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EXPERIMENTAL DISTEMPER IN THE GNOTOBIOTIC DOG

DISSERTATION

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

By

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The Ohio State University

1964

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CHAPTER I

THE GNOTOBIOTIC DOG

Introduction

For the past several years we have been investigating the pathology of viral diseases in the dog. A major problem in this research has been that no two experimental animals were identical. The variations in the microbiological flora of the integumentary, respiratory, and digestive systems have made it difficult to determine which of the organisms were pathogenic and for what portion of a disease process they were responsible.

In order to determine the effects of canine distemper virus on dogs, it became necessary to obtain gnotobiotic dogs as research tools. The objective of this study was to adapt the techniques used for rearing gnotobiotic rodents to dogs.

Review of Literature

There have been no published reports of the successful raising or use of gnotobiotic dogs. Luckey (2) cited Glimstedt as having raised germfree dogs in the 1940's. According to Wagner (3), Phillips raised a group of dogs free of micro-organisms except Toxocara canis for 28 days. The methods were not given.

Materials and Methods

Most of the bitches from which we obtained gnotobiotic dogs were in advanced pregnancy when received from commercial sources. The signs of impending parturition which were most helpful were a change in attitude (restlessness, irritability), the preparation of a nest, swelling of the vulva, a mucoid discharge from the vagina, the onset of milk production, soft or unformed feces, and a drop in the rectal temperature. In some instances all these signs were present for several days prior to parturition and in others whelping began without warning.

At the onset of labor or after the first puppy had been whelped spontaneously, a tranquilizer was administered intravenously and hysterectomy was performed under local anesthesia through the ventral abdominal midline. A puppy presenting in the cervix was expelled into the vagina and the body of the uterus was amputated. The uterus containing the puppies was passed through a trap containing 800 p.p.m. Roccal¹ into a flexible plastic isolator (Figs. 1 and 2). Inside the isolator the puppies were removed from the uterus and stimulated to breathe. More than five minutes were seldom required from the time of hysterectomy until the uterus was opened, and no ill effects were ever attributed to this procedure. Handling of the puppies was facilitated by having two pairs of gloves in each isolator. The bitch was euthanatized and necropsied to determine the presence of spontaneous diseases.

¹Winthrop Laboratories, New York.

Fig. 1.--A six-foot-long flexible film isolator containing a wire cage. The cage is inserted through the "plug" in the end of the isolator at the start of the experiment.

Fig. 2.--An example of a flexible film isolator attached to a stainless steel cage. Note the plastic bag for collection of liquid wastes.

Fig. 1

Fig. 2

The newborn puppies were warmed by application of external heat and the temperature inside the isolator was maintained at 26 to 30 C. for the first week of life. They were not fed until six or eight hours after birth and were then fed four times a day for the first three weeks. Two liquid diets were used; one an experimental diet² formulated to resemble bitch's milk (4) and a commercially available infant formula³ which was chosen because it was advertised and proven in our laboratory to be sterile and because it was lower in carbohydrates than other similar products (Table 1). These liquid diets were administered through four-ounce infant nursing bottles with premature baby nipples. If the

TABLE 1
COMPOSITION OF LIQUID DIETS COMPARED WITH BITCH'S AND COW'S MILK

	Bitch's Milk	Varamel	Exp. Puppy Diet	Cow's Milk
Water	77.8	79.8	80.9	87.0
Protein	7.0	4.9	7.5	3.3
Carbohydrate	3.0	8.3	4.0	4.8
Fat	11.0	5.8	6.1	3.7
Ash	1.2	1.2	1.2	0.7
Cal./gm.	1.28	1.03	1.30	0.68

puppies were smaller than 150 Gm., small plastic puppy nursing bottles were used.⁴ Weak or premature puppies were fed through

²Morris Research Farms, Topeka, Kansas.

³Varamel, The Baker Milk Company, Cleveland, Ohio.

⁴Breeders Equipment Company, Flourtown, Pennsylvania.

a No. 5, French, polyvinyl, premature infant feeding tube.⁵ Up to three weeks of age there was no gag reflex and the tube was readily passed through the mouth into the stomach. The feeding level for the first three weeks was 200 calories per kg. body weight daily. After each meal they were stimulated to urinate and defecate by gentle massage of the anus, perineum, and vulva or prepuce. If housed together they quickly stimulate each other by frequent sucking which often lead to edema and hyperemia of the genitalia. Irritation from sucking occasionally became severe enough to cause dysuria.

Whenever materials were taken in or out of the isolators, at least weekly, tests for sterility were made according to the method of Wagner (5). In addition, examinations were made during and at the end of each experiment for helminth ova by fecal flotation, for mycobacteria and mycoplasma on special media, for rickettsia and psittacosis-lymphogranuloma organisms by inoculation of embryonated eggs, and for viruses by inoculation of canine kidney tissue culture cells and by histopathological examination for lesions. At necropsy, colon, spleen, and lung were routinely tested for sterility.

At about three weeks of age solid food⁶ was gradually added to the diet and the number of feedings was gradually reduced to twice daily.

⁵Pharmaseal Laboratories, Glendale, California.

⁶Hill's prescription diet p/d. Hill Packing Company, Topeka, Kansas.

Results

During the last four years 350 dogs have been raised under gnotobiotic conditions and 143 littermate controls under conventional laboratory conditions for periods up to three months. The puppies raised in a gnotobiotic environment resembled those raised conventionally. They were well developed and playful. The skin was soft and elastic and their hair coat smooth and shiny. The growth curves for both groups were similar, although the conventional littermates sometimes had irregular growth due to the intercurrent diseases. There were no significant differences in growth and development with the two liquid diets.

All the dogs were congenitally infected with the nematode Toxocara canis and were therefore monocontaminated. Larvae could be detected in the tissues of newborn puppies by enzymatic digestion and histopathologic examination. At about three weeks of age, ova were passed in the feces. To prevent reinfection it was necessary to house the dogs in cages with wire floors and to treat them with a sterile vermifuge. Diethylcarbamazine⁷ tablets, ground and suspended in water, were determined to be effective and low in toxicity after autoclaving. The suspension was administered orally at the rate of 50 mg./kg. once weekly for several weeks. Ova were no longer found in the feces if reinfection was prevented.

The results of the first 36 litters raised in a sterile environment are listed in Table 2. The dogs in seven litters remained sterile except for T. canis throughout the experimental

⁷Caricide, American Cyanamid Company, Princeton, N. J.

TABLE 2
RESULTS
THIRTY-SIX CONSECUTIVE LITTERS OF GNOTOBIOTIC DOGS

Terminated at end of experiment	7
Premature	3
Microbic contamination	
Diet	8
Outlet traps	5
Gloves	4
Surgical technique	2
Gaskets	1
Uterine infection	1
Entry	1
Experimental inoculum	1
Unknown	2
Unknown deaths	1

period. All experiments were terminated at three months of age. The puppies from two of the premature litters died singly during the first week of life. A third premature litter was intentionally delivered on the sixty-second day of gestation. None of the puppies survived and it appears that the last day or two of development in utero is necessary for vigorous puppies. The only anomalies found were congenital umbilical hernias with prolapsed intestines in two puppies and unilateral renal agenesis in another.

Most contaminations occurred early in the experimental period while suitable diets were being developed. To conserve the nutritive value, some solid diets, especially those containing eggs, had been minimally heated in the canning process and a few aerobic, gram-negative rods survived. Cultures of aliquots of these canned foods did not always detect the organisms and sometimes several weeks elapsed before they became numerous enough to

be detected in the isolator. This problem was solved by obtaining larger quantities of food from the same manufactured lot which could be adequately tested for sterility before use. A few lots of the liquid experimental puppy diet and several commercially available canned infant formulas which were tested were also contaminated. Another liquid diet tested which had a higher fat content could not be used because it had a shelf-life of less than six months.

