Inactivation of Rabies Virus in Reagents Used for the Fluorescent Rabies Antibody Test

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Procedures for inactivating rabies virus in reagents used for the fluorescent rabies antibody test are described. Mouse brain adsorbing suspensions containing \( \geq 10^6 \) 50% lethal doses of virus per ml were rendered noninfectious by treatment with 0.1% \( \beta \)-propiolactone or by heating at 56°C for \( \geq 30 \) min. Viable virus in tissue impression smears was inactivated by acetone fixation at 50°C for \( \geq 30 \) min or by immersion in 0.1% \( \beta \)-propiolactone at 37°C for 2 h. Inactivated reagents gave specific and sensitive reactions in the fluorescent rabies antibody test.

The fluorescent rabies antibody (FRA) test is the recommended microscopic method for identifying rabies virus antigen in impression smears and in tissue sections (7). Many public health laboratories routinely use the FRA test in examining diagnostic specimens since the accuracy of the test is equal to that of virus isolation by animal inoculation. Although human cases of rabies in the United States have been rare, the incidence of laboratory-confirmed cases of rabies in animals and the potential for transmission to humans have increased steadily during the past several years (3). Impression smears from brains of rabid animals have been reported to remain infectious despite the use of recommended fixation procedures (5, 12). In addition, adsorbing suspensions made from infected brain material are infectious. Therefore, with these reagents, the performance of the FRA test is a potentially hazardous procedure. Inactivation of rabies diagnostic reagents by gamma radiation has been reported (6). A disadvantage of this method, however, is the need for expensive and not commonly available equipment. We conducted investigations to determine additional effective methods for inactivating reagents for the FRA test.

MATERIALS AND METHODS

**Viruses.** Seed preparations of rabies virus strain CVS and street rabies virus (isolated from a fox salivary gland) were prepared as 20% (wt/vol) suspensions of infected mouse brains in phosphate-buffered saline (PBS) (pH 7.2) with 20% normal inactivated rabbit serum. These viruses titrated in suckling mice by intracranial inoculation gave 50% lethal doses of \( 10^6 \) \( \pm \) \( 0.1 \) for CVS and \( 10^6 \) \( \pm \) \( 0.5 \) for street virus.

**Host systems.** Suckling and weanling mice (strain ICR) were supplied by the Veterinary Services Branch of the Centers for Disease Control. Virus titrations were performed in suckling mice inoculated intracranially with 0.02 ml of virus suspension and observed for 21 to 28 days. Impression smears of mouse brains at the endpoint dilution of the titrations were examined for rabies virus antigen by the FRA test. Brains from weanling mice inoculated intracranially with 0.03 ml of virus suspension were used for the production of adsorbing suspensions and for impression smears.

**FRA control reagents.** Normal and rabies mouse brain (NMB and RMB) impression smears, NMB and CVS RMB adsorbing suspensions, and rabies fluorescein isothiocyanate-labeled globulin were obtained from the reagent inventory of the Biological Products Division, Centers for Disease Control. The preparation of these materials has been described previously (4). The procedure for the FRA test and the specifications of the fluorescent microscope have been described previously (1).

**Preparation of adsorbing suspensions.** Weanling mice were inoculated with 1:100 dilution of the CVS seed. The brains were aspirated from moribund and paralyzed mice, and a 20% suspension was prepared in PBS. The RMB was leached for 30 min at 4°C and clarified by centrifugation at 800 \( \times g \) for 30 min, and the supernatant fluid was stored at \(-20^\circ\)C. NMB suspension was prepared by the same procedure from noninfected mice of the same age as the CVS-infected mice at the time of harvest.

