A NEW MEMBER OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP OF VIRUSES THAT CAUSES INFECTION IN CALVES*

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Plate 51

(Received for publication, February 19, 1951)

In the course of inoculating various materials from calves into guinea pigs intraperitoneally, suspensions of feces from apparently normal animals produced a febrile response. When examined during the febrile period, these guinea pigs showed changes in the liver and a fibrinous exudate clinging to the surfaces of abdominal organs. Since this pathology was regularly produced in serial passage, apparently an infectious agent had been obtained which could be transferred in guinea pigs. Films from their peritoneal exudate stained by Macchiavello's method, showed none of the bacteria ordinarily associated with feces, and neither liver nor spleen suspensions showed growth when placed on ordinary bacteriological media. Instead occasional small, coccoid, red-staining bodies, similar in size to members of the psittacosis-lymphogranuloma group of viruses, were seen. The lesions found differed from any hitherto reported as caused by agents infectious for cattle and, since this infectious agent seemed not unlike the viruses of the psittacosis-lymphogranuloma group, further investigation appeared worthwhile, especially in view of the unusual finding of a virus that produced elementary bodies in the intestinal tract of apparently normal calves.

Isolation of Strains

Transmission to Guinea Pigs.—Although suspensions of feces from calves contained many bacteria, they failed to cause peritonitis when inoculated intraperitoneally into guinea pigs. Indeed, it was usually not possible to cultivate any bacteria from the peritoneal cavity 5 days later. Inoculation of guinea pigs, therefore, became the method of choice to procure the agent from feces of calves.

Guinea pigs used in this work weighed from 250 to 375 gm. and came from a stock reared by this laboratory under conditions designed to maintain them as free from infection as possible. Repeated tests made by blind passages of liver, spleen, and kidney into other stock guinea pigs failed to reveal any latent infectious agent. Furthermore, temperature readings of these animals during the period of observation did not exceed 40°C. After inoculation all

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

guinea pigs were kept under conditions of isolation in order to prevent the introduction of any unplanned infection and to avoid spread of any infectious agent to which they might be exposed.

For strain isolations, individual fecal samples were obtained from 1 or more calves in each of 6 different herds located on widely separated farms around Ithaca. A portion of feces was weighed and a 10 per cent suspension made in saline by shaking. After coarse particles had settled, 1 ml. of the supernatant was inoculated intraperitoneally into each of 2 guinea pigs. Thereafter, temperatures were taken twice daily and guinea pigs that showed fever were presumed to be infected and were autopsied. When characteristic lesions were found it was considered probable that a strain had been isolated. Agents that produced fever and characteristic pathology were procured from animals in all 6 herds tested and percentage figures showed that feces from 10 out of 15 calves (about 66 per cent) were positive. From these, 4 strains that represented different herds were selected for further study and were designated strains I, II, III, and IV. Additional strains (V and VI) were secured directly in embryonated hens' eggs by a method to be described later.

For maintenance of the agent in serial passage, guinea pigs were killed while showing fever and portions of liver and spleen were pooled, weighed, and a 10 per cent suspension made in saline with a glass grinder. Into each of 2 guinea pigs, 1 ml. of the suspension was inoculated intraperitoneally.

Three to 4 days following inoculation, guinea pigs showed a febrile reaction that lasted 3 to 4 days. During the febrile period, animals showed a loss of weight that varied from 20 to 40 gm. Serial passages did not increase the severity of the reaction and in no instance did death occur, although strains I, II, III, and IV each were transferred for more than 5 passages. Guinea pigs killed and examined during or just after the period of fever showed excessive peritoneal fluid with extensive fibrinous exudate that covered the liver and spleen. The spleen was not enlarged, but the liver appeared swollen and friable. No lesions were found in the lungs. Histological sections of liver showed in the midzonal region of the lobules areas of focal necrosis with collections of mononuclear and polymorphonuclear leukocytes.

Attempts also were made to transfer the infection by exposing guinea pigs in the same cage to others that had been infected. In one test, 2 guinea pigs were exposed to 5 infected animals for a period of 20 days; in another, 3 were exposed to a series of 10 infected guinea pigs over a period of 50 days; and in a third test, a group of 5 guinea pigs was exposed to an equal number of infected animals for a period of 14 days. In no instance did the agent transfer from infected guinea pigs to others during exposure in the same cage.

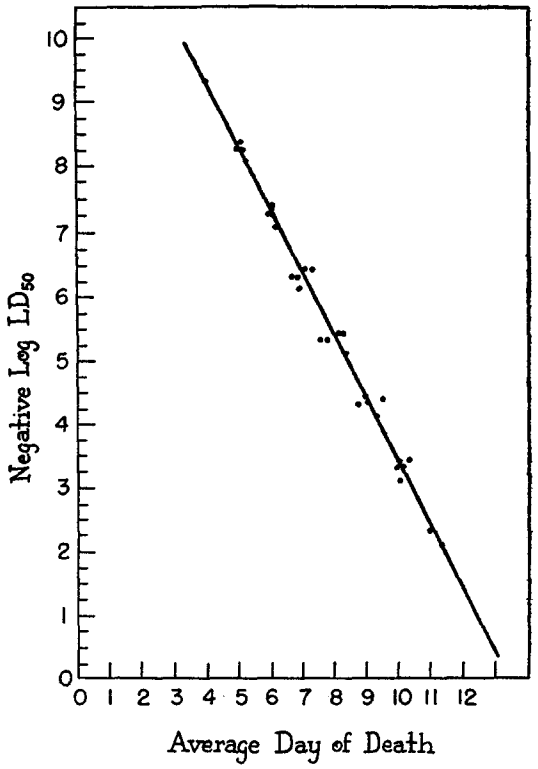
Cultivation in Eggs.—From a specimen of calf feces that had been shown to contain the agent a portion was removed, weighed, and a 10 per cent suspension made in saline. After centrifugation at 2500 R.P.M. for 30 minutes, the supernatant was removed and passed through a Berkefeld V filter. The filtrate was centrifuged again at 4000 R.P.M. for 1 hour and the sediment resuspended to 1/10 of the original volume. From this suspension, 0.5 ml. was inoculated into each yolk sac of a group of 6 hens' eggs that had been embryonated for 6 to 7 days. Thereafter, the eggs were candled daily. The embryos died 8 days after inoculation and an agent morphologically similar to those obtained in guinea pigs was demonstrated (strain V). In 3 other attempts, another strain (VI) was obtained after similar tests.

