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NOTES

Visual Reading of Enzyme Immunofluorescence Assays for Human Cytomegalovirus Antibodies

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Enzyme immunofluorescence assays for cytomegalovirus antibodies could be read satisfactorily using a light box with ultraviolet illumination. Higher antibody titers were obtained with a fluorogenic substrate than with a color-producing substrate.

Quantitive enzyme immunoassays, as introduced by Engvall and Perlmann (2), have been applied to the assay of a wide variety of biological substances, including viral antigens and antibodies (9). Enzymatic activity is usually detected through the use of substrates giving a soluble, colored reaction product, and results are read visually or with a colorimeter or spectrophotometer. Alkaline phosphatase has been most generally employed as an enzyme label for detector antibodies with *p*-nitrophenyl phosphate (PNPP) as the substrate. There has been much interest recently in the use of fluorogenic substrates such as 4-methyl umbelliferyl phosphate (MUP), since the fluorescent end products of the enzymatic reaction can be detected fluorometrically at extremely low concentrations (3, 8). The sensitivity of viral enzyme immunoassays using fluorogenic substrates has been found to be higher than that of assays using the same enzyme label with a color-producing substrate (10).

Although enzyme immunofluorescence assay (EIFA) is recognized to be highly sensitive, a drawback to its rapid and widespread adaptation in diagnostic virology laboratories has been the need for a fluorometer and the relatively high cost of these instruments. We report here the application of a simple, inexpensive light box (1; U.S. patent 4,071,883, September 1976) for visual reading of EIFA results directly in microtiter plates and describe results obtained with EIFA for detection of antibody to human cytomegalovirus (CMV).

Purified CMV antigen and control antigen were prepared as described elsewhere (6). On the basis of preliminary block titrations, a 1:250 dilution of antigen was chosen as the optimal dilution for coating microtiter plates, and a corresponding dilution of uninfected control antigen, processed in the same manner as the viral antigen, was used. Our basic procedure for enzyme immunoassays has been described previously (4, 5). An alkaline phosphatase-labeled conjugate directed against human immunoglobulin G (7) was used at an optimal dilution of 1:4,000. The color-producing substrate PNPP was used at a concentration of 1 mg/ml in 10% diethanolamine buffer (pH 9.8) with 10^{-3} M $MgCl_2$, and the fluorogenic substrate MUP was used at a concentration of 0.025 mg/ml in the same buffer. Action of the enzyme on PNPP was stopped with 0.05 ml of 3 N NaOH, and enzymatic action on MUP was stopped with 0.05 ml of 1 M K_2HPO_4-KOH, pH 10.4 (3).

For tests using the PNPP substrate, spectrophotometric readings were made at 405 nm with a Beckman DB instrument; antibody endpoints were the highest serum dilution giving an optical density of ≥ 0.2 against the CMV antigen with a reading of ≤ 0.05 against the control antigen. For

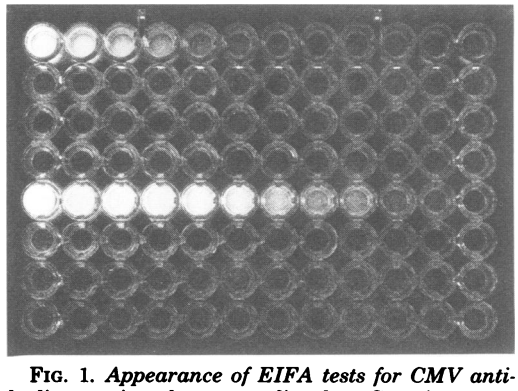

FIG. 1. *Appearance of EIFA tests for CMV antibodies as viewed on a reading box. Sera in rows 1 and 5 show positive results.*

