HISTOLOGICAL CHANGES IN THE CENTRAL NERVOUS SYSTEM FOLLOWING EQUINE ENCEPHALOMYELITIS *

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REVIEW OF LITERATURE

Meyer, Haring and Howitt ¹ have described an infectious disease in horses and mules which they demonstrated to be produced by a filterable virus, and which affects the brain and spinal cord of these animals. They reported no important gross anatomical lesions, but described significant microscopic changes in the brain and cord consisting of perivascular hemorrhages, infiltration of the sheaths of the blood vessels and scattered patches of leukocytic infiltration in the gray, and occasionally in the white, matter.

Haring, Howarth and Meyer ² have described briefly the symptoms of this disease in horses and mules, which can be attributed directly to the microscopic changes in the brain. They stated that the malady is apparently identical with the horse disease that at different times during the past 60 years has caused heavy losses in various parts of the United States, particularly in the west central states, and which has been called Kansas-Nebraska horse plague, and also, incorrectly, cerebrospinal meningitis, forage-poisoning and botulism.

In a subsequent paper Meyer, Haring and Howitt ³ concluded that the disease, as observed in California, differed etiologically from the equine encephalitis described by Moussu and Marchand ⁴ in France, and from the well known Borna disease of Germany. According to Records, ⁵ and Records and Vawter ⁶ the outbreak, which started in horses and mules in California in 1930, had spread by 1932 throughout most of the western states. Newsome ⁷ described its occurrence in Colorado. In 1932 and again in 1933 Meyer ^{8, 9} summarized the existing knowledge of the disease.

Howitt ¹⁰ has shown that the virus may be recovered during the febrile period from various parts of the central nervous system and

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from the blood of experimentally infected animals, including the horse.

Records and Vawter¹¹ have attributed favorable results to the use of antisera in both laboratory and field cases.

Vawter and Records,¹² Kelser,¹³ and Giltner and Shahan¹⁴ have described various ways of artificially transmitting the disease.

Recently Syverton, Cox and Olitsky¹⁵ have shown similarities in the virus of equine encephalomyelitis and vesicular stomatitis, when inoculated into monkeys, guinea pigs and mice. They found that when such animals succumbed to experimental encephalomyelitis or vesicular stomatitis, the gross and microscopic changes in the brain were apparently identical and the same type of intranuclear inclusion body was formed in the neurones. They state that similar tissue changes occurred in the liver and kidney.

The particle size of encephalomyelitis virus in brain suspensions has been estimated at $500\mu\mu$ by Krueger, Howitt and Zeilov.¹⁶ They state that under like conditions of preparation and filtration it is of the same order of magnitude as the causal agent of poliomyelitis and apparently ten times the size of the hoof and mouth disease particle.

MATERIAL AND METHODS

Intensive microscopic study of the brains and spinal cords of eight horses affected by the virus of encephalomyelitis, either experimentally or by contagion, has yielded additional information as to the effects on the nervous system. As controls we have studied the brains and cords of three horses that died from sepsis with no clinical signs of encephalomyelitis, and the brain and cord of one normal horse that was killed by exsanguination. In addition the brains of two guinea pigs injected with filtered virus and killed with chloroform 3 and 4 days after successful inoculation were studied by the same methods. Also sections from various parts of the brain of a farmhand who died with symptoms similar to those of the equine encephalomyelitis, to which he had been exposed, were studied.

Portions of the brains and spinal cords of the experimental animals were fixed immediately after death in 10 per cent formalin, in formol-Zenker solution, or in Bouin's fluid. Material from the field animals was fixed in some cases shortly after and in other cases many hours after death, and was less satisfactory for histological study. The human brain was obtained at autopsy 12 hours after death and fixed in formalin.