Each yolk sac in a group of 6 eggs was inoculated with 0.5 ml. of a suspension of liver and spleen from infected guinea pigs. Immediately after death of embryos or when the embryos appeared moribund, yolk sacs were harvested, weighed, a 10 per cent suspension made in saline, and other groups of eggs inoculated. All strains (I, II, III, and IV) grew readily in eggs and were maintained for 3 or more serial passages. In initial transfers, the embryos usually died 6 to 7 days after inoculation, but in all subsequent passages, died 4 days afterward.

To determine the concentration of the agent in yolk sacs, material was obtained, weighed,

and tenfold dilutions prepared in saline and each dilution was inoculated into a group of 6 or more eggs. For 12 days following inoculation, the eggs were candled daily and all deaths noted. Infective titers were calculated according to the formula of Reed and Muench (1).

The first titration was made with strain I using 10th passage yolk sac material. The dilution end-point was LD_{50} $10^{-8.0}$ and all subsequent titrations with this strain and with strain III resulted in end-points that varied from LD_{50} $10^{-7.4}$ to LD_{50} $10^{-8.4}$. Using the method Golub



TEXT-FIG. 1. Composite plot of titrations on calf agent with average day of death of 10^{-2} dilution plotted against actual negative log end-point obtained and the average day of death at each successive dilution plotted against consecutively lower negative log values.

(2) applied to virus of psittacosis, titration values of various dilutions were plotted on arithmetic paper with the average day of death of the 10^{-2} dilution plotted against the actual negative log end-point and the average day of death at each successive dilution plotted against consecutively lower negative log values. As shown in Text-fig. 1, a straight line was obtained similar to that for the psittacosis-lymphogranuloma group of agents. Since a linear relationship existed between the amount of virus inoculated and the average day of death of the embryo, this graph was used occasionally to simplify measurements of virus concentration.

In other studies, eggs embryonated for 9 days were inoculated on the chorio-allantoic membrane either with 0.1 ml. of a 10 per cent suspension of yolk sac from infected eggs or 0.1 ml. of a pooled suspension of liver and spleen from infected guinea pigs. Serial transfers

were made for 29 passages with strain I using as inoculum a 10 per cent suspension of chorio-allantoic membranes. No deaths of embryos occurred in any of the passages. Titration of the inoculum of the last passage was made by inoculations into the yolk sac and titers of LD_{50} $10^{-4.0}$ to LD_{50} $10^{-5.0}$ were obtained.

Groups of eggs also were inoculated into the allantoic cavity each with 0.5 ml. of a suspension of yolk sac from infected eggs. Undiluted allantoic fluid was used for subsequent serial transfers. All embryos survived inoculations by this route. A titer of LD_{50} $10^{-7.3}$ was obtained in a test of allantoic fluid after 12 passages and, although this was higher than that for the chorio-allantoic membrane, it was less than those found for yolk sacs.

Pathogenicity for Other Animals

Mice.—As all viruses of the psittacosis-lymphogranuloma group of organisms produce pneumonia in mice by intranasal inoculation (3–5), a similar route of inoculation was tried with this agent. Since Nigg (4) has shown that one member of this group of agents produces a latent infection in mice, the stock mice used in these studies were tested for its presence. A 20 per cent suspension of lungs from stock mice was made and 0.05 ml. inoculated intranasally into each of a group of 6 stock mice that were 3 weeks old. With suspensions prepared as above, 20 serial transfers were made at 5 day intervals. No evidence of pneumonia or other lesions was found in any of these mice.

Mice that were 3 weeks old were inoculated intranasally in groups of 6, each with 0.05 ml. of a 10 per cent suspension in saline of yolk sac from eggs infected with strain I. Subsequently, a 20 per cent suspension of lung was used during the first 20 serial passages and then reduced to a 10 per cent suspension. Some pneumonia was seen in mice after initial inoculation but no deaths occurred. It was not until after 20 successive transfers had been made that death was produced. Thereafter, the interval between inoculation and death progressively shortened until only 2 to 3 days were required to kill mice with complete consolidation of the lungs. It appeared that virulence of the agent for mice increased with continued serial passage but as will be shown later, it remained unchanged in its effect on calves. A similar series was completed with strain III.

In 3 separate trials, 6 stock mice were placed in contact with an equal number that had been infected with the agent after it had undergone more than 20 serial transfers in mice. The exposed mice were killed and examined for pneumonia 1 week after the infected ones had died and suspensions of their lungs were passed to other mice for at least one additional passage. In no instance was pneumonia found either in the exposed mice or in those of the subsequent passage.

In 3 different attempts 6 mice each were inoculated intracerebrally with 0.02 ml. of a 10 per cent suspension in saline of yolk sac from infected hens' eggs. After a period of 5 days, the brains from the inoculated mice were removed, made into 10 per cent suspensions in saline, and inoculated into additional mice. Two successive transfers were made in this manner. At no time did these mice show nervous manifestations, and none died.

Also, in 3 separate trials, 6 mice each were inoculated intraperitoneally with 0.3 ml. of a 10 per cent suspension in saline of yolk sac from infected eggs. After 5 days, the spleens of the inoculated mice were removed, made into 10 per cent suspensions in saline, and transferred to other mice for 2 subsequent serial transfers. The mice did not become ill at any time and none died. On autopsy the spleens of the inoculated mice showed no enlargement.