Contaminations due to tears in the rubber gloves and entrance of bacteria through the outlet traps were due to faulty equipment design. The only two contaminations related to hysterectomy occurred when a fetus partly in the cervix was squeezed back into the uterus instead of into the vagina. The puppies in the litter which died from an unknown cause were developing well but died suddenly at about two weeks of age. No microorganisms could be isolated from their tissues and no lesions could be found microscopically.

If puppies were accidentally contaminated in the first two weeks of life they sometimes died. If older, no clinical disease resulted except for three dogs exposed at one month of age to a large rod resembling Bacillus subtilis. All three dogs died within three days. Congenital infections which were anticipated but not yet encountered are toxoplasmosis and viral hepatitis. Even though the hookworm, Ancylostoma caninum, is known to pass the placental barrier and some of the bitches had adults in their intestinal tracts, congenital ancylostomiasis was not found.

At necropsy, as in other gnotobiotic species, the lymphoid tissues and lymph nodes associated with the integumentary, respiratory, and gastrointestinal systems were small. The cecum, which varies considerably in size in conventional dogs, was found to be larger in four two-month-old gnotobiotic dogs than in the control dogs. It retained its spiral shape but measured up to 9.0 cm. in length and 2.5 cm. in diameter. The contents were soft. Most gnotobiotic dogs, however, did not have enlarged ceca.

Of the 143 colostrum-deprived, littermate control dogs raised on the same diets, 85 (59 per cent) died from bacterial diseases caused by Escherichia coli, streptococci, or micrococci. Death could be prevented by administration of antibiotics. The mortality rate was higher when the external temperature was maintained above 32 C.

Discussion

The length of time monocontaminated dogs were maintained in a sterile environment was limited by the experimental design and by the limitations of space inside the flexible film isolators available commercially. With the larger isolators and diets now available, however, there should be no problem maintaining gnotobiotic dogs for prolonged periods. The use of these dogs as research tools requires that they have adequate space for skeletal and muscular development, be accessible for frequent physical examinations, and be kept clean to prevent recontamination with ascarid ova.

The major problem in obtaining germfree dogs was congenital ascariasis. Although the parasites with which they were born could be removed by treatment with vermifuges, the effects of the parasites or chemicals could interfere with an experimental procedure. The ultimate solution is the development of an ascarid-free breeding colony as a source of puppies. The development of such a colony is necessarily lengthy since proving the absence of ascarids requires raising bitches to maturity and the subsequent whelping of uninfected puppies.

The high mortality (59 per cent) of the littermate dogs raised in a conventional environment was about twice that expected (1) and could be explained partly by the lack of colostrum and by the high carbohydrate content of the liquid diets. The absence of disease in the monocontaminated littermates on the same diets emphasized the role of microorganisms in causing these deaths. Similar bacterial diseases in puppies have previously been attributed by veterinarians to metritis or mastitis in the bitch. This belief is untenable in view of the data reported here.

Summary

During the last four years we have raised 350 dogs under gnotobiotic conditions and 143 littermates under conventional laboratory conditions for use as research tools. These dogs have been used principally in short-term experiments to determine the effects of viruses on gnotobiotic animals.

In adapting the techniques which had been developed successfully for raising gnotobiotic rodents to dogs, two problems have been encountered. All of the dogs have been congenitally infected with the nematode Toxocara canis and were therefore monocontaminated. Secondly, the isolator design had to be modified to permit the introduction or attachment of cages large enough for dogs.

CHAPTER II

CONGENITAL ASCARIASIS IN GNOTOBIOTIC DOGS

Introduction

It has been known for more than 40 years that Toxocara canis is capable of penetrating the placenta of a pregnant bitch and causing prenatal infection in the pups (2). The frequency with which this occurs, however, has not been determined. Since visceral larva migrans in man, which is most often attributed to T. canis, is being diagnosed with increasing frequency, it is important that veterinary practitioners be aware of the ways in which pups might serve as a source of contamination for children. The recent development of techniques for raising dogs in a gnotobiotic (germfree) environment (3) has afforded us a unique opportunity to study congenital transmission of T. canis and other parasites in the dog.

Methods and Materials

Ninety-five of 224 pups from 34 bitches were studied, including at least one from each of the 34 litters. Of these, 65 were raised in a gnotobiotic environment and 30 littermate, colostrum-deprived controls were raised in a conventional laboratory environment but on the same sterile diet and isolated completely from exposure to nematode ova. The method for raising

gnotobiotic dogs has been described (3). Hysterectomy was performed at term and the gravid uterus delivered into a previously sterilized, flexible plastic isolator. The air introduced into the isolator was sterilized by filtration and all the supplies including cages, bedding, and feed sterilized. The pups were removed from the uterus within the sterile environment and raised by hand.

Of the 34 bitches, three were Beagle dogs raised in our laboratory. The remainder, of various breeds and ages, were obtained in advanced pregnancy from animal dealers in Ohio and nearby states. Following hysterectomy, 30 bitches were euthanatized and necropsied to determine the presence of intestinal parasitism.

The methods used for detecting parasites in the pups were fecal flotation, gross necropsy examination, histopathologic examination, and digestion of lung, liver, kidney, heart, spleen, mesenteric lymph node, diaphragm, and psoas muscle with pepsin or trypsin. For tissue digestion with pepsin, 50 ml. of a freshly prepared 0.5 per cent pepsin solution adjusted to pH 1 with hydrochloric acid was added to 2-gram samples of finely cut tissue and incubated for 10 to 18 hours at 37 C. Digestion was stopped by the addition of an equal volume of 10 per cent formalin. The entire sediment was then examined microscopically for larvae. For tissue digestion with trypsin, 50 ml. of 1 per cent trypsin in 0.85 per cent saline solution adjusted to pH 8 with sodium bicarbonate was added to 2-gram samples of finely cut tissues and

incubated for 4 hours at 37 C. The minced tissues were placed in a wire-screen receptacle and suspended in the fluid so that the live larvae gravitated to the bottom of the test tube and were prevented from re-entering undigested tissue. Two-gram samples of brain were minced in 0.85 per cent saline solution and incubated for 4 to 18 hours at 37 C. In addition to digestion, replicate lung samples were also teased in saline solution under a dissecting microscope. Larvae were identified according to published criteria (6-8, 11-14, 16).

To determine the location of nematode larvae in the tissues of adult dogs, 2-gram samples of psoas muscle, kidney, liver, lung, heart, spleen, brain, uterus, mammary gland, and mesenteric lymph node were digested. The samples were collected from 17 bitches and 15 male dogs over one year of age presented for necropsy to the Department of Veterinary Pathology. Replicate samples were fixed in 10 per cent isotonic neutralized formalin, embedded in paraffin, sectioned at six μ , stained with hematoxylin and eosin, and examined microscopically.

Results

Congenitally acquired T. canis larvae were demonstrated in all 95 pups. No other parasites were found. In the first few days of life, third-stage larvae were found in the lungs (Fig. 3) and liver by histopathologic or digestion techniques. Fourth-stage larvae were already present in the intestinal contents of one pup on the first day of life. After the first week of life larvae were no longer found in the viscera but fourth-stage larvae and

Fig. 3.--Photomicrograph. Transverse section of a third-stage Toxocara canis larva in a bronchiole of a two-day-old conventionally raised dog. There is no inflammatory response. H & E stain; X 500.

Fig. 3

young adults were found in the intestinal washings. Ova were first demonstrated in the feces at 31 to 50 days of age. At necropsy, from one to 50 adult T. canis were found in the small intestine of various pups. Differences between the gnotobiotic pups and their conventionally raised littermates were not observed in the numbers or rate of development of ascarids.