Pieces from various parts of the nervous system were embedded in paraffin and sectioned at 5 microns. Sections were stained with Loeffler's methylene blue and basic fuchsin, or by Maximow's hematoxylin and eosin azur method. Prior to staining many of the sections were treated on the slide by immersion for 1 to 2 hours in Zenker's fluid and then washed with tincture of iodine for

removal of any precipitate of mercuric chloride. This treatment increased the stainability of the sections, especially of material fixed in formalin.

Other sections from a number of animals were subjected to tests for various pigments, since it was apparent early in the study that diffuse blood pigments in the tissues, and other pigments, especially in the large nerve cells of the brain stems of many of the horses, led to much confusion. It therefore became necessary to take into consideration the age of the animals and to test some of the material for blood pigments, lipochromes and melanin pigments. The tests applied were the alkaline alcohol treatment for blood pigments, the ether and chloroform solubility test for lipochromes and lipofuscin, the osmic acid color tests for lipochromes, and the osmic acid and silver nitrate tests for melanins. The sections subjected to these various procedures were afterward stained lightly with hematoxylin and eosin or with safranin. Through the courteous coöperation of Dr. Gordon H. Scott, of the department of cytology of the Washington University Medical School, the method of incineration on the slide was also used.

The tests for lipochromes leave much to be desired, since they could be applied only on material already subjected to the action of various fat solvents used in preparing paraffin sections. The combined results of the several tests, however, are worthy of some consideration and are described later. Lipofuscins are usually regarded as resistant to solution with alcohol after fixation other than directly with fat solvents. This fact, together with the yellow to yellowish green color of many of the inclusion granules, is considered indicative of lipofuscins.

MICROSCOPIC EXAMINATION

Sections of the brain and spinal cord of horses affected by the virus show the perivascular and leukocytic infiltration, in greater or less degree, already mentioned (Fig. 1). The cell types in the perivascular cuffs are lymphocytes, polymorphonuclear leukocytes and monocytes. In some of the horses many cells are phagocytic, containing numerous granules of brown pigment. Granule-laden phagocytes are also present within the blood vessels in a number of animals. The perivascular cuffs continue along the blood vessels for considerable distances and in some cases can be followed to the brain surface. Outside the layer of infiltrated cells there is a clear zone of varying width which in part, at least, represents shrinkage of the edematous brains, due to treatment with alcohol. The sections from most of the animals indicate considerable edema, especially in the brain stem and cortex. Diffuse blood pigments are present along the course of the blood vessels. These disappear on treatment with alkaline alcohol, as do the pigment granules in the phagocytic cells. The latter are accordingly also interpreted as blood pigments.

The control horses, three of which died from sepsis and one of which was killed, show no perivascular infiltration of cells. One of

the three dying from sepsis was not exsanguinated and shows an unusual amount of blood pigment in brain and cord. The two guinea pigs show no blood pigment, but both have perivascular infiltration, the one killed 4 days after inoculation in more marked degree than the other killed 3 days after being inoculated with virus. In the one human brain examined perivascular infiltration is also present, but little blood pigment is apparent. This brain was very edematous, as judged from the sections.

Many of the large nerve cells of the brain stem and spinal cord, and to a less extent in various parts of the cerebral cortex and cerebellum, show cytoplasmic inclusions in much larger numbers than in nerve cells of animals dying from other causes, as judged from the four control horses examined by us and from the large number of horses ranging in age from $2\frac{1}{2}$ months to 30 years, reported by Kikuchi.¹⁷ The inclusions consist of granule-like bodies which vary from 0.3 to 0.7 microns in diameter. Most of them are rounded but many of the larger ones are somewhat angular in form. In staining properties they show a wide range of variation which appears to depend in part upon the method of fixation and the state of the tissue when placed in fixing fluid. In material fixed in formol-Zenker's fluid immediately after death of the animal the eosin of the Maximow stain gives them a deep pink tint. In sections from blocks fixed in formalin or in Bouin's fluid they fail to stain with Maximow's technique unless previously treated on the slide with Zenker's fluid, and then they show much variability in staining reaction. Usually, with this treatment, many of the granules take a pink tint, but many others remain nearly colorless or yellowish. In the same cell frequently are found granules ranging from pale yellow to pink in color. In much of the material yellowish green to green granules are present, especially after treatment with Zenker's fluid followed by iodine. This suggested that the granules are related to lipochromes and led to further tests for lipochromes and other pigments, as described in another part of this report in further detail.

