A HISTOLOGICAL STUDY OF INFECTIOUS CANINE HEPATITIS BY MEANS OF FLUORESCENT ANTIBODY*

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Plate 3

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The experiments which form the basis of this report were undertaken with two objectives in view: to study in its natural host the mode of spread of a virus disease, infectious canine hepatitis, and to determine whether the prominent, intranuclear inclusions in this disease contained viral antigen. For these purposes, infected tissues taken from dogs in various stages of the disease were studied histologically, using fluorescein-labeled antibody as a specific means of detecting the presence of antigen in the cells. The findings indicate that the inclusions contain high concentrations of specific viral antigen.

Infectious canine hepatitis was first described under this name (hepatitis contagiosa canis) by Rubarth in 1947 (1), although the inclusion bodies typical of the disease had been noted in 1930 in two dogs used for experimental purposes by Cowdry and Scott (2). It is an acute disease of dogs characterized by intense centrolobular necrosis, congestion, and edema of the liver, edema of the gall bladder, widespread hemorrhages, and, occasionally, icterus. It is of sudden onset and short course with an appreciable mortality rate. The most distinctive microscopic feature of the disease is the presence of large intranuclear inclusion bodies within the endothelial cells of the smaller veins and venous sinuses, the liver sinusoids, and in the hepatic parenchymal cells. It is caused by a filtrable virus (1, page 153) and has been transmitted to dogs and other canids but not to other genera. Cultivation in chick embryos has been reported (3, 4). Many aspects of the disease are similar to those of fox encephalitis, as described by Green *et al.* (5–7). The antigenic properties of the virus of the two diseases are probably identical, as shown by Siedentopf (8). Rubarth has contributed a detailed study of the manifestations and pathology of the disease (1).

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Materials and Methods

The material examined consisted of one 6-week-old puppy and one 2-year-old dog which died of the naturally occurring disease at the Angell Memorial Animal Hospital, and of seven Basenji puppies from two litters, 4 and 5 months old, respectively, which had been raised under controlled conditions and which had been immunized against distemper.[1]

Virus.—The seed virus was obtained as follows: A pool of the livers from three dogs dead of the natural disease was prepared in 10 per cent suspension. This material was injected in a dose of 0.13 ml. into the anterior chamber of the eye of two normal dogs, both of which developed fever and leucopenia on the 3rd day. One died on the 7th, the other on the 8th day after infection, and both showed typical inclusions in the liver. The liver of the second dog was stored frozen in 10 per cent suspension and served as the seed virus in the experiments described.

Antiserum.—Commercial "anti-fox encephalitis serum" prepared in dogs by the Fromm Laboratories, Grafton, Wisconsin, lot 677. This serum had a complement fixation titer of 1:10 (9). It was concentrated by precipitation with half-saturated ammonium sulfate and conjugated with fluorescein isocyanate by the method of Coons and Kaplan (10). After prolonged dialysis, it was absorbed in small portions for use twice with acetone-precipitated dog liver powder to remove non-specific material which reacted with normal dog tissue (10).

Animals.—Seven Basenji puppies were inoculated into the anterior chamber of the right eye with 0.13 ml. of the seed suspension of infected dog liver, and into the anterior chamber of the left eye with an equal amount of normal liver suspension. A study of the obvious signs and hematological changes in these animals was also made (11).

Starting on the 2nd day after inoculation, one dog was sacrificed each day. One animal was sacrificed *in extremis* on the 4th day and one spontaneous death occurred on the 5th day. The dogs were anesthetized with pentobarbital sodium and exsanguinated. Tissues were fixed in formalin for routine histological examination and small pieces of the tissue were quick frozen by plunging the tubes containing them into a dry ice–alcohol mixture. The frozen tissue was stored at −20°C. until used.

