A STUDY OF THE TRANSMISSION OF A VIRAL-INDUCED AVIAN NEPHROBLASTOMA (EMBRYONAL NEPHROMA)

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ABSTRACT

A STUDY OF THE TRANSMISSION OF A
VIRAL-INDUCED AVIAN NEPHROBLASTOMA
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By Willard G. Walter

Transmission studies of an unusual avian renal tumor reported previously as an adenocarcinoma were undertaken since the neoplasm occurred in chickens inoculated with an avian leukosis strain (BAI-A) formerly believed to cause only granuloblastic leukemia.

The tumor was successfully transplanted through 12 serial passages. In the majority of cases, a palpable tumor at the site of inoculation developed in about 2 weeks, growth was progressive and was followed by mortality in about 3 additional weeks. The leukemia manifestation characteristic of this strain was eliminated after nine transplant passages.

Viral activity of three of the transplant passages was tested by inoculation of tumor filtrates in leukemia susceptible chickens. Those from the last transplant passage failed to induce leukemia but did cause a high incidence of renal tumors and a moderate incidence of visceral lymphomatosis and osteopetrosis.

Histopathological evidence was presented which justifies considering this tumor a nephroblastoma (embryonal nephroma) rather than an adenocarcinoma.

Although the leukemia manifestation was eliminated from the neoplastic spectrum of strain A in later transplant passages and in the filtrate test of one of them, these experiments are not conclusive

evidence of a separate etiological agent for leukemia. The possibility that avian renal tumors should be included as an additional member of the group of diseases known collectively as the avian leukosis complex is considered.

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INTRODUCTION

Recent long-term experiments (Burmester et al., 1959b) designed to test the tumor inducing potential of two strains of avian leucosis (R of Engelbreth-Holm, and DAI-A) considered pure (Beard, 1957a), i.e., capable of producing only leukemia, showed that both strains caused visceral lymphomatosis as well as the specific type of leukemia characteristic of each strain. In addition, strain DAI-A caused osteopetrosis and a tumor of the renal parenchyma. The latter was considered at the time to be a renal adenocarcinoma.

The renal tumor is of interest because renal epithelial tumors are not considered a manifestation of the avian leukosis complex (Jungherr, 1959). Also strain DAI-A has been extensively investigated (Beard, 1956, 1957b), and has consistently caused only granuloblastic leukemia. Accordingly, efforts were made to propagate the renal tumor from strain DAI-A as a distinct entity.

The purpose of this investigation was to supply information pertinent to the transmission of the renal tumor and to characterize it histopathologically.

REVIEW OF LITERATURE

The induction of renal epithelial tumors by a strain of leukosis virus was considered unusual, but not without precedent. Carr (1956) reported renal adenocarcinomas following inoculation of the ES-4 leukosis strain of Engelbreth-Holm (Rothe Meyer and Engelbreth-Holm, 1933). The designation ES indicates what was previously believed to be the extent of the pathologic expression of this strain i.e.,erythroid leukemia or sarcomas or both. Carr reported a low incidence of renal tumors following inoculation of either cellular or cell-free material injected intramuscularly. All chickens with renal tumors also had leukemia, and in addition some had a sarcoma at the site of inoculation. More recently, Carr (1960) reported transmission of a similar tumor caused by the MH-2 reticuloendothelioma virus (Begg, 1927).

Foulds (1934a, 1934b) also observed renal tumors in chickens inoculated with strain MH-2, but considered the lesion to represent metastatic cells growing within renal tubules and discounted the idea of the lesion being of renal origin and of viral etiology.

Chouroulinkov and Reviere (1959) agreed with Carr that the renal tumors induced by strain MH-2 were viral-induced and of renal origin but in contrast, considered them tobe of two histological types, either adenomas or epitheliomas.

At the U.S. Regional Poultry Research Laboratory, tumors harvested from BAI strain A inoculated chickens were carefully studied histologically. As additional causes became available it was apparent that the tumor was more complex than originally suspected, and that the designation of adenocarcinoma seemed inappropriate. Great variation was observed concerning the relative amount and degree of differen-

tiation of both the stroma and epithelial components. In some cases microscopic areas of cartilage and bone were detected. Since mixed tumors have been previously described in poultry (Feldman and Olson, 1959) e.g., carcinosarcomas, teratomas, and embryonal nephromas, it seemed probably that this renal tumor should be classified as one of the above tumors. To preclude adding confusion to the already complex and vast array of literature on avian leukosis, much of which is contradictory, the staff of the Armed Forces Institute of Pathology was consulted for aid in appropriately classifying this tumor. Accordingly, representative samples of the tumor were submitted, including those of the original donors and some derived from subsequent passage. The conclusion was that the tumor is an embryonal nephroma, histologically comparable with Wilms' tumor of man, and that the preferred terminology in animals is nephroblastoma.

Nephroblastomas in general. Ewing (1941) classified epithelial tumors of humans which affect renal parenchyma as follows: adenoma, carcinoma, embryonal adenomyosarcoma, and in addition those considered to be of adrenal origin and known as hypernephromas. Embryonal adenomyosarcoma of infants is described as a highly characteristic tumor of complex embryonal structure suggestive of abortive renal constituents. It is lobulated, encapsulated, and generally sharply delineated from normal renal tissue. With age it becomes cystic, hemorrhagic and necrotic. Rapid growth and fatal termination are usual. Metastasis is rare. Histopathological variation is such that either spindle cells or epithelial cells may predominate, thus individual tumors or areas within tumors may resemble either adenocarcinomas or adenosarcomas.

The diverse microscopic appearance of renal epithelial tumors in domestic animals has resulted in many different descriptive names being applied, such as, adenoma, sarcomatode, adenomyosarcoma, adenosarcoma, sarcocarcinoma, sarcoadenoma, and rhabdomyosarcoma (Feldman, 1932), and in addition, Wilms' tumor, however, the latter designation has more often been restricted to the literature on neoplasia in man.

McKinney (1931) and Feldman (1932) discussed the origin and histopathology of renal tumors in animals and emphasized that primitive nephrogenic tissue was capable of differentiating into both epithelial and connective tissue. They agreed further that variation in the predominating tissue both as to amount and degree of differentiation was characteristic of these neoplasms as a group. Feeling that the tumors should be named according to origin rather than by histopathological appearance, they suggested the single term embryonal nephroma should be used to designate all renal tumors arising from embryonic tissue. Subsequently this term has gained widespread usage, largely replacing the older terms. More recently, the term nephroblastoma has been introduced and is being used synonymously with embryonal nephroma and Wilms' tumor (Bloom, 1954; Cotchin, 1956; Evans, 1956; Smith and Jones, 1957). Nephroblastomas are relatively rare animal tumors which occur more frequently in swine than in other domestic animals. They have also been observed in cattle, sheep, rabbits, and other small mammals, and chickens (Feldman, 1932; Jackson, 1936; Smith and Jones, 1957). Recent reports indicate that this neoplasm is being recognized in dogs (Weitz and McClelland, 1940; Jones, 1952; Cotchin,1954; Savage and Isa, 1954; Medway and Nielson, 1954; Seibold and Hoerlein, 1957), and in cats (Plummer, 1951; Cotchin, 1952).

