TRANSMISSION OF VISCERAL LYMPHOMATOSIS FROM FIELD CASES

by

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INTRODUCTION

Visceral lymphomatosis is a neoplastic disease in domestic fowl characterized by the accumulation of large numbers of lymphoid cells in one or more visceral organs, and caused by a virus-like agent. Terms which have been used synonomously are lymphatic leucosis (55), lymphocytoma (57), hepatolymphomatosis (66), hemocytoblastoma (78), lymphadenoma (88), and lymphosarcoma (97).

The various forms of the avian leucosis complex gave rise to many names and synonyms in the evolution of the knowledge of the diseases, and in 1941 (Jungherr, et al., 80) a tentative pathologic nomenclature was set forth. In that classification, lymphomatosis was designated as one of four types of the avian leucosis complex; the others being erythroblastosis, granuloblastosis, and myelocytomatosis. These workers further classified lymphomatosis as neural, ocular, visceral, and osteopetrotic. The visceral and neural forms are the most important of the group and occur more frequently than do the other types.

It was estimated that in 1953 (105) more than 53 million mature chickens died of this disease with a total value of at least 73 million dollars. These figures do not take into account impairment of growth, reduction in egg production, and loss of housing space.

This study was undertaken to isolate strains of the virus of visceral lymphomatosis from farm flocks in Kansas and to study the pathological changes produced by these strains.

Infective material was collected from field cases and transmitted to susceptible chickens. These birds were housed at the Veterinary Research Laboratory and Poultry Farm at Kansas State College.

REVIEW OF LITERATURE

History

Visceral lymphomatosis of fowl is a known neoplastic, malignant disease that is spread by contact, although the leukemias and sarcomas of the Rous type are also caused by highly infectious viruses.

Leukemia in man was described by Virchow (quoted by Ellerman, 55) in 1845. His original name for the disease was "leucaemia" and this condition was characterized by the presence of excessive numbers of primitive malignant cells in the blood stream. It was recognized that a leucemia did not always accompany a disfunction of the hematopoietic organs and that this condition sometimes occurred only in the terminal stages of the disease. For this reason Chubb and Gordon (39) preferred the term leucosis, indicating that the disease complex is not solely associated with malignant leucemias.

They credit Roloff as reporting the first case of leucosis in 1868 which was termed "lymphosarcomatosis." Most authors (39, 90) reported that fowl leukemia was first described by Caparini in Italy in 1896 and probably existed in fowls many years before this. Lee, et al. (85) stated that in 1907 Marek

reported on fowl paralysis in Hungary. The first report on this condition in the United States was made by Butterfield (34) in 1905 when he reported it in the District of Columbia and in Michigan. Rous and Murphy (101) in 1914 reported on three transmissible avian tumors which they had studied. These tumors were described as (a) spindle-celled sarcoma, (b) osteochondrosarcoma, and (c) a spindle-celled sarcoma fissured by blood sinuses. The agents causing these conditions were passed through Berkefeld cylinders which suggested that they were of similar size and class. Each agent was a distinct entity since each gave rise only to growths of the kind from which it was derived.

Another transmissible strain of fowl tumor (leukosis) was reported in 1915 by Schmeiser (102) which was the same year that Olson (90) reported that Magnusson also demonstrated transmission of the disease. The term "leucosis" was first introduced by Ellerman in 1920 (quoted by Ellerman, 55). The term was used to describe that group of diseases affecting fowl which was characterized by neoplasia of the hemopoietic tissue. Ellerman (55) presented a comparative description of the three forms of leucosis which were later called erythroid, myeloid, and lymphoid leucosis. The third general type, lymphatic leucosis, was reported in 1922 to be caused by a filtrable agent. The name lymphocytoma was given this disease by Feldman (57) in 1932.

Fowl paralysis (neural lymphomatosis) was prevalent in the station flock of the Massachusetts Experiment Station (105) from 1913 to 1920 and caused a 25 per cent mortality in 1918-19. The disease was characterized by extreme emaciation, possibly due to

paralysis of the digestive tract. In 1921 it was reported (Kaupp, 83) that 15 cases of paralysis had been studied in seven states since 1914. Fowl paralysis was reported in Holland in 1924, Germany and South Africa in 1927, Japan in 1930, Italy in 1931, and in Australia in 1932 (Chubb and Gordon, 39).

In a report by Pappenheimer, et al. (93) in 1926, they stated that fowl paralysis had occurred in all parts of the United States and probably South America, and was endemic in certain areas. Symptoms at that time appeared in affected birds from three to 18 months of age and in some cases, ocular lesions were present. Visceral lymphomata, originating in the ovary, were associated in a certain per cent (10 per cent in one experiment) of the cases. There was evidence that this association was not accidental, but a manifestation of the disease. They suggested the name "Neurolymphomatosis gallinarum."

In studies (94) of lymphomatous birds, it was found that there was not an excessive proportion of lymphoid cells or other leucocytic elements in the vascular blood, even in situations where the infiltration of tissues was most intense. Therefore, the lesions of lymphomatous birds were not associated with a leucemic blood picture. The occurrence of numerous mitoses were considered proof that the cells proliferated in situ and were not derived from the circulating blood.

During the 1920 decade (105), various forms of lymphomatosis were present throughout the United States. Furth (61) commented in 1929 that "It is commonly accepted that lymphomatous tumors constitute the most common neoplastic disease of fowl," but did

not support Ellerman's claim that the same agent caused myeloid and lymphoid leucemia. According to the U.S.D.A. Circular No. 970 (105), neural lymphomatosis caused its highest mortality prior to 1930, and visceral lymphomatosis was much more prevalent after that date. The visceral form (105) is now responsible for approximately 66 to 75 per cent of the losses due to the lymphomatosis. Other forms have never caused a high death rate in the United States.

In a discussion of the etiological concepts of the leucosis complex, Cottral (43) pointed out that prior to 1932, two groups of workers and theories existed. The fowl leucosis group included erythroblastosis, granuloblastosis, myelocytomatosis, and visceral lymphomatosis under the term of fowl leucosis. Ellerman (55), who was a proponent of this group, believed that all these diseases were caused by the same agent. The fowl paralysis group eventually included ocular and visceral lymphomatosis, in addition to neural lymphomatosis, in their concept of fowl paralysis because of the frequent association of the three.

A unitarian view (Johnson, 76) was taken by some in 1932 when it appeared that the common cause of leucosis and lymphomatosis of fowls was a filtrable agent. Those opposed (58, 59, 65) believed that each of the various diseases had its own distinct etiological agent. It was evolved later that there was a type of visceral lymphomatosis (VL) associated with neural and ocular lymphomatosis and another type associated with erythroblastosis and granuloblastosis.

"The avian leukosis complex" was suggested as a term to include the entire group of diseases in 1941 (Jungherr, et al., 80). The classification at that time was an attempt to arrive at a uniform terminology and did not imply any etiological relationships. The terminology became well accepted in the United States even though each group of the leucosis complex may be separate and fundamentally distinct pathologically (8, 35, 36, 37, 48, 111).

Campbell (37) favored the separation of fowl paralysis, ocular lymphomatosis, and osteopetrosis from the leucoses as distinct and unrelated conditions. He maintained that fowl paralysis is not a neoplastic condition but one of a chronic inflammatory nature and that the term "leucosis" be applied only to the neoplastic condition of the hematopoietic tissues. Three groups would then be designated as lymphatic leucosis, myeloid leucosis, and erythroleucosis, depending upon the type of cell involved.

The English workers, Chubb and Gordon (39) and Campbell (37) believed that visceral lymphomatosis, as it is known in the United States, should be divided into two distinct groups; one called visceral lymphomatosis and the other lymphoid leucosis (lymphocytoma). Campbell stated that VL is an inflammatory condition with endothelial proliferation, lymphoid hyperplasia, and areas of necrosis with the infiltrating cells being similar but distinguishable from lymphoid leucosis. They proposed the following classification:

Avian Leucosis and Lymphomatosis

(Modified conception)

Leucosis	(Erythroleucosis		
	(Myeloid leucosis	(1)Diffuse	(2)Discrete (myelocytoma)
	(Lymphoid leucosis	(1)Diffuse	(2)Discrete (lymphocytoma)

Lymphomatosis	(Visceral
	(Neural (fowl paralysis)
	(Ocular

Osteopetrosis

Campbell (37) stated that

> Fowl paralysis is typically a chronic disease associated with inflammatory infiltrations in the nerves and viscera, and a progressive though not neoplastic accumulation of cells of the lymphoid series which eventually produce tumour-like lymphogranulomata.

He based his opinion, in part, upon the absence of blood and marrow involvement and lack of conclusive evidence that leucosis has ever resulted from experimental transmission of fowl paralysis material. It was suggested that the "Burmester transmissible lymphoid tumour" might be an atypical or myeloid leucosis.

Visceral lymphomatosis of chickens bears a close resemblance to the lymphoblastoma group of mammals which includes lymphocytic leukemia, lymphosarcoma, Hodgkin's disease, reticulum cell sarcoma, plasma cell myeloma, and lymphocytoma (Cottral, 44). Comparison is only on their pathological similarities and not on interspecies etiological relationship. Chickens have a much higher incidence (105) of lymphoblastomas than any other animal. The reasons for this are that chickens are exposed at one day of age to large populations of their kind from a variety of sources and commonly raised in crowded quarters.

