Demonstration of Venezuelan Equine Encephalomyelitis in Tissue Culture By Immunofluorescence. (26289)

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With the advent of cell culture technics the possibilities of clinical detection of virus diseases are greatly enhanced. To accomplish this, however, it is necessary to obtain maximal cytopathogenic effect (CPE), numerous passages to assure that the CPE is due to a specific virus, and finally, specific identification by neutralization or other serologic tests. The introduction of immunofluorescent technics has presented a possibility of detecting virus in tissue culture cell lines prior to specific changes in the infected cell. A model virus system was studied. Venezuelan equine encephalomyelitis (VEE) was selected as it can be studied in all phases of cell culture, from its initial inoculation to marked CPE.

Materials and methods. The virus strain was one which had been adapted to a guinea pig heart cell line (GPHC) by Berge *et al.* (1). The starting strain was an egg-adapted Trinidad VEE isolate. During 80 passages in GPHC there had been changes in virulence for various laboratory animals without known alterations in serologic activity. The cells were maintained in Eagle's media(2) with 20% horse serum and antibiotics added. Infected and noninfected cells were grown on cover slips, in a Petri dish, at 37°C under 5% carbon dioxide.

The antiserum, provided by Gochenour(3), was from a burro (*Equus asinus*) that was bled 2 weeks after an intramuscular inoculation of viable VEE. The serum gave a hemagglutinating titer of 1/640 and a complement-fixing titer of 1/520.

The serum was divided into 3 portions: (a) whole serum without any treatment (WS); (b) whole serum in which the globulin fraction was precipitated with methanol (4) and reconstituted to four-fifths volume (GP); (c) whole serum absorbed by adding 1 g of tissue culture cells per 5 ml and incubating 30 minutes at 37°C with intermittent shaking, then adding 50 mg per cc of acetone-dried rabbit powder(5) and shaking at room temperature for 1½ hours, finally centrifuging in the cold at 15,000 rpm (ALS). All serum preparations were conjugated with fluorescein isothiocyanate(6). To serums WS and GP, serum albumin conjugated with Lissamine rhodamine RB 200 was added(7). Serums were stored at 4°C. Preimmunization bleedings were similarly treated.

The cover slips were fixed for 15 minutes in (a) 10% formalin, then washed 3 times with phosphate-buffered saline, pH 7.4, before staining; (b) in acetone, then air-dried; or (c) in methyl alcohol, then air-dried. If the cover slips could not be stained immediately, those fixed in acetone or methyl alcohol were placed at −60°C, and those fixed in formalin at 4°C, either in the formalin or phosphate-buffered saline.

The cover slips were overlaid with the conjugated serums for 30 minutes at 37°C, washed in 3 changes of phosphate-buffered saline, pH 7.2, and mounted in buffered glycerol. A Zeiss fluorescent microscope, with an OSRAM HBO 200 light source and a BG12 exciter and GG4-OG4 barrier filter, was used for all observations.

Results. Conjugated immune and control serums were applied to duplicate sets of infected and noninfected cover slips that had been harvested after 72 hours, when CPE was easily observable, and fixed by one of the 3 technics used. The virus could be demonstrated in all infected preparations. Neither the infected cells treated with control serum nor the noninfected cells treated with immune serum gave specific fluorescence. The viral aggregates were best demonstrated with serums WS and GP to which the counterstain had been added. Formalin-fixed material

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could be stored at 4°C in 10% formalin for periods up to 2 weeks with little or no diminution of fluorescence, while the methanol- and acetone-fixed material had to be examined immediately, as storage even at −60°C did not preserve the antigen. When an inoculum was used that gave maximum virus and CPE at the end of 72 hours but no CPE at 24 and 48 hours, harvested cultures at these times gave specific fluorescence for the viral aggregates; the 24-hour culture showed a marked decrease in number of infected cells, however. With large inocula, virus could be determined as early as 10 hours after infection. Unconjugated immune serum applied to infected cover slips blocked over 90% of the specific fluorescence. Heterologous serums did not produce this effect.

Discussion. An adapted strain of VEE in GPHC can be detected by specific immunofluorescent technic as early as 10 hours when heavily inoculated, and in 24 to 48 hours with an inoculum that does not give a CPE during this period. The whole immune serum (WS) conjugated with fluorescein isothiocyanate with the counterstain added provides the best reagent. Although the methanol-precipitated conjugate (GP) produces similar results, its routine use could present problems, in that extreme care must be taken to prevent denaturation and subsequent loss of specificity. Generally, the stability of viral antigens in formalin has not been studied. It is interesting that our findings show a stability of the antigen fixed in formalin for up to 2 weeks, while with the usual fixatives its stability diminishes rapidly. This is undoubtedly not true of all viral antigens, but it suggests the possibility of studying the pathogenesis of viral diseases in routine pathologic specimens.