Nephroblastomas in fowl. Although these tumors are said to occur relatively frequently in chickens (Feldman, 1932), there are as yet comparatively few reports to be found concerning this aspect. Mathews (1929) presented a detailed histopathological description of 12 cases, 0.6%, observed from 2,000 necropsies. They occurred in chickens 3 months to 2 years old but especially in those over 9 months old. One case of apparent metastasis was seen with secondary tumors located in the thigh muscle and the lungs. However, the renal tumor was predominately sarcomatous, and secondary tumors were exclusively spindle-celled sarcomas devoid of epithelial components. McKinney's (1931) two cases were seen in 1-year old White Leghorns, were malignant in appearance but showed no evidence of metastasis. Feldman and Olson (1933) reported a somewhat unique case in that it contained epithelial "pearls". Although both kidneys were involved, the authors concluded that metastasis was not involved and that the tumors arose independently as the result of a developmental fault of a bilateral nature. Jackson (1936) reported 7 cases, 3.5%, in a series of 203 avian neoplasms. Goss (1940) did approximately 7,400 necropsies and found only 4 embryonal nephromas, 0.3%, in 1,445 birds afflicted with neoplasia. Three of these occurred in chickens less than 9 months of age. Secondary tumors were not observed. Olson and Bullis (1942) classified as embryonal nephroma 14 of 384 tumors in 365 chickens of a total of 2,304 birds examined. Among the chickens which were allowed to die naturally, mortality occurred at an average age of 32 weeks. Epithelial "pearls" were found in six cases and cartilage in one.

Artificially induced nephroblastomas in fowl. Previous attempts to artificially propagate avian embryonal nephromas include those of

Foulds (1940) who described 13 spontaneous avian renal tumors which resembled Wilms' tumor of children. Transplantation of some of them was attempted but was unsuccessful. Duran-Reynals (1946) reported tumors of seven of ten cases to be nontransplantable. Of the remaining three neoplasms, one resulted in a fibroma at the site of inoculation in the first passage only. The second tumor produced a predominately pleomorphic stroma with little epithelial components, and was subsequently nontransplantable. The third case contained both sarcomas and embryonal nephromas. The latter was nontransplantable but the sarcoma was repeatedly transplantable.

Recent successful transmission of viral-induced avian nephroblastomas was reported by Thorell (1958a, 1958b, 1959) in a series of experiments begun at approximately the same time as those conducted at the U.S. Regional Poultry Research Laboratory. The DAI strain A seed virus was supplied to both laboratories by Dr. J. W. Beard, Duke University, Durham, North Carolina.

Thorell refers to these tumors as adenosarcomas, and reported 100% mortality from leukemia when chicks were inoculated at 3 days of age with infectious plasma. In contrast, when 2-week old chicks were inoculated, 50% died from leukemia and 30% of the survivors developed renal tumors. He attempted to develop a renal tumor-producing substrain by using these tumors to prepare cell-free extracts. Experiments were conducted in parallel, using tumor extract as one source of virus and plasma from leukemic birds as the other. Three serial passages were made. Chicks were inoculated intravenously when 3 days old. Infectious plasma continued to cause 100% mortality. Using extracts derived from tumors, the mortality from leukemia decreased from 50%

to 20% and the incidence of nephroblastomas rose from 40% to 80% from the second to the third passage. Within limitations of the experiments other data suggest no detectable change in the pathologic expression of the virus. Plasma from a leukemic bird of the second tumor passage again produced 100% leukemia upon subinoculation of 3-day old chicks.

MATERIALS AND METHODS

Cell suspensions. Nephroblastomas were harvested under aseptic conditions and macerated in an Olson tumor mincer (Olson, 1941). Two parts of Simms' salt solution (Simms and Sanders, 1942) by volume were added to one part by volume of the mince. The suspension was passed through a double layer of coarse sterile gauze and 1,250 units of penicillin G and 2.5 mg of dihydrostreptomycin per ml were added. Cell suspensions were prepared from freshly harvested tumors and processed as rapidly as possible so that inoculation into other chickens was accomplished within 2 to 3 hr.

Cell-free extracts. Filtrates of transplanted tumors were prepared 1 day prior to inoculation from tumors which had been harvested from several transplant passages. The tumors were collected aseptically, sealed in Pyrex tubes and stored in dry ice. One tumor from each of transplant passages 2, 8, and 12a which had been stored for 215, 118, and 32 days, respectively, was selected for test of viral activity. The thawed tumor was processed in a Virtis homogenizer with 19 parts by volume of Simms' salt solution for each gram of tumor. The homogenate was clarified in an International PR-1 refrigerated centrifuge at approximately 1,200 X g for 20 min at 4C. The supernatant fluid was carefully pipetted into a graduated cylinder and 1 g of Celite 512* was added for each 20 ml of extract. The mixture was further clarified by passing it through a pad composed of Celite and Simms' salt solution on No. 1 Whatman filter paper in a Buchner funnel. It was then passed through a Selas 02 filter under negative pressure which did not exceed 50 mm of mercury. The filtrate

*Johns-Manville Products Corporation

was shell-frozen in sealed vials and stored overnight in dry ice.

Inoculation. The cell suspensions were inoculated into the pectoralis muscle except for the first passage in which the intraperitoneal route was used. The filtrates were inoculated into the cubital vein. The inocula were 0.2 cc, using a 1-cc tuberculin syringe fitted with a 20-gauge needle for the cell suspensions and a 26-gauge needle for the filtrates. All inocula were kept in crushed ice during preparation and inoculation. Both types of inocula were used without additional dilution beyond that used in preparation.

Chickens. All studies utilized progeny of an inbred line of single-comb White Leghorn chickens (Line 15I) which had been maintained in isolation for many years in order to reduce natural infection (Waters, 1945, 1951). The spontaneous incidence of both visceral lymphomatosis and neural lymphomatosis in this flock does not exceed 5% and no cases of leukemia or osteopetrosis have been reported. The age of the chickens at the time of inoculation was variable for different cell suspension passages, but was uniform within each passage. All chickens inoculated with filtrates were 7 days old.

Histopathology. Blood smears were stained with May-Gruenwald and Giemsa stains. Tissues were fixed in Zenker-formol solution and stained with hematoxylin and eosin.

RESULTS

Transmission of tumors with nephroblastoma cell suspensions passed serially

Ante-mortem observations. The renal tumor was readily transmitted using cell suspensions in serial passage. Growth at the site of inoculation occurred in the majority of cases, and was progressive. While rapid progressive growth with fatal termination was usual, no cases of unquestionably metastatic renal tumors were observed.

Examinations were conducted three times a week to determine by palpation the time of onset of neoplasia. The data in Table 1 are an approximation of the time at which one-half the tumor-bearing birds developed a palpable mass. The time of development of the tumors was variable and ranged from 10 days to 19 days. Those of passage 5 developed later than the tumors of the preceding or succeeding passages. This discrepancy may perhaps be attributed to the use of a young donor in an early stage of oncogenesis. The tumor used for passage 5 was harvested at 2 weeks postinoculation, in contrast to most of the others which were selected at 3 to 4 weeks.

In spite of the decreased incidence of tumors when older more resistant chickens were inoculated (passages 9, 10, 11, and 12b), no delay in the clinical onset of neoplasia was noted. In those birds which developed a tumor, a palpable mass was detectable in 10 to 12 days. Within the limitations of these experiments, the major effect of inoculation of an older chicken was a decreased incidence of mortality and not a delayed onset of neoplasia. Whether or not the time of mortality is delayed in older tumor bearing chickens cannot be definitely determined from the data. The mean time of onset of palpable

tumors for all 12 passages was 13 days. The mean time of mortality, 33 days, occurred about 3 weeks later.

Mortality of chickens from nephroblastomas. The mortality rate in each passage is shown in Table 1. The age of the host has a direct bearing on both the incidence and the rate of development of many experimentally induced tumors. In these experiments, the age at the time of inoculation was unavoidably variable since passages were made with freshly harvested tumor and it was impossible to have birds of a constant age available for all passages. Older birds were deliberately selected beginning with the 7th passage so that this factor could be appraised in relation to the alterations it might induce in both the type of neoplasia and magnitude of the response. Eckert et al. (1955) reported on the susceptibility of chickens of different ages to the development of granuloblastic leukemia, and results showed the youngest age tested (3 days) to be the most susceptible to BAI strain A. Resistance increased progressively with age.

