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FREE PIG.

The Ohio State University, Ph.D., 1965
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PATHOGENESIS OF PORCINE POLIOENCEPHALOMYELITIS

IN THE GERMFREE PIG

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

John Ervin Holman, B.Sc., D.V.M.

The Ohio State University

1965

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CHAPTER I

PATHOGENESIS OF CLINICAL SIGNS, GROSS, AND MICROSCOPIC LESIONS OF POLIOENCEPHALOMYELITIS IN THE GERMFREE PIG

Introduction

The isolation of viral agents associated with clinical signs and lesions of polioencephalomyelitis was reported in Ohio in 1962 (26). Their cytopathic effect in primary monolayer swine kidney cell cultures was described and the disease was reproduced in germfree pigs (23). Additional study (24) of the six isolates established them as enteroviruses according to the criteria proposed by Betts et al. (5) and Melnick and Wallis (36). Five of the viruses were antigenically distinct. Selected viruses were nonpathogenic for various laboratory rodents, monkeys, and embryonating hen's eggs (41). Lesions of a non-suppurative encephalomyelitis were produced in an initial infectivity study in germfree and pathogen-free pigs by both intracerebral and oral inoculation (29).

Studies with other enteroviruses were occasionally handicapped by intercurrent disease and all faced the possibility of modifying influences due to other microorganisms. The germfree pig with its standardized environment made it possible to study the effects of a pathogenic enterovirus in the absence of other microorganisms. This investigation was initiated to broaden
our understanding of the disease produced by enteroviruses in swine by defining the histopathogenesis in experimentally infected germfree pigs with a view towards determining the portal of viral entry to the central nervous system. Oral inoculation was used to simulate the most likely natural route of exposure. An additional report in connection with this study will deal with virus reisolations from various organ sites during the course of the disease, the development of serum neutralizing antibodies, and electrophoresis of serum proteins.

Review of Literature

Numerous reports dealt with the isolation of porcine enteroviruses, their properties, and the results of natural and experimental infection. Teschen disease, the prototype of swine polioencephalomyelitis, was first described in the region of Teschen, Czechoslovakia, in 1930 by Trefny (46). Subsequent studies by Czech and German investigators were reviewed in English by Kaplan and Meranze (22) in 1948. Innes and Saunders (19) and Jones (21) reviewed the literature on lesions in the central nervous system (CNS). A more recent review by Betts (3) summarized work with porcine enteroviruses up to 1964.

Information concerning the pathogenesis of Teschen disease was reported in several studies. Some studies emphasized virus reisolation results from various body tissues, while others were confined to histopathologic changes in the CNS.

An important contribution to virus reisolation studies was the demonstration of virus multiplication with cytopathic effects
in porcine kidney cultures (28, 34, 35). By this means Hecke (16) was able to study the distribution of Teschen virus in various body tissues following oral inoculation. The entire digestive tract was considered as an entrance port for the virus. A viremic stage was supposed, but not demonstrated. A later study indicated the main site of virus multiplication was in the epithelium of the colon (17). General support for the lower digestive tract as a major site of virus multiplication was indicated in studies with other porcine enteroviruses (2, 4, 9, 20, 25).

Histopathologic investigations suggested various routes of viral spread to the brain and spinal cord. Lesions in the olfactory bulb of orally infected pigs indicated that this region might serve as a site of viral entry from the nasal mucous membrane (13). Manuelidis et al. (30) indicated there was no difference in the distribution of lesions in experimental pigs following intracerebral, intranasal, or oral inoculation. Kötsche (27) related the distribution of lesions in the spinal cord to the regional nerve supply of the intraperitoneal and intramuscular inoculation sites. An ascending viral spread in the CNS was indicated in this study. The severity of lesions paralleled the titer of virus. Fischer's (11) classic study of histopathogenesis presented further evidence for the neural spread of virus via the olfactory route with intranasal infection. However, the distribution of lesions excluded this site as a major portal of viral entry in natural and contact infected animals. There was agreement of early lesions in the reticular formation and nucleus of the vagus nerve in contact infected animals, and following oral, intramuscular, and intravenous infection.
Additional support for the initial involvement of the medulla oblongata was presented by Hecke (16) who indicated possible spread via regional nerves from the pharynx. Lesions in abdominal sympathetic ganglia indicated the digestive tract might serve as a portal of viral entry to the CNS (45).

Viremia was demonstrated during the preparalytic stage of Teschen disease and during infection with other enteroviruses (2, 12, 18, 20, 43, 44, 49). This route of spread to the CNS was emphasized by Bodian (7) in human poliomyelitis.

The present status of Teschen disease infection was summarized by Mayr and Hecke (33). They noted three stages in the disease: (1) viral multiplication in the digestive tract and regional lymph nodes, (2) viremia, (3) neural infection. The vast majority of infections were limited to the initial stages and occurred without clinically detected signs of disease (17, 32).

Materials and Methods

Virus preparation

A porcine enterovirus (03b), previously isolated from the brain of a pig with poliensephalomyelitis, was used. The isolation and characterization of this virus were previously described (23, 24). The second passage of the virus in swine kidney tissue culture was used. The first swine kidney tissue culture passage was a reisolation from a pool of cerebral cortex, cerebellum, and medulla from an orally infected pig. One-hundred ml. prescription bottles of primary monolayer swine kidney cells were inoculated with 0.2 ml. of the first swine kidney tissue culture passage of the virus. The
cultures were incubated for thirty minutes at 37° C., after which
the maintenance medium (23) was added. After two days of incubation
at 37° C. the infected tissue culture fluid was harvested at the time
of maximum cytopathic effect. Tissue culture fluid from additional
bottles inoculated with 0.2 ml. of Hanks' balanced salt solution
was harvested after two days following scraping of the cell sheet.
The infected and uninfected tissue culture fluids were stored at
-70° C. and served as a source of inoculum for all subsequent
experiments.

The culture fluids were titrated in Leighton tubes prepared
as previously described (23). A tenfold dilution series of virus was
prepared and 0.1 ml. was inoculated into each of four tubes per
dilution. Titers were calculated by the method of Reed and Muench (38)
and expressed in 50 per cent end point tissue culture infectious
dose (TCID₅₀). The final titer represented an average of three
separate titration experiments in different lots of swine kidney
tissue culture.

Experimental animals

Three litters of germfree pigs¹ were divided into experimental
(20 pigs) and control groups (14 pigs). Virus inoculated and control
pigs from each litter were maintained in separate flexible film
isolators under identical conditions. Pigs were fed a commercially
available infant formula² six times daily for the first week. The

¹Laboratory Animal Facility, The Ohio State University,
Columbus, Ohio.

²Varema, The Baker Laboratories, Inc., Cleveland, Ohio.
pigs drank readily from a pan within the first twenty-four hours after birth. Solid food$^3$ was added to the diet at three days of age in slowly increasing amounts. The feeding schedule was reduced to five times per day at one week and four times per day at two weeks of age.

Germfree status was established by culturing samples of feed and body wastes from an isolator each time an entry was made. A fecal sample from the rectum and a swab of the oropharynx were obtained from each experimental pig just prior to its removal from the isolator. Samples were cultured by the method of Wagner (46) with the addition of testing for mycoplasma.

