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A Fatal Septicemic Disease of Infant Puppies Caused by Cytopathogenic Organisms with Characteristics of *Mycoplasma.** (29710)

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In many breeding kennels there may occur sudden unexpected neonatal or infant puppy deaths. During an investigation of such unexplained deaths, there were brought to our attention 2 similar occurrences in breeding kennels located in central New York, in each of which young puppies had died from what appeared to be a septicemic disease that could not be attributed to recognized viral, bacterial, or protozoan infections. From dead puppies that were submitted for study, agents were isolated and grown to tissue culture. These proved cytopathogenic for dog kidney cell cultures (DKC) and possessed characteristics of *Mycoplasma*(1,9). In another instance DKC cultures degenerated spontaneously and from them a similar cytopathogenic agent was isolated. This report concerns some of the characteristics of these agents and certain features of this apparently new disease of puppies.

Materials and methods. Source of isolates. (1) An agent cytopathogenic for DKC cultures was isolated in Nov., 1961 from the heart blood, lungs, livers, spleens, and kidneys of 3 Springer Spaniel puppies that died between 2 and 3 weeks of age. All the remainder of a litter of 12 also died. The only sign of illness had been continual crying that began 8 to 12 hours before death. A kidney isolate, designated strain F-205, was selected for further study. (2) A second isolate was obtained in August, 1962 from DKC cultures that originated in Illinois. A history of the puppies that donated the kidneys for cell culture was not available. Because spontaneous cytopathic effects (CPE) resembled those caused by certain viruses, cultures were submitted to the Veterinary Virus Research Institute for study. From them a cytopathogenic agent was isolated and was designated strain A-1. (3) In Oct., 1963 agents cytopathogenic for DKC that produced CPE similar to those caused by strains F-205 and A-1 were recovered from lung, kidney, spleen, and lymph node tissues from 3 littermate Beagle puppies that had died within 2 to 3 weeks after birth. These specimens were brought to us within a few hours after death and from them a spleen isolate, designated T-O, was selected for further study.

Portions of tissues from various organs were selected from all of these puppies at time of necropsy. They were cultured both aerobically and anaerobically on horse blood agar and stored at —60°C in a mechanical freezer. Portions also were fixed in 10% neutral formalin or in Bouin's fluid. Later, the stored tissues were triturated in medium 199 (Difco) to make a 10% suspension and inoculated into DKC cultures.

Histopathological examination. Paraffin

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sections were made of fixed tissues and were stained with hematoxylin and eosin (H and E). Imprint smears were fixed in methanol or Bouin's fluid and stained with Giemsa's solution for several hours.

Culture methods. DKC cultures were prepared from puppies that had been raised in strict isolation and from which repeated attempts to demonstrate mycoplasma or viruses had failed. Cell culture methods were reported earlier(2). Before inoculation with 0.1 ml amounts of test suspensions, sample cell culture batches were examined culturally and tinctorially for mycoplasma. For cytopathological studies, cells were grown on coverslips in flat bottom tubes. When cell sheets were confluent, they were inoculated with fluid from infected DKC cultures. Coverslips were removed from inoculated tubes at intervals, fixed in Bouin's fluid for 15 minutes, rinsed in tap water to remove the yellow color of picric acid, and stained overnight in Giemsa's solution.

Embryonated eggs from a mycoplasma-free flock of chickens were inoculated with suspensions of puppy tissues or infected DKC culture fluid. After inoculation with 0.25 ml amounts of the various materials into the yolk sacs of 4 to 6 day eggs, they were placed in an incubator at 37°C and were candled daily. After 6 to 7 days the yolk sacs were harvested and examined for psittacoid agents and rickettsiae by staining yolk sac smears with Macchiavello's stain. Yolk was tested for bacteria by culturing on blood agar plates and, if no growth occurred, a second transfer to eggs was made. Yolk from inoculated eggs was inoculated also into DKC cultures and artificial media prepared for cultivation of mycoplasma as described below.