Chickens up to 15 days of age proved highly susceptible to the tumor transplant, with mortality associated with the primary neoplasm ranging from 67% to 97%. With chickens up to 60 days of age the incidence of tumors decreased. This is best illustrated in passages 12a and 12b, since the same inoculum was injected into chickens of two different ages. In the 11-day old group, 92% developed the primary tumor. In the 60-day old group, only 17% had primary tumors. In spite of the apparent increased resistance with age, a high response was obtained in passage 11 where 60% of the chickens developed a primary tumor after inoculation at 29 days of age. Comparison of the time range with the median time for mortality in each passage

indicated an atypical rate of development by some individuals of a passage group. This may be an expression of the susceptibilities of individuals to the tumor transplant, and an indication of lack of homogeneity in this highly inbred population. The median time of mortality would seem to be preferable to the mean as an indication of response because of the relatively wide range. The median time ranged from a high of 41.5 days in passage 10 to a low of 20.5 days in passage 12a. In most passages the median time of death occurred in 2 to 4 weeks after a palpable tumor developed in 50% of the chickens, but in passage 12a the interval was only 8.5 days.

Pathology. During the early stages of development, the tumor contained a glistening, gray-white, friable parenchyma enclosed in a firmly adherent thin-walled capsule. As the tumor increased in size, additional islands of neoplastic tissue became evident and were palpable. The cut surface of an entire neoplasm revealed a mass divided into many compartments by septa continuous with the peripheral capsule. Centrally, a large tumor often contained extensive areas of hemorrhage and necrosis (Fig. 1).

Histopathological study revealed these tumors to contain a wide range of morphological patterns. Variations were observed among tumors as well as among different areas within the same tumor.

The least differentiated tumors were composed of anaplastic, generally ovoid, vesicular cells containing finely clumped chromatin and having an indistinct cytoplasmic membrane. Growth was both expansive and invasive with the cells forming a sheet and showing little tendency for organization (Fig. 2). Lymphocytes were fairly prominent in the early lesion. They were seen both as single cells

throughout the lesion and as small foci located generally at the periphery of the tumor. This was apparently one manifestation of the host's defense mechanism against the invasion of the cells contained in the inoculum.

Tumors showing slightly greater organization were composed of masses of cells arranged in nests separated by varying amounts of connective tissue stroma (Fig. 3 and 4). Within cell nests, organoid changes were common. Canalization occurred and tubular structures were formed which were thick-walled initially, being composed of many layers of cells. From these emerged tubules of more definite structure consisting of tightly packed columnar cells and resembling pseudostratified columnar epithelium. Those showing greater maturation were composed of a single layer of columnar cells (Fig. 5).

Initially, the connective tissue stroma was of no greater interest than the limiting membrane of any encapsulated neoplasm. With continued observation it became apparent that the stroma exhibited considerable variation in both the amount and degree of maturation. In some tumors this tissue had the appearance of a malignant sarcoma composed of fibroblasts showing little evidence of fibril formation. At the other extreme the stroma consisted of well maturated connective tissue which was quiescent in appearance with fibrils much in evidence. In a few cases, microscopic areas of cartilage was seen in addition to irregular shaped spicules of partially ossified bone (Fig. 6). The tubules were thin walled and mature. The general lack of rapid activity in this lesion would suggest that the cartilage and bone were of metaplastic origin.

Tumor regression. Only four cases of regression were observed,

one each in a chicken of passages 4 and 10, and two in passage 12b. A small tumor was detected on the chicken of the 4th passage on three occasions from 33 to 37 days postinoculation. The mass then regressed, and the bird lived to 141 days at which time it died of visceral lymphomatosis. On the 10th passage chicken a tumor developed at 17 days, persisted to 43 days, and then regressed. Ultimately this bird also died of visceral lymphomatosis at 212 days. The two chickens of passage 12b developed palpable masses which persisted from 16 to 18 days and 21 to 29 days, and both died of undertermined causes at 187 and 212 days, respectively.

Viral-induced neoplasia. In addition to the tumor which developed at the site of inoculation, some chickens developed other forms of neoplasia which could not be specifically attributed to the growth of the transplanted cells, but probably to viral oncogenesis from a virus or viruses released from the transplanted cells. These were designated as cases of "secondary neoplasia". Four types of "secondary neoplasia" were recognized: granuloblastic leukemia, nephroblastomas (renal), visceral lymphomatosis, and osteopetrosis. The time of mortality attributable to these secondary neoplasms is given in Table 2. In passage 1, the intraperitoneal route of inoculation was used. In this group, no distinction could be made between primary nephroblastoma transplants and tumors which arose secondarily in the kidney. Accordingly, all nephroblastomas of passage 1 were classified as primary transplants. In the remaining passages, nephroblastomas developed either at the site of inoculation or secondarily in the kidney. Since the nephroblastomas were never observed in any other locations, it was suspected that secondary tumors were the result of

viral induced oncogenesis rather than metastasis. Mortality from the tumor in the kidney occurred later than for the tumors in pectoral muscle in the same passage. Secondary nephroblastomas occurred in one chicken each in passages 5 and 10.

Moderate to low mortality from leukemia occurred in chickens of each of the first six passages. None developed in the 7th, two cases occurred in the 8th and one case in the 9th passage. In the remaining three passages, all chickens failed to develop leukemia, including those of passage 12a which were inoculated at a highly susceptible age. Thus, the methods employed were effective in allowing for propagation of the renal tumor as a transplant. Ultimately nephroblastomas were obtained in chickens which showed no manifestations of the leukemia characteristic of BAI strain A.

Beginning with the 4th passage,blood smears were prepared weekly from all birds to minimize the possibility of subclinical cases of leukemia being unrecognized at necropsy. The terminal lesions of leukemia are easily recognized grossly, but subterminal lesions may escape detection. Since both the transplant tumor and leukemia occur during the same interval of time after inoculation and either may be a primary cause of mortality, it is possible for a bird to be afflicted with both, but die from a well-developed transplant tumor prior to the full expression of the grossly visible leukemic manifestation. Blood smears and tissue sections of liver and bone marrow were studied from all donors and any other birds which were killed. In Table 3 the leukemic birds are identified, indicating the day of death, whether sacrificed or allowed to die naturally, the duration of the leukemic blood picture, and the diagnostic method or methods used to classify

the bird as leukemic. The earliest detectable cases are those in which the leukemic process is confined to the bone marrow. This manifestation has been termed incipient leukosis (Olson, 1936) and is seen when death occurs prior to the time that the immature cells gain access to the circulation. Following this stage, the neoplastic cells are found in the blood stream in large numbers and the disease may be diagnosed on the basis of a blood smear only. Shortly after the development of the leukemic blood picture leukostasis occurs in various organs which have a sinusoidal type of circulation; tissue sections of these organs are then of diagnostic significance. Since blood smears were made at weekly intervals in this study, the duration of leukemia could not exceed the observed period by more than 6 days. In these chickens the maximum observed duration of leukemia was for a period of 10 days.

Throughout this series of experiments, the leukemia observed was of the granuloblastic type. No cases which could be unquestionably classified as erythroblastosis were seen.

Nephroblastomas were seen in combination with leukemia in a few instances. They occurred as transplanted tumors in pectoral muscle in 4 cases which occurred in passages 3 and 4. In passage 5, a case of leukemia in combination with a renal nephroblastoma was detected. The bird (T3279) failed to develop a palpable tumor at the site of inoculation. Leukemia was first observed on a blood smear made 113 days postinoculation. The blood became progressively more abnormal until the 121st day when the bird was killed for collection of tissues. At necropsy, a small gray-white nephroblastoma about 1 cm in diameter was discovered on the posterior lobe of the right kidney.

Mortality from lymphomatosis occurred in all passages except 3, 5, and 6 (Table 2). In these passages all birds died prior to the time lymphomatosis mortality could be expected (Burmester et al., 1959a, 1959b). One bird in passage 4 had osteopetrosis.