Inoculation of animals

Twenty one-or-two-day-old Yorkshire pigs were inoculated orally with 2 ml. of virus suspension in tissue culture fluid. The inoculum contained $10^{4.5}$ TCID$_{50}$ of virus per ml. Four control pigs received 2 ml. of virus free tissue culture suspension. Inoculation was accomplished by restraining the pig in a vertical position and injecting the inoculum slowly from the tip of a 10 cc. syringe placed in the left cheek pouch.

Examination of animals and tissues

Results of a daily physical examination and rectal temperature reading were recorded for each pig throughout the course of the experiment. A daily neurological examination was carried out on

$^3$P/D, Hill Packing Co., Topeka, Kansas
each pig and included the following reflexes: flexor, extensor thrust, extensor postural thrust, righting, and placing reactions.

Pigs were euthanatized at intervals from two to twenty-eight days after inoculation. They were electrocuted and exsanguinated by bleeding from a severed axillary artery. Four selected pigs and one control pig were anesthetized by intraperitoneal injection of pentobarbital Sodium and prepared for perfusion. A total of 600 ml. of isotonic saline was perfused through a 14 gauge needle secured in the root of the aorta at a pressure of approximately 100 mm. of mercury. The right atrium was incised to allow for the return outflow. Following this, 3500 ml. of 10 per cent phosphate-buffered formalin was perfused over a period of twelve to fifteen minutes. Selected tissues from the perfused animals were further processed for electron microscopy studies.

The following tissues were fixed in 10 per cent phosphate-buffered formalin, embedded in paraffin, sectioned at 6 microns, and stained with haematoxylin and eosin: transverse sections of one cerebral hemisphere at the level of the mid olfactory bulb, mid olfactory tubercle, optic chiasma and thalamus, corpus mamillare and hippocampus, and mesencephalon. Entire transverse sections were taken through the pons and cerebellum, anterior medulla and cerebellum, and medulla at the obex. Entire sections of spinal cord with attached dorsal root ganglia were taken from the following levels: anterior cervical, cervical enlargement, mid and posterior thoracic, anterior and mid lumbar enlargement. Additional sections of neural tissues included brachial plexus, median nerve,

^Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa.
sciatic nerve, anterior tibial and peroneal nerves, maxillary nerve, optic nerve, gasserian ganglion, stellate ganglion, coeliac ganglion, and spiral ganglion. Sections of non-neural tissues were prepared in a similar manner from the following sites: pituitary, all lobes of the lung, myocardium of both ventricles and atria, thoracic and abdominal aorta with attached lymph nodes, trachea, esophagus, thymus, thyroid, lacrimal gland, lower eyelid, tongue, posterior pharyngeal wall, hard and soft palate, turbinate, mandibular lymph node, mandibular salivary gland, external ear, inner ear, planum nasale, lower lip, diaphragm, liver, gall bladder, spleen, both kidneys, urinary bladder, cardiac, fundic, and pyloric areas of the stomach, duodenum, jejunum, ileum, cecum, spiral colon, descending colon, rectum, mesenteric lymph node, pancreas, adrenal, psoas muscle, ventral abdominal skin, testis or ovary, seminal vesicles or uterus, penis and prepuce or cervix and vagina, rib at costochondral junction, sternal marrow, and head of femur. Eyes were fixed in Zenker's acetic fluid and processed in the usual manner after overnight washing in tap water. Sections at fifty micron intervals from the brain stem and selected areas of the spinal cord were examined from pigs euthanatized seven and eight days following virus inoculation in order to evaluate the nature and distribution of early CNS lesions. Other staining methods employed in addition to hematoxylin and eosin were luxol fast blue B-periodic acid Schiff-hematoxylin, and cresyl echt violet.
Results

Clinical signs of disease were not observed in the nine pigs euthanatized on or before the seventh post-inoculation day (PID). The most consistent sign noted in the eleven pigs maintained beyond seven days was a temperature rise. An elevation of one to two degrees F. was recorded in all eleven pigs. Initial elevations were noted most frequently on the eighth PID (six animals) with a range from seven to ten days. Temperatures remained elevated from three to nine days with peaks up to 106 F. occurring in the early stages of fever. Temperatures returned to normal by the nineteenth day.

The rise in temperature was accompanied or followed within one day by diarrhea in ten pigs. Brownish, semi-liquid stools were observed for two to four days, corresponding closely to the period of highest fever. With the exception of one pig, appetites remained unchanged during this period and the pigs remained active and alert.

Signs of neurological involvement were inapparent in seven pigs. Slight to moderate loss of the extensor thrust reflex was detected in three pigs on the eighth or ninth PID. Signs persisted for approximately one week. Ataxia or abnormalities in other reflexes were not observed. Indications of impaired mobility were somewhat handicapped by the limited floor space in the isolator. One pig became paralyzed on the eighth PID. Spinal reflexes were absent in the hind limbs and moderately reduced in the forelimbs. This pig could drag itself about for short distances by using the forelimbs. It became progressively weaker and required assistance to eat.
Clinical signs in control animals were absent except for a three day period of mild diarrhea in one pig. This occurred when the supply of water available for dilution of the liquid diet was temporarily exhausted.

Macroscopic lesions were not observed in virus inoculated or control pigs.

Consistent microscopic changes were present in several sites in addition to the expected neural changes. These are described by organ system following the recording of developmental lesions in the central and peripheral nervous system.

Microscopic lesions were present in the CNS of all pigs (thirteen) examined seven or more days following virus inoculation. The brain stem, lumbar spinal cord, and associated dorsal root ganglia were the seat of early, but clearly defined changes in one of the seven-day animals. Lesions in the pons and medulla oblongata were sparse and only occasionally present in serial sections. Endothelial and perithelial cell swelling occurred in vessels and was accompanied by intramural cellular infiltration. Neurons within these areas were frequently shrunken and intensely eosinophilic. Cellular infiltrations surrounding degenerated neurons consisted primarily of pleomorphic microglia with intermixed monocytes (Fig. 1). Neutrophils were infrequent. Oligodendroglia surrounding such areas were often slightly swollen with a clearing of the nuclear background and more prominent chromatin granules. Occasional shrunken, degenerating forms occurred.
Dorsal root ganglia in the lumbar area of this same pig were the site of occasional focal cellular accumulations among the nerve cells. Nerve cells in such foci were shrunken and eosinophilic. Cytoplasmic vacuoles were present in some degenerating nerve cells (Fig. 2). Early degenerative changes were apparent in a few scattered neurons in adjacent levels of the lumbar spinal cord. Diffuse chromatolysis was present in slightly swollen nerve cells with eccentrically located nuclei. Cellular infiltrations and perivascular cuffings were absent.

Lesions were more numerous in a second pig on the seventh PID and in one on the eighth PID. Changes were most marked in the pons and medulla oblongata of both pigs. Relatively circumscribed areas of involvement were randomly distributed throughout the gray matter. Vascular infiltrations were more extensive with accumulation of monocytes in the perivascular space. Occasionally diffuse infiltrations of monocytes and perithelial cells occurred in the adjacent parenchyma. Neurons were shrunken and darkly stained in areas of more intensive lesions. Microglia predominated in cellular infiltrations around nerve cells in advanced stages of degeneration. Limited cellular accumulations were noted in the leptomeninges adjacent to superficial parenchymal lesions (Fig. 3). Focal cellular infiltrations occurred in the cervical dorsal root ganglia. Monocytes with occasional large reticular cells formed dense nodules when they were within the Mantel capsule occupied by degenerating neurons. A few scattered nerve cells in the ventral horns of the adjacent cervical spinal cord showed mild degenerative changes. In one pig (eighth PID) the neuronal degeneration was accompanied by occasional
Fig. 1.—Photomicrograph. Two shrunken, degenerated neurons (arrows) associated with extensive infiltration of pleomorphic microglial cells occurring in the medulla oblongata (J609). H & E stain; X 504.

