Standardization and Quantitation of Immunofluorescence in the Rabies Fluorescent-Antibody Test

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The standardization and quantitation of immunofluorescence with a Farrand microphotometer in the rabies fluorescent-antibody technique were determined. The system is useful in maintaining the quality of examinations for rabies in a public health laboratory since quantitation of reagent, equipment performance, and specificity of reaction can be evaluated. An analysis of the results demonstrates the high degree of reproducibility and the accuracy of the techniques described.

The fluorescent-antibody technique (FAT) is employed in many disciplines of microbiology, but in few fields is it as depended upon and as universal in its application as in rabies diagnostic examinations. Since Goldwasser and Kissling (8) adopted the techniques of Coons and Kaplan (2) for rabies identification, the techniques have been modified and improved. Excellent fluorescein isothiocyanate (FITC)-labeled anti-rabies conjugates are produced in many public health laboratories, and several commercial preparations are also available.

The standardization of the rabies FAT has been hindered by the lack of an accurate, commercially available instrument to quantitate fluorescent intensity. Visual quantitations by the conventional estimation (+1 to +4) of relative intensity have been used but are quite subjective and often vary from day to day and between laboratories. Attempts to quantitate immunofluorescent intensity were reviewed by Goldman (7), and the development of several accurate and practical schemes was described, but these require the assembly and tuning of several pieces of equipment manufactured independently. More recently, two suitable instruments commercially produced were described, and their application in research and development was evaluated (9, 11). This paper describes a similar system and its applications in the standardization, monitoring, and quantitation of fluorescent-antibody examinations and production of reagents in a rabies diagnostic laboratory.

MATERIALS AND METHODS

The FAT in our laboratory is essentially that of Dean and Ableth (3) except in the labeling of the antisera. The fluorescein is added by dialysis rather than directly to the globulins (1). Measurements are made on Zeiss fluorescent microscope equipment previously described (4).

The Farrand MSA microscope spectrometer analyzer (Farrand Optical Co., Inc., Mt. Vernon, N.Y.) consists of an eyepiece-monochromator assembly which clamps to the monocular barrel of the microscope and an RCA 1P21 photomultiplier tube and photometer. A beam splitter reflects 80% of the energy to the monochromator and transmits 20% to the eye. Interchangeable exit slits and entrance pinhole diameters allow a choice of target area diameter from 1 to 25 μm.

The standard fluorescent particle for illumination intensity control is standard sample 1023, zinc cadmium sulfide phosphor, the U.S. Department of Commerce, National Bureau of Standards. The crystal is mounted in Diaphane (Will Scientific) on a standard microscope slide. The Zn-Cd fluor fades a total of only 5 to 10% under ultraviolet light (UV) stimulation; this drop takes several minutes to occur. When the crystal is removed from excitation, intensity quite rapidly returns to the original value. The system is standardized from test to test by adjusting the intensity of incident radiation so that fluorescent intensity of the phosphor is at an established level.

The monochromator is set at the characteristic emission wavelength for the dye being used, in the case of FITC, 523 nm. After an initial warming of the lamp and photometer, the standard crystal is arranged by stage manipulation so that it entirely fills the target area. The target area is delineated by a red light in the field of view. The target light is turned off, and the phototube shutter is opened. The condenser
and focus of the microscope are adjusted to maximal intensity on the photometer, and the iris diaphragm on the lamp is regulated so that the intensity of fluorescence is at a predetermined standard reading. The condenser and focus are then adjusted to optimum for each slide during the reading of all measurements.

Virus from naturally infected striped skunks (Mephitis mephitis) was the antigen employed for all quantitative procedures. Smears were prepared from either skunk brain or first or second mouse passages of skunk isolates.

Measurements of fluorescent intensity in the FAT are taken from 10 labeled particles on each slide. A particle is measured only if it is large enough to fill entirely the target area in the eyepiece of the monochromator assembly. Once the particle is positioned under the target area, the photomultiplier tube is opened, and the intensity of immunofluorescence is read from the appropriate scale on the photometer. Representative readings were taken from each portion of the stained area, and the arithmetic mean of these values was used in the calculations. The photometer has nine settings, ranging from 0.1 to 100 \( \mu \)A.

**RESULTS**

Ten test slides were examined to evaluate the reproducibility of results. The arithmetic mean of 10 particles was calculated for each slide. Analysis of the means showed a standard error of 0.276 \( \times 10^{-3} \), with a population mean of 7.85 \( \times 10^{-3} \), or a standard error of 3.5% of the mean. The confidence interval \((P = 0.95)\) was calculated to be \( \pm 0.20 \times 10^{-3} \). The small standard error and narrow confidence limits indicate the degree of reproducibility of results. In applications of the quantitative technique, differences of 5% or greater from the control are significant. The employment of a more uniform standard antigen, such as infected tissue culture cells, should make this figure even smaller.