Cell-free transmission

Having demonstrated the feasibility of propagation of this tumor as a transplant, it was of interest to test the viral activity of tumors of various passages. Representative tumors of several passages were available in the frozen state. A careful study of tissues of donors was made to exclude any which might show evidence of incipient leukosis. Those selected for the test were from chickens of passages 2, 8, and 12a. Recipient chickens were inoculated intravenously at 1 week of age with filtrates of selected tumors. Results are given in Table 4.

Specific disease response included granuloblastosis, nephroblastomas, visceral lymphomatosis, and osteopetrosis. The data clearly show a change in the ability of this strain to cause leukemia following passage as a transplant tumor. Filtrate of a 2nd passage tumor resulted in 65% leukemia mortality; that from the 8th passage, only 5%, and from passage 12a no cases of leukemia developed.

In contrast, the incidence of nephroblastomas showed an increase from passage 2 to passage 8 and was maintained at that high level for the final passage tested.

The uninoculated controls remained normal throughout the entire experimental period of 210 days with the exception of three deaths of a nonspecific nature. At the time of termination all birds were killed and subjected to necropsy. One case of visceral lymphomatosis was found.

Table 4 lists both the specific and nonspecific mortality incidence as well as the survivors. Since it was not unusual for a chicken to have more than one type of neoplasm, the sum of these conditions exceeds the number of birds per experimental group. All possible two-way combinations of neoplastic diseases were observed with the exception of leukemia and visceral lymphomatosis. Also a few cases of three-way combinations were seen.

The time of mortality due to granuloblastosis, nephroblastomas, and visceral lymphomatosis is given in Table 5. There was a tendency for these diseases to occur at different intervals of time after inoculation, although overlapping was considerable. Mortality from leukemia occurred earliest, and few cases were seen after 75 days. Mortality from nephroblastoma occurred as early as 64 days while some cases were not discovered until termination. The majority died between the 100th and the 160th days. Most of the mortality from lymphomatosis occurred after 140 days.

The filtrate-induced nephroblastomas were well encapsulated lobulated tumor masses that originated in the renal parenchyma which they largely replaced (Fig. 7). They occurred, either unilaterally or bilaterally, as distinct separate tumors or more commonly as a single nodular mass of large proportions. They appeared to be embedded in normal renal tissue but were sharply demarkated from it by a fibrous capsule when small. Often the tumors were many times the size of a normal kidney and only small remnants of normal tissue remained. The cut surface showed areas of gray-pink fleshy tumor masses separated by fibrous trabeculae. Various sized cysts containing straw colored tenacious fluid were frequently seen. The center of the neoplasms

was often hemorrhagic and necrotic.

A syndrome which, clinically, resembled that seen in neural lymphomatosis often resulted from pressure exerted on the nerve trunks emanating from the spinal cord dorsal to the kidney.

Microscopically, a great diversity of morphologic patterns was observed in these tumors similar to that already described for the transplants, except that examples of the extreme anaplastic lesion were not seen. This may be the consequence of selection of tumors which were in a more advanced state of differentiation, since tumors growing in the kidney were necessarily quite large before they could be detected by palpation.

These tumors showed a predominence of either epithelial or sarcomatous cells. The origin of nephroblastomas is construed to be from the undifferentiated nephrogenic tissue which is recognized to have pleuripotent capabilities for differentiating into several types of adult tissue. The mixed nature of these filtrate induced neoplasms and various degrees of differentiation are shown in Figs. 8 to 10.

Undifferentiated tumor cells were seen arranged in nests separated by a sarcomatous stroma (Fig. 8). Canalization occurred within cell nests and resulted in the formation of irregular shaped tubules. Some tumors showed a greater degree of differentiation in which well developed tubules were composed of a single layer of cuboidal or columnar cells and were separated by a scanty stroma which also contained nests of undifferentiated cells and structures very suggestive of glomerulus formation (Fig. 9). Masses of sarcomatous spindle cells were the predominating feature of still other neoplasms, some of which contained well differentiated tubules which showed a papillary structure (Fig. 10).

DISCUSSION

The successful serial propagation of nephroblastoma cell suspensions in these experiments, is in contrast to the unsuccessful results of earlier investigations (Foulds, 1940; Duran-Reynals, 1946). The use of Line 15I chickens which are highly inbred and susceptible to the transmissible forms of avian leukosis was quite likely of major importance in providing an environment suitable for propagation of these tumor cells.

Propagation of the renal tumor minus the development of manifestations of leukemia was a major objective, and is of particular interest. Although the chickens used in some passages were sufficiently old that resistance to the development of leukemia could be predicted, those of the last passage (12a) were 11 days old, which is a relatively susceptible age. Experiments of Burmester et al. (1959b) showed that plasma, diluted 10-fold greater than these tumor suspensions, caused over 90% mortality from leukemia in chickens inoculated at 14 days of age.

An experiment was designed to provide a more critical appraisal of the leukemia-inducing potential of the strain after transplantation. A separate filtrate was prepared from a tumor selected from each of suspension passages 2, 8, and 12a, and was inoculated by the intravenous route into chickens 7 days old. As the result of transplantation of renal tumor cell suspensions, the propensity of strain A to induce nephroblastomas increased while its tendency to cause leukemia decreased. A significant incidence of both visceral lymphomatosis and osteopetrosis occurred in some chickens of each of the three groups challenged with filtrates.

The altered oncogenic potentialities of strain A following transplant propagation as a renal tumor could be cited as evidence that the strain represents a mixed population of viruses and the virus responsible for leukemia had been eliminated by selection and passage. An alternate viewpoint is that a single agent can induce more than one histologic type of tumor even to the extent that different cellular systems are affected. As pointed out by Burmester et al. (1959b), many investigators have considered various leukosis strains to contain a single uniform population of virus particles which have multipotent potentialities for induction of distinctly different neoplastic entities. Thorell (1958b) seemed to favor the concept of a single agent possessing multipotent oncogenic capabilities, the expression of which may be influenced by the experimental method. He also considered the other viewpoint, but no mention was made of lymphomatosis or osteopetrosis occurring in his stock. Evidence which he felt was suggestive of separate specific etiology for the tumors induced, therefore, pertained to only leukemia and the renal tumors. The lack of lymphomatosis and osteopetrosis in Thorell's experiments which were of 5 months duration may indicate that his flock was of a different degree of susceptibility for those diseases than the flock of this laboratory.

Baluda and Jamieson (1961) interpreted their results with strain A to be indicative of a single virus being responsible for the various neoplasms induced. Plaque isolated polyoma virus was shown by Sachs and Winocour (1959) to induce neoplasms in the mouse which are of several different histological types. Thus, they added support to the earlier contention of Stewart et al. (1958) that the virus was

probably a single multipotent oncogenic agent. If this is the situation with strain A, the results suggest that the experimental conditions employed created an unfavorable environment for the development of the leukemic manifestation. For reasons mentioned, it is unlikely that the resistance of the host was too great for the leukemic response with strain A (Beard, 1957a; 1958; Burmester et al., 1959b). If a loss of titer occurred as a result of transplant passage this could account for the lack of leukemia in the later cellular passages, and the results of filtrate tests are also compatible with this hypothesis. However, the incidence of renal tumors did not decrease with transplant passage, nor did the filtrate tests indicate decreased renal tumors as a result of passage. One can conclude that if virus titer is the factor responsible for the presence or lack of leukemia, the other neoplasms are less subject to the influence of dosage at least in the dosage level employed in these experiments.

Still another and attractive possibility is that the virus has been modified by transplant tumor passage and rendered incapable of inducing leukemia regardless of the inoculated dose of virus. This could be demonstrated only by further extensive experimentation and collection of negative evidence in an attempt to regain a leukemia inducing virus from experimentally induced nephroblastomas.