Cats.—In tests for the pathogenicity of strain I for cats, 10 cats were given intracardially 1 ml. each of a 10 per cent suspension in saline of yolk sac from infected eggs, and 4 cats were given 1 ml. each of the same inoculum intranasally, and 6 cats were fed approximately 100 ml. each of the same yolk sac suspension. Food was withheld for 24 hours from the cats prior to feeding virus. Temperatures were taken daily and observations made for signs of

illness. Within 1 week after inoculation half of the cats inoculated intracardially, half of the cats fed, and all of the cats inoculated intranasally were killed and examined for presence of virus in the spleen and liver. Histological sections also were made from the liver. Similar examinations were made on all remaining cats 2 to 4 weeks after inoculation. In addition, sera, for complement fixation tests, were obtained from cats at the time of inoculation and when killed 2 to 4 weeks later.

All the cats inoculated intracardially responded with a febrile reaction that lasted from 2 to 4 days. Only 2 of the 4 cats inoculated intranasally and none of the cats that had been fed showed increased body temperatures. During the febrile period, the cats ate little and appeared depressed. Virus was recovered from the spleens of all 5 cats that had been inoculated intracardially and from the spleens and livers of all 3 cats that had been fed when they were examined within 1 week after inoculation. Virus was not recovered in any cat inoculated by these routes 2 to 4 weeks after inoculation. None of the cats inoculated intranasally showed the presence of virus in the spleen and liver.

No gross lesions were seen in any of the cats but, in histological preparations, lesions were seen in the livers of the 5 cats killed within 1 week after inoculation intracardially. These consisted of varying amounts of focal necrosis with collections of leukocytes, predominately monocytic in type, in the periphery and in the midzonal regions of the lobules. No lesions were seen in the livers of any of the other cats.

In the complement fixation tests to be described later, no complement-fixing antibodies were found in any serum that had been obtained at the time of inoculation. From 5 cats inoculated intracardially 4 developed antibodies, with a titer in 1 of 1:4, in 2 of 1:8, and in 1 of 1:16. Sera from none of the other animals fixed complement.

Rabbits, Swine, and Dogs.—Additional tests were made to determine the effect of the agent on other animals. Strain I was used throughout these tests. One ml. of a 10 per cent suspension in saline of infected yolk sac was inoculated intraperitoneally into each of 2 rabbits and intravenously into a third. All animals showed an elevation in temperature 24 hours after inoculation that lasted from 3 to 5 days. When killed and examined 7 to 8 days after inoculation, no lesions were seen, but the agent was recovered from the spleen of 1 rabbit by tests in guinea pigs.

Two pigs were inoculated intravenously with 1 ml. each of a 10 per cent suspension in saline of infected yolk sacs. Within 24 hours after inoculation each had a rise in temperature above 40°C. that lasted for 4 days. One pig was killed and examined on the 4th day and the other on the 12th day after inoculation. The liver and spleen of 1 pig were made into a 10 per cent suspension in saline and 1 ml. inoculated intravenously into another pig. No rise in temperature occurred in this animal after inoculation, although in a guinea pig test the agent was shown to be present in the inoculum. No lesions were seen when the pigs were killed and examined. Histological preparations of the liver showed no pathological changes.

One ml. of 10 per cent infected yolk sac suspension made in saline was inoculated intravenously into each of 2 dogs. No rise in temperature occurred. The animals were killed and examined 2 weeks after inoculation but no pathological changes were seen and the agent was not demonstrated in the liver and spleen when tests were made in guinea pigs.

Characteristics of the Agent

Morphology.—Films prepared of peritoneal exudate from infected guinea pigs stained by Macchiavello's method (6) occasionally showed small coccoid red-staining bodies in a few of the mononuclear cells that constituted the cellular portion of the exudate. Films either of the yolk sac just prior to or just after the death of the embryo, or of the allantoic fluid from infected eggs, when

stained by the same method, revealed numerous small red-staining coccoid bodies that were located both intracellularly and extracellularly and singly or in small clumps. (Fig. 1.) Numerous bodies of similar appearance also were seen in impression films of lungs from infected mice. Individual bodies markedly resembled the elementary bodies of the psittacoid group of organisms (Chlamydozoaceae), but only occasionally were they seen in plaques or vesicles described as part of the developmental cycle of this group (7, 8). Clusters of elementary bodies were seen best in films from allantoic fluid, although examination of films of lungs from infected mice occasionally revealed such arrangements of elementary bodies.

From infected eggs a 10 per cent suspension in saline of yolk sacs was prepared and centrifuged at 2000 R.P.M. for 20 minutes to sediment coarse particles of tissue. The supernatant was removed, centrifuged at 4000 R.P.M. for 1 hour, and the sediment resuspended in saline to the original volume. This differential centrifugation was repeated two additional times, and then resuspension of the elementary bodies was made with 0.1 M phosphate buffer at pH 7.8 and about 0.3 gm. of celite (celite analytical filter aid, Johns-Manville Corp.) added for each initial gram of yolk sac. The celite was removed by centrifugation at 2000 R.P.M. for 20 minutes and the supernatant again centrifuged at 4000 R.P.M. for 1 hour. Finally the sediment was resuspended to 1/10 of the original volume with 0.05 M phosphate buffer at pH 7.0 and formalin added to a concentration of 0.1 per cent. Films of this suspension showed numerous elementary bodies almost entirely free of yolk sac tissue.

