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STUDIES ON INFECTIOUS CANINE HEPATITIS III. STUDIES ON THE INTRANUCLEAR INCLUSION BODIES

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INTRODUCTION

Since RUBARTH described the inclusion body in infectious canine hepatitis (H. c. c.), this body has attracted the attention of many workers. Prior to this, GREEN and his co-workers¹⁰⁾ had fully observed the inclusion body in enzootic fox encephalitis and differentiated it clearly from that of canine distemper¹¹⁾. It is a well-known fact that H. c. c. and enzootic fox encephalitis is the same disease caused by the same virus, but both inclusions show positive to Feulgen reaction.

RUBARTH believed that the character of the inclusions in H. c. c., the degeneration connected with their appearance in the cells and their perfect likeness to previously known intranuclear virus inclusions might be considered to give strong support to the view that these nuclear inclusions in H. c. c. are caused by a virus. COFFIN et al., after experiment by means of specific fluorescent antibody, established it as a fact that the intranuclear inclusions in H. c. c. contain high concentrations of viral antigen.

On the other hand, cytochemical studies on these inclusions have been scarce, but SCHULTE and AKÜN reported that the inclusions consisted of polynucleotides (chains of desoxyribonucleic acids). DI DOMIZIO reported that the virus of H. c. c. is capable of synthesizing nucleic acids, especially thymonucleic and ribonucleic acids, of which the inclusion bodies are apparently composed.

OKUYAMA et al. reported that inclusions showed positive Feulgen reaction, pyroninophilia and slight positive reaction to methylgreen stain, and osmiophilic materials were seen, accumulated in the center of the nucleus under electron microscopy, but the H. c. c. viral materials were hardly identified.

The present author investigated the character of these inclusions by means of cytochemical technique and conducted a differential diagnosis of some similar inclusions.

MATERIALS AND METHODS

Cytochemical studies were made on 8 cases of experimental fatal severe form which

have already been reported⁹⁾. In addition, investigations were also conducted on one case (E 2874, 4 months old, ♀) which was inoculated with purified H. c. c. virus by means of ultracentrifugation and which died manifesting very rapid progression. Specific nuclear inclusions were numerous in almost all parts of the body.

In fixation, fresh tissues were used exclusively. Materials were fixed with Carnoy's fluid, 10% formalin, Zenker's fluids and chilled in absolute acetone-alcohol. Paraffin or frozen sections were prepared. The various cytochemical methods employed are listed in table 2. In order to avoid false interpretations based on these cytochemical tests, controls were run whenever possible in order to avoid the confusion of the genuine reaction with similar reactions of non-specific nature.

Further, in order to confirm the presence of nucleic acids in the nuclear inclusions, the author made use of ultraviolet photography (2600 Å).

OBSERVATIONS

1. General Findings in the Nuclear Inclusions

Two types of inclusions were indicated, the one granular inclusions and the other homogeneous inclusions. These nuclear inclusions were distinct from the nucleolus.

The former type had granular and irregular appearance. Some of them exhibited a diffuse granular appearance or a clear cluster which was accumulated in the center of the nucleus. The internal structure of the inclusions was variable, some appeared to be consist of several small clusters, some contained granules around a large round body or fine radiating bands extending between the periphery of the inclusions and the nuclear membrane. Most of the inclusions were acidophilic, but some were basophilic. In some cases, a halo between the inclusions and the wall of the nucleus was distinctly observed, but was indistinct in others. The nuclei had often one or more nucleoli and some of them were enlarged, but most were atrophic and pressed on the wall of the nucleus.

The latter type of inclusions showed marked margination of the chromatin in the nuclear membrane. The nucleoplasm with inclusion was poor in chromatin, and bright. Bright haloes were also observed between the inclusions and the walls of the nucleus. The nuclear inclusions displaced the nuclear contents to the periphery of the nucleus and often occupied all the nucleus with karyorrhectic nuclear membrane. The tinctorial properties were generally acidophilic, but some were basophilic.

The shape of the inclusions coincided with the nuclear figure—round or elliptical. The nuclear inclusions of the vascular endothelium usually showed spindle or oval shapes. The nucleolus was generally situated in the periphery of the nucleus and often was amalgamated with the nuclear membrane, but some of them were situated separately. In the internal structure of the inclusions, no vacuoles were observed. This type of the inclusions, was referred to as COWDRY's type A⁶⁾.

