Fluorescent Antibody Staining of Street and Fixed Rabies Virus Antigens (23996)

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The diagnosis of rabies in animals is usually based upon the demonstration of Negri bodies in brain smears or sections. However, these inclusion bodies cannot be demonstrated in approximately 10% of naturally infected animals and under certain conditions this percentage may rise to 26%(1). These Negri negative animals may be confirmed as infected with rabies by virus isolation procedures but such technics are too time-consuming to be used as basis for decision regarding vaccination. Because of dangers inherent in the use of the standard anti-rabies vaccines, it is desirable to administer them only to persons who have experienced a real exposure to the virus. Thus there is need for a diagnostic technic which will combine the speed of histological technics with the accuracy of biological examination.

Materials and methods. Strains of rabies virus we used included fixed virus strain CVS 11 obtained from National Institutes of Health, Division of Biologics Standards, and several strains of street virus isolated from salivary glands of dogs and foxes in Alabama. These strains were prepared as 20% infected mouse brain suspensions in a diluent consisting of 10% yolk from 6-7-day embryonated eggs in .05 M sodium phosphate buffered water, pH 7.6-7.8. All suspensions were lightly centrifuged to remove larger particles before storage at −60°C. Mice were inoculated intracerebrally and were sacrificed on showing definite signs of disease; the brains were removed and impression smears made from cross-section which included the hippocampi. Various methods of fixing were tested but only one gave consistently satisfactory results. Freshly prepared smears, while still moist, were dropped into Coplin jars containing acetone pre-cooled in dry ice-alcohol bath. The jars were then transferred to a household type deep freezer kept at −15° to −20°C. After 18-24 hours, slides were removed from the acetone and drained and dried without being taken out of freezer. These slides stained satisfactorily with fluorescent antibody even after prolonged storage. Indirect staining(2), complement staining(3) and direct staining(4) have all been used in this work. The conjugates used for indirect stain were rabbit-antihuman globulins and rabbit antiguinea pig globulins conjugated with use of fluorescein impregnated filter discs as described elsewhere(3). Rabies antiserums were derived from humans who had undergone an experimental course of immunization with duck embryo grown rabies vaccine. Following fixing and drying in the cold, the impression smears were treated with antiserum diluted 1:10 or with a mixture of equal parts antiserum diluted 1:5 and guinea pig complement diluted 1:10. These slides were incubated 1/2 hour at 37°C in a wet chamber, washed 10 minutes in .01 molar phosphate buffered saline pH 7.4-7.6, exposed to the appropriate conjugate at 37°C for ½ hour, washed and mounted in buffered glycerol. For direct stain, a commercial refined and concentrated horse antirabies serum (Lederle) was used. This serum was simply diluted to suitable concentration for labeling without any preliminary treatment, and conjugation was carried out as above. All conjugates were absorbed once with hamster liver powder and again with mouse liver or mouse brain powder (5), to reduce nonspecific staining.

Results. Street virus. Fig. 1 illustrates typical staining obtained in impression smears of brains from mice in late stages of rabies infection. Great variation was seen in size of fluorescent bodies which ranged from size of nuclei of nerve cells or larger, to the barely visible. That these were associated with rabies virus was borne out by the following evidence:

A. Paired (pre-immunization and post-immunization) serums from the same individuals were used to stain duplicate slides. The pre-

