Nat. Inst. Health, USPHS, Department of HEW.

2-week intervals and bled 14 days after the last injection. The sera were stored at −20°C.

Conjugation of RS virus immune globulins with fluorescein isothiocyanate. Monkey immune globulins were separated by precipitation at half saturation with ammonium sulfate and the precipitate was redissolved in distilled water. The globulins were reprecipitated several times until the supernatant fluid was clear. The protein concentration was determined by a quantitative biuret method.

The immune globulins were conjugated with fluorescein isothiocyanate at a ratio of 1:200 (FITC/protein) according to the method developed in this laboratory by Dr. Rex Spendlove(7).

Fluorescence microscopy. A Zeiss fluorescence microscope was illuminated with an Osram HBO 200 mercury-vapor burner. For microscopic observation, 25× and 40× objective lenses were used. For excitation a UG5 filter was used in conjunction with a GG4 barrier filter. A similar filter system was used for photomicrographs.

Infection of cover slip cultures. After complete monolayers of HFD cells had formed, the outgrowth medium was removed from the petri dishes as completely as possible by aspiration. The undiluted viral inoculum, in a volume of 0.03 ml, was carefully pipetted onto the surface of the cover slips, care being taken that the inoculum completely covered the cell sheet without running off the cover slip. The inoculum was allowed to adsorb for approximately 3 hours, then 5 ml of maintenance medium was added and the cultures were incubated at 36°C in a humidified atmosphere of CO_2 for varying periods.

Titration of RS virus-immune conjugate. The optimum working dilution of immune conjugates was determined by preparing dilutions in 0.01 M phosphate buffered saline (PBS), pH 7.5, and staining infected cover slip cultures with the various dilutions of the conjugates. The highest dilution of conjugate producing maximum specific fluorescence with a minimum of background staining was selected as the optimum working dilution.

Specificity of conjugated antiserum to RS virus. The specificity of the conjugates was determined by the absence of specific staining of uninfected HFD cells and HFD cells infected with herpes simplex virus, adenovirus type 7 and a rhinovirus of undetermined type. In addition the conjugated immune sera did not stain cells infected with RS virus and previously treated with an unlabeled reference RS immune guinea pig serum (blocking reaction).

Immunofluorescent staining procedure. The maintenance medium was removed from infected cover slip cultures, which were then air-dried and fixed with acetone for 10 minutes at room temperature. The fixed cultures were then either stored at −20°C for staining at a later time, or were stained immediately with labeled antibody.

For staining, the cells were rehydrated in PBS for 10 minutes and then the entire cover slip was covered with the working dilution of the conjugate. The conjugate was allowed to react with the cells for 1 hour in a moist chamber at 37°C, and then removed by washing the cover slips in PBS. For microscopic observation, the cultures were mounted on clean dry slides, employing a medium of 9 parts glycerin and 1 part PBS.

Source of specimens for virus isolation. Material for virus isolation attempts was obtained from patients in the pediatric ward of San Francisco General Hospital, and from children of military personnel seen at the pediatric out-patient clinic or admitted to the base hospital at Fort Ord, California. Specimens were collected by swabbing the throat and nose with separate dry cotton swabs and immersing both in 6 ml of tryptose phosphate broth containing 0.5% gelatin. The specimens, in screw capped tubes, were sealed tightly in plastic bags, to exclude CO_2, and stored on dry ice until required for testing.

Results. CPE of RS virus in MK and HFD cells. The CPE induced by RS virus in MK or HFD cells differs markedly from that induced in continuous passage cell lines such as Hep-2 or KB. The development of the usual well-defined syncytia, characteristic of RS virus in such cells, is not seen in either MK or HFD cells. The initial CPE produced in HFD cells consists largely of small rounded cells scattered throughout the cell sheet; late

in the infectious process (Fig. 1A) the infected cells appear to lyse or disintegrate leaving behind a scattered debris of cell fragments which imparts to the culture a "salt and pepper" appearance. Giant cell or syncytial formation in HFD cells has not been observed. In contrast the predominant feature of CPE in MK cells(8), Fig. 1C, is the formation of small clusters or groups of cells and the accumulation of giant cells, many of which are obviously multinucleated.

Appearance of immunofluorescent stainable antigen in HFD cells. The first detectable RS virus antigen was seen as minute areas of fluorescing material scattered throughout the cytoplasm between 10 and 12 hours post infection. The number of fluorescing areas then increased in size and number and reached a maximum staining intensity between 20 and 22 hours (Fig. 2). In many of the infected cells, 2 large, brilliantly-fluorescing inclusion-like bodies could be seen near the poles of the nucleus, although intranuclear fluorescence was not observed at any time.