The species of parasites found at necropsy in 30 bitches are listed (Table 3). Only five bitches had adult T. canis in

TABLE 3
INTESTINAL PARASITISM IN 30 BITCHES WHICH WHELPED
PUPS INFECTED WITH TOXOCARA CANIS

Species	No. of Bitches
<u>Dipylidium caninum</u>	13
<u>Trichuris vulpis</u>	10
<u>Toxocara canis</u>	5
<u>Ancylostoma caninum</u>	5
None	7

their intestinal tracts. In seven bitches intestinal parasites were not found although all whelped infected pups. Second-stage larva of T. canis were demonstrated in nine of 17 bitches and eight of 15 adult males by tissue digestion and histopathologic techniques (Table 4). Most larvae were found in the kidneys and skeletal muscle (Table 5). Larvae were not found in liver, spleen, mesenteric lymph node, uterus, or mammary glands. Dogs of all ages were infected; two were 12 years old.

TABLE 4

NEMATODE LARVAE DEMONSTRATED IN THE TISSUES OF 32 ADULT DOGS
BY DIGESTION AND HISTOPATHOLOGIC TECHNIQUES

Species	No. of Dogs
<u>Toxocara canis</u>	15
<u>T. canis</u> & <u>Ancylostoma caninum</u>	2
<u>A. caninum</u>	1
<u>Dirofilaria immitis</u>	1
None	13

TABLE 5

LOCATION OF TOXOCARA CANIS LARVAE IN 17 INFECTED ADULT DOGS

Location*	No. of Dogs
Kidneys	12
Psoas muscle	9
Lungs	1
Brain	1
Heart	1

* None were found in liver, spleen, mesenteric lymph node, uterus, or mammary gland.

Discussion

The finding of 100 per cent congenital infection with T. canis in 34 litters of pups suggests that most, if not all, dogs were congenitally infected. In one study in Russia (9), larvae were found in nine of 11 stillborn puppies and in 26 of 28 puppies up to six days of age. Absence of intestinal infection in the bitch does not mean that the puppies will be uninfected, since only five of 30 bitches whelping infected litters had

intestinal infections. The 53 per cent incidence of tissue infection with second-stage larvae in 32 adult dogs would probably have approached 100 per cent if the entire dog had been examined after digestion. It is interesting that even though hookworms have been demonstrated to pass the placental barrier and five of the bitches had intestinal infections with Ancylostoma caninum no congenital ancylostomiasis was found in the 95 puppies. In a previous report (10) five of six gnotobiotic dogs examined had larvae of T. canis and in addition one newborn pup harbored larvae of A. caninum.

Every puppy had viable T. canis ova in its feces before weaning age. It was not feasible to perform fecal flotations at daily intervals on the gnotobiotic dogs and it is likely that in some dogs ova were present for a few days before they were detected. Two authors (4, 17) have reported T. canis ova in feces of puppies as early as 21 days of age. It is apparent that to prevent reinfection of the dog or possible infection of man, treatment of pups for parasitism must be performed at frequent intervals starting before the infection becomes patent at three weeks of age.

Infection of children with larvae of T. canis results in a chronic but self-limiting disease called visceral larva migrans (1, 5). Fever, hepatomegaly, eosinophilia, hyperglobulinemia, and transient respiratory signs are the most common findings. The migrating larvae may invade the eye and produce endophthalmitis which clinically resembles retinoblastoma and has resulted in

enucleation. Although only 58 cases have been reported in the last 12 years (5), the increasing frequency with which visceral larva migrans is being diagnosed clinically has led some investigators to suggest that dogs and cats not be kept as house pets or their activity restricted to an enclosed area to which children do not have access.

The migration of larvae has little effect on newborn gnotobiotic or conventionally raised pups. Inflammatory response was not observed in the liver or lungs and in a few days all the larvae had migrated to the gut. It is possible, however, that migrating larvae play a role in neonatal bacterial pneumonia in conventionally raised dogs.

Much of the experimental data in the literature based on the administration of ova to dogs must now be re-evaluated, since the experimental dogs may have been infected before the experiment started. The hormonal factors important in increased migration of larvae in the pregnant bitch are still unknown (15).

Summary and Conclusions

Congenital infection with Toxocara canis was demonstrated in all 95 pups selected for study from 34 litters of gnotobiotic dogs. Diagnostic techniques employed were fecal flotation, gross necropsy, histopathologic examination, and tissue digestion. Other parasites were not found. Only five of 30 bitches had adult

ascarids in their intestinal tracts although all whelped infected litters. To prevent reinfection of dogs or infection of children, it is necessary to treat pups for ascariasis before the infection becomes patent at three weeks of age.

CHAPTER III

EXPERIMENTAL DISTEMPER IN THE GNOTOBIOTIC DOG

Introduction

Considerable importance has been attributed to secondary bacterial complications in canine distemper. The recent development of the gnotobiotic dog (14) with its standardized environment has made it possible to study the effects of distemper virus in the absence of other microorganisms. A review of the literature revealed few complete histopathological descriptions of either natural (8, 19, 30) or experimental (26) distemper, and no description of the pathogenesis of the disease.

Review of Literature

Hundreds of articles have been written on canine distemper. Bindrich (2) made an extensive review of the literature in 1954. Gorham's excellent monograph (13) published in 1960 covered most of the pertinent articles. Innes and Saunders (18) have reviewed the literature on lesions in the nervous system.

Canine distemper has been known since the early nineteenth century as a disease of variable severity with protean manifestations in which a fever and all or only a few of the respiratory, intestinal, exanthematous, and nervous manifestations may be present (18). Even after the reports of Carré (3) and Lignieres

(20), who found distemper to be caused by a filterable virus, many workers continued to credit the bacterium Brucella bronchi-septica as the etiological agent (10, 25, 32). It was not until 1926 that Dunkin and Laidlaw (9) first portrayed the disease in its present concept. Realizing the extreme contagiousness and great variability of the clinical disease, they used strict isolation procedures in their experimental studies. Under these conditions they produced a disease characterized by biphasic fever with coryza, severe gastrointestinal signs, variable signs due to inflammation in the respiratory system, and nervous signs in 10 per cent of the cases. The disease was rarely fatal, but was serious enough to predispose to various secondary infections.

The great variation in clinical manifestations reported in experimental canine distemper has ranged from severe pneumonia, convulsions, and death (5, 11, 17) to mild and even undetectable disease (9). Some variation was undoubtedly due to inability to determine susceptibility of the experimental animal prior to inoculation and also to differences in the routes of inoculation and viral strains. Other important causes of variability have been the difference in intercurrent diseases and microbial flora in experimental dogs. In 1905 Carré (13) suggested that skin pustules in canine distemper were not a primary result of viral infection. Dunkin and Laidlaw (9) found no skin pustules in their experimental studies, and purulent conjunctivitis, rhinitis, and pneumonia were rare. In 1952 Hsiung and Stafseth (16) reviewed the earlier work and also contributed their extensive bacteriological study in both infected and uninfected dogs. There was

little difference between the two groups except for Brucella bronchiseptica which was only isolated from distemper infected dogs, where it was present 28.5 per cent of the time. Lauder et al. (19) and McIntyre et al. (23) found that Brucella bronchiseptica was not a common pathogen despite its time honored reputation as a secondary invader in distemper.

In spite of the extensive literature on canine distemper few complete histological studies of the natural disease are available. In 1937 DeMonbreun (8) described the lesions in 12 distemper infected dogs. Scheitlin et al. (31) in 1951 reported their findings on 15 naturally infected dogs, and Lauder et al. (19) described the changes in 50 dogs. Studies of experimental distemper have been numerous, but most have been related to the clinical, immunological, or etiological aspects of the disease. Potel's description (27) of 80 experimentally infected dogs is the only complete histological study found, but he failed to give an adequate description of the pathogenesis.

