Rapid Diagnosis of Mumps Virus Infections by Immunofluorescence Methods

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Mumps and its complications, particularly meningoencephalitis, is an important disease problem, and more rapid diagnostic methods are desirable. A study was made of immunofluorescence methods for the early detection of mumps virus isolated in cell cultures, or adsorbed directly from clinical specimens onto guinea pig erythrocytes. A specific diagnosis could be made in hours to 2 or 3 days utilizing immunofluorescence methods, in contrast to about 6 days by standard methods. Details of the direct immunofluorescence methods are presented, to encourage wider application in clinical virology laboratories.

Despite their potential as viral diagnostic tools, immunofluorescence (IF) techniques have not yet achieved the role they deserve in the clinical laboratory. In the Viral and Rickettsial Disease Laboratory of the California State Department of Health, IF microscopy is now used routinely to identify many viruses isolated in cell cultures or animal hosts, or for rapid diagnosis directly from infected tissues, such as in rabies, psittacosis, herpes, and varicella-zoster infections. We describe here additional IF methods developed in this laboratory and our attempts to use them in the rapid and early diagnosis of mumps virus infections, particularly the central nervous system manifestations which may occur without accompanying parotitis and be impossible to differentiate clinically from other viral diseases. A few earlier studies of IF procedures for mumps virus were unsatisfactory in some respects or were not applied clinically (1-4, 8).

MATERIALS AND METHODS

Mumps antiserum. Mumps antiserum was obtained by immunizing male Hartley strain guinea pigs intranasally with 0.5 ml of allantoic fluid from chicken eggs infected with Enders strain egg-adapted mumps virus (45th egg passage), grown as described elsewhere (5). Three weeks later, they were inoculated intraperitoneally with 0.5 ml of similar allantoic fluid which had been concentrated 10-fold by high-speed centrifugation and stored at -70 C. One week after the second inoculation, the animals were bled to obtain immune serum; the serum antibody titer was $\geq 1:1024$ by the complement fixation test, and $\geq 1:4096$ by the hemagglutination (HAI) inhibition test.

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Anti-mumps conjugate. The globulin-rich fraction of the pooled mumps antiserum was obtained by fractionation with 50% saturated ammonium sulfate and conjugated with FITC by the standard method of this laboratory (7). The precipitate was collected by centrifugation, dissolved in a volume of distilled water equal to the original volume of the serum, and then this precipitation procedure was repeated twice. The product was dialyzed against two changes of 100 volumes of phosphate-buffered saline (PBS), pH 7.2, at 4 C. The protein content of the globulin fraction was found by means of the biuret method; the fraction was then conjugated overnight at 4 C using 1.8 μ g of fluorescein isothiocyanate per mg of protein. Free fluorescein was then removed by passing the conjugate through a column of Sephadex G50. The "optimum" use dilution of the conjugate, defined as one-fourth the highest dilution giving brilliant (4+) specific fluorescence but no nonspecific fluorescence, on 96-h infected cell cultures was determined to be 1:10 in 10% beef brain suspension in PBS, pH 7.2. The conjugate was passed through a 400-nm pore-diameter membrane filter (Millipore Corp.) to achieve bacterial sterility, then was dispensed in 1.5-ml portions and stored at -20 C. After a portion was thawed for use, it was not frozen again, but was kept at 4 C for not longer than 10 days.

Cell cultures. The Vero line of African green monkey kidney cells, primary rhesus monkey kidney (RMK), and human fetal diploid lung (HFDL) and kidney cells (HFDFK) were used in the study. The Vero line was maintained by weekly passage, using Eagle minimal essential medium in Earle balanced salt solution, with 8% fetal calf serum. For the IF procedures, both Vero and RMK cells were grown in Falcon TC-6 plastic cell culture plates, which contain six 35-mm diameter wells; each well was fitted with three 15-mm diameter coverslips. As cell growth approached a nearly confluent monolayer, the growth medium was replaced with medium containing 2% fetal calf serum; on some occasions this medium was supplemented with 20 mM N-

morpholino propane sulfonic acid (Calbiochem, La Jolla, Calif.) buffer, pH 7.1. The buffer was useful in prolonging the time that cell cultures could be held without changing medium. As the cell culture plates were not sealed, they were kept in a 5% CO₂ atmosphere at 36 C. The RMK and HFD cells in tubes were grown by our standard techniques (6).

Virus strains. In addition to the egg-adapted Enders strain used in preparing immune serum, the Ward strain of mumps virus, isolated in this laboratory, was used in its fourth passage in RMK cells. This strain was used in the development of IF procedures and to provide positive controls during isolation attempts from clinical specimens.