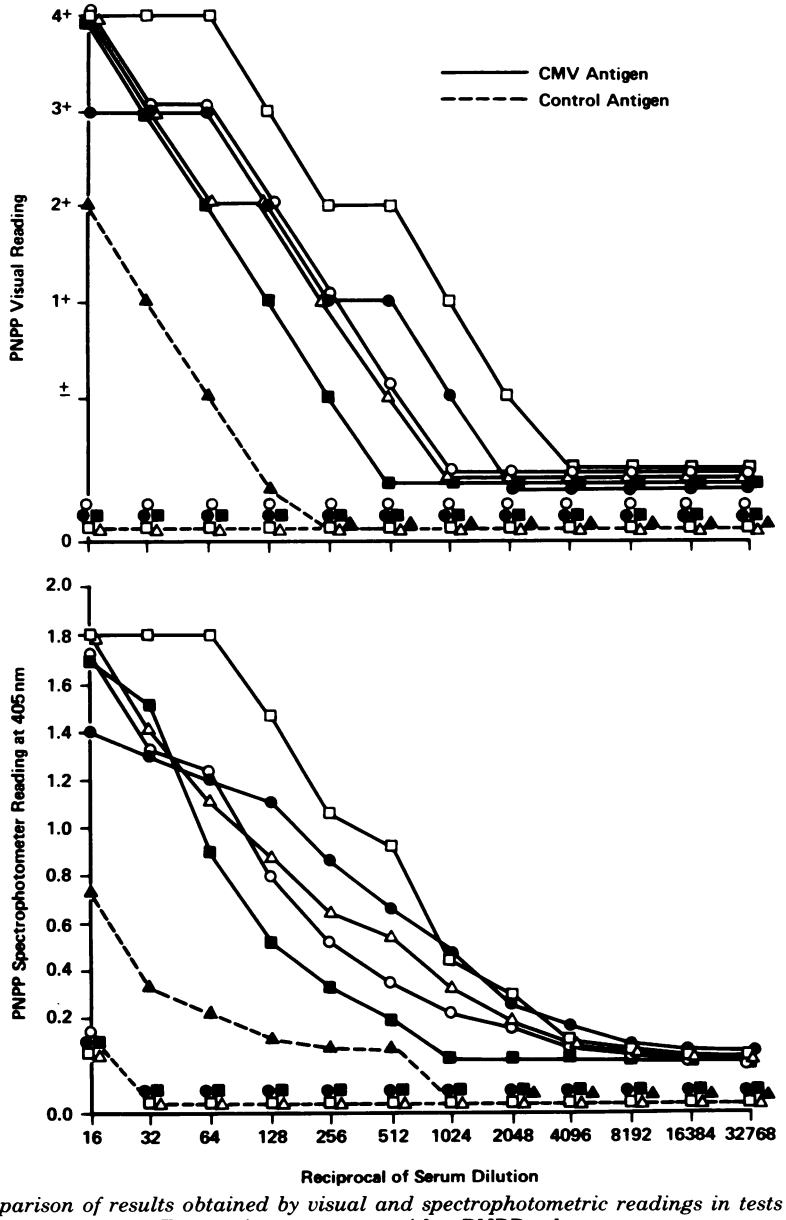

FIG. 2. *Comparison of results obtained by visual and spectrophotometric readings in tests on five CMV antibody-positive human sera. Enzyme immunoassays with a PNPP substrate.*

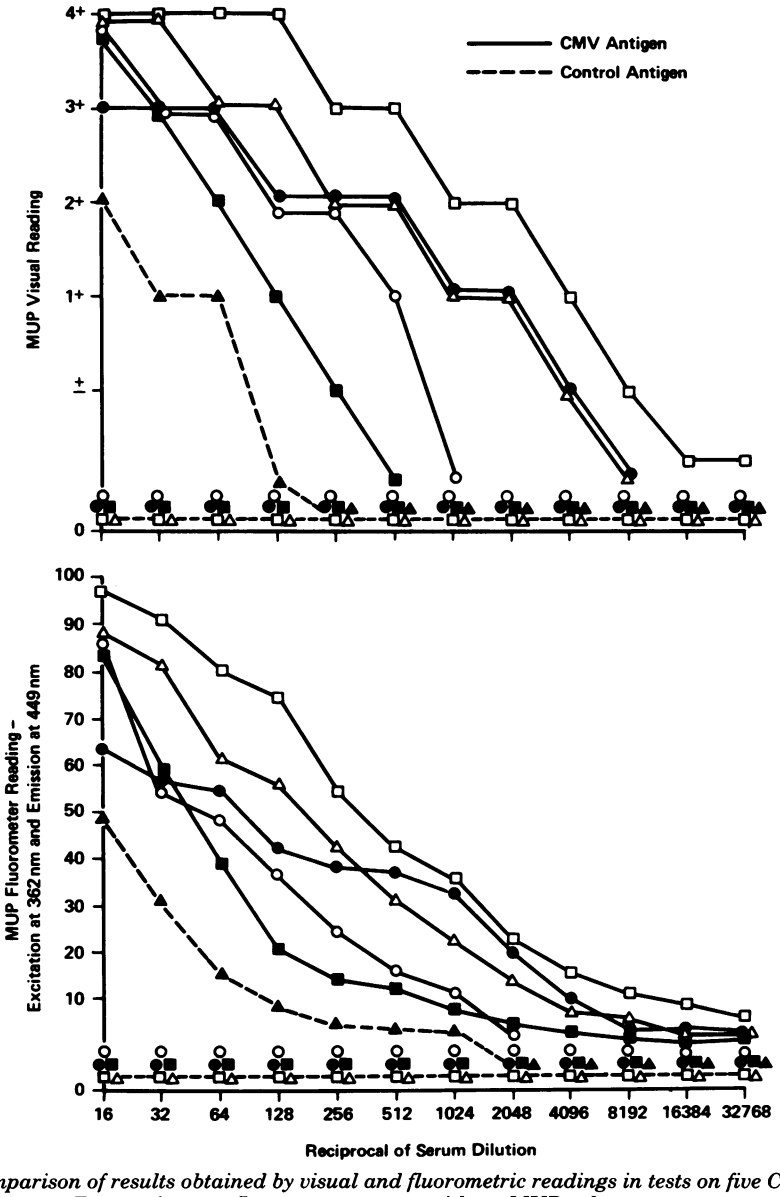

FIG. 3. *Comparison of results obtained by visual and fluorometric readings in tests on five CMV antibody-positive human sera. Enzyme immunofluorescence assays with an MUP substrate.*