Fig. 2.—Photomicrograph. Degenerating neuron with vacuoles (arrow) in a dorsal root ganglion with focal cellular accumulation (J609). H & E stain; X 700.
perivascular cuffing in the gray and adjoining white matter. Small, diffuse accumulations of perithelial cells and monocytes with prominent oligodendroglia were oriented around a few of these vessels. An increased perivascular space occurred around other vessels with slightly swollen perithelial cells. Similar lesions were present in the lumbar spinal cord. Intramural cellular infiltration of small vessels in the molecular layer and rare glial nodules in the mitral cell layer were noted in the olfactory bulb of the same pig.

Five pigs were examined during the period from ten to fourteen days. This corresponded to the most severe stage of CNS involvement and similar lesions were noted in all pigs. Minor variations in distribution occurred in this group.

The spinal cord was consistently the site of lesions throughout its entire length. No regional differences in involvement were apparent. The characteristic features were neuronal degeneration, neuronophagia, perivascular cuffing, and glial accumulations. The mid and ventral horn areas of the gray matter were the usual sites of involvement.

Mild changes were frequent in the large motor neurons of all five pigs. Early degenerative changes were indicated by slight swelling, fine granularity of the Nissl substance, and eccentric nuclei. Shrinkage of nerve cells was associated with an increased affinity for eosin staining, condensation of nuclear chromatin, and loss of nuclear membranes. Cytoplasmic vacuolization with karyolysis was infrequent except in the one pig with clinical paralysis.
Satellitosis of neurons was occasionally noted, but did not appear to be a prominent feature of neuronal involvement.

Cellular infiltrations within vessel walls were common. Perivascular cuffing varied up to two or three layers of cells and mitotic figures were not infrequent among the infiltrating cells. Variable size parenchymal infiltrations were a striking part of the inflammatory reaction (Fig. 4). The predominating cells were pleomorphic microglia with intermixed monocytes and perithelial cells. Limited numbers of neutrophils were present in some areas (Fig. 5). They occurred along with microglia in degenerating neurons undergoing neuromophagia (Fig. 6).

Less prominent cuffing and parenchymal infiltrations were occasionally present in the dorsal horns of the gray matter. Perivascular cuffing was infrequent in the white matter except in association with larger vessels radiating from the gray matter.

Cellular infiltration of the accompanying dorsal root ganglia was regularly present at all levels of the spinal cord. Infiltrates were not as extensive as those occurring in the spinal cord and relatively few ganglion cells were destroyed (Fig. 7). Interstitial accumulations of cells were most pronounced around vessels with infiltration of the adjacent Mantel capsules. Monocytes and large reticular cells with indented, vesicular nuclei were mixed with occasional neutrophils and smaller pleomorphic forms resembling the microglia seen in spinal cord foci. Mitotic figures were not infrequently observed among these infiltrating cells. The layer of flattened capsule cells was generally intact even when a few infiltrating cells were present inside the Mantel capsule. Mild
Fig. 3.—Photomicrograph. Perivascular cuffing (arrows) and neuronal degeneration associated with cellular infiltration and vacuolization of the gray matter in the area of the seventh cranial nerve (J603). Note the overlying meningitis. H & E stain; X 150.

Fig. 4.—Photomicrograph. Marked cellular infiltration occurring in the ventral horn of the spinal cord 12 days after inoculation (J604). Scattered, necrotic neurons are undergoing neuromophagia (arrows). H & E stain; X 150.
Fig. 5.—Photomicrograph. Cellular infiltration including neutrophils occurring in the ventral horn of the spinal cord 12 days after inoculation (J604). Note neuronomophagia (arrow) of degenerating neuron. H & E stain; X 346.

Fig. 6.—Photomicrograph. Higher magnification of neuronomophagia in Figure 5. Neutrophils (arrows) and microglia are present in the cytoplasm. H & E stain; X 850.
degrees of neuronal degeneration could not be differentiated with certainty since considerable histologic variation was apparent in control material. More advanced stages of neuronal degeneration were similar to those seen in the spinal cord and were accompanied by limited cellular infiltrations. Marked cellular accumulations occasionally filled capsular spaces replacing degenerated ganglion cells (Fig. 8).

Lesions similar to those occurring in the spinal cord were found scattered throughout the medulla oblongata and pons. Nuclear groups throughout these areas of the brain stem were involved with no indication of any preferential location. Degenerative changes of the ganglion cells were less marked and neuronophagia was infrequent. One to two layers of cells occurred as cuffs around occasional vessels, but infiltrations were more often confined to the vessel wall with a few perivascular cells.

Cellular infiltrations were even less pronounced in the mesencephalon. Perivascular cuffing and glial infiltrations occurred in the substantia nigra and nucleus ruber. Lesions were infrequent in the gray matter around the aqueduct of Sylvius and in the corpora quadrigemina.

Involvement of the diencephalon was intermediate between that of the mesencephalon and medulla oblongata. Lesions were more frequently noted in the ventral portion of this area. Neuronophagia was infrequent, seldom involving more than one or two ganglion cells per section.
Fig. 7.—Photomicrograph. Cellular infiltration in a dorsal root ganglion 12 days after inoculation (J604). H & E stain; X 162.

Fig. 8.—Photomicrograph. Marked cellular accumulation in one Mantel capsule (arrows) replacing degenerated ganglion cell (J604). H & E stain; X 475.
Mild lesions were consistently present in the cerebellum. Circumscribed glial foci were sparsely scattered throughout the areas containing dorsal roof nuclei. Neuronophagia of ganglion cells in this area was rare and degenerative changes were not striking. Perivascular cuffing was infrequent in the white matter of the gyri. The molecular layer was a more frequent site for scattered, circumscribed, glial accumulations and minute, perivascular, infiltrations. Larger foci extended slightly into the granular cell layer and disrupted the layer of Purkinje cells (Fig. 9). Shrunken, eosinophilic, Purkinje cells were occasionally present in such foci. Purkinje cells in adjacent zones and generally throughout the cerebellar cortex were not the site of definite degenerative changes. Occasionally prominent focal infiltrations of cells occurred in the leptomeninges in association with underlying changes in the molecular layer. The cells were primarily large reticular cells and monocytes.

In only a few sections were lesions noted in the cerebral cortex dorsal to the rhinal fissure. There was no area of predisposition for the intramural cellular infiltrations and small glial foci. Lesions in the basal ganglia were rare. The ependymal cell lining and choroid plexus of the lateral ventricles were always normal.