The economic importance of the leukosis complex to the poultry industry was recognized, and the Regional Poultry Research Laboratory at East Lansing, Michigan was established in 1939 by the authority of Congress to "study the problem of viability of poultry, the first emphasis of which was placed on the avian-leukosis complex" (Winton, 112). The objectives were to determine the etiologic agent of the complex, develop control procedures, and discover prophylactic techniques for the prevention and control of the avian leukosis complex. Rigid quarantine measures and sanitation programs were initiated and maintained.

Characteristics and Properties of the Virus

Ellerman and Bang, in 1908 (as quoted by Ellerman, 55) reported that they had transmitted fowl leukemia with a filtrate. Since that report, others have confirmed this work (Jungherr, 82). The agent was found to be capable of multiplication; of being inactivated by ultra violet light, heat, and x-ray; of survival during storage at low temperatures; and of not being inhibited by penicillin, streptomycin, and sulfamerazine (Burmester and Cottral, 12).

Electron microscopic studies (Burmester, et al., 32) revealed virus particles approximately 90 millimicrons in diameter, with a centrally-located zone approximately 30 millimicrons in diameter and of greater density than the peripheral area. Destruction of cytoplasmic elements was characteristically found in cells of the liver and spleen of birds affected with visceral lymphomatosis. The cytoplasmic changes and virus particles were

not found in control birds.

The agent of VL has been demonstrated (Cottral, et al., 45) in tumor-free birds, therefore, it is not necessary for a donor bird to show evidence of the disease to transmit the entity. Cells from lymphoid tumors have been successfully transplanted (Cottral, 44) to the embryonating egg, and tumors developed in about six days. Osteopetrosis has been transmitted with VL (Burmester and Gentry, 26) and has been postulated to be of virus etiology. There appears to be an age resistance (71, 73, 109) to VL since delaying exposure of the chicks to VL until a month of age decidedly reduces the incidence.

The incubation period for VL is highly variable. If a group of chicks are exposed during the first week of life, the first deaths can be expected around 40 to 50 days, and the rate increases up to about 200 days. After that, a few birds die up to five years of life. Environment plays an important role in the incidence of this disease. The greatest mortality is experienced when a large number of birds are raised on a litter floor, and the least mortality when smaller groups of birds are raised on wire floors in more isolated areas. However, repeated inoculations of birds does not significantly increase the incidence of disease. Cottral (44) suggested a "trigger factor" which is capable of precipitating the tumor formation in the bird infected with the etiological agent.

The causative agent has been demonstrated (21) in the egg, nasal and oral washings, and feces. Chicks may be infected by several routes, including aerogenic, conjunctival, nasal, oral,

cloacal, and parenteral inoculations (6, 16).

In 1932 it was reported (Furth, 64) that the agent of fowl leucosis resisted drying and retained its activity at least 54 days. Preservation could be accomplished up to 104 days by adding glycerin. The agent was not inactivated by freezing in liquid air. By holding the agent at 37.5° C. for 14 days, the activity was lost and some activity was lost by holding it at 4° C. for the same period.

In reports (6, 16) on the viability of a transmissible fowl tumor upon storage at low temperatures, results indicated that such storage did not significantly reduce or alter its capacity to produce typical tumors. The lymphoid tumors were grown in the pectoral muscles of chickens, excised aseptically, and minced before placing in pyrex glass tubes and lusterloid tubes. Only the pyrex tubes which were sealed with a gas-oxygen flame proved satisfactory. The sealed tissues were frozen at the rate of one degree per minute from 0° C. to -20° C., by the use of CO_2 and 95 per cent alcohol, and held in a CO_2 ice box at temperatures no higher than -65° C.

Immunological Research

In a series of experiments showing the effect on immunization against a transplantable lymphoid tumor upon subsequent incidence of VL, Burmester, et al. (9) reported that hyperimmune plasma used in chicks injected with the same tumor strain, reduced the mortality in the test chicks. In the same report, it was indicated that chickens immunized against cell transplants of

lymphoid tumor strains from naturally-occurring cases with a cellular product were no more resistant than nonimmunized chickens with similar parentage and husbandry.

Cross immunity (Burmester and Belding, 11) between some lymphoid tumor strains was demonstrated. It was suggested that birds surviving a lymphoid tumor implant are highly resistant to other lymphoid tumor strains of equal or lower malignancy, but are still relatively susceptible to implants of strains of a higher order of malignancy.

Normal tissues, especially spleen and embryo skin, will increase resistance (Lumsden, 87) to many but not all transplantable tumors when injected into susceptible animals. In the cross immunity studies above, injection of normal tissue suspension did not increase measurably the resistance to implants of lymphoid tumors.

In virus neutralization studies reported (Burmester, 23) in 1955, it was found that antibodies which neutralize the oncogenic VL virus could be produced. In vitro studies in which 9 milliliters of the preparation and 1 milliliter of serum were mixed prior to inoculation showed at the log dose of -9 practically all of the virus was neutralized. At least 99 per cent of the virus was neutralized at log doses of -7 and -5. At log dose of -3, it was believed that there was delay in mortality, indicating a partial neutralization.

In the in vivo studies of the same experiment, chicks were given 2 milliliters of immune serum during the first three days of life and later challenged with the VL virus. In the chicks

receiving the immune serum, only those challenged with a dose of log -3 developed visible VL. These three birds died late in the experimental period, resulting in longer mean latent period than that of any other lot. It was concluded that serum from hens injected into chicks produced a passive immunity.

The first direct evidence that a significant immunity (Burmester, 22) to VL could be demonstrated was reported in 1955. Indirect evidence was presented which indicates that the resistance is due to serum antibodies of the usual type. A group of 14 hens received a series of inoculations with VL virus. Their progeny before and after the inoculations were tested for susceptibility to a strain of virus of known potency. There was a marked difference in the resistance exhibited by the "before" and "after" groups of chicks. It seems probable that the difference was due to the antibodies produced by the dam in response to the series of inoculations, and passed on to the chick via the egg. Vaccination of hens (4, 81) in relation to other viral diseases, has resulted in a passive immunity in the chick.

Parental immunity was studied (30) at the Regional Poultry Research Laboratory when three hatches of chicks were obtained from nine lots of pullets which had been treated by different immunizing procedures. Chicks of hens that had been injected seven times intraperitoneally with live virus were about 3000 times as resistant as chicks obtained from the same hens before immunization. Similar results were obtained in chicks whose dams were given four intramuscular injections or two injections with an

adjuvant. Heat-killed virus increased the resistance by a factor greater than 10. Formalin-killed virus or virus treated with beta propriolactone increased the resistance in the chicks from 200 to 600 times.

Several vaccines (31) for VL were prepared and tested. The RPL-12 strain was the source of vaccinal material and was prepared from lymphomatous livers as a filtrate. The different preparations were administered to hens and their progeny subjected to challenge with dose logs -2.5, -4.5, and -6.5 of the RPL-12 strain of VL virus. It was found that when hens were injected several times with the fully-virulent virus or two times when mixed with an adjuvant, their progeny were more resistant than the control birds. A formalin-killed virus preparation and one treated with beta propiolactone provoked an immune response which resulted in significant passive immunity in their progeny, but not to the extent of that obtained by the live virus preparation. The filtrate was heated to 56° C. for 30 minutes for one preparation and no significant immunity resulted. It was concluded that the VL virus is essentially no different than other viruses with reference to immune responses.

Workers at Cornell University (74) have been conducting experiments since 1935 in an effort to develop strains of fowl genetically resistant to the avian leukosis complex. Their objective was not only to develop resistant stock but also to select for such things as high egg production, adequate egg size, body size, and ability to reproduce. Birds under test were

naturally exposed during the first two weeks of life and held to 500 days of age.

Two strains of White Leghorns have been developed (Hutt and Cole, 74) which are relatively resistant since the mortality rate has been reduced (0.8 to 1.5 per cent). A strain of susceptible birds has also been developed and has an average mortality of 38.8 per cent, much of it the neural type. They also recommend isolation of baby chicks for the first few weeks of life as a control measure. It was concluded that egg transmission is relatively unimportant compared to genetic susceptibility and severity of exposure to the disease after hatching.

Transmission Studies

Egg Transmission. Natural transmission of visceral lymphomatosis can be conveniently viewed from two aspects; one being egg transmission and the other contact transmission. It was suggested by Patterson (96), as early as 1936, that all forms of lymphomatosis were spread by direct contact from bird to bird and by indirect contact with infected litter. Since that time, others (3, 5, 20, 25, 84, 110) have demonstrated transmission from inoculated birds to their contact controls.

Egg transmission (quoted by Jarmai, 75) was investigated as early as 1932 when 20 chicks were hatched from an infected hen. Twelve of these lived to a year or more of age and none of them showed evidence of leukosis. Jarmai, et al., reported that due to the incubator temperature, the causative agent lost its potency in the chicken egg. In a later experiment (75) he injected the

agent of leukosis into eggs on the first, fifth, eighth, twelfth, and thirteenth days of incubation. Chicks with leucosis were hatched only from eggs injected after the tenth day of incubation. He concluded that the agent was not capable of developing until the myeloid tissue of the bone marrow was present.

Chicks hatched from eggs in which the leucosis agent had been carried in serial passage (Van der Berghe and d'Ursell--quoted by Olson, 90) developed a paralysis of the legs within the first three days after hatching. This was suggested as evidence of the ability of the leucosis agent to cause fowl paralysis.