IF procedures. When cell cultures were to be examined, coverslips were removed from the cell culture plates with forceps, rinsed in two changes of PBS, pH 7.2, and dipped in distilled water to remove salts. The coverslips were fixed in acetone at 22 C for 10 to 15 min and then air-dried. They were either stained immediately or stored at -20 C no longer than 2 days before being stained. Diluted conjugate was applied with a Pasteur pipette, one drop to a coverslip; the drops were then spread over the coverslips by means of split wooden applicator sticks. The coverslips were incubated in a moist chamber for 30 min at 22 C, then removed to a slotted plastic rack and washed in PBS, pH 7.2, for 10 min with gentle agitation. The coverslips were then each dipped in distilled water and placed, cell-side down onto a drop of polyvinyl alcohol mounting medium, pH 7.2, on a microscope slide. Infected cells mounted on this medium maintained their fluorescence for several days, if stored at 4 C. A Zeiss dark-field fluorescence microscope with barrier filter 50, interference filter KP-500, and a 100-W quartz Halogen lamp light source were used.

Urine samples were centrifuged at 1,000 × *g* for 15 min at 4 C, to remove cells and debris, which were examined as separate smears. Ten milliliters of the supernatant urine was decanted into a 15-ml centrifuge tube and adjusted to an approximate pH of 6.5 to 7.0 by addition of a few drops of 8.8% NaHCO₃. After the addition of 0.2 ml of 0.4% guinea pig erythrocytes in normal saline, the mixture was incubated for 40 min at 4 C. The erythrocytes were then pelleted by centrifugation at 800 × *g* for 5 min. The supernatant urine was carefully removed, and the cells were suspended in a drop of fetal bovine serum. The suspension was placed on a slide as a smear, which was then air-dried and fixed in acetone for 15 min at 22 C. The fixed smears were then stained by a procedure similar to that followed for the cultured cells. The smears prepared from the urine sediments were dried, fixed, and stained in the same manner.

Clinical specimen processing. A limited number of specimens, consisting of throat swabs and urine samples, were obtained through the cooperation of local physicians. These specimens were delivered to the laboratory in a refrigerated container within 1 or 2 h after collection. No cases of suspected mumps meningoencephalitis were reported to us during the study period, so that fresh cerebrospinal fluid samples were not available. However, tests were also

performed on some specimens previously submitted to the laboratory, and which had been stored at -70 C. Throat swabs were held in 3 ml of Hanks balanced salt solution; upon arriving in the laboratory, 1.8 ml of the Hanks balanced salt solution was added to 0.2 ml of a mixture of penicillin, streptomycin, and Fungizone (final concentrations of 500 U, 2.5 mg, and 5 µg per ml, respectively). The mixture was held for 30 min at room temperature (22 C) before inoculating two wells each of the Vero and RMK cells used for fluorescence procedures (with 0.25 ml each). For comparison, two tubes each of HFDL and RMK were inoculated with 0.2 ml of the sample, and incubated at 33 C according to our routine method (rather than 36 C) for the isolation of respiratory viruses. All cultures were examined for cytopathic effect (CPE) at intervals, and after one week a HAD test was performed, after which the cells were scraped loose and passaged to fresh cell monolayers. The second passage material was examined in the same manner as the first; it was discarded at the end of 1 week if no evidence of infection was seen. Urine specimens were similarly processed, except that 0.4-ml volumes of the supernatant urine were inoculated into two tubes each of HFDL and RMK cells and incubated at 36 C. These cultures were examined daily for cytopathic effect and tested for HAD after 10 days.

RESULTS AND DISCUSSION

Specificity of the conjugate. The anti-mumps conjugate was tested for cross-reactions at a working dilution of 1:10 against Vero cell cultures infected for 24 and 72 h with 500 to 5,000 mean tissue culture infective doses of parainfluenza virus types 1, 2, 3, and 4; no specific fluorescence was seen. If the conjugate was diluted in plain PBS, rather than 10% beef brain suspension, some nonspecific fluorescence was seen on uninfected Vero and RMK cells, appearing as dull, diffuse cytoplasmic and membrane fluorescence, rather than the brilliant cytoplasmic pinpoint foci of specific fluorescence found in mumps-infected cells. Specific fluorescent foci appeared early in the course of infection (usually within 24 h) and gradually grew more numerous and increased in size to become definite disks. The nonspecific fluorescence disappeared when the beef brain suspension was used as diluent for the conjugate; it could also be removed by absorbing the conjugate with a Vero cell suspension. However, the quantity of Vero cells required to absorb the conjugate made it more practical to use the beef brain suspension routinely.

Sensitivity of the test. Virus titers obtained by use of the fluorescent antibody procedure in several types of cells were compared with the titer obtained previously by use of HAD on RMK cells, which proved to be the most sensitive system. A line of BS-C-1 cells was found to

be less sensitive to the Ward strain of mumps virus, which could not be reliably recovered from these cells upon serial passage. By the IF technique, the minimum infectious dose for RMK cells was 20 to 30 mean tissue culture infective doses (as measured by the HAD test) and for the Vero cells it was about 200 mean tissue culture infective doses. Fluorescent foci appeared within 24 to 44 h after infection. The sensitivity of the IF procedure for demonstration of virus concentrated from urine by HAD onto guinea pig erythrocytes was determined by adding known amounts of virus to 10-ml portions of normal urine, which were then treated as described above. About 600 mean tissue culture infective doses (by RMK HAD) of mumps virus was required before the erythrocytes showed enough fluorescence to be reliably detected.