Partially purified suspensions of elementary bodies also were prepared of allantoic fluid from infected eggs by differential centrifugation in the same manner as described for yolk sac suspensions. Celite was not used in the preparation, but the final sediment was resuspended in 1/10 the original volume in 0.05 M phosphate buffer at pH 7.0 and formalin added to a concentration of 0.1 per cent. Films of this preparation also showed numerous elementary bodies relatively free of extraneous material. From these partially purified preparations, films were made and examined by the electron microscope.[1] Both unshadowed photographs (Fig. 2) and uranium-shadowed photographs (Fig. 3) were taken. Studies of these photographs revealed uniformly round bodies that possessed a denser central mass. Shadows cast by the uranium preparations indicated that the organisms possessed a rather loose cytoplasmic material and hence flattened out somewhat in the process of desiccation, as reported by Rake (9) for the members of the psittacosis-lymphogranuloma group of viruses. Measurements of elementary bodies showed an average diameter of about 350 mμ, which is within the range of size described for members of the above mentioned group of viruses.

Suspensions of liver and spleen from infected guinea pigs and of infected

[1] The authors wish to express their gratitude to Dr. B. Siegel, Associate Professor of Engineering Physics, Cornell University, for the preparation of the electron microphotographs and for measurements of the virus.

yolk sacs that contained elementary bodies were placed on infusion agar enriched with whole blood in a concentration of 10 per cent. Both slants and plates were prepared, and incubated at 37°C. either under aerobic or anaerobic conditions. Similar suspensions were also inoculated into thioglycolate broth (Brewer's medium). No growth was demonstrated in any of the cultures.

Physical Properties.—Suspensions of yolk sac from infected eggs were prepared, diluted 1:100, and portions were passed through Berkefeld V or Berkefeld N filters. Broth cultures of *Serratia marcescens* were employed to check the filters for defects prior to use. It was found that the elementary bodies passed through Berkefeld V filters as shown by inoculation of filtrate material into embryonated eggs, although concentration of the agent decreased 1000 LD_{50}. Results with a Berkefeld N filter showed that the agent was present in the filtrate in only 1 attempt out of 3.

A 10 per cent suspension in saline of yolk sacs from infected eggs was prepared and a portion centrifuged at 4000 R.P.M. for 1 hour while another portion was centrifuged at 6000 R.P.M. for 30 minutes. In each instance the supernatant was decanted and the sediment resuspended to the original volume in saline and then 1 ml. inoculated intraperitoneally into each of a group of 2 guinea pigs. Similar inoculations were made with the supernatant. Guinea pigs inoculated with the sediment showed fever after 1 day while those given the supernatant showed fever 7 days afterwards. The sediment showed numerous elementary bodies. Thus it appeared that the elementary body sedimented along with the infectious agent.

In further tests of physical properties of the agent, a 10^{-2} suspension was prepared of yolk sacs from infected eggs in sterile defatted milk, centrifuged at 2000 R.P.M. to remove coarse particles, and the supernatant then dispensed in 5 ml. amounts in thin-walled glass vials. After sealing, one or more vials were placed under several different conditions of temperature and after various intervals of time their contents tested in eggs for viability of the agent. The agent was found to survive a storage period of 3 days at 37°C., 10 days at room temperature, and 1 month at 4°C. Contents of other vials of the same suspension were tested after a storage period of 1 year at −70°C. and showed no loss in activity. When vials were heated at 56°C. for 30 minutes or 60°C. for 10 minutes, the agent was destroyed. Heating at 56°C. for 10 minutes did not inactivate the agent but decreased activity by 1000 LD_{50}.

Toxin Production.—Another property reported by Rake (10) for the psittacosis-lymphogranuloma group of viruses is the possession of an endotoxin.

In a test by Rake's method, heavily infected yolk sacs from recently dead or moribund embryos were harvested and made into a suspension with allantoic fluid and yolk material from normal 7 day old embryonated eggs. After centrifugation at 1500 R.P.M. for 10 minutes to remove coarse particles, the supernatant was decanted and twofold dilutions from 1:5 to 1:40 made with the normal egg material. Three week old mice were given intravenously

0.5 ml. amounts, using a group of 10 mice for each dilution. The mice were observed every few hours and all deaths noted.

All the mice that received the 1:5 dilution died within 5 hours after inoculation. Of the mice given the 1:10 dilution 66 per cent died in 12 hours, 79 per cent died in 24 hours, and 97 per cent within 36 hours. Only 23 per cent of mice inoculated with 1:20 dilution died within 36 hours while none of the mice that received the 1:40 dilution died. No mice died beyond 96 hours, and in the interval between 36 and 96 hours, all mice that were given the 1:10 dilution had died and an additional 60 per cent of the mice died that were given the 1:20 dilution.

In preparation of an antitoxin, a group of 3 rabbits was each given intravenously every 3 days for 3 weeks a series of inoculations that consisted of 0.3 ml. of a 10 per cent suspension in Ringer's solution of yolk sacs from recently dead or moribund embryos.

The inoculum was freshly prepared each time in order to prevent destruction of the toxin. Sera were collected 2 weeks after the last injection. From a normal rabbit, serum was obtained and used as a control. In the neutralization test undiluted immune serum was mixed with an equal amount of a suspension of yolk sacs to make twofold dilutions from 1:5 to 1:40 as used for the toxin tests. After standing at room temperature for 1 hour inoculations were made into mice. Similar preparations were made with normal rabbit serum.

It was found that the antisera prepared in rabbits prevented the deaths of all mice that were given the 1:10 and 1:20 dilutions and delayed death in those given the 1:5 dilution from 5 hours to 36 hours. Suspensions mixed with normal rabbit serum killed mice, as mentioned above for untreated toxin.

Effect of Chemotherapeutic and Antibiotic Agents.—Inasmuch as penicillin (11, 12), sulfonamides (13), and aureomycin (14) have been shown to inhibit various members of the psittacosis group of viruses, tests were undertaken to determine the effect of various chemotherapeutic and antibiotic drugs on this agent.

The tests were conducted in 7-day-old embryonated hens' eggs using the yolk sac route of inoculation. In tests of the various drugs 0.5 ml. of each concentration was inoculated into each of a group of at least 6 eggs and after a 30 minute period 1000 LD_{50} of the agent was inoculated into each egg. The eggs were candled daily for 12 days and deaths of the embryos noted.