Examination for Antigen in Cells.—Histological sections of the frozen tissues were cut at 4 μ in a refrigerated cabinet by a modification of the method of Linderstrøm-Lang and Mogensen (12, 13). Each section, after drying at room temperature, was fixed in acetone for 15 minutes, dried in air at 37°C. for 30 minutes, and then covered with a drop of fluorescein-labelled antiserum. After 20 minutes the fluorescent solution was rinsed off and the slide washed in buffered saline (0.8 per cent NaCl containing 0.01 M phosphate, pH 7.0) with gentle agitation for 10 minutes, mounted in glycerol containing 1 volume in 10 of buffered saline, and examined under the fluorescence microscope for the brilliant yellow-green fluorescence of fluorescein. The details of these procedures and the immunological controls necessary to ensure specificity have been described (10, 12, 13). The two animals which died of the spontaneous disease were similarly examined.

Fluorescence Microscopy and Photography.—Minor changes which have increased the definition and intensity of fluorescence have been made in the arrangement of the microscope from that described previously (10). The arc lamp was increased to 13 amperes D.C. (20 is still better). A standard darkfield condenser has been substituted for the quartz one used previously; no loss of intensity was observed following this change, since it was then possible to use thinner filters (Corning 5840—½ standard thickness only, with a $CuSO_4$-containing cuvette). The filter in the ocular could then be changed to Wratten No. 2B (Eastman). (2A is no longer available, though it is better because it absorbs more blue light and allows thinner filters with a transmitting condenser.)

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[1] This portion of the work was carried on at the Lederle Laboratories, Pearl River, on experimental animals used in the Virus Research Section.

Fluorescence photomicrographs were taken with a 35 mm. film holder (visicam) on super XX film (Eastman) with exposures of 4 minutes for a magnification of 400. The exposure time must be varied somewhat, depending on the intensity of fluorescence of the specimen.

EXPERIMENTAL RESULTS

No symptoms other than those attributable to the local process in the right eye were evident until the 2nd day after inoculation when depression and hyperpyrexia were noted. The course of the disease was progressive to a state of crisis on the 4th and 5th postinoculation days, followed by improvement in the condition of the surviving animals. The last animal, sacrificed on the 8th postinoculation day, was apparently normal.

Although signs of acute infection were present in the first dog (No. 50)[2] sacrificed (2nd postinoculation day) no specific fluorescence was noted, except in the anterior chamber of the right eye where numerous fluorescent inclusion bodies and diffuse fluorescence were noted. Smears from the eye stained by Giemsa's method showed many typical intranuclear inclusions, as noted by Green, Evans, and Dowell (14). No inclusions were found in paraffin sections of other tissues. Specific fluorescence was observed in the remaining animals with the exception of dog 7 (54), sacrificed 8 days after inoculation, in which viral antigen was almost entirely lacking. Detailed data will be presented on one animal and the others compared with it.

In dog 2 (52), sacrificed on the 3rd day, slight gross changes of infectious canine hepatitis were present, but were not as marked as those seen in animals dying of spontaneous infection. Smears prepared from the cut surface of the liver and stained by Giemsa's method (15) contained a few granular inclusions within endothelial nuclei. Numerous homogeneous inclusions were present in smears from the posterior aspect of the right cornea similarly stained but were not present in smears from the left cornea, the pia-arachnoid surface, or the cut surface of the brain.

Specific fluorescence was most abundant in the liver, where it occurred in the endothelial cells lining the smaller radicals of the portal vein and in adjacent sinusoidal cells. No specific fluorescence was present in the hepatic parenchymal cells. Fluorescence was also noted within endothelial cells of the renal glomeruli, the red pulp of the spleen, the veins and sinuses of the lymph nodes, a few cells in the smaller veins in each optic nerve, and a few areas in the interalveolar tissue of the lungs. In addition, diffuse fluorescence occurred in amorphous material within the lumina of the pancreatic ducts and the fibrinous exudate from the posterior surface of the right cornea (the eye inoculated with infective material). The left cornea, the cerebrum, the cerebellum, and striated muscle were negative.