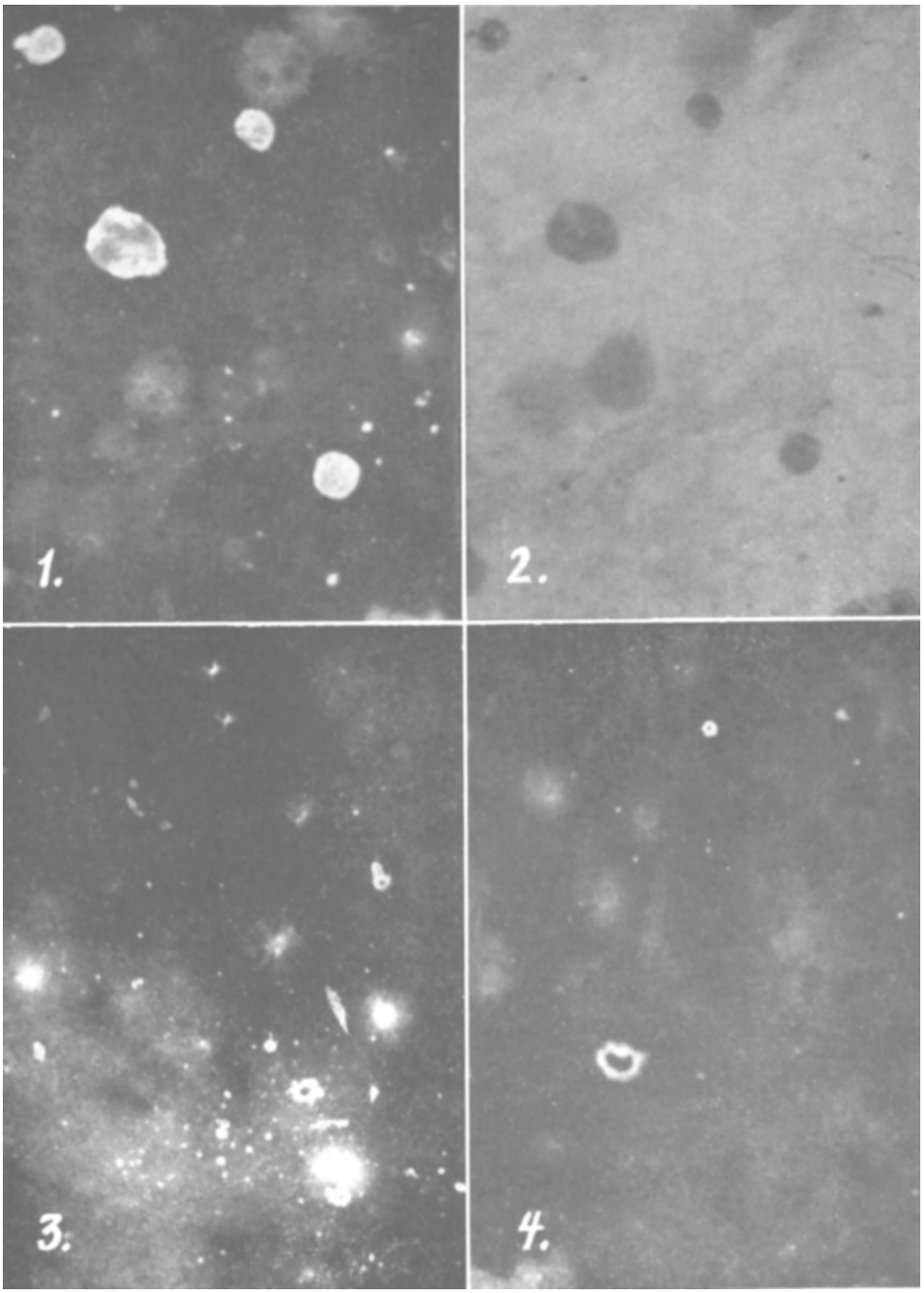

FIG. 1. Impression smear of brain from mouse infected with street rabies virus, stained with fluorescent antibody. × 620.

FIG. 2. Same field as in Fig. 1, stained with Shleifstein's stain. × 620.

FIG. 3 & 4. Impression smear of brain from mouse infected with CVS 11 strain of fixed rabies virus stained with fluorescent antibody. Fig. 3, × 620; Fig. 4, × 530.

immunization serums did not give staining, whereas post-immunization serums resulted in good to excellent staining, depending on individual serum.

B. Since immunization had been carried out with duck embryo grown vaccine, it was considered unlikely that staining was due to production of antibodies against normal mouse proteins, and in fact smears made from normal mouse brains failed to show staining with either pre-immunization or post-immunization serums. The possibility that immune serums contained antibodies against abnormal mouse antigen formed in response to virus-induced encephalitis was investigated. Groups of mice were inoculated intracerebrally with various dilutions of St. Louis encephalitis virus and pseudorabies virus. No staining was obtained in brain impression smears of these mice with any of the rabies immune serums.