Materials and Methods

The methods of raising gnotobiotic dogs have been described (14). Pregnant bitches were obtained from commercial sources. At the onset of labor puppies were removed from the bitch by hysterectomy and passed through a disinfectant trap into a flexible plastic isolator. They were fed a commercial infant milk formula¹

¹Varamel, The Baker Milk Company, Cleveland, Ohio.

for the first three weeks at which time solid food² was gradually added to the diet. Confirmation of the germfree state was accomplished by culturing samples of feed, fresh feces, and body wastes from each isolator. Samples were collected each time an entry was made, which was usually weekly. Cultures were made on tryptose agar plus 5 per cent defibrinated equine blood, and in thioglycolate broth. All cultures were incubated at room temperature, 37 C. and 57 C. in both aerobic and anaerobic environments. At necropsy the colon, lung, and spleen of each dog were cultured in the previously described manner as well as for mycobacteria on Lowenstein's-Jensen Medium³ and for mycoplasma on P.P.L.O. agar⁴ plus 20 per cent equine serum. Microscopic examination of tissues was used to determine the absence of viruses known to infect dogs.

Previous studies in this laboratory have shown that most, if not all, newborn puppies are congenitally infected with Toxocara canis, and that treatment with diethylcarbamazine⁵ will eliminate the infection (15). For this reason daily therapeutic doses (25 mg./kg.) were given for several days prior to the experimental studies.

The schedule of experimental inoculations is listed in Table 6. Twenty six-to-nine-week-old puppies from six litters

²Hill's prescription diet p/d. Hill Packing Company, Topeka, Kansas.

³Baltimore Biological Laboratories, Baltimore, Maryland.

⁴Difco Laboratories, Detroit, Michigan.

⁵Caracide, American Cyanamid Company, Princeton, N. J.

were used. Nine gnotobiotic dogs were inoculated intraperitoneally with 1 ml. of a 10 per cent brain suspension of Snyder-Hill distemper virus⁶ in balanced salt solution. Three other gnotobiotic dogs were inoculated in a similar manner with 1 ml. of a 4 per cent ferret spleen suspension of virulent Lederle distemper virus.⁷ Eight dogs including at least one from each litter were placed in separate isolators and kept as controls. Five of these were inoculated intraperitoneally with a sterile brain suspension.

The Snyder-Hill virus had been passed three times by intracerebral inoculation in conventional dogs before use in this experiment. The Lederle virus had been maintained by alternate dog, ferret passage. The virulence of each virus was demonstrated by intravenous or intraperitoneal inoculation of three conventional dogs which had been raised in isolation. All six dogs developed clinical signs of distemper including fever, watery ocular discharge, and loose stools. In addition, the three dogs inoculated with Snyder-Hill virus developed small red papules approximately 3 to 5 mm. in diameter on the skin of the abdomen and flank. One of three conventional dogs inoculated with the Lederle virus developed nervous signs after 11 days, consisting of whining, forced movement, and attempts to climb the sides of the cage. The nervous signs diminished after two days and the dog was normal at the time of necropsy 15 days after inoculation. Microscopically

⁶Obtained from Dr. James Newbern, Pitman-Moore Company, Indianapolis, Indiana.

⁷American Type Culture Collection, Washington, D. C.

the brain of this dog contained lesions identical with those in the gnotobiotic dogs.

Recorded during the baseline period and throughout the course of the experiment were the results of a daily physical examination, twice daily rectal temperatures, appetite, bowel movement, general appearance, and the appearance of all body openings. A neurological examination conducted daily on each dog included the following reflexes: flexor, patellar, extensor thrust, tonic neck, extensor postural thrust, righting, head down suspension, and placing reactions. Three pups in one litter were examined ophthalmoscopically prior to necropsy. The control dog from this litter and the pup necropsied eight days after inoculation were also subjected to electroencephalographic examination.⁸

The following determinations were made on blood and serum collected prior to necropsy: sedimentation rate by the Wintrobe method, hemoglobin by the oxyhemoglobin method, erythrocyte packed cell volume by the micro-hematocrit method, total erythrocyte and leucocyte counts, differential leucocyte counts, total serum protein using biuret reagent and spectrophotometry, serum electrophoretic pattern using a Beckman-Spinco Model R paper electrophoresis system with 0.1 M veronal buffer at pH 8.6, and distemper antibody titer by neutralization in embryonating eggs.

Euthanasia was performed by electrocution and exsanguination. Animals were necropsied at approximately four-day intervals in order to study the pathogenesis of the disease. The

⁸ Performed by Dr. R. Redding, Department of Veterinary Physiology, The Ohio State University.

following tissues were fixed in 5 per cent phosphate-buffered formalin, imbedded in paraffin, and sectioned at 6 microns: skin of prepuce or vulva, metacarpal foot pad, upper eyelid, membrana nictitans, tongue, tonsil, mandibular lymph node, mandibular salivary gland, esophagus, stomach, duodenum, jejunum, terminal ileum, cecum, colon, ano-rectal junction, anal sac, cecal node, pancreas, liver, gallbladder, planum nasale, turbinate, trachea, bronchial lymph node, right and left apical and diaphragmatic lobes of the lung, kidney, urinary bladder, testis or ovary, prostate or uterus, thyroid, parathyroid, thymus, adrenal, pituitary, rib at costochondral junction, sternal marrow, psoas muscle, diaphragm, myocardium, abdominal aorta, and external and inner ear. The eyes and attached optic nerves were fixed in Zenker's acetic fluid for one hour, diluted half with distilled water and fixed for two more hours, washed overnight in tap water, placed in 70 per cent ethyl alcohol for 24 hours and then embedded and processed in the usual manner. The brain and spinal cord were fixed in Cajal's brom formol solution and processed in a similar manner. Entire transverse sections of both cerebral hemispheres were examined at the level of the anterior sigmoid gyrus, basal ganglia, thalamus and optic chiasma, mesencephalon and hippocampus. Entire sections were taken through the pons and cerebellum, medulla at obex, cervical cord and dorsal root ganglia, thoracic cord, and lumbar cord at the enlargement. Other tissues examined were, posterior cervical sympathetic ganglia, ciliary ganglia, spiral ganglia, and vestibular ganglia.

In addition to hematoxylin and eosin the following stains and histochemical reactions were used: Wilders reticulum stain, Shorr's triple stain, von Kossa's stain, Gram's stain, periodic acid-Schiff reaction, and Hortega's silver carbonate method.

Eight dogs were perfused to insure prompt fixation of brain tissue. Following electrocution, a 14-gauge needle was placed in the left ventricle and 1000 ml. of isotonic saline was perfused at a pressure of 90 cm. of saline. The right ventricle was opened to allow for outflow. Following this 4000 ml. of Cajal's brom formol solution was perfused. The necropsy was then continued in the usual manner.

Results

The only consistently observed clinical sign of distemper in the gnotobiotic dogs was fever. The first temperature rise occurred on the third day after inoculation. All but one of the 12 experimentally inoculated pups had a one to two degree F. rise above their highest baseline recording. The temperature remained elevated for 24 to 48 hours and then returned to normal. In three dogs the temperature remained elevated or fluctuated between normal and abnormal levels for several days. The Lederle virus produced slightly higher and more persistent fevers. Rectal temperatures in the controls were at all times within a range of 100 to 101.9 F.

In all but three dogs a slight decrease in appetite was observed for a few days following the initial temperature rise.

At this time the pups drank milk but ate very little solid food. This decreased appetite continued intermittently in three dogs. Anorexia was not observed.

Slight depression was the only nervous sign and was noted in only one dog. All nervous reflexes were normal during the entire experiment. The electroencephalographic recording conducted on one dog eight days after inoculation appeared identical to its littermate control. Ophthalmoscopic examination revealed no lesions. No coughing, papular or vesicular skin lesions, hyperkeratosis, nasal or ocular discharge, or diarrhea were observed.