There was a moderate intensification of lesions in the rhinencephalon. Perivascular cuffing was more pronounced in the hippocampus, approaching that present in the diencephalon. Glial infiltrations occasionally disrupted the pyramidal cell layer and were found encompassing shrunken nerve cells with eosinophilic cytoplasm (Fig. 10).
Fig. 9.—Photomicrograph. Cellular infiltration in the molecular layer of the cerebellum with disruption of the Purkinje cell layer (J610). H & E stain; X 315.

Fig. 10.—Photomicrograph. Shrunken, degenerated neurons (arrows) surrounded by microglial cells in the pyramidal cell layer of the hippocampus (J610). H & E stain; X 175.
Involvement of the piriform lobe mirrored that in the neocortex, however, lesions were more marked in the ventral cortex of the trigonum olfactorium.

The olfactory bulb was the seat of mild lesions in four of the five pigs at this stage. Perivascular cuffing occurred in the three outer layers, while diffuse glial accumulations were most prominent in the molecular layer. Ganglion cells in these areas and in the mitral cell layer appeared normal. Cellular infiltrations were never present in the inner granular zone.

Lesions were found in the same general areas of the CNS in five pigs, three and four weeks following virus inoculation.

There was a marked diminution in the inflammatory process so that regional differences in severity were no longer apparent. Perivascular cuffing in the gray matter of the spinal cord was usually limited to a few cells. Infiltrations around vessels in the white matter were sometimes more prominent. Small glial foci were occasionally present in the white and gray matter. Accompanying dorsal root ganglia were the site of sparse interstitial cellular infiltrations. Ganglion cells in the spinal cord and dorsal root ganglia appeared normal and there was no detectable decrease in number. Mild perivascular infiltrations and occasional glial foci containing ten to twenty cells were scattered throughout the brain stem. Similar changes were rare in the cerebellum and were not detected in the cerebral cortex.

Lesions occurring in gasserian ganglia were always much milder than those present in dorsal root ganglia. Rare interstitial cellular infiltrations in the gasserian ganglion of two control pigs
made precise interpretation of initial viral effects difficult.
Equivocal interstitial infiltrations of monocytes occurred in one pig examined five days after virus inoculation. More definitive accumulations of monocytes with a few neutrophils occurred in the two pigs on the sixth PID. Cellular infiltrations were oriented in relation to vessels. Indications of neuronal degeneration were not present. Cellular infiltrations were more marked ten to fourteen days following inoculation, corresponding to the height of CNS involvement. Adjacent Mantel capsules were rarely penetrated by the interstitial infiltrates and advanced stages of neuronal degeneration did not occur. Lesions were absent in pigs at three and four weeks.

No significant lesions were found in the coeliac or stellate ganglia. Other autonomic ganglia associated with various organs in the thoracic and abdominal cavities were never the site of lesions. Careful examination of Auerbach’s plexus in multiple sections of the digestive tract did not reveal degenerative changes of ganglion cells or cellular infiltrations. Lesions were not noted in peripheral nerve trunks.

Consistent microscopic changes were detected in the adrenal gland in five out of eight pigs examined four to ten days following virus inoculation. The zona fasciculata of the adrenal cortex was the usual site of randomly distributed, minute, foci of necrobiosis. Swollen cortical cells with vacuolated cytoplasm and pyknotic nuclei occurred singly or in small groups. Adjacent cells were sometimes shrunken with eosinophilic cytoplasm. Karyorrhexis
in such sites was common and fragments of cortical cells containing irregular clumps of chromatin were noted (Fig. 11). Larger foci of involvement were accompanied by extravasation of a few red blood cells and varying numbers of monocytes and lymphocytes (Fig. 12). Cellular infiltrations up to 120 microns in diameter were not uncommon in the adrenal gland from pigs five and six days after inoculation. Cortical degeneration was less marked in older animals and was not observed after the tenth PTD. Lesions were not present in the adrenal cortex of control pigs.

Microscopic changes were noted in the thyroid gland in four out of five pigs examined three and four weeks after virus inoculation. A slight increase in the height of the follicular epithelium occurred and was accompanied by a reduction in the amount of follicular colloid. From one to six desquamated cells in varying stages of degeneration were noted in many follicles (Fig. 13). The follicles remained fairly uniform in size and papillary infolding of the epithelium did not occur. Follicular colloid in the thyroid gland of control pigs was uniform in amount and desquamated cells in the follicles were infrequent.

Lymphoid tissue in virus inoculated and control pigs showed a comparable increase in cellular population with increasing age. Primary follicles became apparent between one and two weeks of age in regional lymph nodes and the tonsil. "Blast" forms were common in the follicles of older pigs and there was marked mitotic activity. Eosinophils were often prominent in the lymph sinuses. A steady increase in the white pulp occurred in the spleen. Demarcation
Fig. 11.—Photomicrograph. Focal necrobiosis in the adrenal cortex 5 days after inoculation (H&E stain). H & E stain; X 346.

Fig. 12.—Photomicrograph. Focal necrobiosis in the adrenal cortex with marked cellular infiltration (H&E stain). H & E stain; X 700.
Fig. 13.—Photomicrograph. Desquamated epithelial cells in follicles containing reduced amounts of colloid 21 days after inoculation (J605). Note the cuboidal epithelial lining of the follicles. H & E stain; X 431.
between the medulla and cortex of the thymus became more pronounced after one week of age, and there was a continuous increase in the width of the densely cellular cortex.

Virus effect could not be detected at any level of the gastrointestinal tract. Lesions were absent from the eye and optic nerve. Focal interstitial infiltrations of plasma cells, lymphocytes, and occasional reticular cells were noted in the kidneys of pigs examined three and four weeks following virus inoculation. The cellular foci were usually noted among collecting tubules at the cortico-medullary junction and in the medulla. Similar foci were noted in the one twenty-six-day-old control pig. An explanation for this finding was not apparent.

Discussion

Oral inoculation of one- or two-day-old germfree pigs with 03b virus regularly produced a polioencephalomyelitis. A rise in body temperature of from one to two degrees F., seven to ten days following virus inoculation, indicated a relatively uniform incubation period. Clinical signs of neurological involvement, with one exception, were mild or inapparent. The one pig with flaccid paralysis of the hind limbs continued to eat and was able to move about for short distances by using its forelimbs. The mildness of the clinical course is generally comparable with that noted in other experimental studies employing Talfan, T 80, T 52A, and related viruses (4, 14, 15, 42). There was no serological relationship between 03b virus and Teschen and Talfan viruses (23). The relationship between the
Ohio isolate and the viruses isolated by Betts (4) from the tonsils of clinically healthy pigs is unknown.

The development of lesions in neural tissues was most clearly depicted in the ventral horn of the spinal cord. Initial involvement in this area was indicated by diffuse chromatolysis of slightly swollen nerve cells. A cellular reaction was absent in this first stage. Neuronal degeneration on the eighth PID was accompanied by intramural infiltrates in vessel walls and swollen endothelial and perithelial cells. Diffuse accumulation of glial cells and monocytes occurred in the adjacent parenchyma and satellitosis was occasionally noted. Mild degenerative changes occurred in many ganglion cells during the height of CNS infection. Complete dissolution of the cytoplasm with karyolysis was relatively infrequent. Perivascular cuffing and accumulations of microglia and monocytes were a prominent feature of ventral horn involvement at this stage. Limited numbers of neutrophils were present in some areas. Residual glial nodules and mild cuffing were noted three and four weeks after inoculation. Ganglion cell changes were not apparent at this stage.