The results, as are shown in Table I, indicated that both penicillin and aureomycin had a pronounced inhibitory action on this agent, while sulfonamides had little or no effect.

Inability of the agent to grow on bacteriological media, its tinctorial, morphological, pathogenic, and other properties indicated that it belonged to the psittacosis-lymphogranuloma group of viruses. This virus was obtained in guinea pigs and in eggs from feces of apparently normal calves. Since this virus was not present in stock guinea pigs or in uninoculated eggs, it appeared probable that it came from calves.

Experimental Infection in Cattle

Production.—As mentioned earlier, since the agent was procured from animals in all 6 herds tested in this vicinity and feces from 10 out of 15 calves were positive, purchase of suitable uninfected experimental animals seemed unlikely. This laboratory maintains a herd of cattle[2] under conditions designed to keep them free of accidental infection. Tests on feces and complement fixation studies of sera from many calves of this herd failed to show the presence of the virus in any animal. Accordingly, 5 calves were removed from the herd and placed in individual isolation units for experimental use. The animals varied in age from 4 to 9 months at the time of study.

TABLE I

Effect of Chemotherapeutic and Antibiotic Substances on the Agent Obtained from Calves

Drugs	Concentration per egg	Mortality ratio
Sodium sulfadiazine	2 mg.	5/5
	1 mg.	5/5
	0.5 mg.	6/6
	0.25 mg.	5/5
Penicillin	1000 units	0/5
	500 units	0/6
	100 units	3/6
Aureomycin	1 mg.	0/5
	0.5 mg.	0/6
	0.25 mg.	0/6
	0.125 mg.	3/6
	0.0625 mg.	6/6
Saline control	None	18/18

Numerator—number of embryos dead; denominator—number tested.

In attempts to produce infection, 2 calves, 2-48 and 2-49, were fed 20 ml. of a 10 per cent suspension in saline of yolk sac from eggs infected with strain III, and 1 calf, 2-45, was fed in a similar manner with a suspension of lung from mice also infected with strain III. In a series of exposure tests another calf, 2-47, was removed from its isolation unit and placed in the same pen with 2-45. Subsequently, calf 2-47 was placed in another isolation unit with still another calf, 2-55, to complete the effort to make 2 serial transfers by contact. Temperatures were taken and leukocyte counts were made daily on all animals. Feces from each calf were tested by guinea pig inoculation for the presence of the virus on 2 occasions before feeding or exposure and thereafter at least twice each week.

[2] Originally established by The Rockefeller Institute for Medical Research and given to Cornell University in September, 1948.

Virus was not present in the feces of any calf before feeding or exposure but appeared in the feces of the 3 animals that had been fed after 4, 8, and 9 days, and in the 2 exposed calves 10 and 25 days afterwards.

It thus appeared that infection not only occurred after ingestion of virus but was maintained through 2 serial passages by contact exposure.

Features of the Infection.—No signs of illness were observed in any of the inoculated or exposed calves and temperatures remained in the normal range. There was a rise above 30,000 in the leukocyte count for 1 day in 4 animals while the leukocytes of the other remained in the normal range. This increase occurred 1 to 11 days after inoculation and in 3 calves preceded by 2 or 3 days demonstration of virus in the feces. In the other, this time interval was 13 days. Although the leukocyte count did not increase in 1 calf, virus was demonstrated in its feces as readily as in the others.

Pathology.—Three experimental calves and 2 naturally infected calves were killed and examined. No lesions were found by gross observation. In histopathological studies on sections from the liver, spleen, kidney, various portions of the small intestine, cecum, and large intestine, no changes were found that could be attributed to action of the virus.

Location and Persistence of the Virus in Calves.—Since this virus was isolated from feces of calves, further tests were made to determine what portion of the intestinal tract was involved and if other organs were infected.

Portions of the intestinal tract at various levels, of the liver, and of the spleen were obtained from animals that had been shown to be infected. Each intestinal piece was washed free of gross fecal contamination by 3 washings in large amounts of sterile saline and then made into a 10 per cent suspension. Similar suspensions were made of the liver and of the spleen. Each suspension was tested in guinea pigs in the usual manner. When a guinea pig responded with a thermal reaction above 40°C. it was killed and inoculations made into 2 additional guinea pigs and into 6 embryonated eggs. This procedure provided sera from inoculated guinea pigs for complement fixation tests and yolk sacs for microscopic examination. From guinea pigs that did not show a rise in temperature, sera were obtained and they were tested for immunity. In this study 3 calves had been experimentally infected while 2 were naturally infected, making 5 in all. These results are shown in Table II.

It is seen in Table II that virus was isolated from the cecum in 4 out of 5, from the ileum in 2 out of 5, and from colon, jejunum, and duodenum in 1 out of 5 attempts. Virus was never isolated from rectum, spleen, and liver of any calf.

The feces of several calves, both experimentally and naturally infected, were tested for presence of the virus at more or less regular intervals during periods varying from 1 to 6 months while being held in strict isolation from other cattle. The results are tabulated in Table III.

It can be seen in Table III that virus was consistently present in the feces of an experimentally infected calf for a period of at least 6 months after inoculation and that virus persisted in 2 calves, that had been infected by contact, for a

period of between 2 and 3 months. In the feces of 2 naturally infected calves virus was present for at least 2 months, while in another for at least 4 months.

Complement Fixation Tests.—Tests were made with sera obtained from all experimental calves before inoculation and from 3 of the calves, 2-45, 2-47, and 2-55, at 15, 30, and 60 days after infection had been demonstrated.

Sera from 7 naturally infected calves were included.