The leucocyte determinations are recorded in Table 6. Leucopenia was present four days after inoculation but had returned to normal by the twelfth day. The leucopenia was due to an absolute lymphopenia and an absolute neutropenia. The sedimentation rate was increased during the first 12 days from a mean of 0.5 mm./hr. in the controls to a mean of 4 mm./hr. in the inoculated group.

Mean erythrocyte and hemoglobin values are recorded in Table 7. A mild normochromic, normocytic anemia was present during the first 12 days of the infection. Mean serum protein values are recorded in Table 8. There was no significant difference between the electrophoretic patterns of inoculated and control animals, except for one dog which had a prominent α_2 peak. The gamma globulin level in gnotobiotic dogs was low.

TABLE 6
SCHEDULE OF INOCULATIONS AND RESULTS

Number	Inoc.	Days After Inoc.	Distemper Titer	Thymus Per Cent Body Weight	WBC Per Cmm.	Neutrophils Per Cmm.	Lymphocytes Per Cmm.
Controls (8) ³	-	-	0	0.47 ± 0.09	11,421 ± 3,452	5,829 ± 2,142	5,183 ± 1,090
G716	SH ¹	4	0	.43	3,850	1,887	1,964
G1523	L ²	4	0	.46	3,400	2,856	476
G878	SH	5	0	.38	3,250	1,333	1,788
G1284	SH	7	-	.38	7,750	5,038	2,635
G741	SH	8	32	.16	-	-	-
G1556	L	8	32	.35	3,500	1,925	1,190
G1571	L	12	32	.12	9,050	3,620	4,435
G762	SH	12	32	.19	8,750	3,150	5,163
G1303	SH	12	32	.11	9,200	4,692	4,324
G909	SH	13	316	.09	-	-	-
G793	SH	17	316	.11	5,350	3,638	1,659
G925	SH	19	147	.08	-	-	-

¹SH = Snyder Hill.

²Lederlee.

³Mean and standard deviation.

TABLE 7
MEAN ERYTHROCYTE AND HEMOGLOBIN VALUES*

	R.B.C. Million Per Cu. Mm.	P.C.V. Per Cent	Hb Gm. Per 100 ML.	M.C.V. Cu. μ	M.C.H.C. Per Cent
Control (7)	4.46 $\pm$ 0.27	33.90 $\pm$ 4.85	10.93 $\pm$ 1.68	76.86 $\pm$ 9.57	32.27 $\pm$ 1.50
Inoc. (8)	3.51 $\pm$ 0.29	26.75 $\pm$ 3.38	8.89 $\pm$ 1.38	76.18 $\pm$ 6.97	33.19 $\pm$ 2.02

*Values obtained during first 12 days of infection.

TABLE 8
MEAN SERUM PROTEIN VALUES (GM./100 ML.)

	Total Protein	Albumin	Globulin	Gamma Globulin
Control (8)	5.15 $\pm$ 0.57	2.63 $\pm$ 0.59	2.52 $\pm$ 0.31	0.12 $\pm$ 0.04
Inoc. (11)	5.06 $\pm$ 0.77	2.49 $\pm$ 0.42	2.57 $\pm$ 0.62	0.14 $\pm$ 0.06

The serum antibody titers against distemper are recorded in Table 6. Titers were first detected eight days after inoculation, and continued to rise during the remainder of the experiment.

The only consistent macroscopic change in inoculated animals was a striking reduction in the size of the thymus which was readily apparent in all animals necropsied eight or more days after inoculation (Fig. 4). An interlobular gelatinous infiltrate was also present in two dogs. The percentages of body weight attributable to the thymus are recorded in Table 6. A progressive reduction in the size of the thymus occurred as the disease progressed. The only other gross finding was the presence of one Toxocara canis adult in the small intestine of one dog and six in another. One control dog also had two T. canis adults in its intestine.

Microscopic examination revealed consistent lesions in the lymphatic and central nervous systems. The lesions caused by both viruses were similar and will be described together.

The first microscopic changes attributable to distemper infection were noted in the thymus five days after inoculation. Numerous reticular cells in the cortex contained phagocytosed nuclear debris. The medulla appeared slightly larger than normal due to a depletion of lymphocytes at the corticomedullary junction. A few degenerating lymphocytes with pyknotic nuclei were present in this area as well as in the medulla. Many of the Hassall's corpuscles appeared to be undergoing degeneration. The cytoplasm

Fig. 4.--Thymic atrophy (G1571, right) 12 days after inoculation compared to normal thymus (G1577, left).

Fig. 4

of the cells composing these corpuscles was very eosinophilic and contained many prominent basophilic granules. The reticular cells in the medulla were prominent because of the loss of lymphocytes. Eight days after inoculation the reaction was more severe with some lobules more affected than others. There was considerable space between the lobules which was filled with loose, poorly-stained connective tissue, edema fluid, and a few scattered lymphocytes. At the periphery of the lobules, reticular cells were becoming numerous and were closely packed beneath the capsule. By 12 days post-inoculation lymphoid depletion was nearly complete (Fig. 6). The lobules were greatly reduced in size and almost devoid of small lymphocytes, although occasionally small foci remained. Numerous lymphocytes could be seen within the lymphatics in two dogs. Occasionally these cells showed degenerative nuclear changes. The interlobular connective tissue had a loose arrangement. In one dog the spaces between the lobules were especially prominent and were filled with widely separated fibrillar connective tissue. In another dog many of the degenerated Hassall's corpuscles had become mineralized. By 17 and 19 days post-inoculation regeneration had occurred, but the division of lobules into cortex and medulla was not yet apparent.

The lymphoid tissue of the gnotobiotic dog appeared similar to that of other germfree animals (22). Secondary reactive centers were occasionally seen in the mandibular and cecal lymph nodes, tonsils, and Peyer's patches. They were not seen in other lymphoid organs. Plasma cells were extremely rare.

Fig. 5.--Photomicrograph. Normal thymus from uninoculated gnotobiotic dog (G1577). H & E stain; X 25.

Fig. 6.--Photomicrograph. Severe lymphoid depletion in thymus 12 days after inoculation (G909). Compare with Figure 5 taken at same magnification. H & E stain; X 25.

Fig. 5

Fig. 6

The first detectable change in the tonsil was early lymphoid depletion which was present seven days after inoculation. This was recognized most easily at the center of the primary follicles where only reticular cells remained. The periphery of the follicle was still composed of closely packed adult lymphocytes although they were decreased in numbers. The rest of the parenchyma also appeared partially depleted as the reticular cells and reticular framework were slightly more prominent. Necrotic lymphocytes and nuclear debris were scarce. An occasional homogeneous eosinophilic inclusion body approximately one to two microns in diameter could be seen in the cytoplasm of reticular cells in one dog. Twelve days after inoculation the tonsils were greatly reduced in size, follicles were scarce, and the parenchyma was composed mainly of reticular fibers and some remaining lymphocytes and reticular cells. The reticular cells were more prominent partly because of the loss of lymphocytes and partly because of hypertrophy and hyperplasia. Their cytoplasm was abundant and quite eosinophilic. They were present in the surrounding tissue as well as in the center of follicles. Reticular cell hyperplasia was so prominent in one dog that it gave the impression of a granulomatous reaction (Fig. 7). No fungi were demonstrated by the periodic acid-Schiff reaction. In this one dog many multinucleated giant cells were seen in the tissue surrounding the follicles. One giant cell contained several inclusion bodies within its cytoplasm (Fig. 8), and an occasional inclusion was noted in the cytoplasm of other reticular cells. In spite of the reticular

Fig. 7.--Photomicrograph. Lymphoid depletion and reticular cell hyperplasia in tonsil 13 days after inoculation (G909). Note giant cell (arrow). H & E stain; X 125.