With the exception of the diffuse fluorescence noted in the lumina of the pancreatic ducts, viral antigen was exclusively intracellular. In certain cells it

[2] Numbers in parenthesis are those used in the work reported in reference 11.

appeared as brightly fluorescent rings corresponding to the position of the nuclear membrane when the same section was stained by hematoxylin and eosin. Frequently one or more bright spots were enclosed by this bright ring. A few larger fluorescent masses, corresponding in size and location to hepatitis inclusions, as seen in paraffin sections, were observed. Most of such inclusion-like areas appeared as collections of minute fluorescent particles, suggesting the granular inclusion bodies seen in paraffin sections from this animal. The nuclei containing the inclusion bodies were enlarged. Faint, specific fluorescence was scattered in an irregular area around some of the Kupffer cell nuclei, indicating the presence of antigen in the cytoplasm.

In dogs subsequently sacrificed the location, brilliance, and abundance of the fluorescent areas differed with the progress of the infection. In the succeeding dog 3 (51), sacrificed on the 4th day, abundant fluorescence occurred in the nuclei of the hepatic parenchymal cells and in the endothelial cells of the cerebrum and lungs. The inclusion bodies were larger, more numerous, and brighter. Many appeared as oval or circular masses, either diffusely fluorescent or more brightly fluorescent around the margins (Fig. 1). Peripheral rings, corresponding to the nuclear membranes, were still evident and some ring forms alone were present (Fig. 2). The nuclei of the affected cells were larger and ballooned out into the lumina of the veins and sinuses and in certain instances were apparently becoming detached from the vessel wall. This was particularly evident in the brain.

Dog 4 (55), which died spontaneously on the 5th day after inoculation, showed similar changes. The postmortem alterations in Nos. 3 and 4 were similar to those in animals dead of the spontaneous infection and the dogs had evidently been sacrificed at the peak of the disease. The fluorescence findings were similar in dog 5 (53) sacrificed 6 days after inoculation, even though improvement was evident. Dog 6 (56), sacrificed 7 days after inoculation, showed less fluorescence. In the liver it was confined solely to the endothelial cells of the veins and sinusoids, thus resembling the liver of the first animal to show fluorescence (dog 2). Fluorescence also occurred in the endothelial cells of the spleen, kidney, and brain. The last animal, No. 7 (54), sacrificed 8 days after inoculation, was devoid of antigenic material except for equivocal fluorescence in one Kupffer cell and some diffuse fluorescence in a renal glomerulus.

The puppy which died of the naturally occurring disease showed hemorrhage and perivascular cuffing in the hypothalamic region and basal area of the brain, with inclusions in the vascular endothelium. When studied for antigen, sections of this brain (as well as those of the brain of normal dogs and the experimental dogs described above) showed yellow-green fluorescence of nuclei, which was diminished but not abolished by absorption of the conjugate with normal dog brain powder (see reference 8 for discussion of "non-specific staining"). This nuclear staining in the central nervous system is usual with fluorescein-labeled

sera. However, no brightly fluorescent nuclei comparable to those found elsewhere could be found in glial, ependymal, or nerve cells; but the inclusions in the vascular endothelium contained high concentrations of antigen. It seems likely, therefore, that the brain lesions were the result of the vascular damage, and not indicative of viral invasion of the central nervous system itself.

DISCUSSION

The nature of the inclusion bodies associated with certain viral infections has long been the object of speculation and experiment. The arguments have ranged over the possibilities that they were viral colonies, reaction products produced by the cell, or both.

The literature up to 1929 was reviewed by Goodpasture (16), who concluded that only in molluscum contagiosum and fowl pox was there clear morphological evidence that the inclusions were composed of viral elementary bodies which were embedded in a hyaline matrix produced either by the cell, or the virus, or both. A more recent discussion by van Rooyen and Rhodes (17) concluded that "the most feasible explanation of the majority of inclusions is that elementary bodies first enter the cell, sometimes reaching the nucleus, and then proliferate to form a colony. The cell itself probably reacts and produces a covering which gives the inclusion a more or less homogeneous appearance" (page 91). They point out that this theory was first suggested by Prowazek (18).