The hatching period is important, as demonstrated at the Regional Poultry Research Laboratory (25) when they hatched in the same incubator, chicks from an infected source and those from a disease-free strain. There was an increase in visceral lymphomatosis in the disease-free chicks; however, a higher rate of transmission occurred during the brooding period. Debri from the above-mentioned incubator was used as an inoculum and caused a high incidence of disease in inoculated birds. Since there can be hens which have a latent or inapparent infection (Cottral, et al., 45), it follows that when their progeny are incubated and hatched with susceptible chicks, the susceptible chicks become exposed to the virus of VL.

The first direct evidence (45) of egg transmission of VL in chickens was reported in 1954. Eight experiments were conducted, testing certain embryonic, chick, and adult bird tissues for the presence of the causative agent. This was accomplished by injecting the tissues to be tested into susceptible chicks and observing

the incidence of VL. The results indicate that at least five of the 12 dams reared in infected quarters, and possibly two of nine dams from isolated pens were carriers of the disease agent and were capable of infecting their offspring. All the dams were clinically normal at the time the test eggs were produced, indicating that normal-appearing chickens are carriers of the VL agent.

Waters' report (107) in 1945 involved over 4,500 White Leghorn chickens which were subjected to several different environmental conditions, to study natural transmission of lymphomatosis. The condition was observed in chickens under four months of age even though there had been no previous direct contact with affected birds. Evidence supporting egg and contact transmission was presented from another part of the work. By careful selection and testing of certain families which were hatched and reared in isolation and kept under rigid quarantine, the occurrence of lymphomatosis was prevented to at least 700 days of age.

In a later experiment at the Regional Poultry Research Laboratory (24), infectivity tests were conducted by using 15-day embryos of various selected dams. The homogenate of the pool of livers from 15-day embryos from each of 22 dams was injected intraperitoneally into groups of 50 susceptible chicks. The resulting data show that the higher proportion of shedders were from lines having a high natural incidence of VL, and the proportion of shedders was much less in the line having a low natural incidence. The shedding of the virus was directly related to the incidence of VL in the sibs of the dams under test; however, its

relation to disease in the progeny was not.

Cottral (45) reviewed the problem of egg transmission and classified the types of studies associated with the problem. Epiornithological observations indicated the spread of VL through the use of hatching eggs from infected flocks, as did flock isolation studies. Egg transmission may have occurred in some family isolation studies and was indicated in incubator exposure studies. Histological studies indicate that normal cells of the dam can be transmitted to the egg by way of blood spots.

It was suggested by Burmester (24) in 1955 that the importance of egg transmission is not in the direct effect on the progeny to which the infectivity is passed, but to other susceptible birds in which the infected chicks might come in contact. It was pointed out that the progeny of infected hens could possibly have a passive immunity to the agent which protects them through the ordinarily susceptible age and remain in a carrier state.

Cornell investigators (40, 41, 72) maintain that egg-borne transmission is of little importance in the natural transmission of leukosis. It was noted (24) that about 75 per cent of lymphomatosis at Cornell was of the neural type and that about 75 per cent of lymphomatosis at the Regional Poultry Research Laboratory is of the visceral type. Since there is evidence (10, 20, 49, 111) that the two types have separate etiological agents, it was suggested that conclusions drawn from data on one type might not necessarily apply to the other.

In egg transmission studies reported (Burmester and Waters, 25) in November 1955, results suggested that the brooding period is far more important than the hatching period in the transmission of VL from infected to noninfected birds when not hatched on the same levels in the incubator. This is the case where chicks from shedding hens are hatched and brooded with chicks whose dams have had no experience with VL. Further, to attempt transmission in the incubator, an aerosol of known viral activity was administered and susceptible chicks could not be infected. However, it must not be forgotten that direct chick-to-chick transmission can occur in mixed (infected and susceptible) populations. With this evidence in mind, the management procedure would be to hatch and brood chicks from separate flocks in different incubators and brooders.

In the same report, another experiment indicated that there was no significant difference between the progeny of shedders and the progeny of nonshedders with relation to incidence of VL. The hens which had previously been classified as shedders, passed the VL agent to the chick through the egg but this did not necessarily result in a high incidence of neoplasia.

It was suggested by Cottral (42) that an important aspect of egg-transmitted diseases might be in the use of contaminated egg-propagated vaccines. Newcastle virus has been demonstrated (70, 113) as a contaminant in fowl pox and laryngotracheitis vaccines. It seemed probable that VL could be transmitted in such a way provided that susceptible birds were involved. A report (Piedrafita, 98) on the spread of VL through the use of a formalized Newcastle

disease vaccine was discounted (Burmester, 18) since the VL virus is quite susceptible to formalin and ultraviolet light.

In three of four experiments (Burmester, et al., 27) at the Regional Poultry Research Laboratory, over a five-year period, VL was transmitted through the use of Newcastle disease vaccines. In experiments one and four, the NDV vaccine was prepared from embryonated eggs from known shedders of VL virus and produced disease at a significant level. In experiments two and three, commercial live virus Newcastle disease vaccines were used. A highly significant incidence of VL was seen in one of the three vaccines used in experiment three. Experiment two did not show significant differences. It was concluded that if NDV vaccines were sufficiently contaminated with VL virus and highly susceptible stock was vaccinated, a significant incidence of VL would occur.

Carr (38) emphasized the desirability of breeding only from two-year-old birds for obtaining replacement stock in controlling VL. Subsequent experiments (Burmester and Waters, 28) indicated that hens which are shedders of the virus of VL do shed less at an older age, but the age at which this occurs is variable. This information is based on data which were obtained on eggs laid at five different periods during two years by hens of three inbred lines, and on eggs laid by the progeny of some of the hens employed in the first series. The decrease in the shedding in the hens of the first series occurred during the age period of 18 to 24 months and in the progeny group at about 9 to 15 months of age. It was observed that the decrease of shedding happened at about the same time for both groups and was suggested that the environmental

conditions might have been a factor.

Contact Transmission. Saliva of infected baby chicks contains infectious levels of the virus (Burmester, 33) of VL through the first three months of age. This is true whether exposed by contact or from the egg. The VL virus can be spread by normal-appearing hens as well as diseased hens by way of the egg, saliva, and feces. The drinking fountain has also been proven to be one means of disseminating the disease. It was found that about one-third as many contact control birds became infected when watered by a rapidly-flowing fountain as contact controls in a similar lot watered by a reservoir-type waterer. In another pen, the virus was placed directly into a nonflowing water fountain and results paralleled the outcome of the pen with the reservoir type waterer. Since the saliva of day-old chicks hatched from infected hens contains the VL virus, reservoir-type waterers could be a likely avenue for infecting susceptible noninfected chicks. It was found in other contact transmission studies (Burmester and Gentry, 20) that the number of contact birds in which VL is produced is directly related to the number of infected individuals in the flock.

A study (21) concerning the infectivity of the virus of VL by different routes, using a liver filtrate and oral washings, revealed that any mucous membrane normally exposed to the external environment may be invaded by the viral agent of VL. The respiratory tract was especially susceptible, but in all cases the dose required was considerably more than that required

intraperitoneally to produce the disease. The tumor filtrate, when injected intraperitoneally, induced the disease in 82.6 per cent of the birds in a 270-day experimental period. Percentages by other routes with the same inoculum are as follows: tracheal, 83.1 per cent; nasal, 73.0 per cent; cloacal, 57.6 per cent; conjunctival, 47.2 per cent; oral, 45.2 per cent; aerogenic, 39.2 per cent; and esophageal, 7.7 per cent. A significant incidence with the use of oral washings was evident only by intraperitoneal injections.

Inoculation Transmission. Patterson, et al. (95) successfully transmitted leucosis in 1932 with the use of cellular inocula prepared from the viscera of affected birds, and the injection of one type apparently produced most of the other types that are considered expressions of leucosis. Their Berkefeld filtrate transmission experiments were inconclusive. They noted that the clinical course was variable, extending to over a year, and that complete recovery of a positively-diagnosed case was rare. It was observed that there was occasional improvement or arrested progress in some cases. The statement is made that "the pathologic manifestations resemble that of a malignant neoplastic process very much, although their close resemblance to an inflammatory one is very striking at times." This is of importance since some English workers regard several of the "leucotic" conditions as being inflammatory in character.

Johnson (76) reported on fowl paralysis in 1932 and suggested the condition was due to an infectious agent which reached the

chick soon after hatching. He believed that many birds which were slightly affected developed an immunity and others developed other forms of leucosis. In egg transmission experiments, it was found that many of a normal hen's progeny developed paralysis. This was thought to be due to a transmitted weakness or susceptibility to the agent. This theory was favored over the possibility of the hen being a carrier.

Furth (65) transmitted lymphomatosis in 1933 by the use of plasma and an emulsion of tumor cells. Of the birds injected with cellular material, 131 of 294 developed leucosis and 60 of the 131 had lymphomatosis. Inoculations with plasma which had not been filtered were successful in approximately 36 per cent of the injected birds and in about 21 per cent of the ones injected with filtered plasma.