Fig. 8.--Photomicrograph. Higher magnification of giant cell in Figure 7. Inclusion bodies (arrow) are present in the cytoplasm. H & E stain; X 1250.

Fig. 7

Fig. 8

cell hyperplasia, the tonsils remained quite small. By 17 and 19 days the tonsils were undergoing lymphoid replacement, primary follicles were again becoming prominent and the parenchyma contained increased numbers of lymphocytes.

Microscopically the spleens of inoculated dogs had changes similar to those in the tonsils. They were first detected seven days after inoculation. At this time a few lymphocytes were missing from the center of follicles and a slight amount of nuclear debris was present. The disappearance of lymphocytes from the red pulp was obscured by the extensive number of erythropoietic cells and megakaryocytes which were also present in the control dogs. One dog had a pronounced lymphoid depletion. In this dog the centers of the splenic follicles also contained considerable eosinophilic material resembling paramyloid. This was considered to be due to proteinic deposition in the area since the reticular framework appeared normal when stained by Wilder's method. In this dog the cytoplasm of an occasional reticular cell contained an inclusion body. The reaction noted at 12 days was quite similar. All dogs had a moderate loss of lymphocytes from the follicles, and one had very severe depletion. The centers of the follicles in this animal contained many hypertrophic reticular cells, and occasional multinucleated cells. Inclusion bodies were rare. Dogs necropsied 17 and 19 days after inoculation had slightly larger and more numerous lymphoid follicles. These follicles measured between 700 and 900 microns in diameter while those of the control dogs measured approximately

500 microns. They were packed with medium and large lymphocytes. Blast cells were rare but mitotic figures were numerous. Congestion of the follicles was not an important feature of the disease.

The viral effect noted in the mandibular, bronchial, and cecal lymph nodes was similar but less striking than in the thymus and tonsil. Changes were first detectable seven days after inoculation. The centers of some of the follicles were undergoing lymphoid depletion. In the rest of the cortex the lymphocyte loss was more uniform and less noticeable. Within the sinusoids an occasional multinucleated cell could be seen. Structures suggestive of inclusion bodies were occasionally seen in the cytoplasm of reticular cells in one dog. By 12 days the changes were more prominent. In one dog the follicular centers contained a few blast cells suggesting lymphoid hyperplasia. By 17 days the nodes were nearly regenerated.

In the Peyer's patches little change was seen at four days although some nuclear debris and occasional phagocytic reticular cells were noted in two animals. By eight days post-inoculation a considerable reduction in lymphocytes had occurred. Small lymphocytes were greatly reduced in number and the follicles were a conglomeration of reticular cells, medium sized lymphocytes, and a few small lymphocytes. The reticulum appeared prominent because of the cellular depletion. Structures suggestive of viral inclusions were occasionally seen in the cytoplasm of reticular cells, and were numerous and prominent in one dog. Occasional

reticular cells contained phagocytized nuclear debris. By 12 days the lymphoid tissue in the terminal ileum was reduced to less than half its normal size. The follicles were primarily composed of collapsed reticular stroma. Only a small portion of the pre-existing lymphocytes remained. Hypertrophic reticular cells were frequent in one dog, and indistinct inclusions were seen in all dogs. By 17 and 19 days the follicles were beginning to assume a more normal structure. Many lymphoblasts and large lymphocytes were present although the lymphoid tissue had not yet regained its pre-inoculation size.

Microscopic lesions were demonstrated in the brains of all dogs necropsied eight or more days after inoculation. Dogs necropsied at four, five, and seven days had no lesions. These early lesions consisted of swelling of endothelial and perithelial cells and a moderate infiltration of vessel walls by lymphocytes and monocytes. Such changes were frequent in the small vessels of the cortical gray matter, particularly in the frontal lobes (Fig. 9). They were occasionally noted in both the white and gray matter of the cerebrum, cerebellum, and brain stem. Larger vessels of the parenchyma were less frequently involved while the vessels of the arachnoidea were unaffected. These intramural changes were occasionally seen in the pial vessels on the brain surface and were occasionally severe in the depths of sulci in the frontal cortex (Fig. 10). A similar reaction was occasionally noted in the vessels between the cerebellar folia. On rare occasions small microglial foci could be seen in the tissue near

Fig. 9.--Photomicrograph. Intramural lymphocytic infiltration of small vessels in the cortical gray matter eight days after inoculation (G741). H & E stain; X210.

Fig. 10.--Photomicrograph. Intramural lymphocytic infiltration in pial vessel deep in a sulcus of the frontal cortex (G741). H & E stain; X 210.

Fig. 9

Fig. 10

affected vessels. In the dog inoculated with Lederle virus several vessels within the cerebellar peduncles were affected. This dog also had a few microglial foci in the molecular layer of the cerebellum and surrounding neurons in the dentate nucleus, brain stem, and cerebral cortex.

All dogs studied 12 days after inoculation had mild vessel changes, but they were reduced in extent and severity from that observed at eight days. Most striking at this stage were the focal accumulations of pleomorphic microglia. The microglial foci were usually composed of closely packed cells, but occasionally the cells were loosely arranged. These foci were occasionally seen as single nodules in the molecular layer of the cerebrum and cerebellum and in the white matter. Most frequently, however, they were found in the gray matter of the cerebral cortex and were most numerous in the frontal lobes. The foci usually surrounded neurons in the deeper layer of the cortex (Fig. 11). In many instances the pleomorphic microglia assumed a whorled arrangement around a neuron. At other times they invaded the neuron in the form of neuronophagia. Many of the neurons appeared normal, but others were undergoing degeneration. The most frequent degenerated form was a shrunken, round neuron with densely eosinophilic cytoplasm which obscured nuclear detail (Fig. 12). Occasionally a neuron had swollen vacuolated cytoplasm (Fig. 13). These microglial foci were usually single, but occasionally occurred in small groups. They were never numerous except in one dog inoculated with Snyder Hill virus. In

Fig. 11.--Photomicrograph. Zone of microglial reaction in deep layers of the cortical gray matter 12 days after inoculation (G762). A, molecular layer; B, nerve cell layer; C, white matter. H & E stain; X 60.

Fig. 11

Fig. 12.--Photomicrograph. Two shrunken, degenerated neurons surrounded by microglial cells (G762). H & E stain; X 500.

Fig. 13.--Photomicrograph. A degenerating neuron with swollen vacuolated cytoplasm surrounded by microglial cells. An inclusion (arrow) is present within the nucleus (G762). H & E stain; X 1250.

Fig. 12

Fig. 13

this animal many areas of focal gliosis and neuronophagia were seen in the gray matter of the frontal cortex (Fig. 11). Structures suggestive of inclusion bodies were noted in two neurons in this same dog. One neuron contained a small dense eosinophilic intranuclear body (Fig. 13) while another contained a small eosinophilic mass in its cytoplasm. In the dog inoculated with Lederle virus cellular infiltration was prominent in the pial vessels and microglial reaction in subpial areas along the surface of the cerebellar peduncles. By 17 and 19 days, vessel changes were no longer present although occasional foci of microglial cells could still be seen. These were composed mainly of rod-shaped cells.

Dark shrunken neurons were frequently seen at the tips of gyri and occasionally in other areas including the Purkinje and granular cell layers of the cerebellum. These were noted at all stages of the disease and were considered to be artifacts as they were also present in the controls. Their numbers were reduced but not completely eliminated by perfusion. No areas of status spongiosus or demyelination were detected.

Macroscopically the sternal marrow appeared normal. Microscopically no changes in cell density or erythroid-myeloid ratio were detected and no degeneration, necrosis, or inclusion bodies were seen. No significant lesions were found in the lungs, skin, or intestine.