Direct evidence of the infectiousness of washed single viral inclusions isolated by micromanipulation was obtained for fowl pox by Woodruff and Goodpasture (19), and for ectromelia (20) and herpes simplex (21) by Baumgartner. Van Rooyen (22) removed inclusions of molluscum contagiosum by microdissection and found that the elementary bodies were embedded in a sticky matrix. Banfield *et al.* (23) have published electron micrographs of ultrathin sections of cells infected with molluscum contagiosum which clearly show the elementary bodies embedded in a less dense material, and Wyckoff (24) has done the same for cells of the chorioallantoic membrane of the chick embryo infected with fowl pox.

The evidence presented here indicates that the intranuclear inclusions of infectious canine hepatitis contain high concentrations of viral antigen. It seems quite unlikely that specific antigen in such an inclusion has become dissociated from active virus, particularly since no antigenic material unassociated with inclusions was found except in the cytoplasm of a few Kupffer cells, and in the pancreatic duct. In both these exceptional locations the concentration was considerably lower than in the inclusion bodies. It therefore seems clear that the intranuclear inclusions are composed in large part of virus particles.

By the use of fluorescent antibody, it has been possible to trace the sequence of virus invasion of the cell and the development of the inclusion body itself. The first indication of the presence of viral antigen in a cell was the appearance of a fluorescent ring corresponding to the nuclear membrane, suggesting ac-

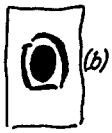
cumulation of virus on or within the substance of the membrane. (This indicates that the thickening of the nuclear membrane described by Cowdry and Scott (2) in paraffin sections and interpreted by them as due to the deposition of chromatin, is at least in part due to virus material.) The next stage in the sequence was the appearance of fluorescent granules in the interior of the nucleus. These increased in number and finally coalesced into granular fluorescent bodies, corresponding to the granular inclusions seen in paraffin sections

TEXT-FIG. 1. The Development and Spread of Antigen in Intranuclear Inclusions

1st stage showing brightly fluorescent nuclear ring due to the presence of specific virus antigen on or within the nuclear membrane (nuclear ring stage).

2nd stage showing the presence of a few fluorescent granules within the nuclear ring, due to extension of the antigen from the nuclear membrane (fluorescent granule stage).

3rd stage showing the aggregation of fluorescent granules to form an organized, granular, fluorescent mass enclosed by the fluorescent nuclear membrane (the granular inclusion body phase).



4th stage, a, and b, showing the fully matured inclusion now diffusely fluorescent either brightly fluorescent in the peripheral zone with a more dimly fluorescent center (*a*), or brightly fluorescent throughout (*b*) (diffuse inclusion body stage).

at this stage. Finally, uniformly fluorescent, homogeneous objects were observed, which were directly related to inclusions when the same section was subsequently stained with hematoxylin and eosin and compared with fluorescence photomicrographs. The end stage of these inclusion bodies apparently existed in two forms: (1) one showed a fluorescent periphery and a non-fluorescent or less fluorescent center and (2) one was uniformly fluorescent throughout. It is thought that the inclusion which was fluorescent only on the periphery corresponds to the inclusion which has a basophilic center and an eosinophilic periphery, as viewed in paraffin sections.[3] This suggests that the central baso-

[3] See Plate II, Fig. 6 (25).

philic portion of such inclusions is chromatin or other products of the cellular nucleus and that the peripheral area is composed of more eosinophilic virus bodies (see Text-fig. 1 for diagrammatic illustration of this sequence).

After inoculation into the anterior chamber of the eye, infectious canine hepatitis virus propagates by growing in the endothelial cells covering the posterior surface of the cornea, producing inclusion bodies in these cells and interstitial edema of the cornea. From this focus spread occurs to cells in direct contact with the blood stream: endothelium and reticulo-endothelium. (There was no evidence of retrograde spread along the optic nerve.) The affected cells either in turn become detached entirely or spill their nuclear contents into the circulating blood, thus further disseminating the virus. The blood tissue barrier is breached only in the liver, where later in the pathological sequence invasion of the hepatic parenchyma takes place. The presence of fluorescent material within the pancreatic duct suggests that the virus here too may pass from the blood stream, but such an interpretation must be advanced with caution, since no virus could be seen in the pancreatic acinar tissue. The use of but a few animals and the fact that they were sacrificed early may explain the absence of chronic interstitial lesions in the kidney, as described by Poppensiek and Baker (26).