Limited numbers of birds were used in experiments in 1934 (Johnson, 77) in which tissue cell suspensions, blood, and filtered blood were employed for inocula, and the results of these experiments presented. Evidence supported the view that neuro-, erythro-, and myeloid-leucosis are different expressions in response to a common filtrable agent. Johnson suggested "that heterotopic blood cell formation may occur in some of the visceral organs as lungs, liver, suprarenals, and kidneys where small amounts of reticular stroma and diffuse lymphoid tissue are present which might be stimulated to hyperactivity by the effects of this agent."

Six transmissible strains of leucosis were established (Jungherr, 79) out of seven transmission trials in 1937. Four of these strains were carried through repeated passages. There were approximately 38.2 per cent takes out of 128 injected chicks. An ultramicroscopic agent was suggested and was shown to be present in blood, bone marrow, nerve, and affected iris. Natural transmission by ingestion of contaminated feces was considered as a possibility. Lymphocytoma of the liver was considered to be nontransmissible and a restrictive term, hepatolymphomatosis, was used to denote the condition.

Cellular inocula was prepared from liver, nerve, ovary, and kidney in experiments in Iowa (Lee, et al., 86) reported in 1937 and transmission of the disease did occur. A filtrate of the above kidney tissue also was prepared and inoculated. About 25 per cent of the birds were affected and there was evidently little difference in the use of Seitz or Berkefeld 3N filters when results were compared. Litter exposure contact over a period of 19 months elicited fatal lesions in 50 per cent of the test birds. Litter had accumulated for two years from affected birds, and susceptible chicks were raised on this litter only after it was dried by raking. Their conclusions were that young chicks are more susceptible than older ones and that the majority of cases occurred between four and ten months. They stated that "neurolymphomatosis gallinarium, associated with eye lesions, hemocytoblastosis, lymphoid, erythroid, and myeloid types of leukosis are different expressions of the same disease and were transmitted by a common etiological agent" and that "this disease is one of the most

important diseases confronting the poultry industry. The most important methods of control are proper sanitation and the use of breeding stock from resistant sources."

It was reported (Stubbs, 104) in 1939 that the shortest period of incubation in baby chicks was an average of 19 days. The highest incidence of successful transmissions and highest mortality was in baby chicks, and all breeds were susceptible. It was found that guinea fowl, turkeys, pheasants, pigeons, ducks, and geese were not affected in transmission experiments. The pheasant-chicken hybrid was susceptible but the condition could not be transferred to pheasants.

Missouri workers (Durant and McDougle, 52) noted that a difficulty in the study of fowl paralysis was that of obtaining a virus that could be transmitted in a high percentage of cases. In their experiment, they transfused whole uncitrated blood from chicks of dams showing ocular lesions, into day-old chicks. Their rate of transmission was 17.1 per cent of 527 chicks inoculated. In the control birds, 3.55 per cent developed the disease. It was stated that it appeared that the "true fowl paralysis virus might cause tumor formation in the ovary, adrenals, and other organs."

In 1945, Durant and McDougle (53) reported further on transmission of fowl paralysis by direct transfusion. They found that birds near six months of age, when visibly affected, are suitable donors and that White Leghorn chicks one to four days old are more receptive to the virus than White Wyandotte chicks of the same age.

Therefore, whole blood, blood plasma, ascitic fluid, or emulsions of organs were used prior to 1940 to transmit leukosis to susceptible birds (1, 55, 56, 61, 62, 63, 75, 89, 102). The infective material was injected either intravenously, subcutaneously, intramuscularly, or intraperitoneally, and disease was produced.

De Ome (50) pointed out that the main properties of a neoplastic growth are: (1) uncontrolled and unorganized growth; (2) metastasis; (3) invading, displacing, or replacing surrounding normal tissue; and (4) a predilection for certain tissues and locations. Burmester (55) indicated that these are all characteristic of avian lymphomatosis, but that the lesions of ocular and neural lymphomatosis have characteristics which are more typical of reactions to foreign infectious agents than of true neoplasms.

Olson (91) accomplished the transmission of a lymphoid tumor which he reported in 1941, by the use of transplants through 30 serial passages, and believed it to be unlike any other transmissible avian tumor described at that time. He reported variable responses to the implants by the host, ranging from failure to grow to progressive growth and metastasis to the visceral organs. It was also noted that in some birds, regression took place after a short period of growth.

In a continuation of his serial passage experiments (92), one line was transferred to a new host every 10 days through the 132nd transfer, and the other line was transferred every 15 days through the 100th transfer. He concluded that the activity potential of the tumor and the resistance of the host determined the

reaction of the host to the implant. It was also found that serial passage enhanced the ability of the tumor to produce a more severe reaction. The growth activity of the tumor transferred every 10 days was more enhanced than the 15-day transfer tumor.

Pentimalli (97) also described a transplantable lymphosarcoma of the chicken in 1941, and apparently it was similar to the one described by Olson. The transplants into the pectoral muscles were carried on a year through 23 serial passages. Filtration and desiccation experiments were negative as was one experiment in which the inoculum was held in glycerine for eight days. He also thought his description of a transplantable lymphosarcoma to be the first.

Hall, et al. (68) of Beltsville, reported in 1941 on 17 serial passages of lymphomatosis. The type changed from the neural type with leucemic tendencies to an apparently pure leucosis type. There was a marked reduction in the incubation period as well as increase in the percentage of takes. Suspensions of neural lesions and blood were used for inoculation material. A filtrate was used in some of the passages, indicating a filtrable agent. In an effort to increase the potency and to stabilize the virulence of the agent, chick embryos were inoculated intravenously for several passages, but results indicated an attenuation rather than enhancement of the infective agent. Transmission from chick embryos to chicks was accomplished by either tissue suspensions or blood.

In a later report (69) they had made 30 serial passages through chick embryos with the use of leucotic blood injected

intravenously. Leucosis developed in 41 per cent of 1089 chick embryos either in the embryonic stage or within a week of hatching. Twenty-three and three-tenths per cent of the injected embryos hatched and 62.6 per cent of these died of leucosis. In the embryos, the incubation period was 7.4 days, and 14.8 days in the ones that hatched. There was no conclusive evidence that the agent increased in virulence as a result of serial passage, since there was no decrease in incubation time and course of the disease.

In transmission experiments reported in 1941, Johnson (78) found that in the chick embryo, intravenous injections resulted in a much higher incidence of transmission than when the material was placed on the chorio-allantoic membrane. Young chicks were more suitable for their work than the embryos since the embryos were no more susceptible than one-to-seven-day-old chicks. Genetic influence was demonstrated when two strains of leukosis was injected into a relatively leukosis-free strain of Leghorns. The birds receiving the inocula influenced to an important degree the incidence, course, and form of leukosis that resulted. Johnson preferred the term hemocytoblastoma to lymphocytoma since he believed the predominant cell to be hemocytoblasts. He presented evidence that this condition was transmissible.

Brandly, et al. (2) reported in 1941 six serial passages of a lymphomatosis-osteopetrosis agent. Blood from Leghorn hens showing ocular and neural lymphomatosis was used for the first passage. Eight hundred and eighty-four chicks, 1 to 22 days of age, were inoculated, and 40.8 per cent incidence occurred.

Lesions of lymphomatosis occurred in 21.2 per cent of the controls. They reported a higher incidence of osteopetrosis in males than females and the opposite in lymphomatosis.

In an interspecies transmission experiment, Pollard and Hall (99) inoculated intravenously, developing embryos of turkeys, ducks, quail, pheasants, and guinea fowls with blood from leucotic chicks and chick embryos. A fatal leucotic dyscrasia developed in 73 of 85 (85.8 per cent) turkey embryos in 8.8 days. In the 57 duck embryos, 16 died of leucosis in 7.9 days; and 88 per cent of the 25 guinea fowl embryos died in 6.2 days. Fatal leucosis also developed in 9 of 10 (90 per cent) of the quail embryos in 5.5 days and in 8 of 9 (88.8 per cent) pheasant embryos in 6.87 days.

The agent of leucosis was transferred in ovo (99) from the chick to the turkey and from the turkey to the duck, guinea fowl, and quail. In a similar experiment, leucosis was transferred from chicks to pheasants and from pheasants to quail; and from chicks to guinea fowl and on to pheasants. The agent was transferred through three generations in turkey embryos, two in ducks, and two in guinea fowl embryps.

California workers (De Ome, 51) inoculated two populations of birds in 1943 known to be susceptible and resistant to spontaneous lymphomatosis. Material from neural lesions was injected intraperitoneally at three levels of exposure. It was determined that the incidence increased with the level of exposure as associated with the degree of susceptibility. The effect of increasing the exposure and the susceptibility was cumulative, and the

incidence increased in both populations by injecting them with lymphomatous material from the parent flock. They concluded that the resistance was due to numerous genetic factors and that additional increases in the level of resistance could be achieved by increasing the level of exposure in the flock to be selected.

Affected lymphomatous organs were used (Burmester and Prickett, 7) at the Regional Poultry Research Laboratory to prepare cellular inocula which resulted in the development of tumor strains reported in 1945. These strains were carried through 15 passages. In the later passages, these strains showed similar characteristics, and some increase in virulence was indicated. Organs showing the greatest frequency of involvement were liver, kidney, gonad, spleen, and the serosa in that order.

The Olson tumor (91) was studied at the Regional Poultry Research Laboratory and they concluded that it did not differ essentially from three other strains under study in most of its pathological manifestations. Lymphocytomas, as referred to by Feldman and Olson (60), also were similar to the condition known by others (78, 80, 82, 85) as visceral lymphomatosis.