The use of cell suspensions to transplant tumors of known or suspected viral etiology usually leaves unanswered questions concerning the origin of secondary tumors. One is ordinarily unable to state whether the secondary neoplasms arise as the result of metastasis from the primary implant or are due to action of oncogenic virus released from implanted cells. Transplantation of the nephroblastoma offered

a unique opportunity to shed some light on the mechanism of spread of this neoplastic disease within the host. The secondary neoplasms in these experiments were predominately manifestations of avian leukosis, which are histopathologically distinct from nephroblastomas and accordingly could not have arisen as a result of metastasis. Only two secondary nephroblastomas occurred in the 12 passages. They could have arisen by metastasis, but viral induction seems more probable since they were observed only in kidneys and mortality from them occurred later than from primary nephroblastomas of the same passage. The results of the cell-free transmission provides additional supporting evidence to this concept.

The histopathological characteristics of the experimentally transmitted renal tumors clearly indicate the complexity of the nephroblastomas. Great variation was found in the type of tissue which predominated and the degree of maturation both among areas of a single tumor and among different tumors. The renal tumor was previously identified as an adenocarcinoma in a report from this laboratory, but in view of the newer information the term adenocarcinoma is considered too restrictive. The term nephroblastoma is considered more appropriate and is in harmony with the previously published, although variously named, nephroblastomas of chickens, other animals and men.

Detailed study of tumors in various stages of development provided information from which a concept of the typical morphogenesis of these tumors could be inferred. The tumor cells grew at the site of inoculation and were barely detectable as palpable masses as early as 10 days after inoculation. Tissue sections of such tumors showed a mass of anaplastic cells which appeared to be growing rapidly and

showed slight semblance of organization. The next recognized developmental stage consisted of epithelial cells organized into nests separated by a loose connective tissue stroma. This was followed by organoid changes including canalization and the formation of tubules. Primitive appearing tubules had thick walls composed of many layers of epithelial cells, but well maturated ones contained just a single layer of epithelial cells. It should be emphasized that most tumors were not highly uniform throughout, since several stages of development might be seen in the same tumor. The stroma was also variable as to its extent and stage of maturation. In a few instances microscopic areas of cartilage and bone were detected.

The filtrate induced nephroblastomas appeared identical to those induced by cell suspensions except that the extreme anaplastic form was not seen. This may be consequential to the collection of filtrate induced tumors at a later stage of development since they were necessarily quite large before they could be palpated because of their location in the kidney.

If a method is devised for proving conclusively that a single leukosis virus is capable of inducing all of these neoplastic diseases, consideration should be given to the inclusion of renal tumors as an additional member of the avian leukosis complex. This suggestion was specifically made by Carr (1960) in reference to his work on renal carcinomas. Olson and Bullis (1942) considered a possible etiologic relationship between embryonal nephromas and visceral lymphomatosis (lymphocytoma) because of the rather high incidence of both diseases occurring spontaneously in the same chickens. However, they concluded that the two diseases were apparently initiated at different

periods of life; because of the widely accepted opinion that nephroblastomas are a result of abnormal embryonic development, and visceral lymphomatosis virus could be assumed to exert postembryonic action making a causal relationship between the two unlikely. The experiments described in the present contribution demonstrate unquestionably, that avian nephroblastomas can be initiated during postembryonic life by inoculation of a viral preparation. The concept that naturally occurring nephroblastomas are a result of a developmental fault should be re-evaluated, for quite possibly viral etiologic agents may be demonstrated for other species if experimental transmission is attempted in suitably susceptible test animals.

Ultrathin sections of BAI strain A nephroblastomas were compared under the electron microscope with infected tissues from chickens with BAI strain A induced leukemia (Dmochowski et al., 1961). Virus particles in the nephroblastomas showed a wider range in size, and had a greater mean size. Also sites of probable virus formation not previously observed in similar studies on avian leukosis were described. The significance to be attached to these findings is unknown at present (Dmochowski et al., 1961).

SUMMARY AND CONCLUSIONS

Transmission studies of an unusual avian renal tumor considered initially to be a renal adenocarcinoma were undertaken since this tumor in addition to visceral lymphomatosis and osteopetrosis occurred in chickens inoculated with a leukosis strain considered previously to cause only granuloblastic leukemia.

The tumor was readily transplantable by inoculation of cell suspensions which resulted in a palpable tumor in about 2 weeks and was followed by mortality in about 3 additional weeks. Serial transplant propagation of the tumor resulted in a loss of the leukemic manifestation characteristic of the strain after nine transplant passages.

Viral activity of tumors of three of the transplant passages was tested by inoculation of tumor filtrates in leukemia susceptible chickens. Those from the last transplant passage no longer induced leukemia but did cause a high incidence of renal tumors and a moderate incidence of visceral lymphomatosis and osteopetrosis.

Histopathological evidence was presented which justifies considering this tumor a nephroblastoma rather than an adenocarcinoma.

Although the leukemic manifestation was eliminated from the neoplastic spectrum of strain A in the later transplant passages and in the filtrate test of one of them, these experiments are not conclusive evidence of a separate etiology for leukemia.

The evidence for viral induction versus metastasis of secondary neoplasms in this system was discussed.

The significance of postembryonic stimulation of nephroblastomas was mentioned as was the possible indications for including these neoplasms as an additional member of the avian leukosis complex.

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Table 1. Duration of primary nephroblastoma and time of mortality related to age of test chickens and donor birds

Passage number	Age (days) at inoc.	Age (days) of donor	Approx. time (days) 50% with palpable tumor	Mortality with tumor no.	%	Time in days Range	Time in days Median
1	21	160	--*	10/16	62.5	36-157	40
2	3	37	19	10/15	66.6	28-59	32
3	6	32	15	14/15	93.2	28-42	35
4	7	28	13	11/15	73.4	22-43	27.5
5	6	14	18	12/15	80.0	33-102	40
6	8	23	10	11/12	91.6	19-37	28.5
7	15	21	13	13/15	96.6	28-61	32.5
8	15	21	10	10/14	71.4	20-80	36.5
9	33	24	11	6/15	40.0	22-62	34
10	26	22	10	3/10	30.0	32-51	41.5
11	29	23	12	9/15	60.0	20-37	33
12a	11	25	12	11/12	91.7	17-35	20.5
b	60	25	11	2/12	16.7	--†	24
Mean			13				33

* Not palpable because of intraperitoneal inoculation.

† Sacrificed at 24 days.

Table 2. Incidence and time of mortality from secondary neoplasms in chickens inoculated with nephroblastoma cell suspensions

Passage number	Age at inoc.(days)	Incidence of neoplasms				Median time to death /		
		Granulo-blastosis	Renal tumor	Visc. lympho.	Osteo-petrosis	Granulo-blastosis	Renal tumor	Visc. lympho.
1	21	1/16	--*	3/16	0	37	--	140
2	3	3/15	0	1/15	0	26	--	197
3	6	2/15	0	0	0	59.5	--	--
4	7	3/15	0	3/15	1/15	37	--	141
5	6	3/15	1/15	0	0	50	121	--
6	8	1/12	0	0	0	63	--	--
7	15	0	0	2/15	0	--	--	146.5
8	15	2/14	0	2/14	0	36.5	--	151
9	33	1/15	0	6/15	0	132	--	130
10	26	0	1/10	5/10	0	--	85	176
11	29	0	0	4/15	0	--	--	157
12a	11	0	0	1/12	0	--	--	121
b	60	0	0	4/12	0	--	--	189

* Data not available.

/ Days postinoculation.