C. In indirect staining procedure as usually carried out, the antigen, which is fixed on a slide, is treated with antiserum diluted in saline and then with a fluorescein-conjugate directed against the antiserum globulins. However it was noted that when an antiserum is suitably diluted not in saline but in a solution or suspension of antigen immunologically related to that fixed on the slide, subsequent staining will be partially or completely inhibited, depending on the closeness of that immunological relationship(6). The rabies-immune serums were therefore diluted 1:10 in a 20% suspension made from brains of mice in late stages of infection with CVS 11 virus, and in a similar suspension made from brains of normal mice. Impression smears from street rabies-infected mice were then treated with each of the above mixtures. The smears treated with antiserum diluted in normal mouse brain showed good fluorescent staining with very low background whereas those treated with antiserum diluted in infected mouse brain gave no visible staining.

D. The number and distribution of the larger fluorescent bodies corresponded well with the number and distribution of Negri bodies seen in duplicate slides stained with Sellers' stain. Some of the fluorescent bodies showed quite uniform fluorescence, but others showed non-homogeneous staining (Fig. 1). In some cases fluorescent inner granules could be seen, but this was the exception rather than the rule, and it was not found possible to determine whether these corresponded to the inner granules, diagnostic of Negri bodies, seen when stains such as Sellers' are used.

Many attempts were made to remove cover slips from smears stained with fluorescent antibody and to stain the same preparations with one of the conventional Negri body stains. The results were invariably poor. Staining was very weak and though the Negri bodies could be seen, the inner granules were rarely visible. When present they were in the Negri bodies in regions where the smear was thickest, areas not suitable for examination with the fluorescence microscope due to high autofluorescence.

Fig. 2 shows the same field as Fig. 1, following the use of Shleifstein's stain. But whereas all large fluorescent bodies stained with above dyes, only some of the smaller ones did so and these could not be distinguished from acidophilic particles which had not shown fluorescent staining.

Fixed virus. Fig. 3 and 4 show some examples of the staining obtained in impression smears of brains from mice in late stages of infection with the CVS 11 strain of fixed rabies virus. No large round fluorescent bodies were seen as with street virus, but many of the smaller particles were identical in appearance to those seen in street virus infections. In the fixed virus, "ring" forms were relatively frequent in appearance, some quite large (Fig. 4). When cover slips were removed from such preparations and the smears stained with Giemsa or Shleifstein's stain, some of these rings and other particles could be found with difficulty since they stained very weakly, but they could not be distinguished from other objects similar in appearance which had failed to show fluorescent staining.

The same tests for specificity of staining were applied as with street virus, with identical results, *i.e.*, normal mice or mice infected with encephalitic viruses other than rabies failed to show staining; pre-immunization serums did not stain with fixed rabies-infected

mouse brain whereas post-immunization serums from the same individuals did so; finally, mixing immune serums with rabies-infected mouse brain suspensions completely inhibited staining of smears but similar use of normal mouse brain suspensions caused no such inhibition.

Discussion. It has long been assumed that Negri bodies contain virus particles and cytochemical studies(7) have presented indirect evidence for this hypothesis. The major part of Negri bodies, however, has been thought to be composed of a matrix derived from cytoplasm of degenerating cells(1). Fluorescent antibody studies, however, indicate that these inclusion bodies contain concentrations of viral antigens which seem to be distributed diffusely throughout the Negri bodies rather than being restricted to the inner granules. The antibodies used in indirect staining of mouse brain smears were produced with virus grown in the duck embryo, thus precluding the likelihood of formation of antibody to mouse tissue. No staining of normal mouse brain was observed, nor did rabies-immune serums produce staining of smears from mice infected with St. Louis encephalitis or pseudorabies virus. Diluting antisera in normal mouse brain suspension reduced background fluorescence to a minimum but had no inhibiting effect on brightness or type of staining, whereas diluting the antiserum in rabid mouse brain suspension caused complete inhibition of such staining.

Small eosinophilic inclusion bodies, which cannot ordinarily be considered as specific because of their similarity to inclusion bodies found in other diseases, are often seen in brains of animals infected with street rabies virus. Fluorescent antibody staining, however, indicates that some of these at least are of rabies virus origin. Actually, staining of particles of all sizes down to the barely visible was seen, and these possibly represented aggregates of various numbers of elementary bodies of the virus. It was not possible to differentiate brain smears of fixed virus-infected mice and street virus-infected mice except that in the latter larger fluorescent bodies appeared, apparently representing the Negri bodies.