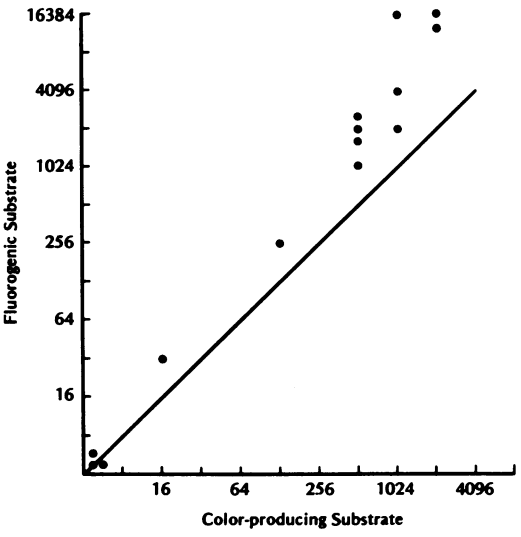

FIG. 4. *Comparison of results obtained with a color-producing substrate and with a fluorogenic substrate in enzyme immunoassays for antibodies to human CMV.*

visual reading, antibody endpoints were taken as the highest serum dilution showing a visible color with the viral antigen and no color against the control antigen. Readings were made on the same light box used for reading EIFA results, but with a white light and against a white background.

For tests with the MUP substrate, readings were made with a Perkin-Elmer MPF-44 spectrofluorometer at wavelengths of 362 nm for excitation and 449 nm for emission; the antibody endpoint was the highest serum dilution giving a reading of ≥ 5 against the CMV antigen and a reading of ≤ 1 against the control antigen. For visual reading of tests using the fluorogenic substrate, the light box was fitted with a long-wave ultraviolet, self-filtering black-light blue fluorescent tube (BLB, General Electric Co.) which emits light at wavelengths of from 310 to 410 nm, with a peak at 350 nm. To enhance the capacity for visual reading, by reducing background light, we placed a Corning 7-60 ultraviolet transmitting visible absorbing filter on top of the box over the ultraviolet light source and under the microtiter plate. According to the manufacturer's specifications, this filter transmits more than 55% of the total emittance of the light source at 365 nm. Although the light box utilized long-wave, rather than hazardous short-wave, ultraviolet illumination, additional safety measures were taken to protect the reader against possible leaks of short-wave ultraviolet light. These included covering the ends of the BLB tube where they were sealed to the metal electrical connectors with black electrical tape and covering the entire opening of the light box with a 0.19-in. (ca. 0.48-cm) Plexiglas panel. Ultraviolet protective goggles can also be worn as a further safeguard against possible leaks of short-wave ultraviolet light. After the microtiter plate was positioned on the filter and the opening of the box was covered with the Plexiglas panel, a stiff cardboard mask made from photographic mounting board, with a cut-out area the size of a test plate, was placed over the panel to reduce background illumination. Positive EIFA reactions showed a bright blue fluorescence, whereas negative reaction mixtures were nonfluorescent and the wells appeared black (Fig. 1).

Figure 2 is a comparison of visual and spectrophotometric readings for five CMV antibody-positive human sera in tests using PNPP as the color-producing substrate, and Fig. 3 shows the results of visual and fluorometric readings in tests on the same sera, using MUP as a fluorogenic substrate. First, these figures illustrate that similar endpoints were obtained by visual and by spectrophotometric or fluorometric readings, with visual readings tending to give two-fold lower titers. Second, it is seen that tests using fluorogenic substrates gave higher antibody endpoints than did those obtained when a color-producing substrate was used. Third, these figures show that individual sera which show similar levels of reactivity at low serum dilutions may differ markedly in the degree to which they react at higher dilutions and in their final antibody endpoints, making it difficult to interpolate antibody titers from spectrophotometric or fluorometric readings on a single serum dilution.

Figure 4 is a comparison of results of tests on additional sera, using MUP and PNPP substrates with visual reading, and further shows that higher antibody endpoints were obtained with a fluorogenic substrate then with a color-producing one.

These studies have demonstrated that EIFA results can be read visually using an inexpensive light box with little sacrifice of sensitivity; thus, laboratories which do not have access to a fluorometer can still take advantage of this new technique with improved sensitivity for detection of viral antigens and antibodies. In addition to economy, light box reading permits rapid screening of large numbers of tests. We have found that EIFA, with its high sensitivity for viral antibody detection, is particularly useful for large-scale screening of cell culture fluids from hybridoma cell populations for viral antibodies.

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