Immunofluorescent identification of RS virus. Viral isolates which induced RS virus-like CPE in MK or HFD cells were presumptively identified as RS viruses; this formed the basis upon which viruses were selected for examination by immunofluorescent staining. Three cover slip cultures of HFD cells, in each of 2 small petri dishes, were inoculated with undiluted infectious fluid from selected cultures, as described above. For control purposes, 2 additional sets of cover slip cultures were used. One was infected with RS virus for a positive control and the second was used as an uninfected control. The test and control cultures were incubated in a humidified atmosphere of CO_2 at 36°C. After 24 hours, 1 of the 2 sets of 3 cover slip test cultures and both positive and negative control cultures were removed from the incubator, and the cells fixed and stained with fluorescein-conjugated RS immune serum. The agent was categorically identified as RS virus if specific cytoplasmic fluorescence was seen in both the test and positive control cultures, and if the uninoculated control culture showed no fluorescence. In the event that specific fluorescence was not observed on any of the 3 cover slip cultures incubated for 24 hours, the 2nd set of cover slip cultures was examined after 48 hours incubation. If careful examination failed to detect specific cytoplasmic fluorescence, the agent was considered to be other than RS virus.

TABLE I. Results of Attempts to Identify Definitively by Direct Immunofluorescent Staining 75 Viruses Presumptively Classified as RS Virus by Nature of CPE.

No. of viruses examined	Specific RS virus fluorescence	
	Present	Absent
75	71	4*

* These 4 viruses were subsequently identified as: 1 adenovirus type 2; 1 adenovirus type 5; 1 mumps virus; and 1 simian virus 40.

The results of attempts to identify by immunofluorescent staining a series of viruses isolated in this laboratory, and presumptively identified as RS viruses by CPE, are shown in Table I. Seventy-one of the 75 viruses examined were identified as RS virus by the immunofluorescent method. Four viruses failed to give specific staining with the RS virus conjugate, and these subsequently were shown to be adenovirus type 2, adenovirus type 5, mumps virus and simian virus 40. (The last virus was submitted to this laboratory as an unidentified agent isolated in monkey kidney cells, and it is assumed that these cells were the source of this virus.)

Discussion. The successful application of the immunofluorescence technic as a reliable and rapid method for the sero-typing of RS virus was not only dependent upon the use of specific fluorescein-antibody conjugates which gave little or no background staining, but also upon the judicious selection of the viruses to be examined. It is thus important that personnel responsible for the periodic examination of cultures for evidence of specific viral-induced CPE be adept at recognizing the CPE induced by RS virus.

Immunofluorescent staining not only provided a rapid method for identification of RS virus, but also one which was highly sensitive. The entire monolayer of cells on the surface of a cover slip could be scanned in a few minutes and the presence of a single

a
b
c
d

FIG. 1. Cytopathic effect of RS virus in HFD and MK cells 7 days post infection. (A) RS virus infected HFD cells. (B) Uninfected HFD cells. (C) RS virus infected MK cells. (D) Uninfected MK cells. Mag. 250 ×.

infected cell noted without difficulty. It is readily calculated from the total volume of inoculum placed on the 3 cover slips in a single petri dish culture that as few as 10 infectious virus particles per ml are easily detectable by this method. The sensitivity of this method could theoretically be enhanced another 10-fold by increasing the volume of the inoculum from 0.03 to 0.3 ml per cover slip (the maximum volume easily contained on a 15 mm diameter cover slip).

The intensity of fluorescing RS virus antigen reaches a maximum at approximately 20 to 22 hours, and recent experiments (to be published) in this laboratory indicate that by 25 hours the appearance of fluorescing antigen from the 2nd cycle of virus multiplication can be detected. Therefore, examination of cover slip cultures 48 hours after inoculation should reveal many more infectious foci than would be expected by examination of similar cultures incubated only 24 hours. The number of fluorescent foci at 24 hours would necessarily be in direct proportion to the concentration of virus in the inoculum, while the number of foci at 48 hours would reflect the virus concentration of the inoculum plus accumulated progeny of the virus. Theoretically one should expect to find at least one fluorescing cell on cover slips incubated 24 hours if fluorescing cells are also observed in similarly infected cultures incubated 48 hours, and so far our limited experience bears this out. However, since it is possible that cells (infected) could be lost from the cover slip cultures during the fixation and staining procedures, even though caution is used to minimize this, it is recommended that replicate cultures be incubated at both time intervals as an added margin of safety.

Antigenic differences between strains of RS virus have been reported(3). However, in this study of over 70 RS virus isolates no evidence of antigenic variation was observed by immunofluorescence. The reported variation between RS virus strains detectable by the serum neutralization test was not observed when the variant strains were examined by cross complement fixation tests(3). If variant strains were present, failure to observe antigenic differences by immunofluorescence is most likely explained by the fact that the RS immune sera used for conjugation were prepared with both the viral antigen and the soluble complement-fixing component. Since the reported variants ostensibly share a common complement-fixing antigen(s), it would seem that conjugates made with soluble complement-fixing antigen would similarly lack the ability to detect antigenic differences between strains.