TABLE II

Distribution of the Virus in Calves

Calf No.*	Organs and secretions tested								
	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum	Spleen	Liver	Feces
2-32	−	−	−	+	−	−	−	−	+
2-33	−	−	−	+	−	−	−	N.T.	+
2-45	+	−	+	−	−	N.T.	−	−	+
2-48	−	−	+	+	+	N.T.	−	−	+
2-49	−	+	−	+	−	N.T.	−	−	+

* Calves 2-32 and 2-33 were naturally infected while 2-45, 2-48, 2-49 were infected experimentally.

N.T.—not tested.

TABLE III

Persistence of Infection in Calves

	Calf No.	Time interval				
		1 mo.	2 mos.	3 mos.	4 mos.	6 mos.
Experimentally infected calves	2-45	+	+	+	+	+
	2-47	+	+	+	N.T.	N.T.
	2-55	+	+	N.T.	N.T.	N.T.
Naturally infected calves	2-08	+	+	+	+	N.T.
	2-32	+	+	N.T.	N.T.	N.T.
	2-33	+	+	N.T.	N.T.	N.T.

Antigen was prepared from yolk sacs heavily infected with virus (strain III) by the method described by Topping (15) for rickettsial antigens. To a 10 per cent suspension in saline of yolk sacs, an equal volume of diethyl ether was added. The aqueous phase was separated after shaking at intervals, centrifuged at 1500 R.P.M. for 20 minutes to remove tissue fragments, and then vacuum was applied to remove residual ether. A normal yolk sac antigen was prepared in the same way. Antigen titrations were made with a 1:10 dilution of sera pooled from 5 convalescent guinea pigs. Two units of complement, 2 units of amboceptor, and 2 per cent sheep cells were used in the test. Each component of the test was employed in 0.2 ml. amounts. Guinea pig sera were inactivated at 56°C. for 30 minutes and bovine sera at 60°C. for 30 minutes. Preliminary incubation was done at 4°C. for 18 hours, the hemolytic

system added and then incubated again for 30 minutes in a 37°C. water bath. The highest dilution of any antigen that showed 4+ fixation of complement was considered 1 unit of antigen. In complement fixation tests 2 units of antigen was used and the same dilution of normal yolk sac antigen served as a control. A serum to be tested was diluted twofold and the highest dilution that fixed complement completely was considered the end-point. The results for the 3 experimentally infected calves are tabulated in Table IV.

Sera from all the experimental animals showed no complement-fixing antibodies against this virus before feeding or exposure. As shown in Table IV, they developed complement-fixing antibodies after infection was established, and antibodies persisted for at least 60 days. Of 7 naturally infected calves, 2 had titers of 1:4, 2 of 1:16, and 3 of 1:32.

TABLE IV

Development of Complement-Fixing Antibodies in Calves Experimentally Infected

Calf No.	Serum before inoculation	Serum after infection*		
		15 days	30 days	60 days
245	0	1:4	1:16	1:32
247	0	1:4	1:4	1:8
255	0	1:8	1:16	1:16

* Count of days began after infection was demonstrated by guinea pig inoculation.

Comparison of Various Strains with Each Other and with Other Viruses

Although studies of the morphological and pathogenic properties of the strains of elementary body virus from calf feces showed them to be similar, further comparisons were made of 4 strains by cross-immunity tests, complement fixation reactions, and neutralization tests. Comparisons also were made with the virus of feline pneumonitis, the virus of lymphogranuloma venereum, *Rickettsia prowazekii*, *Rickettsia rickettsii*, and *Coxiella burnetii* by one or more of these tests. Guinea pigs were inoculated with each strain, allowed to recover, and 18 to 21 days later given a second inoculation with the same strain. After a further waiting period of 14 to 21 days, guinea pigs then were tested with each of the different strains in complete reciprocal cross-immunity tests. In addition, similar tests were made with *C. burnetii* and the virus of feline pneumonitis. Results are tabulated in Table V.

As can be seen in Table V, each strain of the virus from calves immunized against itself and against all the others. Although the virus of feline pneumonitis and *C. burnetii* immunized against themselves, they failed to provide an immunity against the virus from calves.

Complement fixation tests were made on each of the 4 strains of virus from calves, with the viruses of lymphogranuloma venereum and feline pneumonitis and with *R. rickettsii*, *R. prowazekii*, and *C. burnetii*. Each immune serum was titrated in serial twofold dilution in tests with its own antigen and with all

the other antigens. The antigens and immune sera for each strain of the calf virus, feline pneumonitis, and *C. burnetii* were prepared by the method described earlier, while the remaining antigens and immune sera were obtained through the courtesy of other laboratories.[3] All the sera used were of guinea

TABLE V

Cross-Immunity Tests with Strains of Virus from Calves and with Other Agents

Immune guinea pigs	Challenging virus					
	Strain I	Strain II	Strain III	Strain IV	F.P.	Q Fever
Strain I	0/8	0/3	0/6	0/4	N.T.	N.T.
Strain II	1/9	0/4	0/2	0/4	N.T.	N.T.
Strain III	0/10	0/2	0/3	0/5	2/2	10/10
Strain IV	0/5	0/3	0/3	0/3	N.T.	N.T.
F.P.	N.T.	N.T.	2/2	N.T.	0/2	N.T.
C. burnetii	N.T.	N.T.	8/8	N.T.	N.T.	0/10

Numerator—number of guinea pigs reacting to challenge with a rise in temperature following inoculation; denominator—number of guinea pigs tested.

F.P.—feline pneumonitis.