Artificial media consisted of beef heart infusion broth or agar (Difco) to which was added various enrichment materials. Several media were employed initially, all of which have been described in detail by Barber and Fabricant(3). Eventually a medium was employed that consisted of beef heart infusion broth supplemented with 25% puppy brain-liver-heart-muscle infusion, 10% horse serum, and 1% yeast hydrolysate (Nutritional Biochemicals). For solid media, Bacto agar was added to a final concentration of 1.2%. Media usually were supplemented with 1000 units of penicillin per ml and 0.1% thallium acetate, except for certain tests where bacterial inhibitors intentionally were omitted. Broth cultures were incubated aerobically at 36°C and agar plates were incubated aerobically and anaerobically in a moist chamber, or in an increased CO_2 atmosphere obtained by placing inoculated plates in a sealed jar that contained a moist cellulose acetate sponge and a lighted candle.

Antibiotic sensitivity. Sensitivity of the isolates to certain antibiotics was examined by incorporating graded doses of kanamycin sulfate (Kantrex, Bristol), penicillin, tetracycline hydrochloride, streptomycin, and crystalline tylosin tartrate (Eli Lilly) into cell culture media prior to inoculation with infective DKC culture fluid that had been ultrasonicated for 1 minute at maximum frequency with a Biosonik (Bronwell Co.) probe. Approximately 10^3 $TCID_{50}$ were inoculated into tubes that contained 6 ml of the various antibiotic media and also into identical media from which the antibiotics had been omitted. After 4 hours at room temperature, DKC cultures were inoculated with 2 ml of the various test suspensions. Cultures were observed for one week for antibiotic cytotoxicity and for specific CPE.

Serological tests. Antiserums were prepared in rabbits that were given repeated inoculations with ultrasonicated infective DKC cultures. After ultrasonication, cell debris was removed by centrifugation at 1500 rpm for 10 minutes. Then, the infectious supernatant fluids were centrifuged at 20,000 rpm for 30 minutes in a Spinco ultracentrifuge and the sediment fractions were pooled and resuspended to an optical density of 2.5 at 550 M. At time of initial inoculation, rabbits were bled for serum and then each animal was inoculated twice weekly with doses that ranged from 1 to 5 ml. After a total of 40 ml of antigen had been given by 15 inoculations, each rabbit was bled for serum, which was stored at −20°C.

Tests for growth-inhibiting antibody(4) were made with heated serums incorporated

to a final dilution of 1:20 in maintenance medium. As determined by previous titration, approximately 300 $TCID_{50}$ were mixed with 6 ml of each dilution, and incubated for 2 hours at room temperature. Then, the mixtures were inoculated in 2 ml amounts into each of 3 DKC cultures. In addition, growth-inhibition tests were performed with serums prepared as described above against the three established canine *Mycoplasma* types(5). These types, supplied through the kindness of Dr. W. H. Kelton, Ames, Iowa, were designated PG13 (alpha), PG14 (beta), and PG15 (gamma).

Routine virus neutralization tests(2) were performed for antibodies of canine distemper and infectious canine hepatitis (ICH) viruses, using serums from dogs that had been inoculated with infective tissue suspensions from puppies that had died, and sera from dogs that had been inoculated with infectious DKC culture fluids. Also, sera that possessed high titers against distemper and ICH viruses were used in tests for inhibition of CPE caused by the puppy isolates.

Dog studies. Beagle puppies were used that varied in age from 24 hours to 3 weeks. Newborn puppies were obtained as soon after birth as possible from mothers which had been maintained in strict isolation. These were kept in incubators and bottle fed at 8 hour intervals with Esbilac (Borden) for the first 2 weeks of life; afterwards they were gradually accommodated to solid foods.

