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Localization of Frog Virus Multiplication in Chick Embryo Cells by Immunofluorescence.* (31776)

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In recent years numerous viruses resembling herpes viruses in general appearance have been isolated from Wisconsin and Vermont frogs(1,2). Two of these viruses, designated FV1 and FV3 have been isolated from normal and tumor-bearing frogs(1), respectively. A recent report from this laboratory has shown that these viruses contain DNA which has a buoyant density in the range characteristic of herpes viruses(3). However, electron microscopic and acridine orange studies of FV1 and FV3 indicate that they differ from herpes viruses in the site of replication(1,4,5,6). This paper deals with cellular sites of accumulation of antigens of FV1 and FV3 and with their immunologic relationship.

Materials and method. Chick embryo coverslip cultures grown in Leighton tubes at 37°C were inoculated with FV1 or with FV3 obtained from Dr. Rafferty, Department of Anatomy, Johns Hopkins University School of Medicine, Baltimore, Md. The infected coverslips were then placed at room temperature. At 1-2 hour intervals after infection, the coverslips were fixed and stained with fluorescein-labeled antibody. The procedures for preparation and infection of chick embryo cultures and for staining coverslips with labeled antibody were the same as described elsewhere(3,7) except that methanol was used to fix the coverslips at —60°C. The rabbit anti FV1 and FV3 sera were prepared as follows. Once a week for 6 weeks rabbits were given multiple intramuscular injections of chick embryo cells infected with FV1 or with FV3 and homogenized in Friend's adjuvant. The sera was collected one week after the last injection. The globulin fractions of the sera were conjugated to fluorescein isothiocyanate by the method of Riggs as described by Marshall *et al*(8), then absorbed repeatedly with uninfected chick embryo cells. The absorbed, conjugated antibody preparations do not stain uninfected cells. The preparation of the two types of conjugated antibody against herpes simplex virus was reported elsewhere(9).

Results. The earliest specific fluorescence

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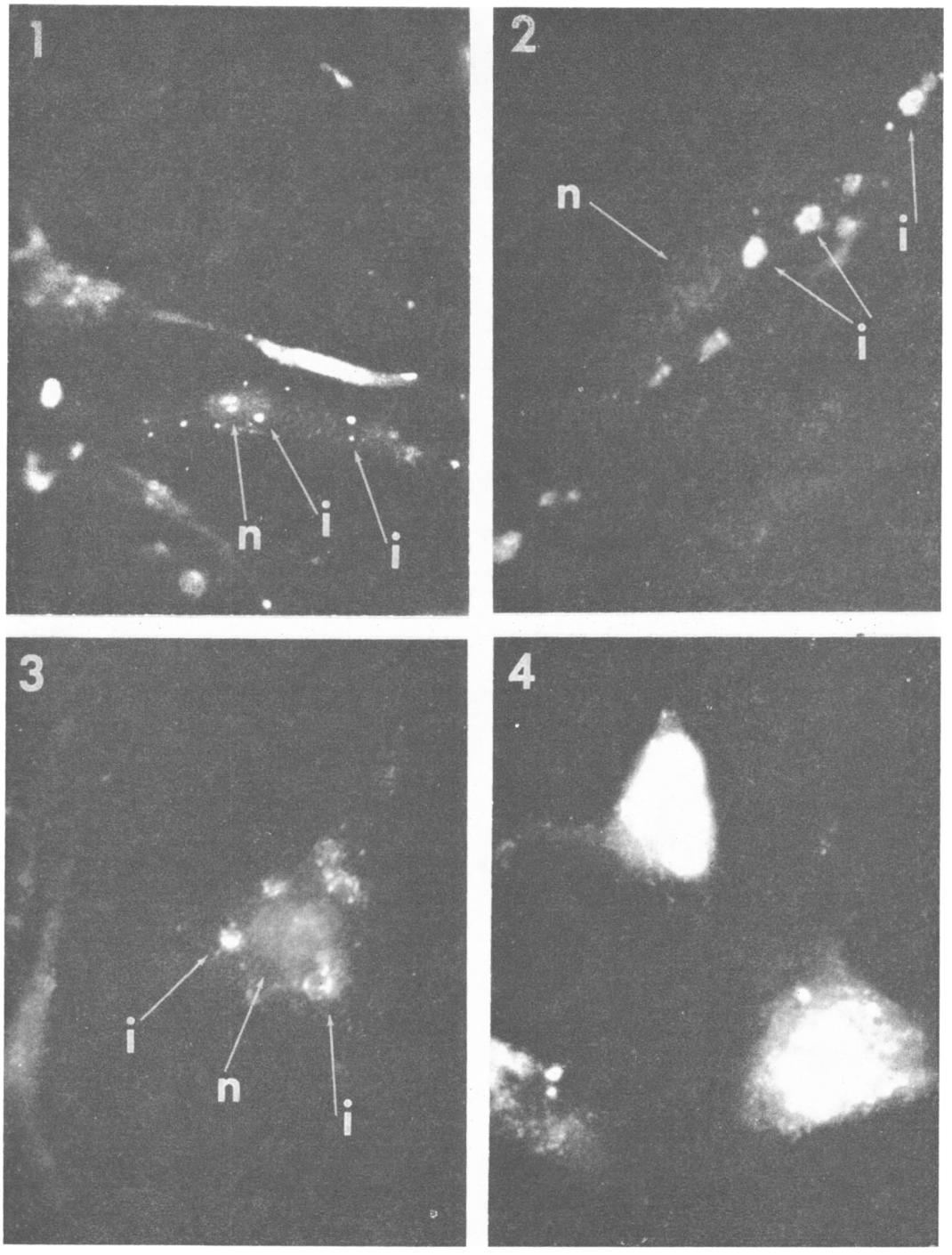