The basic fuchsin stain colors the granules a brick red, irrespective of fixation. This stain was useful in locating groups of affected cells but was of little value otherwise, as compared with the other methods employed.

The distribution of affected cells in sections of material most

favorable for careful microscopic study is somewhat irregular. There are considerable areas of apparently normal cells, while at the margins of such areas are found groups of nerve cells showing inclusions and various stages of cell degeneration. In other sections considerable areas of affected cells occur, varying from nearly normal ones to cells in the last stages of disintegration. In general, nerve cells near blood vessels have progressed farthest in the degenerative process, suggesting that the destructive agent is brought by the vessels or along their sheaths.

PHAGOCYTOSIS OF NERVE CELLS

Some of the injured nerve cells show the effects of marked activity of phagocytes (Figs. 3 and 7). Such cells are necrotic with shrunken eccentric nuclei whose nucleoplasm is basophilic and whose nuclear membrane usually has disappeared. The cytoplasm of these cells is shrunken and altered and Nissl bodies are markedly chromatolytic or absent. Inclusion bodies, however, are usually demonstrable. They have the same staining reaction and position as in slightly damaged cells. Phagocytes frequently may be seen burrowing into the surface of the nerve cells from all sides (Fig. 3) and affecting both perikaryon and cell processes. Extreme cases are encountered where only fragments of the nerve cells remain, surrounded by phagocytes. No phagocytes have been found deeply embedded within the nerve cells, as described by Da Fano and Ingleby¹⁸ (see page 358), but the burrowing action of phagocytic cells is evident from Figure 3.

Many nerve cells, in which nuclei are still nearly or quite normal in appearance, show small vacuoles in the outer layers of their cytoplasm (Fig. 3). The vacuoles vary somewhat in size and distribution. Inclusion granules present in such cells lie in the deeper part of the cytoplasm. There is no apparent direct relation between vacuoles and granules.

NUCLEI OF AFFECTED NERVE CELLS

The fuchsinophile nuclear inclusions found by Joest¹⁹ in the nerve cells of Borna's disease of horses, a disease that has many points of similarity to the horse encephalomyelitis of Haring and Meyer, naturally led to a careful search for intranuclear bodies in our material. Some cell nuclei in horse material (A147 and A252,

fixed immediately after death) contain rounded bodies of about the same size as those in the cytoplasm but, as a rule, of bluish color. Occasionally acidophilic granules appear in the nuclei. In animal A 41, a pregnant female 6 years old, the Maximow stain reveals reddish granules in the nuclei of many of the nerve cells. The granules are indistinct individually, but give a reddish tint to the nucleus. In this animal the cytoplasmic inclusions are small and are largely hidden by the Nissl substance. There was also but little perivascular infiltration in this animal, although clinically it had encephalomyelitis.

In guinea pigs injected with filtered virus numerous reddish granules are brought out by the Maximow method in many nerve cell nuclei, but most of the nuclei are clear and normal in appearance, even when the cells contain cytoplasmic inclusions. In these guinea pigs the cytoplasmic granules, as in horse A 41, are small and more difficult to find than in most of the horse material. The greater part of the material examined, however, including the human, shows very little indication of anything but the normal chromatin granules in the nuclei by the methods employed. In view of the reported discovery by Syverton, Cox and Olitsky¹⁵ of intranuclear inclusions in various animals subjected to the virus of equine encephalomyelitis, our failure to find nuclear inclusions constantly may be due to variations in the condition of the tissue when placed in fixing fluid, caused by differences of the time interval after death, and to other factors of technique which were not controllable in field animals and in human autopsy material. Many nuclei are found in various stages of destruction, with loss of nuclear membrane, and with acidophilic nucleoplasm, but only in markedly chromatolytic and otherwise necrotic appearing cells.