Serial transmission through 15 passages was reported by Brewer and Brownstein (5) in 1946. Cellular inocula prepared from visceral organs were injected either intraperitoneally or subcutaneously. The original donor had no blood dyscrasia, and the liver and spleen were used for inoculation. Fifty per cent of the inoculated birds in the 15th passage died within 24 days and 59 per cent had died by the 100th day. Chicks raised in contact with inoculated birds developed higher incidence of

lymphomatosis than did the controls. Chicks given an oral dose of the infective material had a higher incidence than did the controls.

Positive results of a filtration experiment (Burmester, et al., 8) in 1946, involving birds, demonstrated the presence of a filtrable agent. This was the tumor originally studied by Olson and obtained by the Regional Poultry Research Laboratory in June, 1942. It was later designated as the RPL-12 strain. Cellular inocula induced death of all birds in an average of 10.2 days while the filtered material induced a high incidence (81 per cent) of osteopetrosis and visceral lymphomatous tumors in six months. It was suggested that the agent was propagated in close relation to the neoplastic cells since it was taken from a strain that had been propagated through 200 serial passages by transplantation. Plasma also contained the agent because blood was taken from a bird eight days after implantation and the plasma filtered. The plasma filtrate induced as many positive cases as the filtrate of the affected organ.

In 1947, Burmester (10) reported centrifugation studies on the agents of osteopetrosis and lymphoid tumors. The preliminary data indicate that there is not a more complete separation of the tumor agent at 125,000 g. than at 27,000 g. and a similar incidence of tumors occurred from the sediment and the supernatant. However, the osteopetrotic agent did appear to be more concentrated in the sediment as reflected by the incidence of 32 per cent in the birds receiving the supernatant, compared to 62 per cent incidence in those that received the sediment. Further, it took 174 days for

the appearance of osteopetrosis in those receiving the supernatant as compared to only 117 days in birds receiving the sediment. On this basis, it was inferred that the agents may be separate entities.

The RPL-12 strain was propagated (Burmester and Cottral, 12) through six serial passages in chickens by the use of a filtrable agent. About 81 per cent of the inoculated birds showed some gross involvement and died in an average of 137 days. The results were quite uniform for the different filtrate inoculations and passages. Of the positive cases, about 87 per cent had VL, 55 per cent osteopetrosis, and 6 per cent neurolymphomatosis. They suggested that there was no relation between the malignancy of the tumor cells and the virulence of the agent. Filtrates produced more osteopetrosis, and after the first passage, the filtrates were as effective as cellular inoculum in producing visceral tumors. The intraperitoneal and intravenous routes of inoculation of filtrates were tested and were found to be equally effective, and the filtered plasma and filtered extracts of organs were about the same in producing tumors. It was found that donors showing only osteopetrosis produced visceral tumors at about the same rate as donors having only visceral lesions, or those in which both osteopetrosis and VL was present.

Experiments reported (Burmester and Denington, 13) in 1947 which were conducted with a uniformity of environment and host that had not before been attained demonstrated the variation in the transmissability of VL by cellular inocula from different naturally-occurring cases. In these transmission experiments,

naturally-occurring VL was tested by the use of cellular and filtrate inoculums. Chicks used were 13 to 21 days of age. Visceral lymphomatous tumors were produced in 8 of 10 cases tested. These tumors were induced in 74 to 85 per cent of the chicks in 93 to 183 days. Filtrate experiments produced similar tumors from four of the original cases in 39 to 94 per cent of the chicks in an average of 183 days. None of the controls developed VL. Therefore, the agents producing VL will pass through bacteria-retaining filters. Evidence of neurolymphomatosis was present in 7 of 10 of the donors, but there appeared to be no direct relation between the presence of the lesions in the donor and in the recipient.

It was suggested that in cases of naturally-occurring VL (Burmester, 14), showing only one form of the disease, there may be in a masked or inactive form, several other tumor agents of the leucosis complex which may become apparent under favorable conditions.

Four tumor strains (RPL-18, -19, -20, and -21) which were developed (14) from naturally-occurring cases were studied by transmissions with cellular inoculum and cell-free preparations. Chicks 2 to 75 days of age were used, and cells injected intraperitoneally produced a high incidence of visceral lesions within four weeks. Passages were made 5 to 15 days after inoculation. Filtrates of tissues and plasma also produced visceral tumors.

Tumor cells in three strains (strains 18, 20, and 21) were of the extravascular type, and filtrates of these strains had a long, latent period with an average survival period of 116 to 162

days. Strain 19 was the intravascular type and had a comparatively short incubation period which is a feature associated with transmissible erythrogranuloblastosis. These experiments were presented as conclusive evidence that tumors of VL may be reproduced by a filtrable agent and that these agents may be propagated in serial passage either by cellular or cell-free preparations. It was demonstrated that filtrates (Burmester, 14) prepared from these naturally-occurring cases of VL, when injected into susceptible birds, would produce lesions indistinguishable from other similar cases of VL.

In a review (Davis, et al., 48) of 13,669 postmortem diagnoses, from 1934 through 1945 at Purdue, it was found that the incidence of visceral lymphomatosis was higher in Barred Plymouth Rocks than in White Plymouth Rocks, White Leghorns, or Rhode Island Reds. An inverse correlation was noted between the incidence of neurolymphomatosis and the incidence of visceral lymphomatosis, with White Leghorns having the highest rate. This idea supported the theory of different disease entities.

Indiana workers (46) transmitted leucosis in 1947, using 301 40-day-old White Plymouth Rock chicks which were inoculated with material prepared from liver, heart, spleen, ovary, kidney, and defibrinated blood. Visceral lymphomatosis developed in 38.8 per cent of these birds at an average age at death of 214 days. There was a 9.6 per cent incidence in the controls. They concluded that there was no marked concentration of the causative agent in any certain organ; also that by serial passage, the incidence can be increased and the incubation period decreased. The typical cell

found in the lymphomatous lesions varied in maturity from lymphoblasts to lymphocytes, with the lymphoblasts predominating.

A similar experiment (49) was conducted in 1949, using larger groups of chicks in the serial passages, making the work more valid statistically. Results supported their earlier work.

In liver biopsy studies (Davis and Doyle, 47), it was found that a moderate amount of lymphoid infiltration is normal in a chicken. This is especially true when the areas are perivascular and well-defined without outward diffusion. Fatal cases of visceral lymphomatosis evidently develop very rapidly since such fatal cases of VL had shown no abnormalities by biopsy three or four weeks prior to death. There were apparently some mild cases that recovered after lesions were observed microscopically from biopsy tissue. They stated that "it (biopsies) has a definite use in experimental work, and possibly a limited use in diagnosis in special cases."

Propagation of lymphoid tumors in the anterior chambers of the eye (Burmester and Belding, 15) was accomplished by injecting a cellular suspension into the eye. Lesions appeared in 5 to 7 days when the anterior chamber became enlarged, yellowish gray material appeared, and the cornea showed some cloudiness. There was about a 50 per cent take in the first passage and 80 to 90 per cent in later passages. Material taken from the eye was used for serial passages. Visceral metastasis occurred in most of the birds with lesions in the eye, and death resulted in 5 to 19 days after inoculation.

Three years later, tumors were produced (17) in the iris, ciliary body, and adnexa by the inoculation of cellular suspensions of two strains of VL virus into the anterior chamber and propagated through four and six passages. In some cases an abnormally gray iris was produced in three to six weeks after inoculation and disappeared in about eight weeks. This abnormal greying of the iris was due to a nonspecific reaction to the foreign tissue injected, and not an early stage of ocular lymphomatosis.

In a report from India (Rao, et al., 100), it was indicated that their findings did not deviate much from workers cited above. Their strain of virus did have a marked affinity for the spleen. Leucosis was transmitted by inoculation and contact, and the visceral form did give rise to other forms of the disease. An increase in monocytes was reported as an aid to early diagnosis of the disease.

Transmission studies (Burmester, 16) were used to determine the effect of low temperature storage on the viability of several strains of VL. Inoculation of suspensions of cells into 1350 chickens revealed that there was considerable variation among samples and strains in the viability of frozen tumors. The frozen samples were compared to the fresh material and there appeared to be a direct relation between the growth potential of fresh tumors and those that were frozen and stored. One sample which was tested after 2,028 days of storage at -65° C. or lower produced tumors in inoculated chicks in an average of 8.2 days, indicating that there was no change in the growth potential during an extended storage

period at low temperatures.

In the same paper it was pointed out that rapid freezing and dissolution of the cells reduces the possibility of tumors developing at the site of inoculation in the form of a transplant. Therefore, material which was frozen slowly and later used for injection gave rise to lymphoid tumors due to the viability of the cells and not due to the activity of the virus.

Chickens inoculated intraperitoneally with RPL-12 (19) were compared with noninoculated, naturally-exposed sisters to obtain the relationship of the response to the agent. Of 671 inoculated chickens held to 300 days of age, 31.73 per cent died of the disease as compared to 12.54 per cent of 561 naturally-exposed chickens held to 500 days of age. These results support the theory that intraperitoneal injection of the causative agent is an effective means of increasing the exposure to the VL agent. This process may be employed to identify families of chickens having a greater resistance to VL.