The progression of lesions in the brain stem was less clearly defined. The occurrence of scattered, dark, shrunken, ganglion cells in all stages of disease and in controls obscured the early detection of viral effects. Significant changes in the brain stem were indicated by perivascular cuffing, increased numbers of glial cells, and advanced stages of neuronal degeneration. Histologic variation in neurons was particularly noticeable in dorsal root ganglia. Cellular infiltrations in these ganglia were
considered an indication of viral infection since they were never noted in controls. This contrasts with findings in dorsal root ganglia of conventional control animals in some studies (8, 30, 37).

Bodian (6), in a study of poliomyelitis in monkeys, described diffuse chromatolysis of Nissl substance in motor neurons of the spinal cord without accompanying cellular infiltrations. This was not confirmed in Teschen disease by Fischer (11) or Manuelidis et al. (30), who placed more emphasis on perivascular cuffing and a glial response in the initial stages of infection. The general microscopic features of a polioencephalomyelitis described in their studies were similar to the CNS lesions produced by 03b virus. The frequency and severity of lesions were less than that depicted for Teschen disease and more nearly paralleled those observed with less virulent porcine enteroviruses (4, 15, 39, 42, 45). The demyelination reported in two of these investigations (4, 15) was not a feature of spinal cord involvement in this study.

Lesions in this study were most pronounced in the gray matter of the spinal cord and adjacent dorsal root ganglia. Next most severely involved were the medulla oblongata and pons followed by the diencephalon and mesencephalon. Lesions were consistently noted in the cerebellum during the height of CNS infection, but were never extensive. Perivascular cuffing and glial accumulations were usually mild in the ventral cortical areas of the rhinencephalon and the hippocampus, while they were always rare in the neocortex.

The flaccid limb paralysis in one pig was correlated with the severity of CNS damage. Widespread necrosis of ganglion cells
in the ventral horn of the spinal cord explains the loss of motor function. The absence of paralysis in the other pigs is consistent with the mildness of degeneration in most motor neurons and the more limited indications of cell death. Bodian (6) indicated up to one-third of the motor neurons might be destroyed without signs of limb weakness.

Lesions associated with porcine enterovirus infection have been confined to neural tissues with few exceptions. Necrobiosis and focal cellular infiltrations occurring in the adrenal cortex have not been previously reported. These changes were confined to five out of eight pigs examined four to ten days following virus inoculation. Lesions were noted in pigs representing each of the three litters used in the study. The cellular infiltrations were clearly differentiated from foci of extramedullary hematopoiesis occurring in the spleen and liver of all virus inoculated and control pigs. The extensive study on the histomorphology of the adrenal gland by Matthias (31) indicated the zona fasciculata was the site of focal cellular infiltrations and necrobiosis in more than half of the examined adrenal glands from clinically healthy swine. This was not confirmed in the adrenal glands of control pigs from this study nor in adrenal glands of control pigs available from a previous investigation utilizing germfree pigs (29).

Accompanying virus reisolation studies indicated high levels of virus in the adrenal gland from all ten pigs examined two to ten days following inoculation. Thus necrosis of the adrenal cortical cells may have reflected a direct viral effect in an extraneural
site of multiplication. The possibility of cell exhaustion seems less likely since necrobiosis was most marked several days before the stress indicated by elevated temperatures and diarrhea. Support for in vivo multiplication of poliovirus in the adrenal gland was indicated by the study of Wenner et al. (48). Baron et al. (1) studied poliovirus inhibitory material in various organs and found monkey brain gave 60-90 per cent inhibition of thirty plaque forming units and adrenal gland gave 70 per cent inhibition. Other organs tested, except for the testis (50-70 per cent inhibition), gave less than 50 per cent inhibition.

The thyroid gland changes in older virus inoculated pigs followed the initial temperature elevation and were probably a reflection of increased functional demands for thyroid hormone.

The role of the adrenal gland as a possible site of virus multiplication was previously discussed. Information concerning other possible sites of extraneural virus multiplication was not obtained in the histopathogenesis study. Accompanying virus reisolation results were in general agreement with Hecke's study (16) with Teschen virus, showing early virus localization throughout the digestive tract and regional lymph nodes. Viremia was detected in one pig four days following inoculation.

Routes of virus infection of the CNS have centered around neural spread via peripheral nerve trunks and direct invasion through the blood vascular system. Both routes have their advocates in studies with human polio viruses (7, 10, 40). The multitude of studies and conflicting views in this area indicate the difficulties
in determining precise relationships. More limited data are available in porcine enterovirus studies where spread via neural pathways was suspected in some investigations (13, 16, 27).

Thordal-Christensen's study (45) indicated involvement of abdominal sympathetic ganglia in some spontaneously diseased pigs, suggesting the alimentary canal as a primary site of virus multiplication and portal of entry. Fischer (11) ruled out direct spread via the olfactory tract as the chief pathway of CNS entry in all except intranasally infected pigs. Her work tended to incriminate the medulla oblongata as the initial site of infection. This was supported by Betts (4) with colostrum-deprived pigs infected with T 80 and T 52A viruses. He suggested direct invasion of the brain stem via regional cranial nerves or through a selective area of increased vascular permeability. The demonstration of viremia in the early stages of infection with several porcine enteroviruses has emphasized the possibility of direct spread to the CNS through the blood-brain barrier.

Histopathologic evidence in this study indicated an early virus effect in the gasserian and dorsal root ganglia. Such involvement could have resulted from direct neural spread from centers of virus multiplication or invasion during the stage of viremia. The widely disseminated lesions in the CNS, occurring at the earliest stage of infection, suggest the blood vascular system as the portal of viral entry. The possibility of centripetal spread to the CNS at scattered points from sensory cranial ganglia and dorsal root ganglia cannot be eliminated by the results of this study.
The results did not suggest a localized area, such as the medulla oblongata, as an entry port of virus invasion with subsequent spread to adjacent levels of the brain and spinal cord.

The use of germfree pigs in the study of polioencephalomyelitis permitted a more precise characterization of the effects produced by a single microbiological agent than was previously possible. Further applications of this technique may be expected to widen our understanding of other porcine disease problems.

Summary

Twenty germfree pigs were used to study the disease produced by oral inoculation of 03b porcine enterovirus. Four litter mates served as germfree controls. Elevated temperatures and diarrhea were noted in pigs euthanatized after the seventh post-inoculation day. Neurological signs of disease were mild or undetected except in one pig with flaccid paralysis. Macroscopic lesions were absent. Histologic lesions of polioencephalomyelitis occurred in all pigs examined seven or more days following inoculation. The initial lesions emphasized varying stages of neuronal degeneration. Perivascular cuffing, cellular infiltrations, and neuromophagia were most marked ten to fourteen days following inoculation. Neutrophils participated in the reaction around degenerating neurons. The spinal cord and associated dorsal root ganglia were the sites of most severe lesions. A decreasing frequency of lesions was apparent in anterior areas of the brain stem. Changes in the cerebellum and rhinencephalon were sparse and they were rare in the cerebral cortex. Lesions in the
adrenal gland were noted during the initial stages of virus infection and suggested a possible site of extraneural multiplication. The pathogenesis of central nervous system infection was discussed and the blood vascular system was favored as the portal of viral entry.
CHAPTER II

VIRUS REISOLATION, SEROLOGY, AND SERUM ELECTROPHORESIS OF 
POLIOENCEPHALOMYELITIS IN THE GERMFREE PIG

Introduction

Results of virus reisolations from various organs were reported in several experimental studies of porcine enterovirus infection (1, 2, 3, 5, 6, 9, 11, 32). There was general agreement that the lower digestive tract served as a major site of virus multiplication. Neutralizing activity against the infecting virus was usually demonstrated in the serum of inoculated animals. Viremia was demonstrated during the early stages of infection with several of the porcine enteroviruses (1, 4, 8, 9, 24, 25, 32). Isolation results were not generally correlated with respect to possible routes of spread to the central nervous system. One exception was Hecke (5), who indicated initial virus penetration occurred in the medulla oblongata and suspected spread via regional cranial nerves.