CYTOPLASMIC INCLUSIONS

The inclusion bodies in the nerve cells occur, as a rule, as clusters of granules near the nucleus of the cell. Most frequently they are found at one side of the nucleus (Figs. 2, 3 and 5). Sometimes they occur in several clusters, which may be situated at opposite poles of the nucleus (Figs. 4 and 6). Not infrequently the granules are scattered throughout the cytoplasm without reference to the nucleus. In the larger nerve cells they have been encountered in clusters in

the dendritic processes at considerable distances from the perikaryon. Occasionally they occur as compact, well circumscribed masses of granules, either near the nucleus or elsewhere in the cytoplasm.

Such inclusions are found in nerve cells that show only slight indications of degeneration (Fig. 4), and they also are present in cells that are in the last stages of destruction and that are undergoing phagocytosis (Fig. 3). The hematoxylin-eosin azur method is particularly favorable for staining these granules and the Nissl substance at the same time, when the latter still remains. The Nissl granules take a grayish blue tint, in marked contrast to the unstained, or pink inclusions. In Figure 4 the large, flaky Nissl bodies are shown throughout the perikaryon, appearing in the photomicrograph as gray masses. The inclusion granules, somewhat masked by the Nissl bodies, are shown in the photomicrograph as smaller black dots at either pole of the nucleus. The nucleus itself has been but slightly affected in the cell shown in this figure. Another cell is shown in Figure 5, in which Nissl bodies still are present at the periphery of the perikaryon, but in which chromatolysis has progressed much farther. The nucleus also is pyknotic and eccentric in position. The inclusion granules, however, are altogether similar to those of Figure 2, and show more clearly in the photomicrograph because they are not partially hidden by Nissl bodies. The nerve cells shown in Figure 6 have degenerated markedly, but the inclusion bodies have the same appearance as in the other cells.

Many publications have appeared on intracellular and intranuclear inclusions in relation especially to virus disease. Findlay and Ludford²⁰ have reviewed this literature up to 1926 and Cowdry²¹ has again covered the field up to 1927. Another survey is, therefore, unnecessary.

Hueck²² has differentiated lipofuscin from other lipid inclusions in nerve cells and other tissues. He states that there is considerable variation in the amount of lipofuscin in different types of nerve cells. Kikuchi,¹⁷ as already noted, in 1928 described lipofuscin and other inclusions in the nerve cells of horses ranging widely in age. He considers lipofuscin to be a normal constituent of the nerve cells, but shows that it is more abundant in older animals. Bielschowsky,²³ agreeing in general that lipofuscin is present in normal nerve cells, holds that under certain pathological conditions there is a great in-

crease of this pigment. The nucleus in cells undergoing such pathological formation of pigments may remain well preserved for a long time but eventually succumbs, preliminary to destruction of the cell.

Da Fano^{24, 25} has described minute bodies in herpetic encephalitis of rabbits. So far as can be judged from his descriptions and excellent figures these minute bodies are probably not identical with the cellular inclusions of the present account. Da Fano found bodies in various cells of the brain, including nerve cells. In our material the granules are found only in the nerve cells. There are also certain differences in the appearance of the granules themselves, as compared with the minute bodies of Da Fano. The latter are described²⁴ (see page 97) as a "single or double minute granule, roundish or slightly oval, surrounded by a clear halo; when in pairs the granules are so closely attached one to the other as to convey the impression of dumb-bell shapes; these are surrounded by a common and somewhat wider halo." Only occasional granules in our material show any sign of a halo and there are few if any double granules. In form the range in our material is from rounded to angular, as above described. Da Fano's minute bodies, furthermore, stain a deep purple-blue or purple-red with polychrome methylene blue and Giemsa's stains. Subsequently Da Fano²⁵ and Da Fano and Ingleby¹⁸ reported minute bodies in epidemic encephalitis in man, but with negative results as respects their presence in nerve cells.