Several incidental lesions were found in the uninoculated gnotobiotic dogs. Frequently a drop of cloudy, yellowish, viscous

fluid was seen at the ventral tip of the vulvar lips in all of the females. Microscopic examination revealed purulent exudate between the epithelial folds of the clitoral fossa. Numerous neutrophils could also be seen in the mucosa and submucosa. In the males a few individual neutrophils were scattered in the mucosa and submucosa of the prepuce. On the mucosal surface they occurred in small foci associated with clusters of degenerating epithelial cells. A few neutrophils were always present in the tonsillar epithelium and were sometimes numerous in the depths of the crypts. No impacted ceca were noted in the gnotobiotic dogs, but there was considerable variation in cecal size.

Discussion

Experimental distemper in the gnotobiotic dog was an extremely mild disease and would probably have been undetected if temperatures had not been recorded. Clinical signs of the disease were practically non-existent. Since the disease produced in conventional dogs by the viruses used in this study was similar to that reported by Dunkin and Laidlaw (9), the microbial flora appears responsible for the development of most of the clinical signs of distemper.

The presence of an absolute leucopenia in experimental distemper has also been reported by Bindrich (1) and Gillespie and Rickard (11). Scheitlin et al. (31) and Schalm (30) have reported similar findings in natural cases. These two authors have also noted absolute lymphopenias similar to the findings in this experiment. Scheitlin et al. (31) have also recorded

neutropenia and mild anemia in natural cases similar to that found in gnotobiotic dogs. In many naturally occurring cases, however, neutrophilia is common (30). The absence of neutrophilia in experimentally infected gnotobiotic dogs indicates that it is probably caused by secondary bacterial infection.

With the exception of one dog, serum protein values were not greatly altered by distemper infection. The striking α_2 peak in this dog was similar to the findings of Mebus (24) who reported a consistent increase in this fraction in dogs inoculated intracerebrally with Snyder-Hill distemper virus. The only other report of serum electrophoretic patterns in distemper was that of Polson and Malherbe (26). They claimed to have been unable to detect significant changes in distemper. Some of the patterns which they illustrated, however, did show high α_2 peaks. We have demonstrated high α_2 peaks in naturally occurring cases of encephalitic canine distemper. The only similarity among these cases appeared to be severe nervous disease. This suggests that the increase in the α_2 fraction may be associated, either directly or indirectly, with lesions in the central nervous system.

Serum antibody titers in gnotobiotic dogs developed at the same time and to the same degree as those reported in conventional dogs (12, 28, 29). This indicates that the antibody-forming mechanism of the unstimulated gnotobiotic dog is as capable of response to viral infection as that of the conventional dog.

The gelatinous appearance of the thymus in distemper infected dogs has been reported by other workers (8, 19, 27). No

one has, however, previously reported the striking reduction in size. The prominent lymphoid depletion and reticular cell hyperplasia so noticeable microscopically in all lymphoid organs, has previously been described in natural cases by McMonbreun (8) and Lauder et al. (19). Lymphoid hyperplasia, however, was described by Potel (27) in experimental cases and Scheitlin et al. (31) in natural cases. Lauder et al. (19) speculated that this lympholysis might have been due to cortisone release following stress. Our results suggest that lymphoid tissue may be the primary site of viral multiplication. The first detectable changes were noted in this tissue and it is the only tissue in which inclusion bodies were distinct. Such an assumption is compatible with similar findings in ferrets (6, 7, 21). It is interesting that lymphopenia was present before lesions were noted in lymphoid tissue and that the leucocyte counts had returned to normal levels before lymphoid regeneration was detected in the tissues.

It is interesting that there were lesions in the brains of all dogs eight days after inoculation. The initial intramural lymphocytic infiltration has been noted by many investigators. The scattered microglial foci and neuronophagia in the cortical gray matter, although frequently mentioned by many workers, are usually given very little emphasis. The virus is usually stated to have an affinity for the white matter (18). In this experiment the virus appeared to have a greater affinity for the gray matter although the white matter was also affected. The Lederle virus produced more prominent lesions in the cerebellar peduncles than

the Snyder Hill virus. This suggests that the Snyder Hill virus may have been altered by prolonged serial intracerebral passage. The brain lesions were mild in all but one dog and it is questionable whether any of these animals would have developed more severe brain lesions if the disease had been allowed to progress. The factors necessary for the production of brain lesions severe enough to cause nervous signs are still not known, however, viral lesions in the brain may be responsible for the initiation of an allergic encephalomyelitis. The distribution of lesions within the brain suggested entrance of the virus following a viremia. The virus may have spread through vessel walls into the parenchymal cells as well as affecting the superficial tissues by way of the cerebrospinal fluid. Campbell (4) suggested that the virus entered the brain by way of the cerebrospinal fluid because he noted prominent subpial and ependymal lesions in naturally infected dogs.

The absence of lesions in the respiratory system was unexpected. Further investigation is desirable to determine the cause of giant cell pneumonia in spontaneous distemper.

Many workers have described inclusions bodies with regularity in natural cases (13). They have been given much less prominence in experimental distemper and have not been commonly reported in the brain in experimental studies (5, 9, 11, 17). The viral inclusions noted in this experiment were scarce and usually indistinct. It is doubtful that a diagnosis of distemper could

have been made in any of the dogs on this basis. Occasional distinct inclusions could be seen, however, in the cytoplasm of reticular cells.

Summary

A study of experimental distemper in the gnotobiotic dog revealed that in the absence of other microorganisms distemper is an extremely mild disease. The only significant clinical sign was a mild transient fever which appeared the third day after inoculation. An absolute lymphopenia and neutropenia were present on the fourth day and had returned to normal by 12 days. Serum proteins remained in the normal range except for one of 12 dogs which had a high α_2 peak. The only macroscopic change detected was a great reduction in the size of the thymus. Microscopically all lymphoid organs underwent severe lymphoid depletion. This was first detected in the thymus after five days. A few days later it could be seen in other lymphoid organs. Reticular cell hypertrophy and hyperplasia were commonly noted in lymphoid tissues other than the thymus. Inclusion bodies were occasionally seen in the cytoplasm of reticular cells. Mild microscopic lesions were present in the brains of all dogs eight or more days after inoculation. These initial lesions consisted of endothelial cell swelling and intramural lymphocytic infiltration. Microglial foci and neuronophagia became prominent 12 or more days after inoculation. Neuronal lesions were prominent in the deep layers of the cortical gray matter. No lesions were

present in the lungs, skin, or intestine. Both Snyder Hill and Lederle viruses produced similar disease. The Lederle virus, however, produced higher and more persistent fevers and caused changes in the cerebellar white matter as well as in the cortical gray matter.

CHAPTER IV

DISTEMPER VIRUS IN BRAIN TISSUE CULTURE MONOLAYERS

Introduction

At present there is no satisfactory method for the primary isolation and titration of virulent distemper virus. No description of the lesions produced by distemper virus in tissue cultures of the central nervous system have been reported. Since the virus frequently produces lesions in the central nervous system of infected dogs, a study was made of the effect of the virus on brain tissue culture monolayers. Because of its adaptation to brain by intracerebral passage in dogs, the effects of the Snyder Hill virus (6) were compared with the Lederle spleen-passage virus.¹

In the past ferret inoculation (5) has been the only reliable method for the detection and isolation of virulent distemper virus. This animal develops a characteristic disease when exposed to the virus. Besides being expensive, this animal is difficult to obtain and maintain free from distemper infection. In 1955 West and Brandley (13) reported distinct lesions in mice on initial intranasal inoculation with virulent distemper virus. Pneumonic changes persisted through 40 passages and could be inhibited by distemper antiserum but not by normal serum. No other

¹American Type Culture Collection, Washington, D. C.

workers have reported on this technique. Several workers have used the complement fixation test to titrate virulent virus in tissues (7), but this does not allow for isolation of the responsible virus. Haig (8, 9) and Cabasso and Cox (3) adapted distemper virus to the chorioallantoic membrane of the developing chick embryo, but this took several passages.