Porcine enterovirus isolations from sites other than the alimentary canal and central nervous system have been infrequently reported. Wenner et al. (31) indicated an intermediary period in the pathogenesis of poliovirus infection for the cynomolgus monkey characterized by high levels of virus in the blood and widespread distribution of virus in practically all tissues studied.
This study was carried out to determine the distribution of a porcine enterovirus in various organs of the body at different stages of infection, and by correlating reisolation results with the previously described histopathologic changes, to contribute to the understanding of the pathogenesis of porcine polioencephalomyelitis. Terminal blood samples were obtained to determine the serological response to virus inoculations. Serum proteins were studied by paper electrophoresis and immunoelectrophoresis for indications of virus infection.

Materials and Methods

Virus

A porcine enterovirus (03b), previously isolated from the brain of a pig with polioencephalomyelitis (10), was used. Preparation of the virus in swine kidney tissue culture was outlined in detail in Chapter I. Virus for animal inoculations and for use in serum neutralization tests was obtained from a single virus pool.

Virus titration was performed in Leighton tubes prepared as previously described (10). A tenfold dilution series of virus was prepared and 0.1 ml. was inoculated into each of four tubes per dilution. Titers were calculated by the method of Reed and Muench (19) and expressed in 50 per cent end point tissue culture infectious dose (TCID₅₀).
Experimental animals

Germfree pigs\(^1\) were selected to prevent the possible modifying influence of other microorganisms. Three litters of pigs were divided into experimental (20 pigs) and control groups (4 pigs). Pigs were inoculated orally at one or two days of age with 2.0 ml. of tissue culture suspension containing \(10^{4.8}\) TCID\(_{50}\) of O\(\text{3b}\) virus. The four control pigs received 2.0 ml. of virus free tissue culture suspension.

Virus inoculated and control pigs from each litter were maintained in separate flexible film isolators under identical conditions. Pigs were fed a commercially available infant formula\(^2\) to which solid food\(^3\) was added at three days of age.

Germfree status was established by culturing samples from the isolator according to the method of Wagner (28), with the addition of testing for mycoplasma.

Collection of tissues

Pigs were euthanatized at intervals from two to twenty-eight days following inoculation. They were electrocuted and exsanguinated by bleeding from a severed axillary artery. Blood samples were collected at this time and held at 0 C. until the serum was separated. Serum samples were stored at -30 C. until used. Portions

\(^1\)Laboratory Animal Facility, The Ohio State University, Columbus, Ohio.

\(^2\)Varamel, The Baker Laboratories, Inc., Cleveland, Ohio.

\(^3\)P/D, Hill Packing Co., Topeka, Kansas.
of the blood clot were stored at -70 C. for virus reisolation.

Cerebral spinal fluid was aspirated from the cisterna magna following euthanasia and samples were stored at -70 C. for virus reisolation.

Samples of tissues for virus reisolation were collected from sixteen virus inoculated pigs and three control pigs. Samples from the following sites were routinely collected, taking precautions to prevent contamination: olfactory bulb, cerebral cortex, cerebellum, medulla, lumbar spinal cord, turbinate, tonsil, thymus, mandibular salivary gland, heart, lung, diaphragm, adrenal gland, liver, spleen, kidney, mesenteric lymph node, ileum, and spiral colon. All specimens for virus reisolation were stored at -70 C. until used. No more than one month passed until the collected specimens were used for tissue culture inoculation.

Examination for virus

The tissues collected at necropsy were ground in glass tissue grinders following the addition of a small volume of Hanks' balanced salt solution (BSS). Additional Hanks' BSS was added to make an approximate 10 per cent tissue suspension. The suspensions of ileum and spiral colon containing feces were centrifuged for thirty minutes at 2100 g. and the supernatant fluid was used for inoculation. The liquid suspension of ground blood clot and cerebral spinal fluid were used without additional dilution.

Leighton tubes with a confluent monolayer of pig kidney cells were used for virus reisolation. For each sample, two tubes were inoculated with 0.1 ml. and 0.05 ml. of tissue suspension. The cultures were incubated for thirty minutes at 37 C. and medium added.
The maintenance medium was Hanks' BSS containing 2.0% bovine serum, 5.0% sodium bicarbonate solution (1.1%), 100 μg. of streptomycin, 200 units of penicillin, and 50 units of nystatin per milliliter. The cultures were observed daily for cytopathic effect (CPE). The cultures were harvested between the seventh and tenth day following inoculation or at the peak of CPE. All samples were passed two times in swine kidney culture to confirm the presence of the viral agent.

Serum neutralization test

Sera were inactivated at 56 C. for thirty minutes before use. Twofold dilutions of serum were made in Hanks' BSS and mixed with an equal volume of virus suspension diluted to contain 100 TCID50 in 0.1 ml. After mixing and incubating for one hour at 37 C., 0.2 ml. of each mixture was added to each of four tubes of swine kidney tissue culture. After adsorption for thirty minutes at 37 C., 2.0 ml. of the maintenance medium was added to the tubes.

The cultures were observed microscopically each day for CPE and were discarded after one week. Neutralizing titers were based on readings done on the fourth day after inoculation. This was at least twenty-four hours after complete degeneration had occurred in tubes inoculated with an equivalent amount of virus without serum. In most of the tests the neutralizing end point drifted progressively towards lower values. A complete virus titration was included in each test. Tubes inoculated with dilutions

^Mycostatin, E. R. Squibb and Sons, New York, N. Y.
of serum in Hanks' BSS and with Hanks' BSS alone served as additional controls. Serum neutralizing titers were calculated by the method of Reed and Muench (19) and titers were expressed as the serum dilution which protected half of the tubes from CPE of the inoculated virus-serum mixture.

Serum electrophoresis

Serum protein fractions were determined using a Beckman Model R paper electrophoresis system. Samples of 0.01 ml. were applied to 3.0 x 8.0 cm. strips of Whatmann 3MM paper. Separations were of six hours duration at room temperature using veronal buffer at pH 8.6, 0.075 ionic strength. Strips were stained in a solution of 0.1 per cent bromphenol blue dye in methanol and rinsed in three changes of 5.0 per cent glacial acetic acid. The strips were exposed to ammonium hydroxide vapors for at least fifteen minutes. Stained strips were scanned and integrated with a Model RB Analytrol.5

Total serum protein was determined using biuret reagent and spectrophotometry. The amount of each serum protein component was determined by multiplying its relative percentage by the total serum protein value.

