Simultaneous Use of Contrasting Fluorochromes to Separate Measles and Canine Distemper Viruses in a Common System

JAMES A. GOURLAY AND JOHN R. PEMBERTON
Veterinary Biologics Division, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010

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Measles and canine distemper viruses were grown together in a Vero monkey kidney cell line. Each virus could be identified and individually titrated by using the color contrast produced by the reddish tetra-methyl rhodamine isothiocyanate-tagged antimeasles conjugate and the green fluorescein isothiocyanate-tagged antidistemper conjugate. Both blue light and green light were used for the excitation of the fluorochromes. Incident light was transmitted to the specimen by a vertical illuminator of the Ploem type.

The viruses of measles, canine distemper, and rinderpest have been shown to closely resemble one another in size, structure, and type of cytopathology produced (10). An antigenic relationship between measles and distemper viruses has previously been demonstrated (4). It has also been demonstrated that a sufficient quantity of measles virus when inoculated into a dog will prevent clinical signs of distemper when the dog is subsequently challenged by a natural route with virulent distemper virus (2). In view of this close relationship and because Ackermann has suggested the combining of measles and distemper viruses into a bivalent vaccine for distemper prophylaxis (1), a study was undertaken to determine whether the two viruses could be identified and separately quantified when grown in a common system.

MATERIALS AND METHODS

Viruses. A strain (3) of canine distemper virus passaged in chicken embryos and chicken embryo fibroblasts and the Edmonston strain (6) of measles virus were used.

Serum. Anti-canine distemper serum was obtained from the blood of dogs after vaccination with commercial canine distemper vaccine and intracerebral challenge with the Snyder Hill strain of canine distemper virus.

Antimeasles serum was obtained from the blood of a horse hyperimmunized with the Edmonston strain of measles virus (kindly provided by S. Musser, Philips-Roxane, Inc., St. Joseph, Mo.).

Fluorochrome-serum conjugation. The conjugates were prepared by precipitating the gamma globulin of the immune serum three times with equal volumes of saturated ammonium sulfate and redissolving the globulin in 0.5 volume of 0.01 M phosphate-buffered saline (PBS), pH 7.4. The globulin was dialyzed for 24 hr at 2 to 4 C against several changes of PBS to remove the excess ammonium sulfate. The protein content of the globulin solution was estimated by the biuret test. The globulin solution was divided in half, and each portion was treated individually with a different fluorochrome.

The fluorescein conjugate was prepared by adding 0.5 M carbonate-bicarbonate buffer (pH 9.0) in an amount equivalent to 15% of the volume of the globulin solution followed by the addition of fluorescein isothiocyanate (FITC) equivalent to 0.02 mg of FITC per mg of protein. After the conjugate had been stirred for 4 to 6 hr at 2 to 4 C, the free FITC was removed by passing the conjugate through a column of Sephadex G-25 which had previously been equilibrated with PBS. The FITC conjugate was further purified by a procedure described by Appel (personal communication) in which the conjugate is passed through a column of diethylaminoethyl-Sephadex previously equilibrated with 0.1 M tris(hydroxymethyl)-aminomethane buffer, pH 8.7.

The rhodamine conjugate was prepared by adding tetramethyl rhodamine isothiocyanate (TRITC) equivalent to 0.02 mg of TRITC per mg of protein to an amount of carbonate-bicarbonate buffer equivalent to 15% of the volume of the globulin solution. After the TRITC was completely dissolved, the globulin was added to the buffered TRITC solution. After the conjugate was stirred for 16 hr at 2 to 4 C, the free TRITC was removed by passing the conjugate through a column of Sephadex G-25 which had been previously equilibrated with PBS. The conjugate was then treated for 16 hr with rabbit liver powder. The tissue powder was removed by high-speed centrifugation, and the supernatant portion was dialyzed against PBS.

Thimerosol was added at a concentration of 1:10,000 to preserve both types of conjugates.

Cell culture. The Vero line (9) of African green monkey kidney cells was grown on glass cover slips
in Leighton tubes by using minimum essential medium (Eagle) with nonessential amino acids and Earle's salts. Ten per cent fetal bovine serum was added to the growth medium with the serum level reduced to 2% during cell maintenance periods. Chlortetracycline (50 μg/ml) was added to all cultures.

**Cell monolayer infection and staining.** Tenfold dilutions of individual viruses or virus mixtures were used. Confluent cell sheets were infected with 0.1 ml of inoculum per tube. Tubes were incubated at 36°C for at least 4 to 7 days after infection and stained at intervals during this period. The cells on the cover slips were dried and fixed for 10 min in anhydrous acetone at 20°C and then stained with 0.1 to 0.2 ml of the mixed fluorochrome conjugate which had been previously diluted to an optimum fluorescent color balance for each fluorochrome. After 30 min in a humid atmosphere at 20°C, the cover slips were washed with 0.2 M phosphate buffer (pH 8.0), dried, and prepared for microscopic examination by using a mounting fluid with low autofluorescence (Unimount, Brunswick Laboratories, St. Louis, Mo.).