2. Distribution of the Inclusions

It has been already emphasized that the inclusions in H. c. c. occurred in hepatic cells and endothelia, but also they occurred in various other tissues.

The list is a long one. They occurred in the vascular endothelia in the liver, the spleen, the kidneys, the myocardium, the lungs, the adrenal glands, the lymph nodes, the tonsils, the pharynx, the larynx, the salivary glands, the tongue, the thymus, the pancreas, the urinary bladder, the ovary, the skeletal musculature, the bone marrow, the brain and the spinal cords (mesoderm), adventitia cells, reticulum cells and histiocytes (mesenchyma), liver cells, bronchial epithelia⁸⁾ and bile ducts epithelia (entoderm), epithelia in the tonsils and the *membrana nictitans*⁸⁾ (ectoderm) and serosa epithelia in the digestive canals and urinary bladder, and epithelia in the cardiac valves and the adrenal cortex (mesoderm). Frequency of nuclear inclusions in the various tissues were as listed in table 1.

From the above findings, the author believes that the virus in H. c. c. is not only a hepato-tropic and endothelio-tropic virus, but also a pantropic virus.

3. Development of Inclusion Bodies

It may be considered that the granular inclusions grow into homogeneous inclusions. The former type showed diffuse acidophilic granules in the nucleus in the earlier stages of H. c. c. In this stage, the nucleolus was often enlarged with rich ribonucleic acid (RNA) and showed slight desoxyribonucleic acid (DNA) around the nucleolus. The granules were gradually accumulated and amalgamated, occupying a central position. At the same time, the chromatin network in the nucleus became rough and a bright halo between the inclusions and nuclear membrane became noticeable. The inclusions became larger round clusters and their periphery often showed granular appearance. Their end result was clearly demarcated homogeneous inclusions. These inclusions fully occupied all the nucleus in marked cases, and were then accompanied by nuclear degeneration.

It seemed that with the development of the inclusions, the nucleolus showed atrophic degeneration and was pressed on to the nuclear membrane, and finally amalgamated with the nuclear membrane.

TABLE 1. Frequency of Intranuclear Inclusion Bodies in the Various Cells

CELLS	%
Liver, parenchymal cell	100
Liver, endothelium	"
Glomerulus, endothelium	"
Myocardium, endothelium	"
Lymph node, reticulum cell	"
Lymph node, endothelium	"
Brain, endothelium	"
Thymus, endothelium	80
Lungs, endothelium	75
Spleen, endothelium	71.4
Spleen, reticulum cell	"
Tonsils, epithelium	62.5
Adrenal endothelium	50
Tongue, endothelium	"
Stomach, endothelium	"
Tonsils, reticulum cell, endothelium	37.5
Intestine, serosa epithelium	25
Skeletal muscle, histiocyte, endothelium	"
Bladder, endothelium, serosa epithelium	"
Bone marrow, endothelium, reticulum cell	"
Adrenal cortex, epithelium	16.7
Spleen, adventitia cell	12.9
Bile duct, epithelium	12.5
Cardiac valve, endothelium	"
Pharynx & Larynx, endothelium	"
Salivary gland, endothelium	"
Oesophagus, endothelium	"
Intestine, endothelium	"
Ovarium, endothelium	"
Bronchial epithelium	0

These granules were all positive to Feulgen test and the ribonuclease test for RNA. Consequently, both inclusion types indicated the same character. It is considered that the granular inclusions are the developing type and the homogeneous inclusions are the mature type.

4. Cytochemical Tests for the Nuclear Inclusion Bodies

The nuclear inclusions in H. c. c. were positive to Feulgen test (DNA) as noted in table 2.

TABLE 2. *Cytochemical Reaction and Staining Properties of the Intranuclear Inclusion Bodies*

SUBSTANCE	METHODS	RESULTS	STAINING PROPERTIES
DNA	Feulgen	+	Violet red
DNA, RNA	Methylgreen-pyronin	+	Red
RNA	Ribonuclease digestion	+	Disappear
DNA, RNA	Thionine stain	+	Blue violet
DNA, RNA	Ultraviolet resorption (2600 Å)	+	
Polysaccharides	MCMANUS'S PAS	—	—
Neutral Fat	Sudan III	—	—
Alpha-Amino-Acid	Alloxan-SCHIFF reaction YASUMA-ICHIKAWA's method	+	Violet red
RNA, -SH	WEIGERT's fibrin method (GRAM stain)	+	Dark blue
Alkaline Phosphatase	GOMORI's method	+	Black
Inorganic Iron	Berlin blue stain	—	—
	BIELSCHOWSKY-MARESCH, PAP method	—	—
	Phloxin-tartrazine stain	+	Deep red
	Hematoxylin & eosin stain	+	Acidophilic or basophilic