Inoculums consisted of organisms isolated in DKC cultures which either had been transferred 9 times in cell cultures or transferred 5 times in broth media after 4 passages in DKC cultures. Of 28 dogs used, 23 were inoculated with 0.5 ml of various doses of infectious DKC culture fluid that varied from 10 to 100,000 $TCID_{50}$. Also, 5 puppies less than 5 days old each were inoculated with 1 ml of 4 day broth cultures that contained viable organisms. Inoculations were made by the oral-nasal route with a DeVilbis nebulizer, by the intraperitoneal (i.p.) route, or intravenously (i.v.). Following inoculation, puppies were observed at least 3 times daily for signs of illness or death. Puppies that were moribund were euthanized with chloroform and from them, and from puppies that died naturally, tissue samples were collected for culture. Portions of various organs were fixed in formalin or Bouin's solution for histopathological examination. Impression smears of various tissues were made and stained by Giemsa's method.

Results. Isolation. Growth of the isolates was obtained consistently only in DKC cultures. Later, it was found that growth occurred in serum broth enriched with yeast hydrolysate. After inoculation of pathological materials on blood agar, a few colonies of *Escherichia freundii* were isolated from the spleen of one puppy (F-205 series) that had been dead for several hours. *Proteus mirabilis* was isolated from the spleen of a second puppy of the same litter, and streptococci were isolated from the lungs of 2 puppies in the second outbreak (T-O series). No bacteria were recovered from the internal organs of other puppies or from the DKC cultures that had degenerated spontaneously. Examination for toxoplasma by Giemsa-stained impression smears of puppy tissues and by intraperitoneal mouse inoculation with suspensions of tissues from puppies did not reveal these organisms. Also, attempts failed to demonstrate a pathogenic agent for embryonated eggs after 3 serial transfers, and examinations of yolk sac smears by Macchiavello's method did not reveal rickettsiae or psittacoid agents.

Sera from dogs that contained antibodies to distemper and ICH viruses failed to neutralize the CPE caused by the puppy isolates, and dogs inoculated with suspensions of puppy tissues that were infectious for DKC cultures did not develop antibody for distemper or ICH viruses. These viruses, therefore, were not considered responsible for the disease in puppies or for the CPE observed in cell cultures.

Growth in DKC cultures. Growth of the isolates in DKC cultures was attended by CPE that initially occurred as proliferating foci of rounded and swollen refractile cells that detached from the glass and left plaque-like cleared areas surrounded by degenerating cells (Fig. 1). Cytopathic effects occurred 3 to 4 days after inoculation with suspensions