In 1958 Rockborn first demonstrated the effect of distemper virus in tissue culture monolayers. He found cell degeneration, inclusion bodies, and multinucleated giant cells in canine kidney cell cultures 42 days after inoculation. Following this report numerous investigators confirmed Rockborn's findings in canine and ferret kidney (1, 10, 11) and chick embryo fibroblast cultures (2, 4). Vantsis (12) reported cytopathic effects as early as six to seven days in cultures of organs from infected animals. Cultures of cells from unaffected animals required extremely long incubation periods or several passages to demonstrate the presence of virulent virus.

Materials and Methods

The cultures used in these experiments were prepared from the cerebral hemispheres of newborn puppies. The hemispheres were collected in an aseptic manner and washed several times in Hank's balanced salt solution (HBSS) containing 2 mg. per cent phenol indicator. Antibiotics were added to make a final concentration of 200 units of penicillin, 50 units nystatin, and 100 mg. of dihydrostreptomycin per milliliter. The pH of the solution was adjusted to approximately 7.8 with a 4.4 per cent sodium bicarbonate

solution. The leptomeninges and choroid plexus were stripped from the tissue, which was then minced with scissors to a gelatinous pulp.

The cells were dispersed by digestion in four to five volumes of 0.12 per cent trypsin in HBSS. This was performed in an Erlenmeyer flask at approximately 32 C using a magnetic stirring rod. After approximately 15 minutes the cell suspension was decanted through a layer of gauze into a beaker. The remaining fragments were again trypsinized for another 15 minutes and the process repeated. If the process was repeated more than twice there was a great reduction in the number of viable cells obtained with each digestion period.

The suspended cells were then sedimented by centrifugation at 300 g. for seven minutes. The supernatant was discarded and 1 ml. of sediment was diluted to 10 ml. with growth medium. This cell suspension was distributed in aliquots of 0.2 ml. into sterile Leighton tubes containing glass coverslips, and 10 ml. into sterile 125 ml. prescription bottles. Two ml. of growth medium was then added to each tube and 20 ml. to each bottle. The containers were closed with sterile, white, rubber stoppers and the cultures incubated at 37 C in the lying position.

The growth medium consisted of Hank's lactalbumin hydrolysate (HLH) medium plus 10 to 20 per cent bovine serum. The HLH medium was composed of HBSS plus 500 mg. per cent lactalbumin hydrolysate and 2 mg. per cent phenol indicator. The growth medium was also adjusted so that each milliliter contained

a final concentration of 200 units penicillin, 50 units nystatin and 100 mg. of dehydrostreptomycin. The pH was adjusted to approximately 7.4 with sodium bicarbonate solution. After the monolayer was well formed the serum content was reduced to 5 per cent. The media was changed whenever the pH indicator began to show some acidity to the medium.

By five days the cultures were usually growing well, although the monolayers were not yet complete. At this time the old medium was decanted and the cultures were inoculated with virus. Two strains of distemper virus were used in these experiments, the brain adapted Snyder Hill virus, and the Lederle ferret spleen virus. The material used for inoculation was identical to that used in producing distemper in gnotobiotic dogs. Bottles were inoculated with 1.0 ml. and the tubes with 0.2 ml. of either a 10 per cent brain suspension of Snyder Hill virus in HBSS or a 4 per cent spleen suspension of Lederle virus in HBSS. The cultures were incubated for 30 minutes and new media added. After the cultures had been inoculated for approximately eight days, the monolayers were detached by trypsinization. These free cells were then sedimented by centrifugation and resuspended in 6 ml. of old media. This material was used to inoculate new cultures in the manner previously described. Such a procedure will be referred to as a serial passage. Material from the primary cultures were also serially subcultured. This was done by adding 4 ml. of the cell suspension to new bottles and 0.5 ml. to new tubes, and then adding more new media.

Every 48 hours a set of coverslips from both control and inoculated tubes was fixed in 100 per cent methyl alcohol and stained by the May-Grünwald-Giemsa technique. Coverslips were also fixed in isotonic phosphate buffered 5 per cent formalin and stained with Shorr's triple stain and Seller's stain to demonstrate inclusion bodies.

Results

Both the Snyder Hill and Lederle viruses produced cell degeneration, syncytium formation, and viral inclusions in the third serial passage and in the second subculture. Inclusion bodies were also found in primary cultures 30 days after inoculation.

The brain tissue culture monolayers were usually growing well by the fourth or fifth day, at which time they were inoculated. By the sixth or seventh day the monolayer was usually complete. These monolayers were composed mainly of glial and fibroblastic Schwann cells, with a few neurons scattered throughout (Fig. 14). The processes of the glial elements were quite distinct and many of the cells could be identified by their processes. By about 12 days many axons could be seen extending for considerable distances across the monolayer.

In the primary cultures no changes were noted until 30 days after inoculation. At this time small inclusion bodies were noted in the cytoplasm of some of the glial cells. These inclusions were surrounded by a distinct clear space or halo and were a pale blue color with May-Grünwald-Giemsa stain, quite

Fig. 14.--Photomicrograph. Uninoculated 16-day-old brain culture monolayer. Several cell types are present. May-Grünwald-Giemsa stain; X 150.

Fig. 14

similar to the color of the cell cytoplasm (Fig. 15). These cultures were followed for a total of 37 days at which time they were showing severe degenerative changes. Many multinucleated giant cells were present and contained either several small inclusions or large plaque-like inclusions which accounted for a great portion of the cell cytoplasm (Fig. 16). The degenerative changes appeared in plaques in the monolayer where the cells had fused into one or several syncytial giant cells leaving a large space.

No changes were noted in the second serial passage, which was observed for about eight days. Viral effect was always noted on the third passage eight to ten days after inoculation. Inclusions were usually noted with the Snyder Hill strain a day or two before they were seen in the Lederle virus infected cultures. After the initial appearance of inclusions four to five days usually elapsed before extensive degeneration of the monolayer occurred. This experiment was repeated a second time with similar results.

No viral effects were noted in the first subculture, but a change in the predominant cell type occurred. The fibroblastic Schwann cells overgrew the other neural elements. The second subculture was composed entirely of these fibroblastic cells. Numerous inclusions appeared on the third day after the second subculture was made but multinucleated giant cells were still rare. This was 21 days after the initial inoculation of the primary cultures. This subculture was harvested on the fourth

Fig. 15.--Photomicrograph. Cell degeneration and inclusion body formation 15 days after inoculation of the third serial passage. May-Grünwald-Giemsa stain; X 600.

Fig. 16.--Photomicrograph. Inclusion bodies and syncytial formation in the third serial subculture. Shorr's triple stain; X 300.

Fig. 15

Fig. 16

day and a third subculture made. Many giant cells and inclusions were noted in this culture after 24 hours.

Discussion

The results of this study correlate well with the works of Rockborn (10), Vantsis (11), and Bittle et al. (1). Although these workers used canine or ferret kidney cortex, and there was some variation in the length of time before changes were detected, the fact that considerable periods of incubation were necessary before evidence of viral effect seemed to be rather uniform. Also the presence of syncytial giant cells and the appearance of the viral inclusions in these experiments were quite similar to the previous reports.

Summary

The Snyder Hill and Lederle strains of distemper virus produced similar changes in brain tissue monolayers. Viral effects were detected eight days after the third serial passage was made. Cytopathic changes consisted of inclusion body formation, plaques of cell degeneration, and syncytium formation. Similar changes appeared in the initial culture 30 days after inoculation. Brain tissue monolayers offered no advantage over the more commonly used kidney cell cultures. Neither method is adequate as a means of primary isolation and titration of virulent distemper virus.

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