Immunoelectrophoresis

The micro-immunoelectrophoresis technique described by Scheidegger (20) was used. The agar bed was prepared by placing

5Beckman Instruments, Inc., Spinco Division, Palo Alto, California.
2.0 ml. of veronal buffered agar on 75 x 25 mm. microscope slides. Serum samples (0.002 ml.) were subjected to electrophoresis for one and one-half to two hours using LKB 6800-A immunoelectrophoresis equipment. The pH of the veronal buffer was 8.6, 0.05 ionic strength. Anti-porcine gamma globulin (0.005 ml.) obtained from a commercial source7 was placed in the center trough following electrophoresis. Slides were placed in a closed, moist chamber for twenty to twenty-four hours to develop the precipitation arcs. Following washing in 0.85 per cent sodium chloride and distilled water, the slides were stained with 0.1 per cent amido black 10B in water, acetic acid, and methanol (45-10-45).

Results

Virus reisolation

Virus reisolation results are summarized in Table 1. Virus was demonstrated in the turbinate area, tonsil, ileum, spiral colon, mesenteric lymph node, and adrenal gland from one pig on the second post-inoculation day (PID). Virus was consistently isolated from the tonsil, ileum, and spiral colon from pigs examined up to the twelfth PID. The blood clot and cerebral spinal fluid contained virus from one pig on the fourth PID. Virus was isolated from the turbinate area, thymus, mandibular salivary gland, lung, heart, liver, spleen, diaphragm, kidney, adrenal gland, and mesenteric lymph node from most of the animals examined from three to seven days following

6LKB - Produkter AB, P. O. Box 12220, Stockholm, Sweden.

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<tr>
<td>J501</td>
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</tr>
<tr>
<td>E2479</td>
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<td>-</td>
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<tr>
<td>J502</td>
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**NE** = not examined
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TABLE 1. (Continued)

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<td>Olfactory Bulb</td>
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<td>Spiral Colon</td>
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<tr>
<td>Blood Clot</td>
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<td>Cerebral Spinal Fluid</td>
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inoculation. Particularly noteworthy were the isolations in all seven animals from the adrenal gland, liver, and diaphragm. Virus was demonstrated in the tonsil, adrenal gland, liver, and kidney on the eighth PID and tonsil, adrenal gland, diaphragm, lung, and mandibular salivary gland on the tenth PID.

Virus was recovered from one or two sites in the central nervous system (CNS) on the fourth, fifth, sixth, and seventh PID. The olfactory bulb, cerebellum, medulla, and lumbar spinal cord contained virus in the two pigs examined eight and ten days following virus inoculation. Virus was demonstrated in the medulla and lumbar spinal cord from one pig on the twelfth PID.

Virus reisolations were confined to the ileum or spiral colon in the five pigs examined from fourteen to twenty-eight days following inoculation. Virus was not recovered from the control pigs.

Serum neutralization

Serum neutralization results are summarized in Table 2. A moderate delay in CPE was noted with serum obtained on the sixth and seventh PID, however, neutralization was not obtained at a dilution of 1:10. A titer of 1:14 was noted on the eighth PID. Progressive elevations of neutralizing activity were recorded with serum from pigs after this time. No neutralizing activity was detected in the serum of control pigs.
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<th>Globulin</th>
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<td>0.38</td>
<td>----</td>
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<td>1.74</td>
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<td>1.07</td>
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<td>0.34</td>
<td>0.52</td>
<td>0.96</td>
<td>0.37</td>
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<td>&lt;1: 10</td>
<td>2.7</td>
<td>0.52</td>
<td>0.37</td>
<td>1.44</td>
<td>0.37</td>
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<td>&lt;1: 14</td>
<td>2.8</td>
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<td>0.43</td>
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<td>3.4</td>
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<td>Number</td>
<td>Days After Inc.</td>
<td>50% Serum Neutralization Titer</td>
<td>Total Protein</td>
<td>Albumin</td>
<td>Alpha&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Alpha&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Beta</td>
<td>Gamma</td>
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<td>--------</td>
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<td>1: 28</td>
<td>3.7</td>
<td>1.00</td>
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Serum electrophoresis and immunoelectrophoresis

Serum electrophoresis results are summarized in Table 2. Total serum protein values showed a progressive elevation with increasing age of the pigs. This was mainly the result of the marked increase in the albumin fraction. Elevation in beta and gamma globulin values were noted in pigs at three and four weeks of age. The absolute amount of alpha_2 globulin remained relatively constant although its percentage of total protein dropped markedly in older pigs. A separate protein component with mobility in the alpha_1 globulin range was not detected in pigs after the tenth PID.

The study of serum proteins by immunoelectrophoresis also revealed changes in protein components in older pigs. Four protein arcs were consistently noted in the alpha and beta globulin area with serum from pigs up to the seventh PID. An identical pattern occurred with serum from controls on the seventh and tenth PID (Fig. 14 a). An additional blurred protein arc in the beta globulin area was noted in a virus inoculated pig on the eighth PID and the control pig examined on the fifteenth PID. This band was separated into two distinct arcs which became more prominent in serum obtained ten to fourteen days following virus inoculation (Fig. 14 b). The heavy inner protein arc was located in the gamma globulin area with serum from pigs at three and four weeks of age. The pattern in virus inoculated pigs and the one control at this age was similar to that occurring with serum from a mature sow (Fig. 15).
Fig. 14.—Immunoelectrophoretic pattern with serum from a control pig (J612, above) and virus inoculated pig (H2486, below) 10 days after inoculation. Note 4 arcs in alpha and beta globulin area with serum from both pigs and the two additional arcs (1, 2) in the beta globulin area with serum from H2486.

Fig. 15.—Immunoelectrophoretic pattern with serum from a pig 21 days after inoculation (J605, above) and a mature sow (below). Note arcs in beta globulin area (1) and heavy arc in gamma globulin area (2) which correspond to numbered arcs in Figure 14 b.
Discussion

The results of virus reisolation studies from germfree pigs infected with a porcine enterovirus (03b) indicated a widespread distribution of virus in the body during the early stages of infection. Virus was consistently demonstrated in the turbinate area, posterior pharyngeal wall, ileum, spiral colon, and mesenteric lymph node for the first week following virus inoculation. Virus persisted in the tonsilar area and lower alimentary canal on the eighth and tenth PID. Virus was only demonstrated in the ileum and spiral colon during later stages of infection. These results are similar to studies with other porcine enteroviruses indicating a relatively transient period of virus localization in the oropharynx and persistence in the lower alimentary canal (2, 3, 11).

In addition to reisolations in the alimentary canal, virus was demonstrated in the majority of other extraneural tissues examined during the period from three to ten days following virus inoculation. Results were particularly consistent through the seventh PID. Virus was demonstrated during this period in the liver, diaphragm, and adrenal gland from all seven pigs, and in the lung and spleen from six animals. Reisolations from the thymus, mandibular salivary gland, heart, and kidney were slightly less frequent. Virus was not demonstrated in any of these extraneural sites after the twelfth PID. Consistent virus reisolations from sites in addition to the alimentary canal and CNS have not been previously demonstrated following oral administration of a porcine enterovirus. Hecke (5) examined seventeen pigs over a period of
one to fifty-eight days following oral administration of Teschen virus. Of ten animals examined three to ten days following virus feeding, reisolations were made from a hepatic lymph node in two animals on the fourth PID and hepatic lymph node, liver, spleen, kidney, and diaphragm from one pig on the fifth PID. Similar studies using oral or other routes of inoculation have either indicated an absence of virus or reisolations from various parenchymatous organs in relatively few animals (1, 3, 32).