Tissues stained by the methylgreen-pyronin method exhibited these bodies with red in pyronin. The ribonuclease tests for RNA in the inclusions were consistently positive. Tissues were hydrolyzed for 1 hour at 65°C, in 0.2 mg/ml crystalline ribonuclease. Controls were incubated without the enzyme. Test and control tissues were then stained by the methylgreen-pyronin method. Control tissues were stained with pyronin, but test tissues were not stained. Therefore, these inclusions contain RNA.

These inclusions were also stained with thionine in blue violet color as a basophilic pigment (DNA, RNA). With WEIGERT's fibrin stain (GRAM stain), the inclusions assumed

a blue-black color.

It was demonstrated that these inclusions showed marked absorption of ultraviolet ray in 2600 Å by means of ultraviolet photography (DNA, RNA). From these results, the indication was that the inclusions contain DNA and RNA.

To detect polysaccharide, the inclusions were stained by the periodic acid SCHIFF method (PAS) and showed negative.

Neutral fats with Sudan III stain and inorganic iron with Berlin blue stain, could not be demonstrated in the inclusions.

PAP's method of BIELSCHOWSKY-MARESCH's silver impregnation test for argyrophile character in the inclusions proved negative.

Preparations tested for alkaline phosphatase by GOMORI's calcium-cobalt method showed positive results in these inclusions whilst control tissues showed negative.

Testing for protein was not always accomplished with satisfactory results, but using YASUMA-ICHIKAWA's method of Alloxan-SCHIFF reaction for alpha-amino-acid groups, the inclusions took the violet red stain characteristic of a positive test.

The inclusions were phloxinophil with phloxin-tartrazine stained in a deep red color, which was clearly demarcated around the tissues.

5. Differential Diagnosis

1) Intranuclear crystal: The presence of acidophilic crystals within the nuclei of hepatic cells and epithelia in the kidneys in normal dogs has often been encountered. These crystals only occurred in the nuclei of hepatic and renal cells and were absent in the cytoplasm, hepatic sinusoidal endothelium, KUPFFER cells, bile duct epithelium and other tissues. Histological pictures of cases were different from those of H. c. c. and these crystals were often found in normal dogs. But in cases of mixed infection of H. c. c., differentiation is needed. The crystals were shown with rod-like or rectangular appearance and were extensively acidophilic. The crystals occupied a clear area or vacuole in the nucleus and the nuclear membrane had margined chromatin. The crystals often partially extruded into the nucleus of the epithelium. They were free from fat and iron and so did not give a positive result to Feulgen's technique. These crystals may have no relation to virus.

2) Differential diagnosis to canine distemper: It is already known that GREEN and EVANS (1939) reported the differences in the inclusions of canine distemper and those of enzootic fox encephalitis. The present author has listed the differences in the inclusions in H. c. c. and in canine distemper in table 3.

DISCUSSION

It is already well-known that inclusion bodies are characteristic of viral disease, but the occurring cells in the disease are limited by each virus. The inclusions occurring in H. c. c., however, occur not only in the limited cells, but they occur in almost all germinal layers of the body. It is considered that the inclusions have no general commonness and they have considerable differences with each virus.

TABLE 3. *Comparison of the Inclusion Bodies of Canine Distemper and Infectious Canine Hepatitis*

LOCATION	CANINE DISTEMPER	H. C. C.
Placement	Cytoplasmic and intranuclear	Intranuclear
Germinal Layer	Entoderm and Ectoderm	Entoderm, Ectoderm, Mesoderm and Mesenchyma
Staining Affinity	Acidophilic	Acidophilic or basophilic
Number	1~10, rarely 20	In general 1, sometimes 2~3, others particulate
Inner-structure	Homogeneous or vacuolated	Homogeneous or particulate
Nucleic Acid	Negative	Positive (DNA, RNA)
Cell	Mainly, discharged epithelia ; Epithelia (skin, bronchi, bladder, kidneys, adrenal medulla and bile ducts) and neurons	Mainly, liver cord cells and endothelia ; Mesodermal epithelia (serosa, myocardium and adrenal cortex) and endothelia Mesenchymal cells (advantitia cells, reticulum cells and histiocytes) Entodermal epithelia (liver, bronchi and bile ducts) Ectodermal epithelia (tonsils and <i>membrana nictitans</i>)

morphologically and chemically.