TABLE VI

Comparison of 3 Strains of Virus from Calves with Each Other and with Other Agents by the Complement Fixation Test

Agents tested	Antisera							
	Strain I	Strain II	Strain III	L.G.V. 1	L.G.V. 2	F.P.	*C. bur.*	Normal g.p.
Strain I	1:128	1:16	1:64	1:64	1:8	1:8	Neg.	Neg.
Strain II	1:32	1:32	1:64	N.T.	N.T.	N.T.	N.T.	Neg.
Strain III	1:64	1:16	1:32	1:64	1:8	1:8	Neg.	Neg.
L.G.V.	1:32	N.T.	1:8	1:64	1:32	1:4	Neg.	Neg.
F.P.	1:32	N.T.	1:8	1:32	1:8	1:8	Neg.	Neg.
C. burnetii	Neg.	N.T.	Neg.	N.T.	N.T.	Neg.	1:32	Neg.
R. prowazekii	Neg.	N.T.	Neg.	N.T.	N.T.	Neg.	N.T.	Neg.
R. rickettsii	Neg.	N.T.	Neg.	N.T.	N.T.	Neg.	N.T.	Neg.

L.G.V.—lymphogranuloma venereum.

C. bur.—C. burnetii.

pig origin except those of lymphogranuloma venereum which were from human beings. Tests were conducted as described above and the results are presented in Table VI.

[3] Dr. Geoffrey W. Rake, Director of The Squibb Institute for Medical Research, kindly supplied the lymphogranuloma venereum antigen and antisera. Dr. H. R. Cox of Lederle Laboratories Division, American Cyanimid Co., was also generous in supplying the rickettsial antigens.

Complement was fixed by each antigen of calf virus at approximately the same dilution with its own antiserum, with sera prepared against each of the other strains, and with antisera of the viruses of feline pneumonitis and lymphogranuloma venereum. Also antigens of the virus of lymphogranuloma venereum and of the virus of feline pneumonitis fixed complement with their homologous antiserum and with antiserum of each strain of calf virus in almost the same dilution. *C. burnetii* antigen fixed complement with its own antiserum but failed

TABLE VII

Comparison of Different Strains of the Virus from Calves with Each Other and with the Virus of Feline Pneumonitis by Neutralization Tests in Mice

Neutralizing serum		Virus		
		Strain I	Strain III	F.P.
Strain I	T.S.	0.9	1.1	16.5
	N.S.	12.8	12.4	1.0
Strain III	T.S.	1.2	1.3	17.0
	N.S.	12.5	12.2	0.5
Strain IV	T.S.	1.5	1.6	17.2
	N.S.	12.2	11.9	0.3
F.P.	T.S.	13.4	13.4	8.3
	N.S.	0.2	0.1	9.2
Normal serum	T.S.	13.7	13.5	17.5
	N.S.	0	0	0

T.S.—total score—an infectivity score was used to indicate the extent of disease (5 death; 4 consolidation of all lobes; 3, 3/4 of lung infected; 2,1/2 of lung; 1,1/4 or less of lung). The score for each dilution of virus was divided by the number of mice used at that dilution to provide a mean infectivity score. This score was added to give a total score for each serum tested.

N.S.—neutralizing score—difference between total scores of normal and immune serum.

to do so with antisera of the strains of calf virus, lymphogranuloma venereum, and feline pneumonitis. While it was not possible to test the antigens of *R. prowazekii* and *R. rickettsii* with homologous antisera, these antigens did not fix complement when mixed with any other antisera.

In preliminary studies with serum from guinea pigs that were convalescent from disease produced by inoculation of strains of calf virus, neutralization of calf virus was not obtained when tested in mice. Accordingly, antiserum was prepared in roosters by the method described by Hilleman (16) for the psittacosis group of viruses.

In groups of 3 roosters that were 6 months old, each was given intraperitoneally 2 ml. of a 20 per cent suspension in saline of yolk sacs from infected eggs. Inoculations were repeated at 3 day intervals until a series of 25 injections had been made. Antisera were prepared against strains I, III, and IV. Control birds were inoculated with a 20 per cent suspension in saline of yolk sacs of normal eggs. In addition, a group of 3 roosters was given a similar series of inoculations with a 20 per cent suspension in saline of lungs from mice infected with the virus of feline pneumonitis. The roosters were bled 1 week after the last inoculation and the antiserum of each strain of virus pooled, inactivated at 56°C. for 30 minutes, and tested for neutralizing capacity. Neutralization tests were conducted with strains I and III of the calf virus that had been adapted to mice, and with feline pneumonitis virus. Each virus was prepared in serial tenfold dilutions from 10^{-1} through 10^{-6} and a portion of each dilution mixed with an equal part of its own undiluted antiserum. Similar mixtures were prepared for all the other antisera in reciprocal neutralization tests. Groups of 6 mice were used for each serum-virus mixture and 0.05 ml. was given intranasally to each mouse. The mice were observed daily for 15 days and all deaths noted. At the end of this time all surviving mice were killed and examined for lung lesions. Extent of the lesions was scored similar to Horsfall's (17) method. The results for these tests are presented in Table VII.

It is seen that strain I immune serum neutralized strain I calf virus in significant amounts. Immune serum of strain III and IV also had definite neutralizing capacity against strain I virus. Results with strain III calf virus were the same in tests with each immune serum. Feline pneumonitis immune serum had no effect on either strain I or strain III of the calf virus, although it did neutralize its own virus in significant amounts.

DISCUSSION

The evidence produced permits the conclusion that the agent obtained in guinea pigs and in embryonated eggs from feces of apparently normal calves is a virus that produces elementary bodies. Furthermore, its morphological, pathogenic, and serological properties place it in the psittacosis-lymphogranuloma group of viruses.

Experimental work with calves has shown that this virus induced an infection which seemed identical with the natural infection, inasmuch as no visible signs of illness were produced, virus was recovered from the intestinal tract only, and persisted for a long period of time. That this virus readily established itself in calves was shown by the production of infection by feeding. Furthermore, infection was maintained in calves through 2 serial passages by contact. It appears that ingestion of virus previously disseminated in feces from infected animals represents the natural means of spread. The method of spread and persistence of virus in infected calves suggested that the virus should be widespread and more than 60 per cent of the calves tested in this vicinity were found to be infected.