Table 3. Leukemia duration and related pathology in chickens inoculated with nephroblastoma cell suspensions

Passage number	Bird number	Days postinoculation: Time of death*	Days postinoculation: Leukemia duration	Diagnostic Methods: Gross necropsy	Diagnostic Methods: Blood smear	Diagnostic Methods: Tissue sections	Concomitant neoplasm
1	SP100	37 k	-- /	±	+	+ liver, marrow	
2	T2479	23 d	--	+	-- /	-- /	
	T2424	26 d	--	+	--	--	
	T2420	37 d	--	±	--	+ liver	
3	T2542	28 k	--	-	-	- liver, + marrow	pectoral nephrobl.
	T2538	91 d	--	+	--	+ liver, marrow	
4	T3251	29 k	--	-	-	+ marrow	pectoral nephrobl.
	T3248	37 d	35-37	±	+	+ liver	pectoral nephrobl.
	T3244	43 d	42-43	+	+	--	pectoral nephrobl.
5	T3281	34 k	28-34	+	+	+ liver	
	T3274	50 d	49-50	±	+	+ liver	
	T3279	122 k	113-121	+	+	+ liver	renal nephrobl.
6	T3719	63 d	61-63	-	+	- liver	
7	none						
8	T3955	36 d	26-36	+	+	--	
	T3960	37 d	33-37	+	+	--	
9	T6032	134 d	128-139	+	+	--	
10	none						
11	none						
12	none						

* d = natural death; k = killed.

/ -- data not available.

Table 4. Filtrate induced mortality incidence of selected passages of transplanted nephroblastomas

Test Passage	Disease incidence										
	Granuloblastosis		Nephroblastomas		Visc. lympho.		Osteopetrosis		Nonspecific	Survivors	Total
	no.	%	no.	%	no.	%	no.	%	no.	no.	no.
2	26/40	65.0	12/40	30.0	5/40	12.5	3/40	7.5	2/40	1/40	49/40
8	2/40	5.0	25/40	62.5	14/40	35.0	7/40	17.7	4/40	0	52/40
12a	0		24/37	64.9	7/37	18.9	7/37	18.9	3/37	2/37	43/37
Controls (noninoculated)	0		0		1/38	2.6	0		3/38	34/38	38/38

Table 5. Time of mortality related to type of disease induced by filtrates

Test passage	Days postinoculation					
	Granuloblastosis		Nephroblastomas		Visc. lympho.	
	Median	Range	Median	Range	Median	Range
2	32	24-108	117	75-192	174	111-190
8	67.5	59-75	127	84-210	151	121-210
12a			134.5	65-210	160	126-203

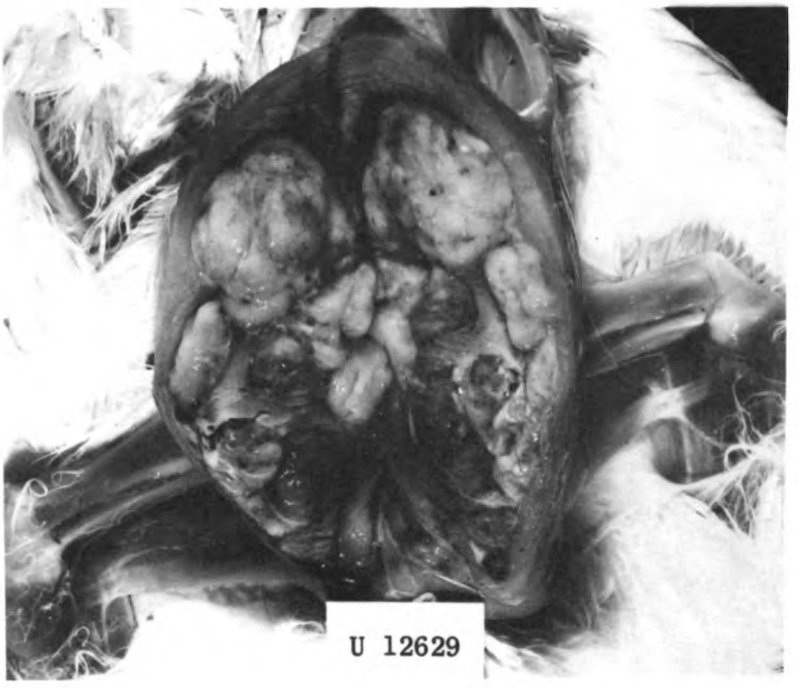

Fig. 1. Nephroblastoma-pectoral; cell transplant. Sagital section shows lobulated tumor masses encased in thin remnant of pectoral muscle. Necrotic tissue prevalent in lower portion.

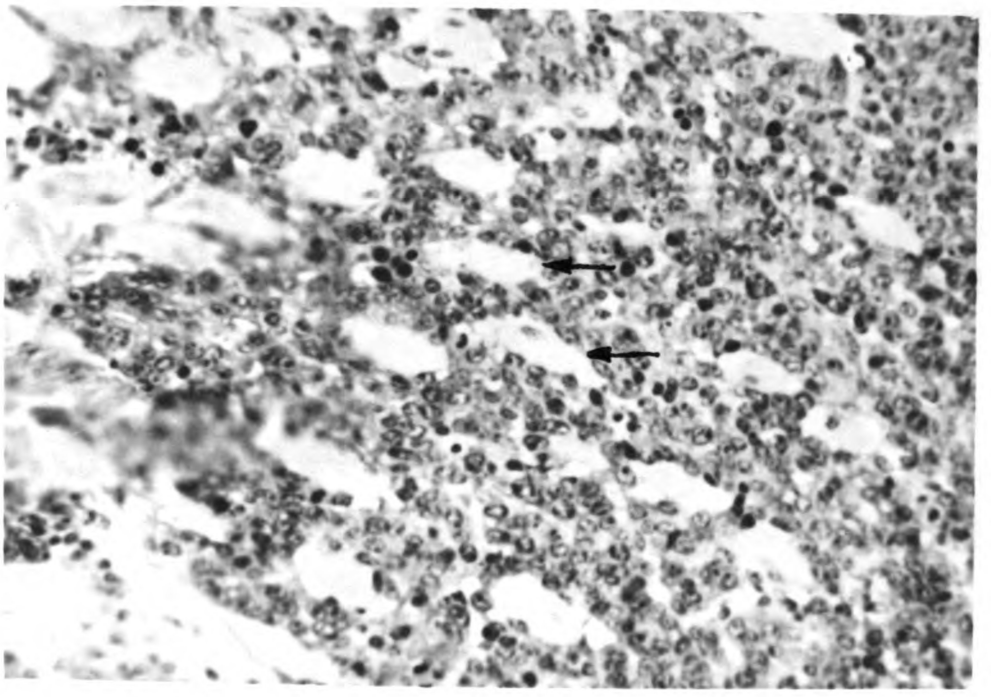

Fig. 2. Nephroblastoma-pectoral; cell transplant. Anaplastic cells growing in an unorganized manner among remnants of isolated muscle fibers (arrows) [X455 approx].

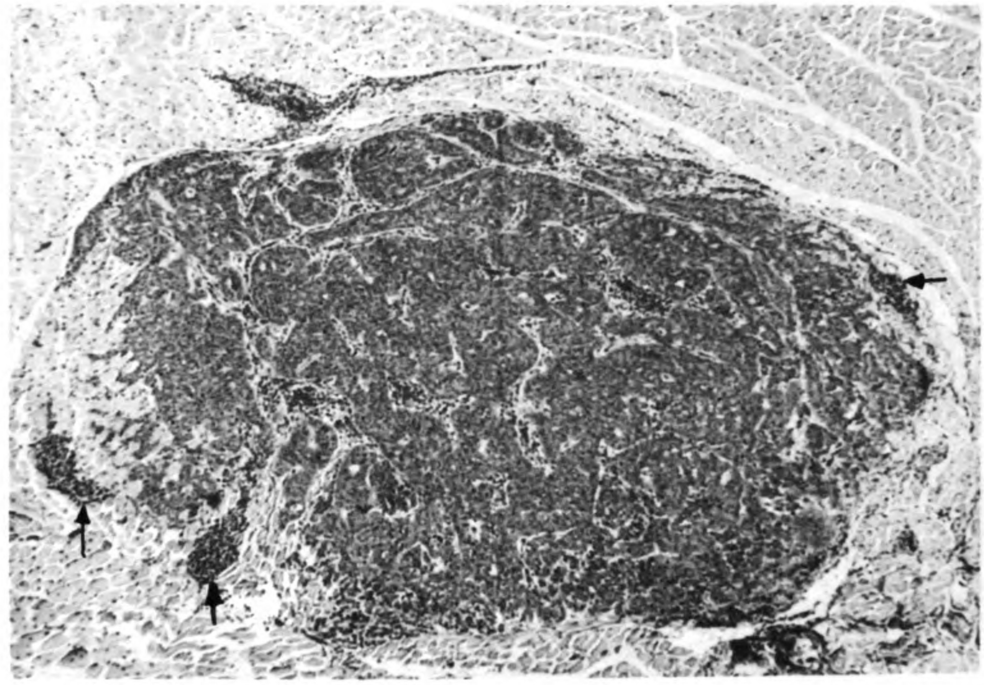

Fig. 3. Nephroblastoma-pectoral; cell transplant. Small tumor with minimal connective tissue septa and slight evidence of cell nest formation. Peripheral lymphocytic foci (arrows) [X85 approx].