Since it was possible to demonstrate specific staining of fixed virus which does not form Negri bodies, as well as of street virus which was not included in Negri bodies, it appears that this type of staining might be useful for rapid diagnosis of rabies in Negri negative animals. Furthermore, the fact that excellent staining was obtained with antisera derived from humans immunized with a rabies vaccine, indicates that this might furnish a useful tool for studying development of immunity in man or animals undergoing rabies vaccination.

Preliminary studies have shown that the methods described may be suitable also for demonstrating the presence of rabies virus in the salivary glands of naturally infected animals. Staining occurred even in glands which had been immersed in glycerine for periods as long as 3 years. Small pieces of glands were rinsed free of glycerine and then used to make impression smears. In some cases triturated tissues were used. The smears were dried and stained as described above. Although large amounts of autofluorescent material were usually present in these smears, the yellow-green fluorescence of the stained antigens could be readily distinguished. The antigens in the glands were similar in appearance to those observed in the brain smears except that the fluorescent bodies tended to be smaller.

Positive glands from foxes, dogs, cows, skunks and one wildcat have been examined so far by this method and a field trial is now in progress in collaboration with the Alabama State Health Department laboratories to determine the reliability of this procedure for determining the presence or absence of virus in the salivary glands of suspected animals.

Many strange and bizarre forms of fluorescent staining material were found in smears of both fixed and street virus in addition to those shown in the photographs. It seems probable that these were due simply to artificial distortion of the antigenic material during the making of impression smears. Staining of frozen sections should make it possible to determine whether these bizarre forms or the ring forms shown in Fig. 3 and 4 exist as such in the animal tissues or whether they are artifacts resulting from the methods used in

making and fixing the smears.

Summary. Methods of fixing and staining of rabies-infected mouse brain smears with fluorescent antibody are described. Evidence is presented that Negri bodies are of viral origin. The possible usefulness of this method for diagnosis of rabies in Negri negative animals and for serological determinations following immunization against rabies is indicated.

Preliminary studies indicate that this technic may be useful in the detection of virus in salivary glands.

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Growth of Rabies Virus in Non-Nervous Tissue Culture. (23997)

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Successful attempts to grow rabies virus in tissue culture have been summarized by Sanders and co-workers(1). The essential feature of each report was the use of brain tissue from embryonic or very young mice, rabbits, or chicks. Previously, attempts to propagate rabies virus in non-nervous tissue cultures have been unsuccessful(2,3). Vieuchange and co-workers(4) reported that fixed rabies virus could be recovered from mouse kidney cultures inoculated 20 days previously. However, they did not demonstrate an increase in virus nor present adequate evidence that the virus could be serially propagated in mouse kidney cultures.

Having demonstrated successful propagation of several neurotropic viruses in hamster kidney cultures(5) these tissues were used in an attempt to propagate rabies virus.

Materials and methods. Cell suspensions of hamster kidneys (HK) were prepared as described previously(5). During the earlier phase of this study the nutrient medium consisted of 3-5% calf serum and 0.5% lactalbumin (LA) hydrolysate in Hanks' balanced salt solution (HBSS) plus antibiotics. Later, the nutrient medium used for infected cultures consisted of 20% horse serum, 0.5% LA in HBSS, although the original medium was still used for growth of the cells until the time of inoculation with virus at 5-14 days of age. Tissue cultures were inoculated with 0.2 ml volumes. Mice used for detection and titration of virus were 3 weeks old, of the CFW strain, and were inoculated intracerebrally with volumes of 0.03 ml. The CVS strain of fixed rabies virus was used. The rabies street viruses used were in the form of naturally infected salivary glands, 2 from dogs and one from a cow, all cases occurring in Alabama during the summer of 1957.

Results. Fixed rabies virus. Hamster kidney cell cultures were inoculated with 0.1 ml of a 10^{-3} dilution of fixed rabies-infected mouse brain representing approximately 10,000 mouse LD_{50} units. The nutrient fluid was harvested and replaced with fresh fluid at 2-4-day intervals over a period of 91 days. No cytopathic changes occurred during this period. Twenty-seven such changes of fluid were made during this period and rabies virus was demonstrated in each harvest, the mouse LD_{50} titers varying between $10^{0.6}$ and $10^{5.3}$. In several instances the amount of rabies virus recovered was somewhat greater than the amount originally inoculated, the greatest in-