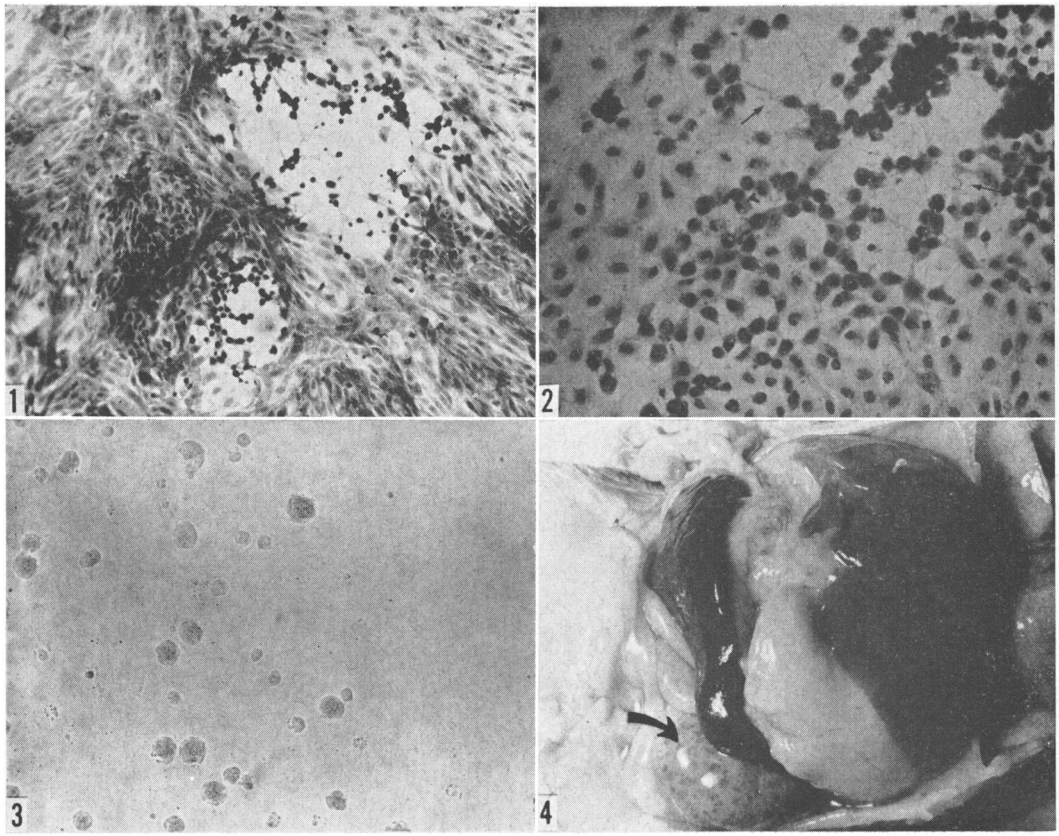

FIG. 1. Cytopathic effects produced in DKC cultures by puppy isolate F-205. H & E × 72.
FIG. 2. Focus of DKC degeneration 72 hr after inoculation with Strain F-205. Giemsa × 135.
FIG. 3. Coarse, vacuolar colonies isolated from DKC culture inoculated with strain F-205. *In situ* × 18.
FIG. 4. Gross lesions of puppy inoculated orally at 1 day of age with strain F-205. Mottled appearance of liver, enlarged spleen, and subcapsular renal hemorrhages are characteristic.

of puppy tissues or cell culture fluids that had been stored frozen, but after further transfer of undiluted cell culture fluid, CPE occurred regularly after 18 to 24 hours. With an inoculum of approximately 50 $TCID_{50}$, maximum titers occurred in 4 to 5 days. Titers ranged from $10^{3.0}$ to $10^{5.0}$ per 0.1 ml. At lower dilutions CPE progressed to complete degeneration of the cultures. At higher dilutions however, foci usually remained localized as discrete plaques and often were overgrown later by normal appearing cells.

Cytopathological changes in infected cells grown on coverslips and stained with Giemsa solution (Fig. 2) consisted of cytoplasmic vacuolization and stranding with marked nuclear changes. Organisms were not seen in preparations stained with hematoxylin and eosin, but they were clearly evident in cells stained with Giemsa's solution. Most cells contained organisms that were both coccoid and coccobacillary. Organisms were present throughout the cytoplasm and also within vacuolar sacs that often were juxtaposed against nuclei. Extreme variation in form was the most characteristic morphological feature of the organisms. Some cells contained distinct filamentous forms that stained dark purple, while filamentous forms within cytoplasmic vacuoles were often violet-pink and barely visible. Microcolonies occurred in the cytoplasm of some cells and less frequently in nuclei. Minute coccoid bodies that stained violet to pink and which were approximately 180 mμ in diameter were present in the cytoplasm of most cells. These forms occupied

intracellular spaces which created a "pavement effect." Short branching filamentous forms emanated from cytoplasmic strands and from cell membranes. Nuclear changes consisted principally of karyorrhexis and pyknosis and were most severe in cells that immediately surrounded foci of degeneration. Small coccoid and globular structures with refractile centers were present about the nuclear membrane and within the nucleoplasm of degenerating cells. Some of these forms stained homogenously purple-violet with Giemsa, and others occurred as "ring forms" with pale pink centers. Occasional microcolonies partially occupied nuclei of a few cells. These often gave the appearance of inclusions similar to those seen in cells infected with certain viruses.

Growth in artificial media. Consistent growth of organisms from puppy tissues and from infected DKC cultures was obtained only in broth media. Three or 4 days following inoculation of broth media with ultrasonicated infectious puppy tissues or DKC cultures a floccular material appeared at the bottoms of inoculated tubes. Smears of broth cultures stained with Dienes' or Giemsa revealed abundant growth of minute coccoid organisms and delicate branching nonseptate filamentous forms that contained granular bodies within the filaments and at their terminal ends. Morphology of the individual organisms was revealed most clearly by phase or darkfield microscopy. Similar structures were not seen in uninoculated cultures or in broth media inoculated with normal sonicated DKC cultures. The coccoid bodies were 180 to 200 mμ in diameter and there was little affinity of the coccoid or the polymorphic filamentous forms for Gram's stain.