Isolation attempts from clinical specimens. A comparison of results obtained by routine isolation methods and by use of IF procedures on clinical specimens is summarized in Table 1. Mumps virus was isolated from seven specimens. In all but one, the IF test procedures were successful in detecting the virus and did so earlier than the standard isolation procedures. It should be noted that Table 1 shows the times at which mumps virus was first detected (and identified) by the IF procedure. In the standard procedure, mumps virus is identified by the HAD inhibition test, requiring 4 to 7 additional days. In addition to the specimens listed in Table 1, virus was isolated by routine methods from urine to which the laboratory strain of mumps virus had been added. Isolation of virus

required four days, whereas virus detected by the HAD concentration and IF procedures required less than 2 h. In addition to mumps virus, two rhinovirus strains were recovered from throat swabs, and an apparently new enterovirus strain was recovered from the urine of an infant hospitalized with a severe illness. In one case the IF procedures did not detect virus that was later recovered by standard methods (no. 1), and in three cases IF procedures required nearly as long a time for virus detection as did the standard methods (no. 5-7) due to a low virus content (only one inoculated tube was positive). In contrast, when the IF procedures detected mumps virus within a few days, all of the tubes were positive by the standard methods. Although comparative titrations for virus in the clinical specimens were not performed, the results thus seem consistent with those found in using laboratory-adapted strains; the IF methods, though more rapid, were a little less sensitive than standard methods.

IF procedures are relatively simple to perform, are rapid, and when properly carried out, are usually sensitive and specific. As it is now common for diagnostic laboratories to possess some fluorescence microscopy equipment, IF procedures could be more effectively used. The procedures developed in this study show promise of utility for the viral diagnostic laboratory. Mumps (and its complications) is common enough that it is desirable to have rapid laboratory confirmation, particularly for the central nervous system manifestations of the disease. We encourage such further development and clinical application of IF procedures.

TABLE 1. *Detection of mumps virus in cell cultures inoculated with clinical specimens^a*

Specimen	Results of:	
	IF procedures	Routine isolation procedures
1. Cerebrospinal fluid	Not detected in 5 days	HAD at 12 days—2nd passage
2. Throat swab	70 hours	HAD at 6 days; later CPE
3. Throat swab	70 hours	HAD at 6 days; later CPE
4. Throat swab	46 hours	CPE at 5 days; also HAD
5. Throat swab	6 days, RMK only	HAD at 6 days; no CPE
6. Throat swab	6 days	CPE (minimal) at 9 days
7. Throat swab	4 days in RMK 5 days in Vero	CPE at 5 days; HAD at 6 days
8. Urine	Negative	Enterovirus, new type
9. Throat swab	Negative	Rhinovirus, probable new type
10. Throat swab	Negative	Rhinovirus, possible new type
11-26. Throat swabs + 26 urines from 8 patients	Negative	Negative

^a For IF procedures, Vero and RMK cell cultures were inoculated and held 7 days, although the first few specimens were only held for 5 days. Coverslips were removed and stained each day, from day 2 through 7. Routine procedures used two tubes each of RMK and HFDL cells, which were examined daily for cytopathic effect (CPE) and tested by HAD on day 6, then passaged once. Nonmumps virus were detected by CPE.

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LITERATURE CITED

1. Bjorvatn, B., and S. Wolontis. 1972. Laboratory diagnosis of mumps by immunofluorescence on experimentally inoculated cell cultures. *Scand. J. Infect. Dis.* 4:105-108.
2. Blaskovic, P., and N. A. Labzoffsky. 1973. Immunofluorescence methods for detection of influenza and mumps antibodies using infected shedded allantoic cells and impression smears of organ cultures as the sources of antigens. *Arch. Gesamte Virusforsch.* 41:354-359.
3. Boyd, J. F., and V. Vince-Ribaric. 1973. The examination of cerebrospinal fluid cells by fluorescent staining to detect mumps antigen. *Scand. J. Infect. Dis.* 5:7-15.
4. Frankova, V., J. Kolatorova, and A. Nevarilova. 1970. Application of the method of immunofluorescence for rapid diagnosis of mumps virus. *J. Hyg. Epidemiol. Microbiol. Immunol.* 14:19-25.
5. Henle, W., E. H. Lennette, and N. J. Schmidt (ed.). 1969. Mumps virus. *In* Diagnostic procedures for viral and rickettsial infections, ch. 13. American Public Health Association, Inc., New York.
6. Lennette, E. H., F. W. Jensen, R. W. Guenther, and R. L. Magoffin. 1963. Serologic responses to para-influenza viruses in patients with mumps virus infection. *J. Lab. Clin. Med.* 61:780-788.
7. Lennette, E. H., J. D. Woodie, K. Nakamura, and R. L. Magoffin. 1965. Diagnosis of rabies by fluorescent antibody method (FRA) employing hamster immune serum. *Health Lab. Sci.* 2:24-34.
8. Lindeman, J., W. K. Muller, J. Versteeg, G. Th. A. M. Bots, and A. C. B. Peters. 1974. Rapid diagnosis of meningoencephalitis, encephalitis. Immunofluorescent examination of fresh and in vitro cultured cerebrospinal fluid cells. *Neurology* 24(2):143-148.