Cowdry and Nicholson²⁶ describe and figure inclusions in the nerve cells of rabbits subjected to herpetic virus, which in size and general characteristics are not unlike those here described. In staining reaction, however, the granules of these investigators were colored a deep blue by the Giemsa stain on air-dried films.

It is apparent from Table I that there are at least three, and probably four, kinds of pigment in some of the brains studied by us. The blood pigments, while confusing because of their wide diffusion in the sections, are easily eliminated by the alkaline alcohol treatment. The melanin pigments can usually be recognized without difficulty in hematoxylin and eosin sections, and on treatment with silver nitrate they show the blackening that is characteristic of this test. With osmic acid they assume a deeper brown coloration. They are not dissolved in ether and chloroform. In a normal horse, 12 years of age, such granules are present in considerable numbers in the

TABLE I
Results of Various Microchemical Tests Applied to the Cytoplasmic Inclusion Bodies

Material	Age	Brain part	Silver nitrate	Osmic acid	Ether and chloroform	3% Sodium hydroxide in 80% alcohol	Incineration at 600° C
Normal horse	yr. 12	Medulla oblongata Midbrain	Black granules Black granules	Brown granules			
Horse No. A147	Aged	Various parts	Yellow granules	Dark brown granules	Pale to yellowish granules	Blood pigments dissolved, other granules present	Granules disappear save for a slight ash
Horse No. A252	16	Various parts	Yellow granules	Dark brown granules	Yellow granules	Blood pigment dissolved, pale granules in nerve cells	Granules disappear save for a slight ash
Horse No. 1683	Young	Midbrain	Some black granules	Brown granules	Granules faint to fairly distinct		
Horse No. 1684	Young	Midbrain Olfactory bulb	Yellow to black granules Diffuse precipitate	Brown granules	Yellowish granules Pale granules		
Human No. 1513	43	Cerebral cortex Deep cerebellar nuclei Purkinje cells Hippocampus	No blackening Some black granules No blackening	Brown granules, vacuoles Yellow and brown granules Light brown granules Brown granules, some vacuoles	Yellow to brown granules Brown granules Pale granules, some solution Brown pigment	No blood pigment, pale granules in nerve cells No blood pigment No blood pigment No blood pigment, pale granules in nerve cells	

brain stem. Horses A147 and A252, although older than the normal horse, do not show any brown granules except after treatment with osmic acid. In sections treated with silver nitrate only yellowish granules are visible in the nerve cells. Treatment with ether and chloroform is followed by some indication of solution of some of the inclusions, but most of the pale to yellowish granules still remain. The poor solubility and the color of these inclusions point to lipofuscins. On incineration of thin unstained sections, mounted on the slide with distilled water only and subjected to a temperature of 600° C in an electric oven, the organic substance of the cells, including the granules, entirely disappears. Only a reddish ash suggesting iron is left in the position of the granules, according to Dr. Scott, to whose generous coöperation we owe the application and interpretation of this test. The younger horses, Nos. 1683 and 1684, show some melanin in the midbrain but very little if any elsewhere. Inclusion bodies are widely distributed, however, in this material, appearing as yellow granules after all the tests, save the osmic acid which turns them brown.

Sections from various parts of the human brain which had been fixed in formalin show a considerable range of reactions to the tests applied. Only in the deep cerebellar nuclei are there granules that give positive indications of melanin, but sections through the deep part of the midbrain were not available. Cerebral cortex, hippocampus and the Purkinje cells show yellow to brown granules, with some solution by ether and chloroform. The yellowish green to green color of granules observed in some of the horse material was also observed in some of the human nerve cells.