Several solid media were employed before growth with colony formation was obtained, and results of isolation attempts from infective tissue suspensions or DKC cultures have been inconsistent on solid media. Nevertheless, similar colonies were isolated from the kidneys of puppy F-205 and also from DKC cultures inoculated with strains F-205, A-1, and T-O. Colonies did not occur on solid media inoculated with normal DKC cultures. Isolations were made initially in broth media that contained yeast hydrolysate and 10% horse serum layered over a horse blood slant which, after 6 days, was transferred to the solid medium described in *Methods*. Growth was initially scant, and occurred only on plates incubated in an increased CO_2 atmosphere however, after further transfers, abundant growth was obtained under aerobic conditions. Similar colonies were obtained from puppy tissues that had been stored at —60°C for more than 2½ years. Colonies were 0.02 to 0.1 mm in diameter and initially were coarse and vacuolar (Fig. 3). After further transfers, colonies had characteristic dense centers with light peripheral zones. None of the isolates reverted to bacterial forms after 3 transfers on media from which bacterial inhibitors were omitted. Although good growth occurred in DKC cultures inoculated with organisms grown on artificial media, CPE did not occur.

TABLE I. Antibiotic Sensitivity (Strain F-205).

Antibiotic	Toxic dose for DKC cultures*	Min inhibitory dose†
Tylosin tartrate	2.0	.75
Kanamycin sulfate	>3.0	>3.0
Oxytetracycline	.3	.1
Tetracycline	.3	.1
Streptomycin	>3.0	>3.0
Penicillin	—	>100,000

* Minimum dose that produced cytotoxicity (mg/ml).

† Minimum dose that completely inhibited CPE (mg/ml, except for penicillin, expressed in units/ml).

Antibiotic sensitivity. The cytopathogenic agents were sensitive to oxytetracycline, tetracycline, and tylosin tartrate but were resistant to penicillin, streptomycin, and kanamycin at the dosage levels used (Table I).

Serological comparisons. Strains F-205, A-1, and T-O were identical by growth-inhibition tests performed with immune rabbit serums (Table II). In contrast, sera produced in rabbits against the 3 recognized canine mycoplasma types: PG13 (*M. canis*), PG14 (*M. spumans*), and PG15 (*M. maculosum*), and shown to inhibit the homologous strains, did not inhibit growth of the cytopathogenic isolates or colonies derived from infected DKC cultures.

Pathogenicity for puppies. The responses

TABLE II. Comparison of Puppy Isolates with Canine Mycoplasma Strains by Growth Inhibition Tests.

Strain	Hyperimmune rabbit serum			
	F-205	PG13*	PG14	PG15
F-205	+†	—	—	—
A-1	+	—	—	—
T-0	+	—	—	—
PG13	—	+	—	—
PG14	—	—	+	±
PG15	—	—	—	+

* See text for designation of strains PG13, PG14, and PG15.
† + = inhibition of CPE or growth on agar containing 5% immune serum. — = no inhibition of CPE or growth.

of puppies inoculated with strain F-205 propagated in DKC cultures are shown in Table III. Of the 5 infant puppies inoculated with isolates that had been transferred 5 times in broth media, none developed signs of illness, but puppies 1 day to 1 week of age were uniformly susceptible to oral or intraperitoneal inoculation with organisms propagated in DKC cultures. In puppies older than 2 weeks a mild febrile reaction that lasted 2 or 3 days was the only sign of illness. These puppies all subsequently developed antibody that inhibited growth of the cytopathogenic agent.