Summary. An immunofluorescence method for rapid identification of RS virus is de-

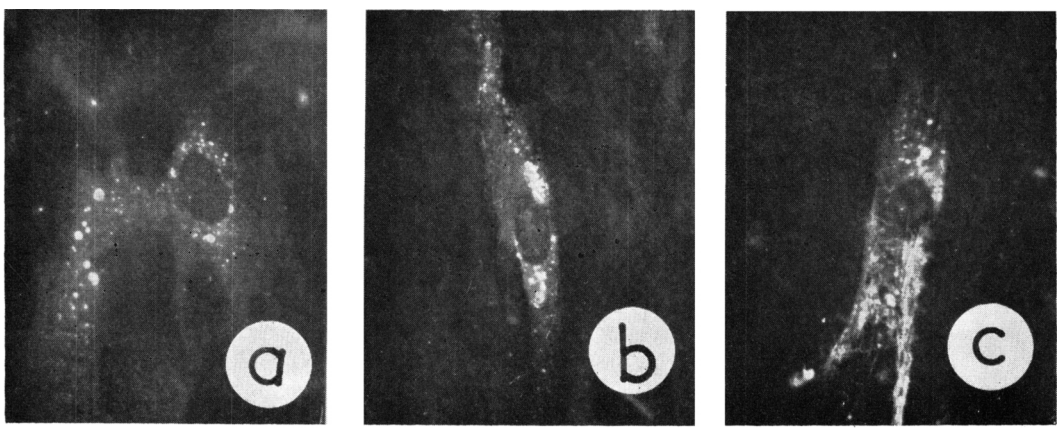

FIG. 2. Immunofluorescent stainable RS virus antigen in HFD cells approximately, (A) 12 hr, (B) 17 hr and (C) 22 hr post infection. Mag. 880 ×.

scribed; results of the identification tests were available in approximately 24 hours. With respect to sensitivity, it appears that as few as 10 infectious virus particles per ml of inoculum are readily detectable within a similar period. Seventy-one of 75 viral isolates examined by the immunofluorescence method were identified as RS virus. The remaining viruses were subsequently identified as 1 mumps virus, 1 simian virus, and 2 adenoviruses. Although serum-neutralization tests indicate the existence of antigenic differences between strains of RS virus(3), no differences in antigenic composition between strains were noted in this study by immunofluorescent staining. A possible reason for this is discussed.

1. Chanock, R. M., Kim, H. W., Vargosko, A. J., Delva, A., Johnson, K. M., Cumming, C., Parrot, R. H., J.A.M.A., 1961, v176, 647.

2. Bennett, C. R., Hamre, D., J. Inf. Dis., 1962, v110, 8.

3. Coates, H. V., Kendrick, L., Chanock, R. M., Proc. Soc. Exp. Biol. and Med., 1963, v112, 958.

4. Bodian, D., Virology, 1956, v4, 575.

5. Hayflick, L., Moorhead, P. S., J. Exp. Cell Res., 1961, v25, 585.

6. Leibovitz, A., Am. J. Hyg., 1963, v78, 178.

7. Spendlove, R. S., Lennette, E. H., Knight, C. O., Chin, J. N., J. Immunol., 1963, v90, 548.

8. Wulff, H., Kidd, P., Wenner, H. A., Proc. Soc. Exp. Biol. and Med., 1964, v115, 458.

Received June 28, 1965. P.S.E.B.M., 1965, v120.

Effects of Cerebral Ischemia in Various Strains of Rats.* (30487)

HUSHANG M. PAYAN, SEYMOUR LEVINE AND RALPH STREBEL
Department of Pathology, New York Medical College, Center for Chronic Disease, Bird S. Coler Hospital Division, Welfare Island, New York City

The effects of carotid artery ligation vary among individual patients(1) and among different species(2,3). The present investigation is aimed at elucidation of genetic factors in the causation of this variation, and at development of reproducible model systems for study of cerebral ischemia. The results indicate that survival following bilateral carotid occlusion of rats varies from 0 to 100% according to the strain of origin. Furthermore, mortality can be greatly decreased by allowing a time interval between ligations of the two arteries.

Methods. Female rats of 5 inbred and 3 random bred strains, initial weight 130-190 g, were maintained on Purina Laboratory Chow and tap water in air conditioned quarters at 70°F. Common carotid arteries were doubly ligated and cut under ether anesthesia through a midline longitudinal incision in ventral neck. Injury to nerves was avoided. Left carotid was ligated first. Right carotid was ligated immediately thereafter (hereinafter referred to as "simultaneous"), or after an interval of 1-27 days, through the original incision. Animals that died during or a few minutes after surgery were excluded. Survivors were sacrificed 5 days after the second ligation. Brains were fixed in formalin, cut in frontal slices, embedded in paraffin, and stained with hematoxylin-eosin and Luxol fast blue.

Results. Mortality after simultaneous bilateral carotid ligation varied from 0% to 100% depending on the strain (Table I). The difference between 5 groups with low mortality and 3 groups with high mortality was statistically highly significant ($p<.001$). Early deaths were caused by massive, bilateral, acute cerebral infarction. Among the 44 survivors, there were 20 focal ischemic brain lesions in 16 rats. The distribution was similar to that reported previously(4): cortex 9, corpus striatum 9, hippocampus 1, corpus callosum 1. No deaths followed unilateral (left) carotid ligation and only 2 animals had focal lesions; these occurred in strains distinguished by 100% mortality after bi-

* Supported by Grant 317-2 from National Multiple Sclerosis Society.