Attempts to reisolate virus from the blood were confined to single samples obtained from pigs at the time of necropsy. In view of the few samples examined, it is remarkable that a viremia was detected in one pig on the fourth PID. The single example of virus recovery from the cerebral spinal fluid from this same pig is explained by the viremia, since samples were invariably blood tinged. Previous demonstrations of viremia with other porcine enteroviruses were also confined to a few samples during the early phases of infection (1, 4, 8, 9, 24, 25, 32).

Virus was recovered from one or two of the examined sites in the CNS on the fourth through the seventh PID. The absence of virus in a fraction of a given area during the initial stages of neural infection does not preclude its presence, since the sampling error for tissues containing small amounts of virus may be quite large. Reisolations from the olfactory bulb, cerebellum, medulla, and lumbar cord were obtained eight and ten days after virus administration. This distribution of virus in the CNS was in close agreement with the early microscopic lesions noted at this stage of infection.
The lumbar cord and medulla contained virus on the twelfth PID corresponding to the general areas of predilection for histopathologic changes. Virus was not isolated from the CNS during later stages of infection. These observations on virus recovery from the CNS are similar to those in experimental polioencephalomyelitis of pigs produced by the T 52A, T 80, and S180/4 porcine enteroviruses. Betts and Jennings (2) recovered virus from several sites in the brain and spinal cord six and seven days after oral inoculation. Isolations were confined to one or two samples of CNS tissue in three of eleven pigs examined at later stages. Recovery of S180/4 virus from the CNS by Sibalin and Iannek (23) was limited to pigs examined seven to fourteen days after inoculation. Patočka et al. (17) reported that most of the virus in Teschen disease was concentrated in the CNS at the time of the first clinical signs. The virus content subsequently decreased rapidly and could not be demonstrated after the fifth to seventh day of the paralytic stage.

Serum neutralization titers were determined for each pig from a single serum sample obtained at the time of necropsy. The absence of neutralizing activity in serum from control pigs and pigs during the initial phase of disease indicates that the progressive elevations in titer during later stages represented a specific response to the viral infection. A moderate delay in CPE was first noted with serum obtained on the sixth and seventh PID. Neutralizing activity at levels higher than 1:10 coincided with the reduction in number of virus reisolations from sites other than the alimentary canal. Other serological tests were not employed in this study. Mayr and Wittmann (15) noted neutralizing antibodies
after feeding Teschen virus as early as five to nine days before paralysis, while precipitating antibodies first appeared on the second or third day of illness. They indicated the two types of antibody were not identical and were produced independently.

The ability of the young, colostrum-deprived pig to produce antibodies has been demonstrated with other porcine enteroviruses (22, 23, 32). Beran et al. (1) detected neutralizing antibodies as early as eight days after inoculation of six-to-nine-day-old pigs with the MF strain of virus. Pigs inoculated with F7 virus produced serum neutralizing antibody detected first after seven days with peak titers three weeks after inoculation (11). Investigations using other antigens have indicated differences in the ability of the colostrum-deprived pig to produce detectable antibody. Kim et al. (12) reported measurable neutralizing antibodies forty-eight hours following inoculation with MSP-8 phage in serum from germfree pigs. The primary response was a 19 S immunoglobulin. Antibodies were demonstrated as early as the sixth postnatal day in pigs inoculated with erysipelas antigen on the day of birth (30). Staub and Boguth (26) did not detect antibody formation with Brucella abortus vaccine before the tenth week of life. Segre and Kaeberle (21) demonstrated antibody formation with tetanus toxoid, but not diphtheria toxoid following inoculation at birth. Pigs under eight weeks of age did not produce a measurable response to the four antigens utilized by Hoerlein (7). Differences in the sensitivity of the assay systems may be partially responsible for these variations. Uhr, et al. (27) indicated that the nature of the
antigen may be the most important factor responsible for the differences in the immunizing capacity of diphtheria or tetanus toxoid compared with bacteriophage $\phi X174$ in newborn, premature infants.

Significant differences in serum protein components attributed to virus infection were not detected in this study by paper electrophoresis or immunoelectrophoresis. Using similar methods Wellman and Engel (29, 30) also indicated there were no significant differences between uninoculated pigs and pigs receiving erysipelas antigen on the day of birth. The alpha$_2$ globulin fraction comprised the major portion of the serum proteins in pigs less than two weeks of age. Progressive elevations of the albumin component occurred, while elevations in beta and gamma globulin values were noted at three and four weeks of age. These results in the virus inoculated and control animals are similar to those previously reported for colostrum-deprived pigs (13, 18). A delay in the maturation of the serum protein profile in pigs raised artificially compared with nursing pigs was reported by Lecce and Matrone (13).

A blending of the alpha$_1$ globulin fraction with albumin after the first few days of life was noted by Miller et al. (16). The only protein component detected by Lecce et al. (14) in immature serum that was absent in mature serum had a mobility slightly slower than albumin. This protein arc became undetectable by immunoelectrophoresis between three and four weeks of age. The same investigators described the appearance of additional protein
components from birth to maturity in nursing pigs and colostrum-deprived pigs (14). The appearance of a protein arc in the gamma globulin region (designated arc 18) two to three weeks after birth corresponds to observations in this study.

With regard to pathogenesis, the results of this study indicate that primary virus multiplication occurs in the alimentary canal. Absorption of virus with spread to regional lymph nodes provides a route for entry to the blood stream. Viremia is accompanied by virus reisolations from parenchymatous organs throughout the body. The relative importance of various extraneural sites as secondary centers of virus multiplication was not determined. It is interesting, however, that the adrenal gland was the only organ from which virus was reisolated in every animal two to ten days following inoculation and the only extraneural organ with demonstrated histologic lesions during the same period.

The varied pattern of reisolations during the initial stage of CNS infection did not suggest a specific point of viral entry. The indication by Hecke (5) that primary entry occurs in the medulla with Teschen virus, based on an isolation from this area in one pig at eight days, does not seem justified. The correlation of widely scattered virus reisolations and the distribution of histologic lesions in this study support a hypothesis of viral entry through the blood-brain barrier at many levels of the CNS.
Summary

Sixteen germfree pigs were used to study the distribution of a porcine enterovirus in selected tissues following oral inoculation. Three litter mates served as germfree controls. Virus was consistently reisolated from sites in the alimentary canal during the initial stages of infection. Viremia, detected on the fourth post-inoculation day, was accompanied by virus reisolations from parenchymatous organs throughout the body. The adrenal gland was a particularly consistent site for virus reisolation. The pattern of reisolations from the central nervous system suggested initial virus penetration through the blood vascular system. Virus reisolations were confined to the lower alimentary canal from fourteen to twenty-eight days following inoculation. Progressive elevation of serum neutralizing activity occurred from the sixth post-inoculation day. Differences in serum protein components attributed to virus infection were not demonstrated by paper electrophoresis or immunoelectrophoresis.
REFERENCES

Chapter I


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Chapter II