**Experimental inactivation procedures for RMB and NMB suspensions involved the use of heat and \( \beta \)-propiolactone (BPL). Split samples were tested by each procedure, and the results with the treated and untreated materials were compared. One sample was buffered with 0.01 M Tris (pH 8.5) and treated at 4°C with 0.1% BPL. Samples taken after treatment for 4, 7, and 24 h were examined for infectivity in mice and for specificity in the FRA test. A flask containing a second sample was submerged in a water bath at 56°C. After
the temperature of the suspension reached 56°C, samples were removed at intervals of 15, 30, 60, 90, and 120 min and were examined for mouse infectivity and for specificity in the FRA test.

Preparation of mouse brain impression smears. Weanling mice were inoculated with a 1:100 dilution of either the CVS or the street rabies virus seed. Paralyzed and moribund mice were killed, and the brains were sectioned as described by Johnson (8). Two impressions were made on each glass microscope slide (75 by 25 mm). Multiple slides were prepared from each section, air dried at room temperature, and stored at −60°C. Representative slides from each brain were fixed in acetone at −20°C for 4 h and examined in the FRA test. Infectivity titers were determined on the brains used for preparation of the impression smears. Smears were used in the inactivation experiments only if the fixed smears were satisfactory by IF examination and if the infectivity titers of the brains demonstrated 50% lethal doses of ≥10^−5/ml for CVS and ≥10^−6/ml for street rabbies.

Experimental procedures for the inactivation of rabies virus in impression smears were conducted with groups of 15 slides prepared from three to five different infected brains and handled as experimental units. The two procedures investigated were (i) immersion in different concentrations of BPL and (ii) fixation in acetone at different temperatures and time intervals. Groups of slides were immersed for 2 h at 37°C in PBS containing BPL at a concentration of 0.05, 0.1, or 0.5%. After treatment, the slides were air dried and fixed for 4 h in acetone at −20°C. Other slide groups were immersed for time periods of 0.5, 1, 2, or 4 h in acetone warmed in a water bath to 22, 37, or 50°C. After treatment for the designated time interval, the slides were air dried. Additional acetone fixation was not required.

A group of control smears fixed in acetone at −20°C for intervals of 2, 4, 7, and 24 h was tested for the presence of infectious virus. Tissues scraped from untreated smears were also titrated for infectivity. Smears prepared by each experimental procedure variation were examined in the FRA test.

After treatment, the tissues from each group of impression smears were scraped from the glass surfaces into a chilled alumina mortar. The pooled tissues were homogenized in 1.5 ml of Eagle minimal essential medium with 2% fetal bovine serum, and infectivity titrations were determined in BHK-21/WI-2 cell cultures.

RESULTS

Mouse brain adsorbing suspensions. The untreated RMB suspensions had infectivity titers (50% lethal dose) ranging from 10^−1.9 to 10^0.2/ml and gave satisfactory reactions in the FRA test. The results of the inactivation experiments are presented in Table 1. The rabies virus was successfully inactivated with 0.1% BPL during all three time periods. The BPL-treated RMB and NMB suspensions gave satisfactory FRA results that were comparable to those of the untreated suspensions.

RMB preparations heated at 56°C for 15 min had reduced infectivity titers but after being heated for 30 min or longer were not infectious for suckling mice. Satisfactory inhibition of staining in the FRA test was obtained with conjugates diluted in all of the heat-treated RMB suspensions. Conjugates diluted with heat-treated NMB suspensions gave specific staining at a 4+ intensity and of acceptable quality without nonspecific or background fluorescence. All FRA tests were performed in parallel with untreated RMB and NMB suspensions, and the specificity and intensity of fluorescence were comparable in the treated and untreated suspensions.

Excessive heat treatment (90 to 120 min) resulted in a clumping of the brain material, giving a granular appearance to the suspension. Homogenization with a tissue grinder made a more uniform suspension but did not eliminate the granular precipitate. Additional washings were necessary in the FRA test to assure complete removal of the granular suspension from the smear. Attempts to clarify the RMB suspensions by centrifugation removed excessive amounts of virus, resulting in incomplete inhibition of staining.