Lesions indistinguishable from those found in the original natural cases occurred in all of the inoculated puppies that died. Detailed histopathology will be reported elsewhere(6). Characteristic pathological changes included enlarged and congested lymph nodes, greatly enlarged spleens, diffuse pneumonic consolidation, and renal cortical necrosis. Striking subcapsular renal hemorrhages were present in all puppies. Liver lesions consisted of disseminated focal necrosis and hemorrhage. Lesions in all organs appeared to be related to cellular necrosis and dissolution of connective tissue ground substance with consequent ischemic necrosis and hemorrhage. Fig. 3 illustrates the appearance of the visceral organs of a puppy that had received inoculum orally at 1 day of age and which died 6 days later.

Imprint smears of lymph node, lung, kidney, spleen, or liver tissues stained with Giemsa's solution revealed great numbers of coccoid, coccobacillary, and slender, short-branched filamentous forms that occurred both intracellularly and extracellularly. Within cells, organisms were seen in the cytoplasm as coccoid elements, sometimes enclosed within a vacuolar structure, or as microcolonies. Coccoid bodies were numerous in some nuclei, and were especially abundant at the nuclear membrane. Extracellular organisms usually appeared in stained imprint smears as small clusters of branching polymorphic filamentous bodies or as minute coccoid forms approximately 200 mμ in diameter. Macrophages and undifferentiated reticulum cells, often engorged with organisms, were seen in all organs. Organisms were not seen in histologic sections stained with hematoxylin and eosin.

The organisms were readily isolated in DKC cultures and in broth media from all internal organs and from heart blood. As noted above, growth on solid media with colony formation was inconsistent.

Discussion. Mycoplasma have been iso-

TABLE III. Responses of Beagle Puppies to Strain F-205 Grown in Dog Kidney Cell Cultures.

Puppy age (days)	Inoc route	Dose ($TCID_{50}$) inoculated	No. died/ No. inoc	Interval between inoc & death (days)	Reisolation of organisms*	Growth-inhibiting antibody†
1–3	oral	10^2–10^5	4/4	8– 9	Yes	—
	i.p.	"	6/6	6– 8	Yes	—
7	oral	10^4	2/2	9–10	Yes	—
15	oral	10^4	0/2	—	—	2/2
	i.p.	"	0/2	—	—	2/2
	i.v.	"	0/2	—	—	2/2
22	oral	$10^{4.5}$	0/3	—	—	3/3
	i.p.	"	0/2	—	—	2/2

* Organisms reisolated in DKC cultures and broth media from kidneys, spleen, liver, lung and lymph node suspensions.
† Serums tested 3-4 wk after inoculation. Preinoculation serums were all negative.

lated frequently from the pharnyx and lower urogenital tract of dogs(5,7,8) although full proof of pathogenicity of canine mycoplasma strains for dogs has not been obtained. Nevertheless, ever since the early association of these organisms *(Astrococcus canis)* with canine distemper(9), a disease later shown to be caused by a virus, mycoplasma have been suspected of causing a variety of disease conditions in the dog. From the throat and vagina of dogs Edward and Fitzgerald(1) isolated mycoplasma which, on the basis of morphological and serological characteristics, were classified into 3 types. One type *(M. canis)* has been associated with chronic epididymitis. Grieg(7) and Gutekunst(8) isolated mycoplasma from canine respiratory illness ("kennel cough"), although the pathogenicity of these isolates cultivated in mice and in broth media was not proved conclusively for dogs.

The cytopathogenic isolates from puppies and from naturally infected DKC cultures reported here were serologically and culturally identical. Also, pathological manifestations of the experimental disease were uniformly characteristic and were not distinguishable from lesions seen in the natural cases. Principal pathological changes consisted of disseminated necrosis and hemorrhage. The causal organisms were small, pleomorphic, coccobacillary bodies that were demonstrated readily in Giemsa-stained infected cell cultures and in imprint smears of tissues from infected puppies. In their variable morphology, size, sensitivity to antibiotics, cultural characteristics, and inhibition by antibody, the organisms possessed characteristics of the *Mycoplasma* group(1,8). Cultural and serological characteristics of the puppy isolates were distinct, however, from those described for the 3 classified canine mycoplasma types (5,9).