A study (El Dardiry, et al., 54) of response of inbred lines of Single Comb White Leghorns of both sexes to tumor transplants was reported in 1952. Of eight lines (2,027 birds) tested, three were bred for resistance and the other five for susceptibility. Lymphoid tumor cells were inoculated into the pectoral muscle of all the birds, and if death did not occur in 27 days, there was a regression of the tumor in nearly all cases. The best measurement of resistance proved to be the involvement of the viscera through metastasis of the pectoral tumor or possibly transportation of the

viable cells via the blood stream to the viscera at time of inoculation. The conclusion was that the pectoral tumor formation was not a reliable index to the resistance of the bird to naturally-occurring lymphomatosis.

The relationship between the dose of the agent and the character of the response was reported (Burmester and Gentry, 26) in 1956 when serial dilutions of filtrates of the VL virus were injected into susceptible chicks. Total incidence of the disease increased with an increase in dose up to a log dose of -3. At that point the maximum effect apparently was reached. Deaths occurred as early as 22 days after inoculation in the birds receiving a high dosage and continued in other lots for the time of the experiments (200 to 270 days). In the low dosage lot, the incidence was 55.5 per cent with an average age at death of 128.2 days as compared to the high dosage lot in which the incidence was 90.1 per cent and had an average age at death of 55.6 days.

The positive cases were classified as extravascular or intravascular, depending upon the location of the neoplastic cells microscopically. The intravascular type of VL increased rapidly with an increase in dose while the extravascular cases were inversely related to the dose. There was a low but significant number of osteopetrosis cases in all inoculated lots. Their results indicated that the males were slightly more susceptible to osteopetrosis and the females were similarly more susceptible to VL.

"The average latent period, or days-to-death, and percentage of deaths due to lymphomatosis are directly related to the dose

of the virus" (Burmester, 29). Deaths may occur as early as four weeks of age when chicks are inoculated at one day of age, and deaths may continue for the next 9 or 10 months. The third month of life was considered to be the peak of the losses. This point was the reason for an experiment concerning the biological activity for assay of the virus of VL. Bioassay of the agent was studied in six titrations, using a total of 3,566 chickens. Under the conditions of the experiment, he considered nine weeks to be the optimum experimental period for bioassay. It was pointed out further that titrations under other conditions and even similar conditions might require different experimental periods for the greatest accuracy.

MATERIALS AND METHODS

Donors

Donor birds for first passage experiments were selected from a farm flock near Manhattan, Kansas and from the flock at the Kansas State College Poultry Farm. The farm flock had a history of high mortality (35 to 40 per cent in 1957) due to visceral lymphomatosis and the Poultry Farm had lower (approximately 17 per cent in 1957) but persistent losses. Other forms of the leucosis complex also had been diagnosed in these flocks but not to a high degree. These adult birds were selected while alive and were sacrificed by cervical disarticulation. Tissues for inoculation were taken aseptically and kept under refrigeration until processed for inoculation that same day. The extravascular type of VL

was present in all the primary donors, and various organs, especially the liver, were used for inoculation (Table 1).

The experiments in which donors were obtained from the farm flock and the Kansas State College Poultry Farm flock were designated G- and PF-, respectively, with numbers following to indicate the particular experiment. Experiments G2B1 and G2B2 were serial passage experiments in which the donors were selected from experiment G-2. The donors for the serial passage experiments were chosen on the basis of their symptoms which were general unthriftiness, weakness, lethargy, and soiled feathers, especially around the vent. They were sacrificed and tissues processed in the same manner.

The tissues which served as the inocula for experiments G-4 and G-5 (farm flock) were taken aseptically, frozen and stored until processed for inoculation. Approximately 10 grams of tissue was placed in sterile pyrex test tubes and sealed in a natural gas-oxygen flame. The tube was sealed, leaving as little air space as possible above the tissue. These sealed tubes were placed in an alcohol bath and dry ice (CO_2) was added in small amounts so that the temperature dropped 1° C. per minute from 0° C. to -20° C. The frozen material was immediately transferred to a dry ice chest and was held at temperatures no higher than -65° C. until used in the preparation of an inocula.

Table 1. Summary of chicks per treatment and tissues used for inoculation.

Experiment No.	Age of recipient at inoculation in days	No. of control chicks	No. of chicks inoculated			Total	Tissues used
			Filt	Plas	Cells		
G-1	17	25	25	25	25	100	Liver
G-2	14	34	45	--	45	124	Liver, kidney, ovary
G-3	1	32	32	32	32	128	Liver, kidney
G-4	6	28	--	28	2(28)*	112	Liver
G-5	12	30	--	--	30	60	Liver, spleen
G2B1	3	25	25	25	25	100	Liver, spleen
G2B2	13	29	28	--	28	85	Liver, spleen, kidney
PF-1	1	35	33	33**	33	134	Liver
PF-2	1	29	30	30	30	119	Liver, pancreas, ovary

* Two dosages of cells were administered in this experiment.

** A portion of the blended material in the preparation of filtrate was substituted for plasma in this lot.

Recipients

An inbred line of Single Comb White Leghorns, referred to as Line 15 (106, 108), was the source of birds for this experiment. This line was developed for susceptibility to lymphomatosis at the Regional Poultry Research Laboratory at East Lansing, Michigan. The extent of natural infection (26) of lymphomatosis has been reduced in the past 14 years to an incidence of less than 3 per cent when maintained for periods of about a year. This has been accomplished at the laboratory by maintaining strict isolation of the birds. Eggs from this line were shipped to Kansas State College and were hatched at the Veterinary Research Laboratory. The chicks were maintained in isolation at the Veterinary Research Laboratory and the Poultry Farm throughout the experimental periods.

The chicks were banded at one day of age and kept in four-deck brooders until they were approximately eight weeks old and then were moved to isolation cubicles at the Veterinary Research Laboratory or to isolated range houses on the Poultry Farm. These range houses had been subdivided into four pens. They were reared in these quarters until the termination of the experiments. A necropsy examination was conducted on both experimental and control birds.

Feed used on the experiments was mixed by the Kansas State College Milling Department, according to the formula used at the Regional Poultry Research Laboratory. The starter formula was fed until the birds were three to four weeks of age and then

changed to the grower formula.

Inoculums and Inoculations

The inoculums used in these experiments were prepared from field cases in seven instances and from inoculated birds on two occasions. The different types of inoculums (filtrate, plasma, and cells) were prepared in a similar manner for all experiments with only minor variations.

Tumor filtrate was prepared by homogenizing lymphomatous tissue with 19 parts of Simms' salt solution (103) or physiological saline in a Waring blendor in which the air had been displaced within N_2 gas for 20 minutes in experiments G-1 and G-2 and 3 minutes in the others. The homogenate was centrifuged at 3000 r.p.m. for 15 minutes in a refrigerated centrifuge which was held at one to three degrees Centigrade. The supernatant was decanted and filtered through a bacteria-retaining filter (Ford centrifuge filter in experiments G-1 and G-2 and a Selas 02 filter in the others). The filtrate was held in a refrigerator until injected into chicks the same day. The intraperitoneal route was used in all experiments except G-1 in which the filtrate was administered intravenously. In experiment PF-1, unfiltered blended tissue was used for inoculation in one pen.

Plasma was obtained from the donor by bleeding from the heart into a one-tenth volume of a 6 per cent solution of sterile sodium citrate. The blood was centrifuged in the refrigerated centrifuge, and plasma decanted. In the G-1 experiment, an equal volume of Simms' solution was added and filtered through a Ford

centrifuge filter. In the other experiments (G-3, G-4, G-2B1, PF-2) in which plasma was used, it was injected without dilution or filtering. All injections were intraperitoneal except in experiments G-1 and G-4 which were intravenous.

Cellular inocula was prepared by macerating the lymphomatous tissue in four parts of either physiological saline or Simms' solution (saline was used in experiments G-1, G-2, G-3, and PF-1). This material was filtered through cheesecloth and centrifuged at 1500 r.p.m. for 15 minutes. The route of injection was intraperitoneal in all experiments; the dose being 0.2 milliliter except in experiment G-2 when 0.06 milliliter was injected and in experiment G-4 when the second pen receiving cells was injected with 0.6 milliliter. The inoculations in the majority of cases were intraperitoneal with aforementioned exceptions in experiments G-1 and G-4, which were intravenous. Chicks ranging from 1 day to 17 days of age were inoculated, using a one ml. tuberculin syringe fitted with a 27-gauge, 1/2-inch needle for intravenous inoculations. Intraperitoneal injections were made with a 19-gauge, 5/8-inch needle.

Management

Restrictive management procedures were in effect throughout the experimental periods. One caretaker was assigned to the isolated areas and care was taken not to introduce contaminating diseases by either direct or indirect contact. Contact between pens on the part of the caretaker was minimized.

Personnel traffic was limited to those who were required in conducting the experiments, and adequate precautions were taken to prevent contamination from external sources and cross contamination between lots and experiments.

One reservoir waterer and one gravity flow feeder were used in each pen. All equipment and pens were disinfected with a quaternary ammonia solution or pine oil solution before the birds were started on experiment or moved to other quarters.

Necropsies

Birds that died during the experimental period were necropsied and the diagnosis based on gross findings with confirmation by a microscopic study of sections of suspected tissues. The remaining birds were killed on termination of the experiments and an examination was made for evidence of the developing disease.

RESULTS

Mortality from lymphomatosis which occurred during the experimental periods is summarized and presented in Table 2. Experiments were designed to have no less than 25 birds per pen or treatment with at least one-fourth of the total number serving as untreated controls. Depending upon the number of chicks available, two to four pens of birds (Table 1) were used for each experiment.