Comparative studies revealed that the virus shared an antigen or antigens in common with other members of the psittacosis-lymphogranuloma group of viruses (18), but, since it came from a new host species, efforts were made to

determine whether it represented a new member . It was found by neutralization tests to be distinct from the virus of feline pneumonitis. It differed from all other members of the group in that it did not readily establish itself in mice by inoculations intranasally, and from the viruses of psittacosis, lymphogranuloma venereum, and meningopneumonitis by its inability to produce disease in mice by inoculations intraperitoneally or intracerebrally. In addition, failure of the virus to be inhibited by sulfonamides assisted in separating it from the virus of mouse pneumonitis. Further evidence of individuality was its ability to transfer serially in guinea pigs and the characteristic pathology it produced. Also, its rather specific trophism for the intestinal tract and particularly the cecum of calves was different from the tissue affinities of other known viruses in this group. It appears, therefore, on the basis of this study that the virus from calves represents a new member of the psittacosis-lymphogranuloma group and, in keeping with the classification in Bergey's Manual prepared by Rake (19), it is suggested that this virus be called *Miyagawanella bovis*.

SUMMARY

From portions of intestine and feces of apparently normal calves, a virus that produces elementary bodies was procured in guinea pigs and in embryonated eggs. Morphologically and tinctorially this virus closely resembled members of the psittacosis-lymphogranuloma group of viruses and it shared a common antigen or antigens with them. Comparison of serological, pathogenic, and other properties indicated that this virus from calves is a new member of the psittacosis-lymphogranuloma group and in keeping with classification practices it is provisionally named *Miyagawanella bovis*.

Miyagawanella bovis when fed to experimental calves established an infection in the intestinal tract that resembled the inapparent infection seen in natural cases but failed to produce evident disease. Ability of the virus to infect experimental animals by feeding, and its presence in feces of infected animals indicate its natural mode of spread. This method of dissemination and persistence of virus for long periods of time in infected animals suggested the virus should be widespread and more than 60 per cent of the calves in the vicinity of Ithaca were found to be infected.

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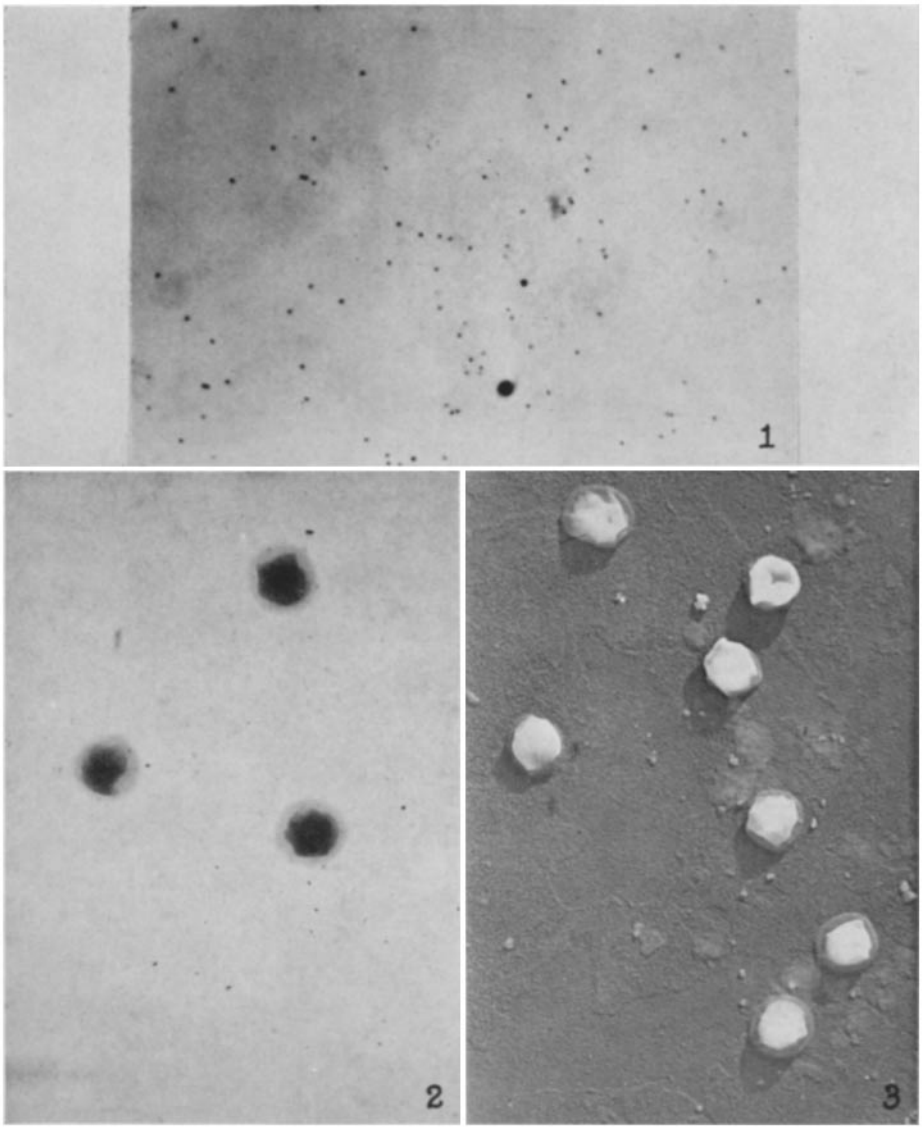
EXPLANATION OF PLATE 51

FIG. 1. Film of partially purified suspension of yolk sac from infected eggs showing elementary bodies of the calf virus. × 1492.

FIG. 2. Unshadowed electron photomicrograph of elementary bodies of the calf virus. × 25,000.

FIG. 3. Uranium-shadowed electron photomicrographs of elementary bodies of the calf virus. × 25,000.



(York and Baker: New virus of psittacosis-lymphogranuloma group)