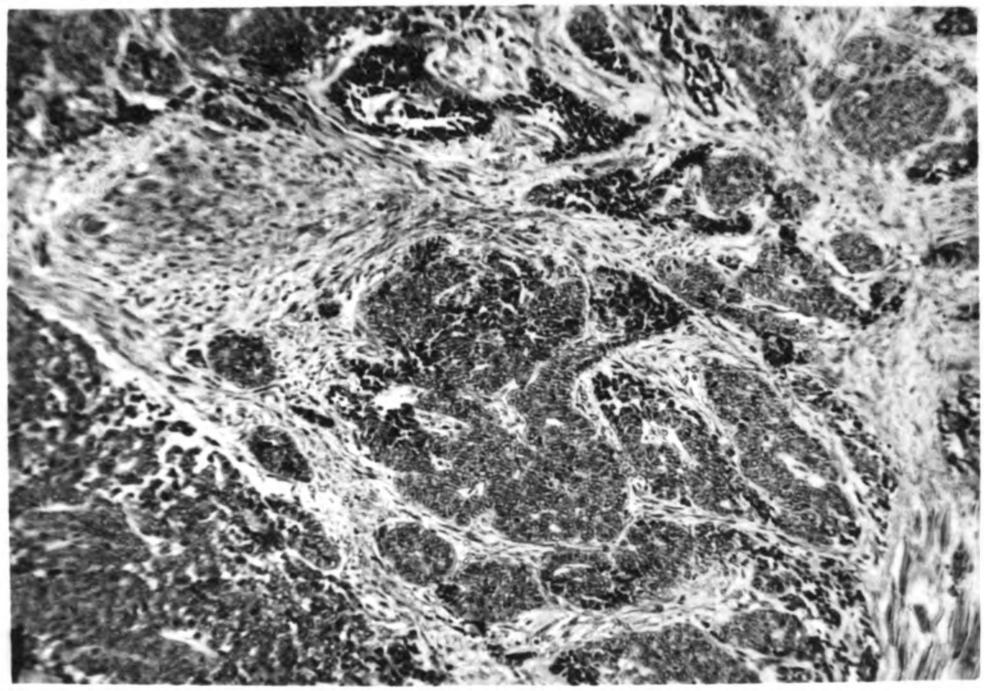

Fig. 4. Nephroblastoma-pectoral; cell transplant. Cell nests separated by abundant sarcomatous stroma; note formation of primitive thick-walled tubules [X175 approx].

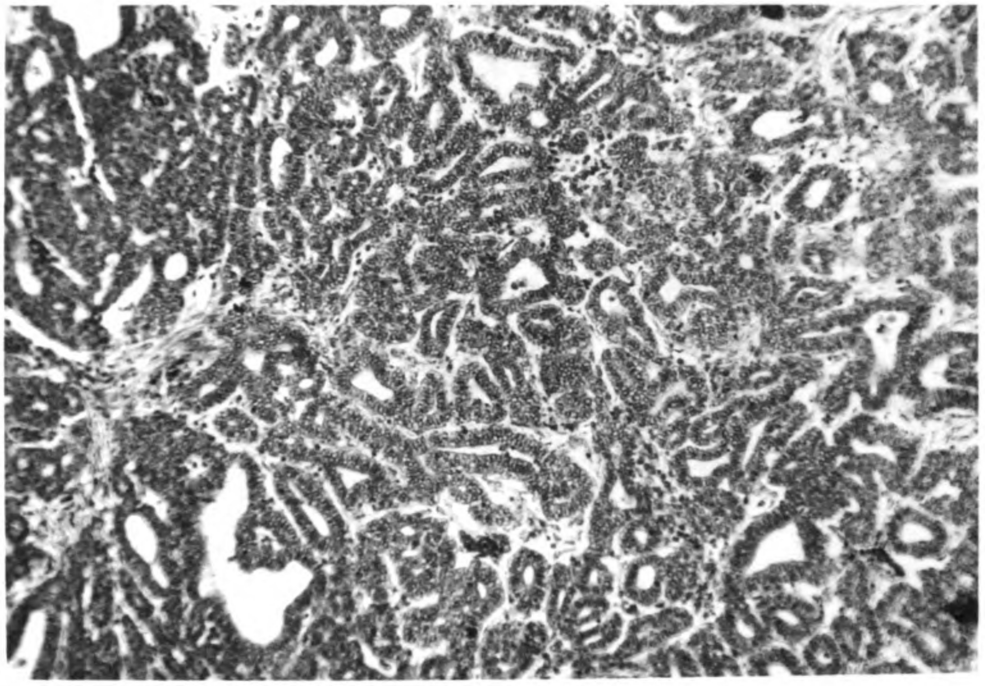

Fig. 5. Nephroblastoma-pectoral; cell transplant. Well defined tubules with basement membranes and scant interstitial stroma [X175 approx].

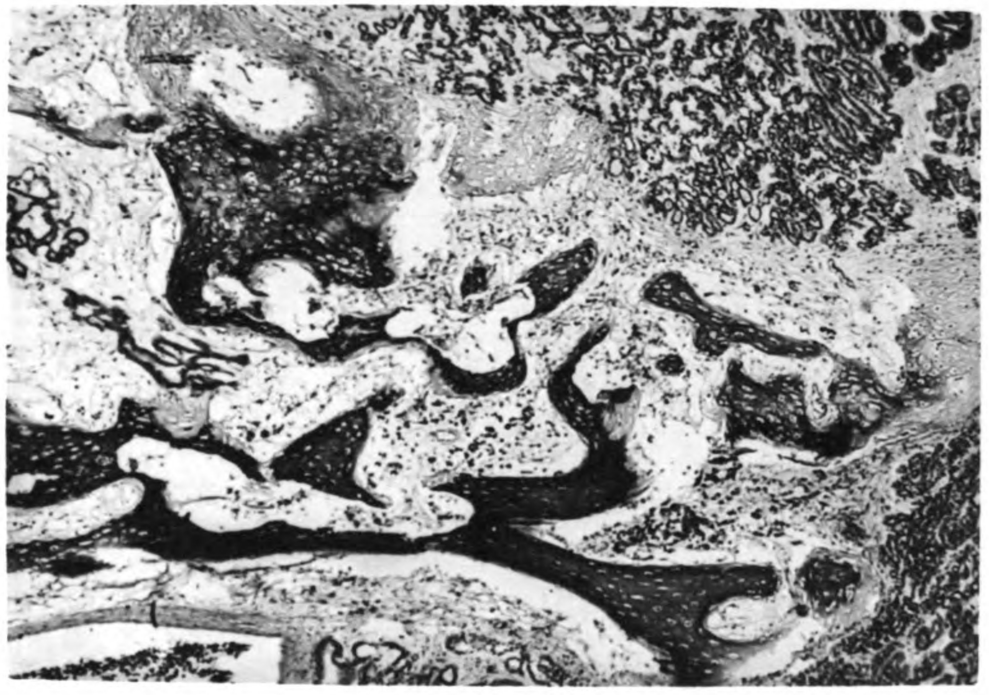

Fig. 6. Nephroblastoma-pectoral; cell transplant. Metaplastic bone in interstitial stroma bordered by area of thin-walled tubules [X85 approx].