**Optical system.** Fluorescent excitation was provided by a 150-w Xenon lamp with incident light transmitted through a vertical illuminator (Ploem design; reference 8). The filters used are listed in Table 1.

**Photomicroscopy.** A Leitz Orthomat camera with automatic exposure control was used. High-speed color transparency film (GAF 500 Color Slide Film, GAF Corp., New York, N.Y.; ASA500) gave satisfactory color reproduction.

### RESULTS

Both viruses could be identified by their distinctive fluorescent colors in separate foci of infection within the same monolayer (Fig. 1 and Fig. 2).

Measles virus infection was detected slightly before that of distemper virus (3 days), and foci of measles infection were more numerous in the systems used in this study. Therefore, it was deemed better to conjugate the antimeasles serum with TRITC and the antidistemper serum with FITC. The apple green color of FITC is more easily discerned by the human eye than is the red color of TRITC and permits the scattered foci of distemper infection to be more easily identified. Each lot of conjugate had to be balanced with its companion conjugate to obtain optimal contrasts of color. With one combination, FITC conjugate was diluted 1:10 and TRITC conjugate was diluted 1:4. With different conjugates, the ratio had to be changed to a 1:2 dilution of FITC conjugate and a 1:10 dilution of TRITC conjugate to obtain comparable contrasts.

With the use of green light excitation (after blue light excitation on identical fields), the staining of distemper virus foci by TRITC-measles conjugate could be seen (Fig. 3 and Fig. 4). The cross-neutralization of distemper virus with antimeasles serum has been previously established (5). The effective suppression of FITC-detectable fluorescence by using green light excitation was verified when preparations were stained with FITC conjugate and viewed with both blue light and green light excitation (Fig. 5 and Fig. 6).

Mixtures of measles virus and canine distemper virus could be individually quantified by using the contrasting fluorochromes. A typical titration is presented in Table 2. End-point estimates did not increase after the fourth day postinfection in the system studied, but foci enlarged and were

### TABLE 1. Filter combinations used

| Exciting radiation | Filters* in lamp housing | Dichroic beam splitting mirror* | Built-in suppression filter* in illuminator | Filters* in microscope body near oculars | Fluorescent colors detected |
|--------------------|--------------------------|---------------------------------|----------------------------------------|----------------------------------------|-----------------------------|
| Blue               | 4-mm BG-38               | T > 495                         | K 495                                   | K 530                                  | Green                       |
|                    | 3-mm BG-12               |                                 |                                        |                                        | Salmon red                  |
|                    | 1.5-mm BG-12             |                                 |                                        |                                        |                             |
|                    | 1.5-mm VG-9              | Interference filter, 546 nm     |                                        |                                        |                             |

* Schott classification.

### TABLE 2. Measles virus and canine distemper virus titered individually and when combined

| Virus                      | FITC-tagged antidistemper conjugate only | FITC-tagged antimeasles conjugate |
|----------------------------|------------------------------------------|-----------------------------------|
| Canine distemper alone     | 3.5a                                     | 3.3                               |
| Measles alone              | NDb                                      | ≥4.5                              |
| Combined                   |                                          |                                   |
| Canine distemper Measles   | 3.0                                      | 3.5                               |
| Measles alone              | ND                                       | ≥4.5                              |

* Titer per 1 ml expressed as log base 10.  
*b Not detected.
FIG. 1. Focus of measles virus infection stained with TRITC-conjugated antiserum (salmon red color) and foci of canine distemper virus infection stained with FITC (green color). Grown in Vero line of African green monkey kidney cells. Approximately × 160. Blue light excitation. Combined TRITC-FITC conjugate.

FIG. 2. Canine distemper virus infection in Vero cells. Approximately × 160. Blue light excitation. Same FITC conjugate as in Fig. 1 but not combined with TRITC.

FIG. 3. A focus of canine distemper virus with blue light excitation.

FIG. 4. Same field as Fig. 3 but with green light excitation. Note evidence of cross-fluorescence with antimeasles conjugate. Approximately × 100.

FIG. 5. Massive measles infection with isolated focus of canine distemper virus infection. Approximately × 160. Blue light excitation.

FIG. 6. Same field as Fig. 5 but with green light excitation. Note evidences of FITC have been completely suppressed.

easier to detect on the fifth and sixth days post-infection.

DISCUSSION

Use of contrasting fluorochromes to separate and identify closely related antigens has been previously reported for differentiating immunoglobulins (7; J. S. Walker et al., in preparation) and species of mammalian cells (10). In this study, the feasibility of using a similar method to identify and titrate closely related viruses is described.

Vertical illumination by using the Ploem design
plus a Xenon lamp allows effective excitation of TRITC. Green light in the 555-nm range enhanced emission characteristics and provided good separation of FITC and TRITC fluorescence. Use of vertical illumination with fluorescence microscopy also provides better illumination at higher magnification, because the optics of the microscope objective serves to concentrate the area of illumination as well as enlarge the image.

Use of the technique described should allow evaluation of individual titers of either measles or canine distemper virus in a combined vaccine.

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