Arguments have ranged over the possibilities that they were viral colonies, reaction products produced by the cells, or both. At any rate, it is a fact that there are close connections between inclusion bodies and viral infections.

From the distribution of the inclusions, it is considered that the virus in H. c. c. makes its primary attack in the vascular endothelium and then spreads out hematogenously. All viruses, as a rule, consist of DNA or RNA. But most animal viruses usually consist of DNA. It is already known that in most viral diseases, inclusion bodies contain DNA in some developing stage of the inclusions. The workers who support the DNA theory are supposing that DNA-containing inclusions are viral colonies. A biological system which shows self-multiplication, namely, cells, mitochondria and virus, always contains nucleic acids. This fact shows that virus multiplies by accumulation of nucleic acids.

It has recently been considered that the multiplication of viruses occurs at first with the accumulation in the host cells of viral materials, consisting chiefly of nucleoprotein, and further, that accumulated viral materials are made up of viral particles. Therefore, in the cells infected by a virus, accumulations of this

nucleoprotein, viral matrix, occur.

In this interpretation, the inclusion bodies in H.c.c. showed a matrix, which is considered to be an accumulation of nucleoprotein. It has been found cytochemically that these matrices contain DNA and RNA.

Some of the DNA in the inclusions are derived from chromatin, but it can not be considered that all DNA in the inclusions consist of simply clusters of chromatin, for abnormal accumulated figures differ from normal nuclei and the degeneration of cells with inclusions was found. Nucleolus was present independently in the nucleus and at the same time accumulation of RNA was observed apart from nucleolus. These facts indicate the occurrence of abnormal metabolism; a large quantity of nucleic acids accumulated in the nucleus.

In the self-multiplied system, at least one more sort of nucleic acid is present, and, to some extent, in order for a self-multiplication to occur apart from other self-multiplication systems, at least both types of nucleic acids, such as DNA and RNA, are needed. It is already known that a self-multiplication system which contains only one type of nucleic acid can not be a multiplication without the co-existence of another self-multiplication system which contains at least other type of nucleic acid.

In view of these considerations, it may be suggested that accumulated figures of DNA and RNA will indicate the multiplication of H.c.c. virus in the matrix. Specifically, after the virus invades the objective cells, the metabolism of those cells is disturbed by viral multiplication. It is considered that this process is observed as "inclusion bodies."

The activity of alkaline phosphatase is found in these inclusions and the above enzymatic activity suggests some relationship with viral multiplication in the nuclei.

In general, synthesis of nucleic acids is regarded as self-multiplication of nucleoprotein granules and it must be that synthesized protein is combined with the synthesis of nucleic acid molecules.

As the protein of the general cell component or other secreted proteins is increased, with self-multiplication of nucleoprotein, the possibility of synthesis in protein, which is not combined with nucleic acids, exists.

Test for protein in the inclusions in H.c.c. showed the existence of some protein. Alpha-amino-acid groups and GRAM's stain show positive results.

In recent years, the mechanism of GRAM's stain has been clearly understood; HENRY and STACEY, BARTHOLOMEW and UMBREIT, etc. believe that a GRAM positive substance probably consists of RNA magnesium salt combined with some protein containing rich arginine. But at any rate, a GRAM positive substance is related to SH-protein and RNA. In this respect, it is of interest to note that GRAM

positive results in these inclusions suggest a part of its character. But in today's cytochemical reactions, specific reactions to protein or amino acids do not exist and this is a common reaction to the chemical compound with groups of some kind of base. Depending on the proportion of amino acid contents in protein, cytochemical reactions may be somewhat intensified or may disappear altogether. Consequently, a positive result is significant, but negative results do not offer proof that no protein exists. Cytochemical reaction for protein reacts only giant molecular compound. Consequently, investigations on protein in cytochemistry are largely left for future study.

It is known that in the rapid synthesis of protein, nucleoli in various cells are enlarged. In H. c. c. infection, enlargement of nucleoli is conspicuous before the formation of inclusions. RNA in nucleoli is increased, then positive reaction to Feulgen's technique appears and inclusions are developed.