Impression smears. CVS smears fixed in acetone at −20°C for 2, 4, 7, and 24 h were examined for virus viability. Scrapings from these smears all contained infectious virus, thus confirming previous reports that acetone fixation at −20°C does not inactivate rabies virus. The infectivity titers ranged from 10^−1/0.1 ml in suckling mice to 10^5.1/0.1 ml in BHK-21/WI-2 cultures.

Tissues scraped from impression smears after acetone fixation were examined for virus viability in BHK-21/WI-2 cell cultures. The results of fixation at 22, 37, and 50°C for time periods of 0.5, 1, 2, and 4 h are compared in Table 2. Untreated CVS-infected tissues gave infectivity
titers of 10^{3.0}/0.1 ml, whereas untreated street-virus-infected tissues had infectivity titers of 10^{1.0}/0.1 ml. Acetone fixation at 22°C and for less than 4 h at 37°C had little effect on the viability of either virus. Fixation at 37°C for 4 h and at 50°C for at least 30 min rendered both viruses noninfectious. The lack of viral replication in BHK-21/WI-2 cultures inoculated with these materials was confirmed by direct IF examinations. All three temperatures gave satisfactory tissue fixation for use in the FRA test.

CVS and street virus impression smears were immersed for 2 h at 37°C in PBS containing 0.05, 0.1, or 0.5% BPL. Control smears immersed in PBS without BPL had infectivity titers in BHK-21/WI-2 cultures ranging from 10^{2.0} to 10^{3.0}/0.1 ml with CVS and from 10^{1.5} to 10^{2.4}/0.1 ml with street virus. Only partial inactivation occurred with 0.05% BPL, but 0.1% BPL effectively inactivated the virus.

Satisfactory results were obtained in the FRA test with the inactivated smears as well as with the normal impression smears treated in a similar manner. Specificity and sensitivity were unaltered by inactivation, and nonspecific staining was not observed.

**DISCUSSION**

The Biological Products Division of the Centers for Disease Control prepares and distributes reference diagnostic reagents. These reagents are supplied to state and international health organizations and to commercial producers of diagnostic reagents. Reagents are inactivated whenever possible without interfering with the specificity, sensitivity, or potency of the antigens.

Numerous reports (9, 13, 14) on the preparation of experimental vaccines with high levels of immunogenicity have described the inactivation of rabies virus. The effects of various physical and chemical methods of inactivation have been compared by complement fixation, hemagglutination, and infectivity assays.

The exposure of medical personnel to rabies from either infected animals or human cases is not unusual, but laboratory-acquired infections have been rare. However, in recent years two cases which indicate an increased risk after exposure to airborne virus have been reported (2, 15). The best protection for the laboratory worker is to use careful technique, avoid accidents, and have preexposure immunization with documented antibody responses. Nevertheless, the removal of potential hazards from the diagnostic laboratory should not be overlooked, and the use of inactivated reagents helps decrease the risk of exposure.

We investigated the use of infectious and inactivated reagents in the FRA test, and compared infectivity assay results were obtained. Inactivated reagents gave results which were specific, sensitive, and devoid of nonspecific IF staining. Based on the results presented in Table 1, we believe that the inactivation of virus in the mouse brain adsorbing suspensions is best accomplished by treatment with 0.1% BPL at 4°C for at least 4 h. Heat treatments exceeding 15 min at 56°C satisfactorily inactivated the virus but caused discoloration and clumping of the suspensions. The inactivation of virus in mouse brain impression smears was investigated by using two procedures. The results showed effective inactivation with acetone fixation at 50°C for ≥30 min and with 0.1% BPL at 37°C for 2 h. The use of acetone at 50°C has the advantage of simultaneous virus inactivation and tissue fixation.

Although BPL has been recognized as a carcinogen (16), it may still be safely used when proper laboratory techniques are employed. We have presented two procedures that should improve the safety of laboratory personnel working with large volumes of rabies virus diagnostic reagents.

**LITERATURE CITED**

1. Centers for Disease Control. 1974. Specifications and evaluation methods for immunological and microbiologi-
References