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Fig. 7. Nephroblastoma-renal; filtrate induced. Large lobulated encapsulated tumor attached to remnant of left kidney; smaller neoplasm on posterior aspect.

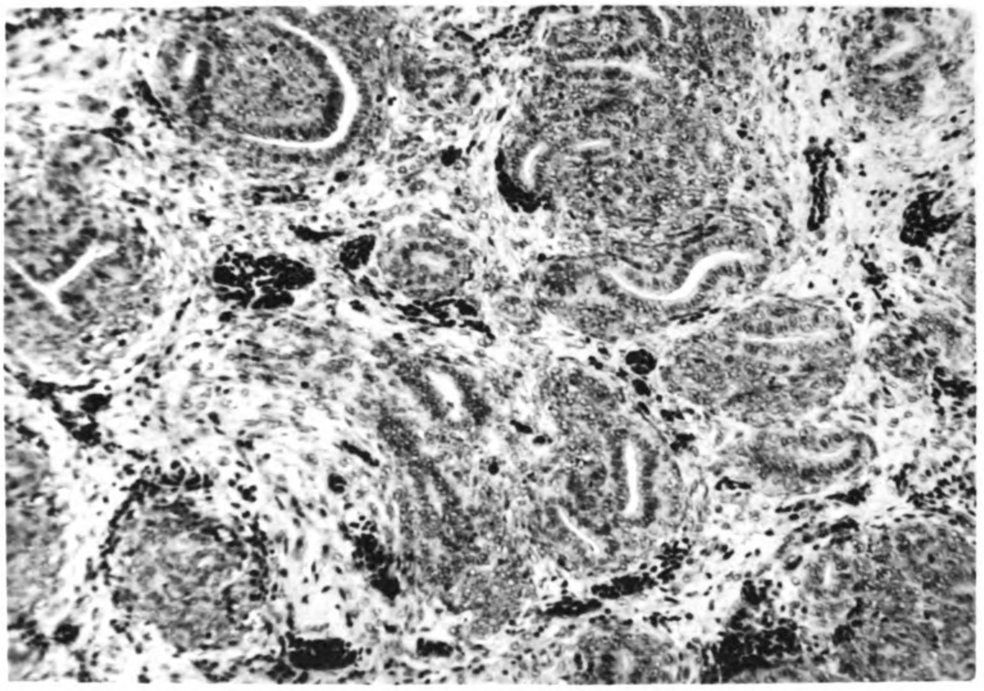

Fig. 8. Nephroblastoma-renal; filtrate induced. Primitive cells arranged in nests separated by sarcomatous stroma. Within nests the formation of tortuous tubules is seen [X220 approx].

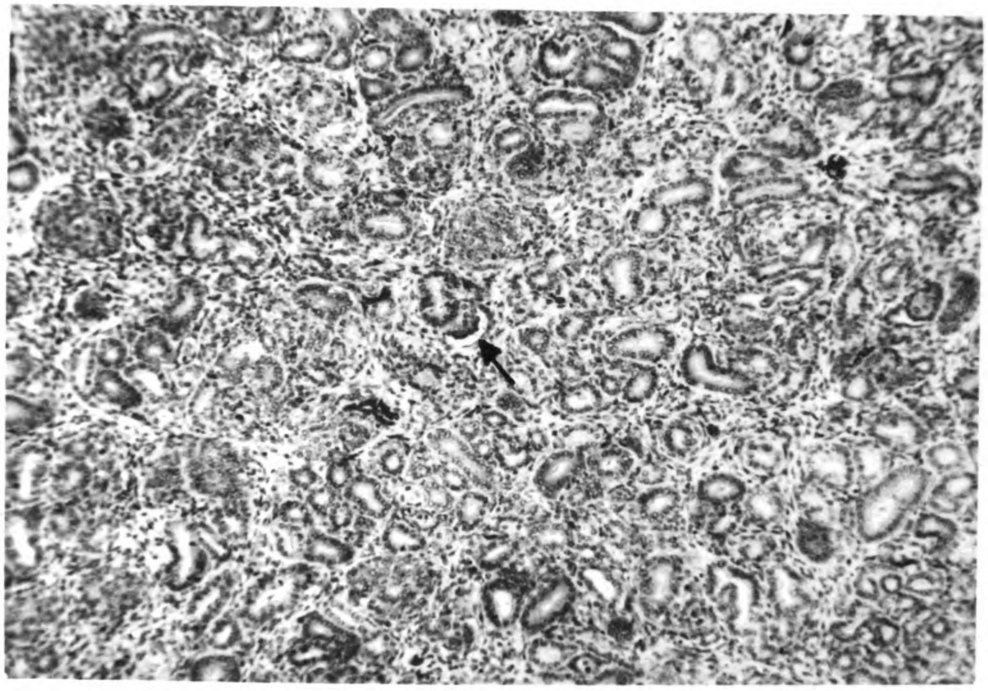

Fig. 9. Nephroblastoma-renal; filtrate induced. Tumor composed of well differentiated tubules which remarkably simulate renal tissue, including glomerulus-like structure (arrow) [X175 approx].

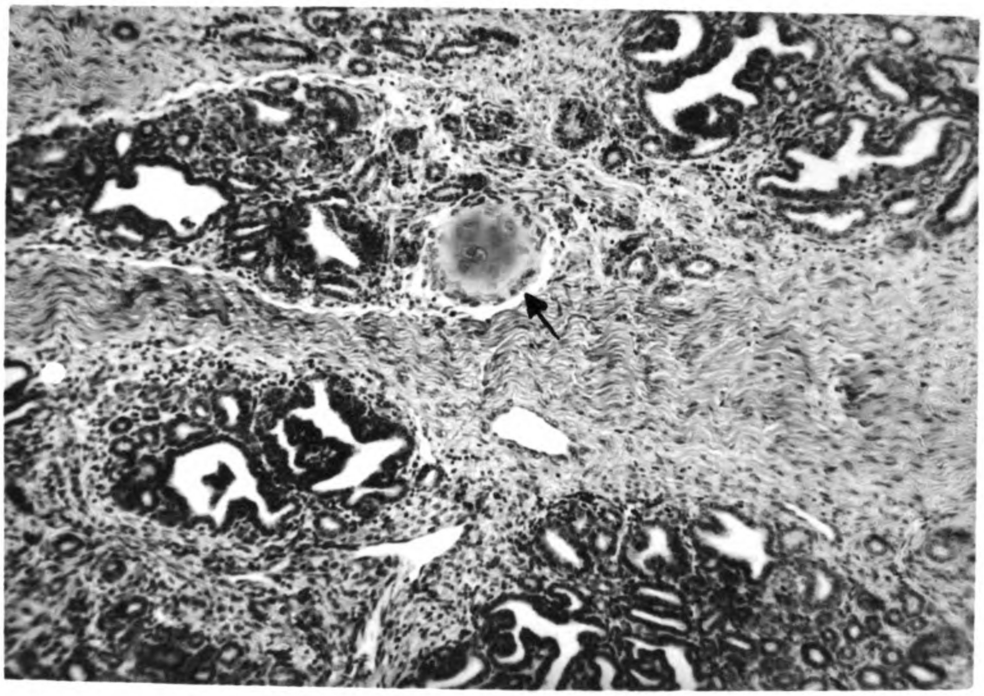

Fig. 10. Nephroblastoma-renal; filtrate induced. Sarcomatous stroma composed of relatively mature fibrous connective tissue, with small area of cartilage (arrow). Tubules are thin-walled, well differentiated and in some cases show papillary structure [X175 approx].