The results of the tests as to the nature of the inclusions point in the direction of lipochromes and lipofuscin. The variations in staining qualities appear to depend not only upon the stain employed and the preliminary treatment, but also upon changes that must take place within the granules themselves, as shown by the presence in many cells of granules that range from pale yellow to deep pink in color when treated by Maximow's eosin azur stain. There is, however, no apparent relation between the stage of destruction of the nerve cells containing them and the staining qualities of the granules, since in material fixed immediately after the death of the animals, *e.g.*, horses A147 and A252, nerve cells in various stages of necrosis are present in which no differences are apparent in the granules.

SUMMARY AND CONCLUSIONS

Histological and cytological study of the brain and spinal cord of horses, guinea pigs and humans subjected to the virus of equine encephalomyelitis shows characteristic pathological changes. The most constant feature is the perivascular infiltration already pointed out by Meyer and Haring. This is found in all horses affected by the virus, but in none of the controls. It is also present in the guinea pig and human brains that were affected by the virus.

There are suggestions of intranuclear inclusions in some of the nerve cells in several animals, but in our material this feature is too inconstant to permit of considering them as characteristic features of the affected cells.

There is considerable degeneration of Nissl substance in many nerve cells of virus-infected animals and also in the human brain. Nerve cells in various stages of necrosis are present, especially in the brain stem and spinal cord. Many nerve cells are in process of phagocytosis by leukocytes.

Cytoplasmic inclusions are present in many nerve cells of all the animals studied, which were affected by the virus, and in the human brain. Similar inclusions are found in smaller numbers in three horses that died from an unknown sepsis and also in a normal horse 12 years of age. The number of inclusions in the nerve cells of the virus-infected animals is considerably greater than in the control animals, and appears to be increased by the pathological conditions of the disease.

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DESCRIPTION OF PLATES

PLATE 97

FIG. 1A. Photomicrograph showing the perivascular infiltration following encephalomyelitis in the brain of a horse.

FIG. 1B. Photomicrograph with higher power showing perivascular infiltration and also nerve cells in various stages of necrosis.

FIG. 2. Brain stem of horse No. A147, showing nerve cell and small blood vessel with cytoplasmic inclusions in a necrotic cell and perivascular infiltration, respectively. Hematoxylin and eosin azur stain after Zenker fixation. Section 5 microns.

en = endothelium; In = cytoplasmic inclusions; N = Nissl bodies.
× 820.

FIG. 3. Horse No. A147. Nerve cell surrounded by phagocytes. The cell is necrotic and also shows inclusions. Hematoxylin and eosin azur stain after Zenker fixation. Section 5 microns.

In = cytoplasmic inclusions; Nu = nucleolus; ph = phagocytes.
× 820.

1A

1B

2

3

PLATE 98

FIG. 4. Horse No. A147. Photomicrograph of nerve cell in brain stem showing Nissl bodies (grayish) and cytoplasmic inclusions (black). Hematoxylin and eosin azur stain after Zenker fixation. Section 5 microns.

In = cytoplasmic inclusions; M = precipitate of mercuric chloride; N = Nissl bodies; Nu = nucleolus.

FIG. 5. Horse No. A147. Photomicrograph of nerve cell in later stage of destruction than that shown in Fig. 4. Nissl bodies are found only at the periphery of the cell and the nucleus is eccentric and shows considerable degeneration. Hematoxylin and eosin azur stain after Zenker fixation.

In = cytoplasmic inclusions; M = precipitate of mercuric chloride; N = Nissl bodies; Nu = nucleolus.

FIG. 6. Photomicrograph of nerve cells markedly degenerated with advanced chromatolysis. Hematoxylin-eosin azur stain after Zenker fixation. Section 5 microns.

In = cytoplasmic inclusions; M = precipitate of mercuric chloride; Nu = nucleolus.

FIG. 7. Brain stem of horse No. A252 showing a group of nerve cells undergoing phagocytosis. Hematoxylin and eosin azur stain after Bouin fixation. Section 10 microns.

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