SUMMARY

A study of experimental infectious canine hepatitis in dogs by means of specific fluorescent antibody indicates that the intranuclear inclusions of this disease contain high concentrations of viral antigen.

The increase in virus in the nuclei, as indicated by the accumulation of specific antigenic material, begins on the nuclear membrane and spreads from there to the interior of the nucleus, with the gradual formation of larger granules. Subsequently there appear the homogeneous inclusion bodies characteristic of this infection.

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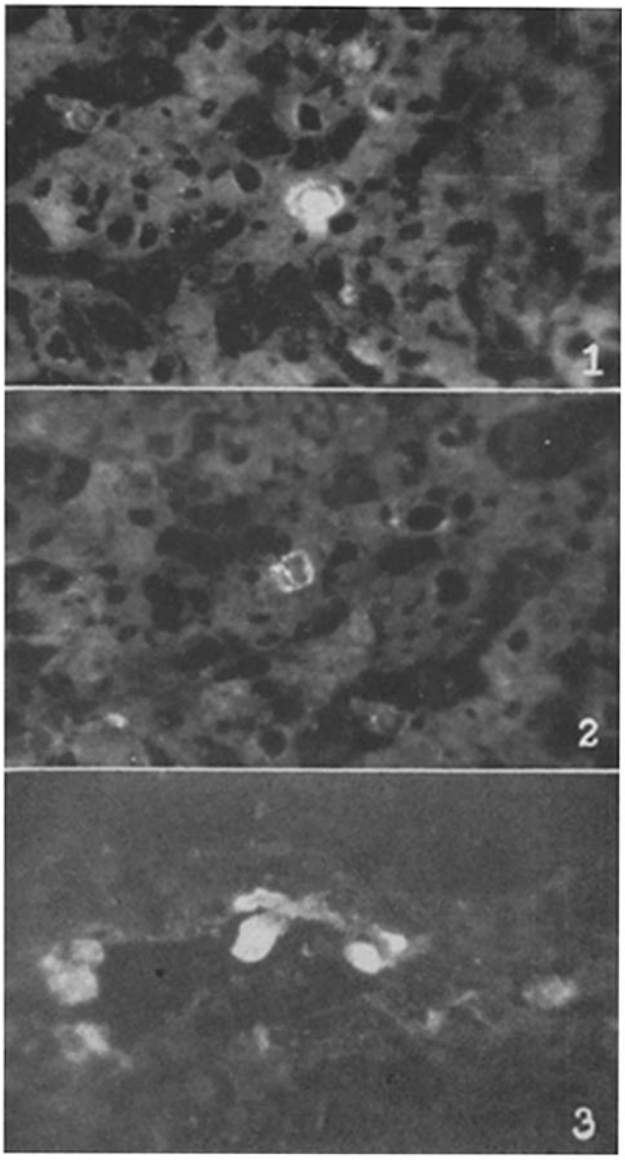
EXPLANATION OF PLATE 3

FIG. 1. Fluorescence photomicrograph of liver tissue showing a fully matured, diffusely fluorescent inclusion body surrounded by a brightly fluorescent nuclear membrane, denoting the presence of virus antigen in both structures. The cytoplasm of the parenchymal cells is faintly illuminated by blue-gray autofluorescence. The black areas within the liver cells are normal nuclei. The sinusoids appear as black areas between the liver cords.

FIG. 2. Fluorescence photomicrograph of liver showing an early or ring phase inclusion body. Only the nuclear membrane and a few antigenic particles within are fluorescent.

FIG. 3. A photomicrograph of a tangental section of a thalamic vein showing fluorescent inclusions within endothelial nuclei. Note that the fluorescent masses project into the lumen.



(Coffin, Coons, and Cabasso: Infectious canine hepatitis)