The nine experiments involved a total of 962 birds of which 695 were inoculated. The incidence of deaths due to visceral lymphomatosis was highest in the G-1 experiment where 8.0 per cent

Table 2. Summary of incidence and mortality due to lymphomatosis in nine experiments.

Experiment No.	No. of chicks inoculated	No. with tumors of the bone	viscera	nerve	Total No. of positive cases all types	Per cent incidence all types	Per cent mortality visceral	Average age at death in days visceral	Length of experimental period in days
G-1	75	2	6	0	8	10.66	8.0	186.5	232
G-2	90	2	3*	0	5	5.55	3.33	100.5+	232
G-3	96	1	3	0	4	4.17	3.13	130.3	216
G-4	84	0	0	0	0	0.00	0.00	--	192
G-5	30	0	0	0	0	0.00	0.00	--	224
G2B1	75	1	1	0	2	2.66	1.33	80.0	213
G2B2	56	0	0	0	0	0.00	0.00	--	199
PF-1	99	0	5	1	6	6.06	5.05	158.2	210
PF-2	90	2	4	1	7**	7.77	4.44	172.75	210

* 1 case intravascular.

** 1 case of erythroleucosis not included in calculation.

\+ 1 donor not included.

of the birds died of that form. No response was evident in three other experiments (G-4, G-5, G2B2). Total incidence of all types of lymphomatosis ranged from 10.66 per cent to no response in three experiments. The visceral form predominated since 22 cases were diagnosed as compared to 8 cases of osteopetrosis and 2 cases of neurolymphomatosis.

The average age at death, due to the visceral form, for each experiment ranged from 80 to 186.5 days. All experiments were in progress for a period of at least 192 days with two extending to 232 days.

In the first three G series experiments, tissues from sacrificed birds were used without a freezing and storage period. The inoculums were prepared and injected on the same day as taken from the donor bird. The first death in experiment G-1 due to VL was in 143 days, and three of the later deaths were in four-day period from the 176th to the 179th day after inoculation.

The first death in experiment G-2 occurred on the 60th day and was the only intravascular type diagnosed in all nine experiments. Two donors were selected from this group for passage to young chicks (experiments G2B1 and G2 B2).

The three deaths due to VL in experiment G-3 occurred within a 13-day period, averaging 130 days after inoculation.

Inoculums from stored material were used in experiments G-4 and G-5. Material used in experiment G-4 had been frozen as previously described and stored in a dry ice chest for 74 days before being removed for processing. A storage period of six days was

the case in experiment G-5 after which a cellular inocula was prepared. Neither of these two experiments yielded a single case of VL.

In the two trials to make a passage from the G-2 experiment, donor birds were selected, killed, and tissues taken asceptically. The inoculums were prepared and injected on the same day the donor was sacrificed. There was one positive case in experiment G2B1 which died on the 80th day after inoculation. There were no deaths due to VL in experiment G2B2.

In the PF-1 and PF-2 experiments, fresh tissues, asceptically procured, were used for inoculums and injected on the same day the donors were sacrificed. The first VL death in experiment PF-1 was 124 days after inoculation, and the succeeding four deaths were evenly dispersed up to 191 days. Deaths occurred in 136, 163, 186, and 206 days in experiment PF-2 with an average of 173 days.

In all experiments, the liver was most frequently affected with VL and the spleen and kidney were also affected in most of these cases. Occasionally the ovary, pancreas, and intestine were involved. Positive cases of VL occurred primarily in chicks inoculated with cellular suspensions. There was one death each in the filtrate-injected lots in experiments G-1, G-3, and PF-1 and one in experiment G-2 besides the donor bird for experiment G2B1. The one bird in experiment G2B1 that died of VL was inoculated with plasma.

Histopathologically, the liver lesions were extravascular with the one exception which was intravascular (Plates I and II,

Appendix) and ranged from the diffuse type of lymphoid hyperplasia to the discrete nodular form (Plates III, IV, V, and VI, Appendix). Lesions in affected organs were characterized by the presence of large groups of lymphoid-like cells which had replaced the functional tissue (Plates VII, VIII, IX, and X, Appendix). The type of cell involved was predominantly lymphoblastic although mature cells were present.

DISCUSSION

Visceral lymphomatosis was transmitted by inoculation to susceptible chicks in a low per cent of cases and it is evident that active transmission was accomplished in experiments G-1, G-2, G-3, PF-1, and PF-2. The only positive case of VL diagnosed in control birds was on necropsy of experiment G2B1 at the termination of the experiment. Since there had been no apparent transmissions with plasma inocula in four other experiments and because of the one possible case in the control group, no significance was placed on the two positive cases of lymphomatosis in experiment G2B1.

The tissues taken for the preparation of the various inoculums were obtained from adult birds in active or terminal stages of the disease. Since the disease usually develops rapidly (18, 47) once the tissue changes begin to occur, tissues were assumed to contain the viable etiological agent. The tissues were processed in such a manner that excessive temperatures would not have killed the agent. Inoculation procedures were similar to those employed by others with favorable results. Therefore, it appears that the agent was either avirulent after processing or, in the

case of the parent farm flock (G experiments), the birds were more susceptible or the conditions on that farm linked with the susceptibility of the birds, resulted in a high incidence on those premises.

Periods of storage apparently rendered the agent nonviable as evidenced in experiments G-4 and G-5 where there were no deaths due to VL. Even though freezing and storage at temperatures below -65° C. has been reported with favorable results (16), this agent must have become inactivated by this procedure.

Osteopetrosis and neurolymphomatosis were evident in these experiments and add support to previous reports (2, 10) that the etiological agents of the leucosis complex were either the same or similar in character. In addition to the cases of neurolymphomatosis recorded, it is possible that other unclassified deaths in these experiments might have been due to neurolymphomatosis. There were cases in some lots in which birds apparently starved to death. Necropsy of these birds revealed no gross lesions of VL, but the birds were dehydrated and emaciated. The presence of the "peck order" (67) was also considered as a reason for this emaciated condition since there was only one waterer and feeder per pen and the birds became crowded as the experiments progressed.

The object was to use chicks as young as possible, ranging from 1 day to 17 days of age, in these experiments. Apparently the age of the recipients up to 17 days of age had no effect on the incidence of the disease.

The latent period or incubation period was long, extending to 232 days and quite variable. This was to be expected since

natural cases continue to succumb until 500 days of age or more (19, 48). The majority of the cases died in the four- to six-month interval after inoculation which is similarly the case in losses in farm flocks, and would be expected in initial isolation studies.

SUMMARY AND CONCLUSIONS

1. Visceral lymphomatosis was transmitted to a limited number of susceptible chicks primarily by the use of cellular inoculums.

2. Transmission was accomplished in five primary isolations in seven experiments.

3. Transmissions did not occur in two trials at serial passages or when stored tissues were processed for use as inoculums in two experiments. The nine experiments involved 962 chickens.

4. Osteopetrosis and neurolymphomatosis were diagnosed and believed to be closely associated with the visceral form.

5. Age range of the recipients (1 to 17 days) at inoculation apparently was not a factor in the incidence of transmission of VL under the conditions of these experiments.

6. The experimental periods extended from 192 to 232 days following inoculation. The majority of the losses due to VL occurred during the four- to six-month period.

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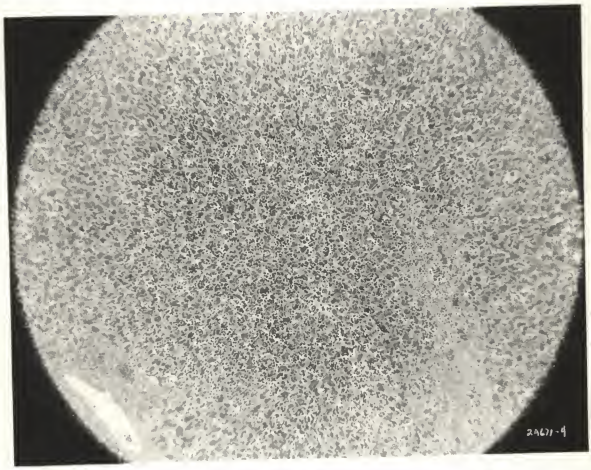
APPENDIX

EXPLANATION OF PLATE I

Section of liver tissue from a chicken with intravascular lymphomatosis, Experiment G-2 (100x).

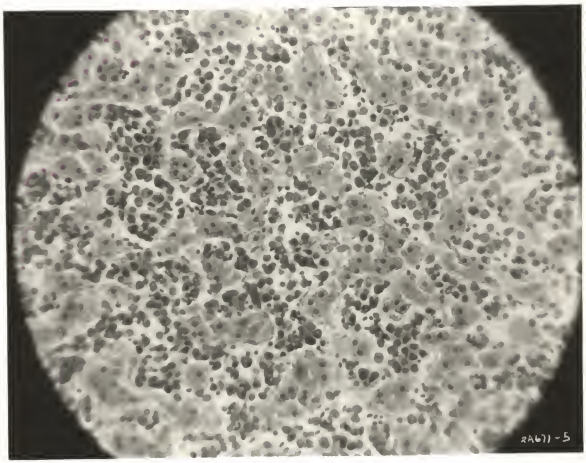
PLATE I



EXPLANATION OF PLATE II

Section of liver tissue from a chicken with intravascular visceral lymphomatosis, Experiment G-2 (450x).