**DISCUSSION**

Reagent production is an integral part of the activities of a rabies diagnostic laboratory. Antisera labeled with fluorescein show a characteristic linear relationship between fluorescent intensity and conjugate intensity and conjugate determination. The optimal dilution of conjugate used for examinations is described as the highest dilution that combines a high degree of fluorescence with low background staining. The optimal working level of this contrast remains subjective, but, once it is determined and measured with the photometer, production of future labeled antisera can be evaluated, and optimal dilutions can be determined quantitatively. Conjugate produced in our laboratory is measured to determine the working dilution which has the best contrast between specific and background fluorescence.

Equipment function can also be monitored by the quantitation of fluorescence. The mercury vapor lamps have a life generally between 100 and 200 hr. Previously, bulb life was monitored by keeping a log of use, and as a bulb approached 200 hr it was discarded. Some bulbs become dim much sooner than others and require replacement after less service. However, because the output of a bulb decreases slowly (7) if several consecutive hours are spent at the microscope, the decreased bulb intensity cannot be perceived adequately (the quartz-iodide lamps coming into use feature the advantages of significantly longer life, constant output until an abrupt drop at the end of bulb life, and a lower initial cost than the mercury vapor lamps). In the standardization procedure, a level of intensity was selected at which the immunofluorescence was very bright visually but well below the capabilities of the new bulb. Therefore, with the installation of a new bulb, the iris diaphragm was closed to some degree. As the bulb grows dim, the iris diaphragm is progressively opened to attain the standard
level of intensity. When the iris diaphragm is left completely open to achieve the standard intensity, the bulb should be replaced.

With each FAT performed, a positive control slide with known rabies antigen was tested. The quality of immunofluorescence on this slide monitors the variables of the FAT. Staining time and temperature, mounting medium pH and viscosity, antigenic characteristics, and background intensity can all be monitored by evaluation of this control slide. An intensity of 5 \times 10^{-4} \text{  } \mu \text{w} \text{ has been established in our laboratory as the minimal level that each control slide must attain.} 

Our laboratory examines approximately 2,500 animals each year for rabies virus by the FAT. This work necessitates reliable equipment and bright, cleanly labeled antisera. The high degree of success of the rabies FAT in diagnostic laboratories results from the efforts of skilled and conscientious microscopists and technologists who maintain the quality of these reagents and instruments. However, because of the facility with which the human eye adjusts to the degree of intensity, adherence to strict criteria has been subjective. Bulb life, conjugate production, and control evaluation are all factors that can and should be monitored by quantitation of immunofluorescent intensity.

The apparatus described has the important feature of being easily installed into fluorescent microscopy systems presently in use. Once the equipment is in place, readings can be taken at any time without disrupting the system.

Some fading of immunofluorescent intensity occurs as soon as the labeled particle is exposed to UV (5, 10). Previous techniques have eliminated this problem by the use of visible light for the targeting of fluorescent particles, thereby not directing the UV stimulation onto the particle until measurement is made. With rabies virus, however, the particles become visible only under UV. This requires that the particle be located while the lamp is on. Approximately 15 sec of illumination are required before measurements are taken. We have found that by reading only one particle per microscopic field, the fading for each particle is constant and causes little variation in the test. Fading continues while the reading is made, but is minimized by recording the highest point of the photometer needle deflection for each particle.

The standardization procedure was employed to reduce the effect of changes in lamp output since fluctuations caused by bulb age and voltage variations occur. Goldman (6) measured incident radiation directly and incorporated this into calculations of fluorescent intensity. The procedure reported here apparently compensates very well for long-range changes in bulb output. Small transient fluctuations were reduced by using a step-up transformer to power the lamp rather than a direct 220-v line which is subject to power surges and depressions.

The development of a better standard antigen is desirable. Because the thickness of the tissue smear is critical and directly affects the intensity of illumination, the use of a single sheet of infected tissue culture cells would be preferable. The production of the necessary large granules and coalescent fluorescent antigen appears feasible but requires additional study. Furthermore, the development of full-cell fluorescence and a very high percentage of infectivity would eliminate the fading problem, since visible light could then be used to locate cells in the target area. The nature of the monolayer and the consistency and ease of production of standard lots of infected cells should make this the antigen of choice.

The quantitation of immunofluorescence has additional applications in research and development. Pittman et al. (11) have shown that a microphotometer can be used in assessing the intensity of nonspecific staining of conjugate preparations. A microfluorometric system is also an effective tool for distinguishing specific fluorescence from autofluorescence and particles of uncombined fluorescein. Unreacted fluorescent materials have a higher intensity than globulin-bound dye (12). Thus, particles that give intensity measurements well above those encountered with known antigen can be identified as free dye. Autofluorescing particles have emission spectra that vary from those of FITC. By rotating the monochromator and looking for the peak of fluorescent intensity, the characteristic peak wavelength of the fluorescent particle can be determined. If the peak of a particle does not match that of the labeled antibody, it can be confirmed as being auto-fluorescent.

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