FIG. 1. Chick embryo cells infected for 9 hr with FV1 and stained with labeled rabbit anti FV1 serum. Arrows indicate viral inclusion (i) and cell nucleus (n). The 2 nucleoli appear as white spots. They do not show specific fluorescence.

FIG. 2. Chick embryo cells infected for 12 hr with FV1 and stained with labeled rabbit anti FV1 serum.

FIG. 3. Chick embryo cells infected for 12 hr with FV1 and stained with labeled rabbit anti FV3 serum.

FIG. 4. Chick embryo cells infected for 18 hr with FV1 and stained with labeled rabbit anti FV1 serum.

was observed in chick embryo cell cultures fixed and stained with labeled antibody 8 hours after infection with FV virus. The fluorescence took the form of small, discrete cytoplasmic inclusions with no discernible internal structure (Fig. 1). The number of inclusions varied considerably; as many as 24 were counted in one cell but most cells contained less than 10. These inclusions often persist unmodified for several hours. In many cells, however, they become altered with time after infection as follows: (a) Between 9 and 12 hours after infection the fluorescent inclusions increased in size and, moreover, internal structures consisting of fluorescent granules could be readily seen (Fig. 2). (b) Further modifications of the inclusion were seen in cultures fixed and stained between 10 and 12 hours after infection. The inclusion again increased in size and, moreover, the fluorescent granules lined the boundary of the inclusion forming a "necklace." The area bounded by the necklace did not show specific fluorescence and no internal structures could be discerned (Fig. 3). (c) At approximately 13-15 hours after infection the granules appeared to be scattered in the cytoplasm forming patches of fluorescent material. By 16-18 hours most of the cells were rounded. At this stage of infection the entire cell fluoresced a very intense yellow-green color and intracellular details were no longer discernible (Fig. 4). It is noteworthy that identical results were obtained with FV3 virus. Moreover infected cells stained with labeled heterologous anti-viral serum could not be distinguished from those stained with the homologous serum. However, no specific fluorescence was seen in infected cultures stained with labeled antibody made against herpes simplex virus. These findings indicate that FV1 and FV3 are related in that they share common antigenic determinant sites. The two frog viruses are not, by this criterion, immunologically related to herpes simplex virus.