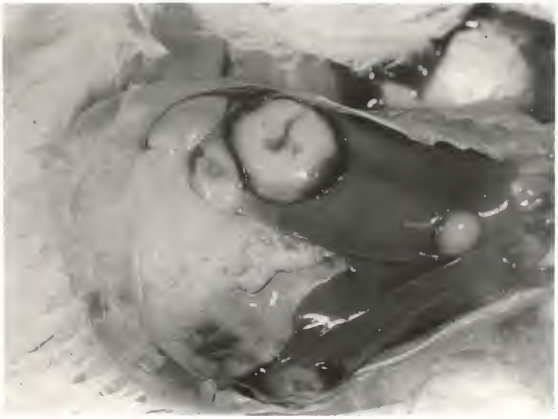
PLATE II



EXPLANATION OF PLATE III

Liver of a chicken with the discrete type of visceral lymphomatosis (field case).

PLATE III



EXPLANATION OF PLATE IV

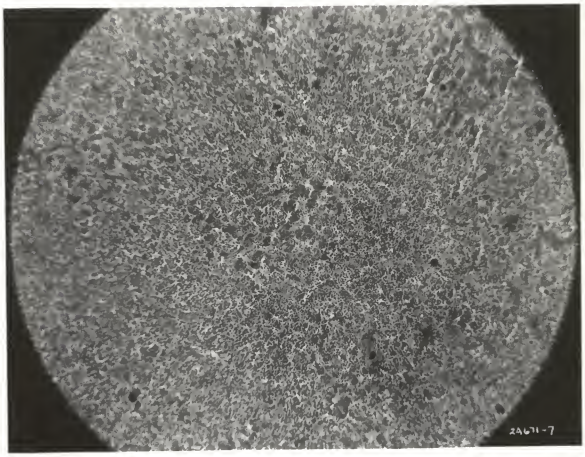
Liver of a chicken with the diffuse type of visceral lymphomatosis (field case).

PLATE IV

EXPLANATION OF PLATE V

Section of liver tissue from a chicken with extra-vascular visceral lymphomatosis, Experiment G-1 (100x).

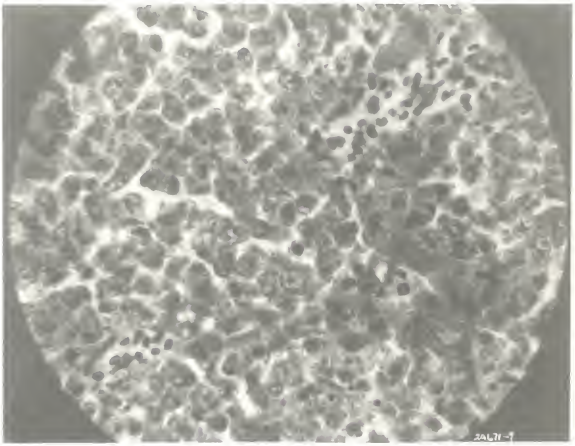
PLATE V



EXPLANATION OF PLATE VI

Section of liver tissue from a chicken with extra-vascular visceral lymphomatosis, Experiment G-1 (970x).

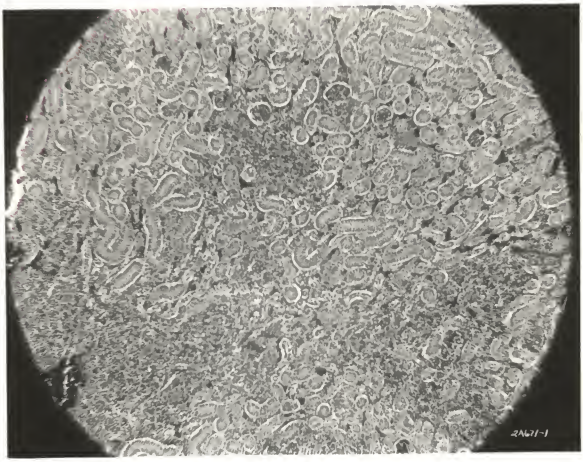
PLATE VI



EXPLANATION OF PLATE VII

Section of kidney tissue from a chicken with visceral lymphomatosis, Experiment G-1 (100x).

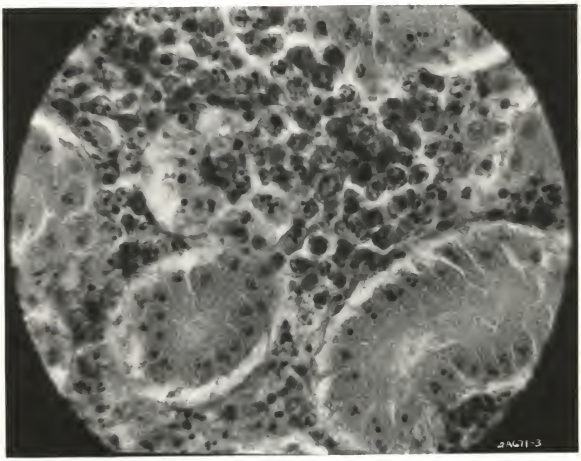
PLATE VII



EXPLANATION OF PLATE VIII

Section of kidney tissue from a chicken with visceral lymphomatosis, Experiment G-1 (970x).

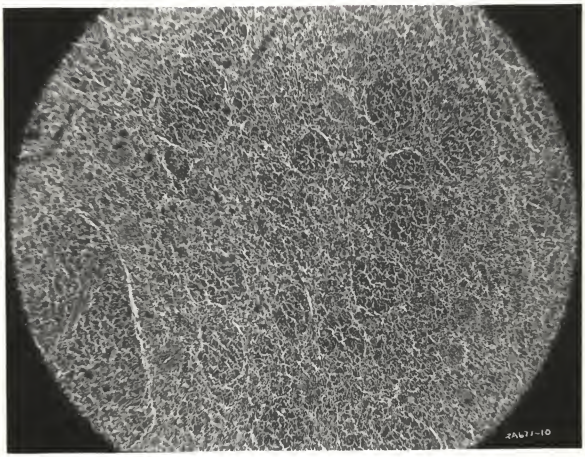
PLATE VIII



EXPLANATION OF PLATE IX

Section of splenic tissue from a chicken with visceral lymphomatosis, Experiment G-1 (100x).

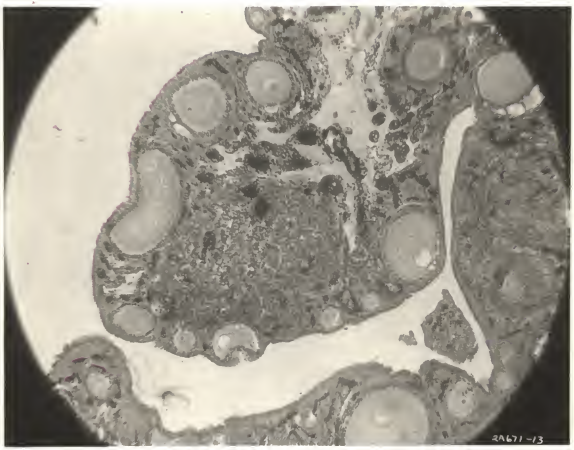
PLATE IX



EXPLANATION OF PLATE X

Section of ovarian tissue from a chicken with visceral lymphomatosis, Experiment PF-1 (100x).

PLATE X



TRANSMISSION OF VISCERAL LYMPHOMATOSIS FROM FIELD CASES

by

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This study was undertaken to isolate strains of the virus of Visceral Lymphomatosis (VL) from farm flocks in Kansas, and to study the pathological changes produced in these strains. Infective material was collected from field cases and transmitted to susceptible chickens. VL is a known neoplastic, malignant disease that is spread by contact, although the leukemias and sarcomas of the Rous type are also caused by highly infectious agents.

Visceral lymphomatosis has been the prevalent form of the avian leucosis complex in the United States since 1930 and is now responsible for up to 75 per cent of the losses due to lymphomatosis. It was estimated that in 1953 more than 53 million mature chickens died of this disease with a total value of at least 73 million dollars.

Nine experiments were conducted in which infective material from advanced or terminal cases of VL was processed and injected into susceptible chicks. These chicks were held under quarantined conditions the entire experimental period. Necropsies were performed on all birds and the diagnosis based on gross findings, with confirmation by microscopic study of suspected tissues.

Visceral lymphomatosis was transmitted to a limited number of susceptible chicks, primarily by the use of cellular inoculums. Other types of inoculums employed were tissue filtrates, plasma filtrates and plasma. The incidence of deaths due to visceral lymphomatosis was highest in the G-1 experiment where 8.0 per cent of the inoculated birds died. No response was evident in three other experiments. Total incidence of all types of

lynphomatosis ranged from 10.66 per cent to no response in three experiments.

Of nine experiments involving 962 chickens, transmission was accomplished in five primary isolations. Transmission was not evident in two trials at serial passage or when stored tissues were processed for use as inoculums in two experiments. Osteopetrosis and neurolymphomatosis were diagnosed and believed to be closely associated with the visceral form.

Age range of the recipients (1 to 17 days) at inoculation apparently was not a factor in the incidence of transmission of VL under the conditions of these experiments. The experimental periods extended from 192 to 232 days. The majority of the losses due to VL occurred during the four-to-six-month period following inoculation.