Primary isolation consistently was obtained only in DKC cultures, where typical CPE occurred which were inhibited by serums from inoculated dogs and from hyperimmunized rabbits, but not by immune ICH or canine distemper serums. The sensitivity of the cytopathogenic agent to certain antibiotics also indicated that the CPE were not caused by a viral agent. Although growth of organisms occurred in broth media, growth on solid media with colony formation was erratic. The vacuolar colonies that were obtained were small and their morphology indicated that satisfactory conditions for growth had not yet been devised. The possibility cannot be excluded, however, that the broth cultures and colonies of mycoplasma isolated from the puppy tissues and from cell cultures were not the pathogenic organisms that produced CPE and disease in puppies, but were other mycoplasma species also present in these materials. Alternatively, the lack of a suitable artificial medium may have prevented the growth of pathogenic organisms. The recovery of identical organisms in artificial media from 3 sources separate in time and locality suggests the latter possibility. Also, immunofluorescent studies(6) have shown that conjugated antiserums prepared against the broth isolates gave brilliant fluorescence when reacted with infected cell cultures and with tissues from infected puppies. Serums prepared against organisms grown in cell cultures also gave bright fluorescence with organisms grown in artificial media. Difficulties in obtaining growth of mycoplasma from pathological materials and the maintenance of natural pathogenicity have been major problems encountered with these organisms(10), and experience with bovine(11) and human(12) strains indicate that tissue culture propagation may provide a growth environment that more closely maintains natural strain characteristics, especially as regards pathogenicity.

Although the isolates reported here represent a new and apparently undescribed cause of illness and death in infant puppies, it is felt that the assignment of taxonomic status to the organisms must await further cultural characterization.

An additional cytopathogenic isolate (A-463) has been made by Capt. R. O. Spertzel, Walter Reed Institute of Medical Research, Washington, D. C.(13) from dog kidney cell cultures that spontaneously degenerated and which in young puppies produced pathological changes that were virtually identical with those produced by the isolates reported here. An exchange of isolates was made and com-

parative studies indicated that isolates A-463 and F-205 were serologically identical. They were similar also as regards pathogenicity and cultural characteristics.

Summary. Pathogenic organisms were isolated from 2 outbreaks of a fatal septicemic disease of infant puppies and from dog kidney cells that degenerated spontaneously. The isolates were indistinguishable serologically and possessed characteristics of *Mycoplasma*. The pathogenic organisms were cytopathogenic for dog kidney cell cultures, and in inoculated puppies, produced pathological changes that resembled those seen in natural cases. Lesions consisted principally of necrosis and hemorrhage. The isolates were culturally and serologically distinct from recognized canine *Mycoplasma* species.

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Influence of Hypothalamic Fragments and Extracts on Lactogen Production *in vitro*.* (29711)

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A number of investigators, using different experimental techniques, have presented results which indicate that the hypothalamus plays a major role in control of anterior pituitary lactogen production. Hypophyseal stalk severance results in deciduomata formation by the traumatized uterus(1), maintenance of luteal function(2), and prevention of mammary gland involution(3). Anterior pituitary transplantation also results in deciduomata formation(4), initiation of mammary growth (5) and secretion(6), and retardation of mammary gland involution(7). Electrolytic lesions placed in the hypothalamus initiate lactation in estrogen primed rabbits(8), result in persistent diestrus and deciduomata formation(9), and retard mammary gland involution(9). Everett(4) suggested that the hypothalamus may produce a substance which inhibits lactogen production, and there are reports that addition of the hypothalamus to anterior pituitary cultures decreases lactogen production(10,11,12). The purpose of this study was to determine the effect of hypothalamic fragments and extracts from various sources on anterior pituitary lactogen production *in vitro*.

Materials and methods. Anterior pituitaries (AP) were obtained from 3-month-old, female, hooded Norway rats and divided into either 6 or 8 pieces. In one experiment AP were obtained from rats in mid-lactation. A piece from each anterior pituitary was placed in each group of an experiment until an equivalent of one AP was present per culture dish (6 to 10 dishes per group). The culture techniques were similar to those previously reported(13) and AP were cultured at either 35 ± 1.0°C or 37 ± 1.0°C for 3 days.

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