Inclusion bodies in H. c. c. were free from carbohydrate, fat and iron. From this, it is considered that the inclusion bodies in H. c. c. contain chiefly DNA and RNA, and are a matrix which co-exists with some protein. Also activity of alkaline phosphatase exists in these inclusions. The author assumes that H. c. c. virus probably multiplies in the inclusion bodies.

Intranuclear crystals, as indicated in differential diagnosis, appear within the nuclei of hepatic cells and renal cells in normal dogs. They do not react to Feulgen's technique and have no relation to virus. SZYMONOWICZ (1901) first described this formation and considered them to be hemoglobin crystals. On the other hand, BRANDT'S (1909) contended that they were an erythrocyte substance, BERG (1929) considered them to consist largely of allantoin, NICOLAU and KOPCIOWSKA (1936) connected them with saprophytic virus, and WEATHERFORD and TRIMBLE (1949) considered them as uric acid excretions. In recent years, such crystals have been reported by BLOOM (1943), RUBARTH (1947) and INNES (1949). They are easily differentiated morphologically.

In canine distemper, two types of inclusions, such as cytoplasmic and intranuclear inclusions, are found. The former type of inclusion has high incidence, but neither inclusion contains DNA or RNA according to cytochemical observations. Canine distemper is easily differentiated from H. c. c. (See table 3).

Diseases other than H. c. c. which are already known to show nuclear inclusions include: viral abortion in mare, canine hard pad disease, Borna disease, herpes simplex, yellow fever, the 3rd virus in rabbits, salivary gland disease in guinea pigs and infectious fowl laryngo-tracheitis.

In cases of virus diseases, the nuclear inclusions show a marked similarity to another in different diseases. Therefore, they have a limited diagnostic value, except for the cytoplasmic inclusions which have the greatest specificity in the

various diseases.

The nuclear inclusions observed in H. c. c., however, are fairly characteristic of H. c. c. in morphological character and localization. In connection with other pathological findings, the ability to diagnose in cases of H. c. c. must be considered to be increased by the study of these inclusions.

The studies on the life cycle of this virus hinge on inclusions. Enzymo-chemical studies and electron microscopic studies which must be strictly compared with staining figures, are to be considered important problems for the future.

SUMMARY

The author investigated the nuclear inclusions in H. c. c. from the view points of histopathology and of cytochemistry. The findings may be summarized as follows: The nuclear inclusions of the disease were fell into two types, the one, granular inclusion bodies and the other, homogeneous inclusion bodies. It is believed that the former grow into the latter. From the distribution of the inclusions, the author believes that the virus in H. c. c. is a pantropic virus. The inclusion bodies observed in H. c. c. were free from carbohydrate, fat, inorganic iron and crystals. They consisted chiefly of DNA and RNA. They formed a matrix which co-existed with some protein, and showed activity of alkaline phosphatase.

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EXPLANATION OF PLATES

PLATE I.

- Fig. 1. Granular inclusion (Initial stage). Nucleolus is conspicuously enlarged. Small clusters are diffusely distributed in nucleus. Hematoxylin-eosin stain (H.-E.) $\times 2,000$.
- Fig. 2. Granular inclusion. Granular materials are accumulated in the center of nucleus and nucleolus is situated in the periphery of nucleus. H.-E. $\times 2,000$.
- Fig. 3. Granular inclusion. H.-E. $\times 2,000$.
- Figs. 4~6. Shows fine radiating bands extending between the periphery of inclusion and nuclear membrane. H.-E. $\times 2,000$.
- Figs. 7~9. Homogeneous inclusion. Distinctly observed a halo between inclusion and the wall of nucleus. Inclusions showing homogeneous appearance and sharp demarcation. Nucleolus situated in nuclear membrane where chromatin shows marked margination. H.-E. $\times 2,000$.

PLATE II.

- Fig. 10. Feulgen reaction. Inclusions exhibit violet red color. $\times 1,000$.
- Fig. 11. Methylgreen-pyronin stain. Inclusions stain in red with pyronin. $\times 1,000$.
- Fig. 12. Phloxin-tartrazine stain. Inclusions stain in deep red. $\times 1,000$.
- Fig. 13. Ultraviolet resorption ($2,600 \text{ \AA}$).
- Fig. 14. GOMORI's method of alkaline phosphatase. Inclusions assume a black color. $\times 1,000$.
- Fig. 15. Intranuclear crystal in liver cell. H.-E. The crystal show rectangular appearance and is extensively acidophilic. $\times 1,000$.