Fig. 1 shows the infected cell treated with the immune conjugate. In the infected controls, viral aggregates are demonstrated on the periphery of the cell. Undoubtedly there is other virus present, but it evidently is not in a degree of maturation to be detected. It is believed that there is no problem of penetration of the antibody into the cell, as evidenced by the fact that yellow fever virus can be detected in the perinuclear region(8),

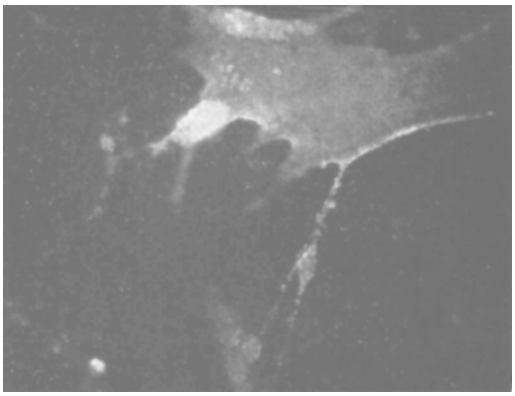

FIG. 1. Infected GPHC treated with conjugated immune whole serum (WS).

and infectious canine hepatitis virus in the intranuclear inclusion bodies(9). The virus cannot be detected prior to 10 hours, possibly because of an eclipse phase. With this virus there may be a breakdown of the virus particles, with a lack of antigenic sites for antibody attachment.

Further studies are in progress to determine the time required for cultures of nonadapted strains of VEE to grow before being demonstrated by this method. If nonadapted strains can be detected with equal facility this procedure could provide a valuable diagnostic tool at the clinical level.

Summary. In a guinea pig heart cell line, infected with an egg-adapted strain of Trinidad VEE, the virus could be demonstrated by immunofluorescence prior to development of CPE. Formalin fixation preserved the antigenicity, and the cover slips could be stored in this fixative at 4°C for periods up to 2 weeks without diminution of specific fluorescence. A whole burro serum conjugated with fluorescein isothiocyanate with a counterstain added proved a more reliable reagent than fractionated or absorbed sera. The model as described has the possibility of obtaining a specific viral diagnosis at the clinical level in the period normally required for initial isolation by conventional cell culture technics.

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Immunologic Studies of Prolactin.* (26290)

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Although the capacity of prolactin to induce antibody formation has been demonstrated(1), no recent studies have been reported in which the antigenicity of relatively small doses of more purified prolactin preparations have been evaluated. A study was therefore designed to evaluate the antigenicity of a relatively pure preparation of prolactin and to investigate the possibility that such antibodies could be employed in a useful assay for this hormone. The present study confirmed the ability of prolactin to induce antibody formation. The antisera that were obtained yielded hemagglutination reactions which were inhibited by small quantities of added prolactin, but which were not inhibited by much larger amounts of other pituitary hormones.

Materials and methods. Prolactin: A highly purified preparation of ovine lactogenic hormone with a stated potency of 15 I.U./mg† was used. *Immunization.* Adult white rabbits were injected subcutaneously with saline solutions of prolactin suspended in equal volumes of Freund's complete adjuvant. Each of the following amounts of prolactin was given to a different animal: 0.1 mg (Rabbit 1), 2.5 mg (Rabbit 2), 12.5 mg (Rabbit 3). Eleven weeks later boosters of 0.25 mg of prolactin were given to rabbits 2 and 3, and one week subsequent to initial injection, a 0.5 mg booster was given to rabbit 1.‡ *Sampling*: Peripheral blood samples were obtained from all 3 animals just prior to immunization. Rabbit 1 was bled again 3 weeks later, while multiple samples were obtained from Rabbits 2 and 3 from the third to 12th week after the initial immunization. The antiserum obtained on the 12th week from Rabbit 2 was used in evaluation of the inhibition of hemagglutination. *Hemagglutination titers*: All serum samples were heated to 56°C for 30 minutes to inactivate complement and absorbed with sheep erythrocytes to prevent nonspecific hemagglutination. Antigen-antibody reaction was evaluated by the hemagglutination technic(2). Each 1.0 ml volume of a 2.5% suspension of washed, tannic acid treated sheep erythrocytes was sensitized with 0.2 mg of prolactin. Of this suspension 0.06 ml was added to tubes containing either normal rabbit serum, serial dilutions of antisera, or serial dilutions of test materials in antiserum. Significant hemagglutination was considered to have occurred if at the end of 3 hours the erythrocyte suspension appeared as a granular layer of cells covering the bottom of the tube. Less marked agglutinations were classified as negative.

Results. All animals lacked demonstrable anti-prolactin antibodies prior to immunization; all developed such antibodies after im-

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‡ Boosters for Rabbits 2 and 3 were in saline. Booster for Rabbit 1 was in Freund's complete adjuvant.