Discussion. The data obtained in this study bear on 2 aspects of frog virus replication. First, several reports have recently shown the presence of frog viruses in the cytoplasm and, conversely, their absence from the nucleus of infected cells(1,4,6). However, immunofluorescence studies have not been previously reported and the question arises whether the patterns of viral antigen accumulation concur with the interpretation of electron photomicrographs. The results obtained in this study show that, within the limitations of the method, viral antigens accumulate in the cytoplasm. Moreover, the antigens are not dispersed throughout the cytoplasm but rather they are confined to cytoplasmic structures which increase in size and undergo other changes during the reproductive cycle of the virus. It should be noted that in two respects, the lack of a nuclear phase in multiplication and localization of antigens in discrete areas of the cytoplasm, the multiplication of frog viruses differ from that of herpes simplex(5, 10). The second aspect concerns the relationship between FV1 and FV3 on one hand, and between frog viruses in general and herpes viruses which they resemble. Although there have been numerous isolations of viruses from frogs, because of lack of immunologic data, it has not been clear whether the isolates are different from each other or simply multiple isolates of the same virus. We chose FV1 and FV3 because they have been isolated from normal and tumorigenic frogs, respectively. The data show that by immunofluorescence tests, the two viruses are related and may well be identical. Moreover, the two viruses, by the same test, are unrelated to herpes simplex virus.

Summary. Chick embryo cells infected with frog viruses No. 1 and 3 were fixed at various times after infection and stained with rabbit antibody labeled with fluorescein isothiocyanate. The data show the following: (a) Viral antigens accumulate beginning about 8 hours after infection in discrete cytoplasmic structures which increase in size and become altered with time. (b) Frog viruses No. 1 and 3 cannot be differentiated by immunofluorescence with respect to site of antigen accumulation and with respect to reactivity with heterologous antibody(3). Frog viruses differ from herpes simplex with respect to antigenic determinant sites and with respect to pattern of intracellular antigen accumulation.

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Experimental Equine Influenza in Chincoteague Ponies. (31777)

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In the past decade, influenza in horses has been shown to be caused by 2 immunologically distinct type A influenza viruses. The first type was isolated from horses with respiratory disease in Czechoslovakia in 1956, and is currently classified as A/Equi 1/Prague/56(1). The second was obtained from horses during an epizootic of equine influenza which occurred in the United States in 1963, and is designated as A/Equi 2/Miami/1/63(2). Information relevant to infection and disease in horses due to these viruses has been obtained almost exclusively by the observation of naturally-occurring epizootics. Epidemiologic investigations have provided considerable information about natural equine infection. However, an experimental model has been needed to obtain added information on host-virus relationships and clinical and immunologic responses in equines under controlled conditions. Since previous reports on experimental equine influenza in the natural host indicated failure to produce clinical disease or were lacking detailed information (3,4), experiments were undertaken in Chincoteague ponies using a strain of A/Equi 2 virus for the purpose of developing a satisfactory system for the study of equine influenza in the natural host and to provide materials for studies of equine influenza virus in man. This report describes the results of the pony experiments. The results of human volunteer studies employing materials from the pony experiments will be reported separately(5).

Materials and methods. Subjects. The study was conducted with 24 random-sexed ponies (*Equus caballus*) from a wild herd on Assateague Island, Virginia. The animals were obtained from a population relatively isolated from humans and had no known previous contact with other equines. The approximate age of the ponies employed in the experiments was 1 to 1.5 years. A serologic survey for neutralizing antibody using representative serum specimens collected in recent years from Chincoteague ponies of various ages and those used in this study indicated a complete lack of prior experience with A/Equi 2 virus.

Plan of study. Studies in ponies were conducted in isolation units at the Grayson Laboratory, University of Maryland, College Park. Seven groups of ponies without detectable neutralizing antibody ($<1:2$) were administered A/Equi 2 virus (Table I). The initial inoculum (E-1) was prepared with an isolate derived from a naturally-infected horse and given to 2 ponies. Thereafter, 5 successive groups of ponies were given a viral inoculum prepared with an isolate obtained from a subject in each preceding group. Be-