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Cultivation in Tissue Culture of the Virus of Avian Lymphomatosis. (23870)

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The demonstration by Burmester, Prickett and Belding(1) that a cell-free extract of the Olson(2) transplantable lymphoid tumor could transmit avian visceral lymphomatosis, firmly established the fact that an agent or agents other than cells could induce the disease. Further studies(3) have shown that filterable agents are present in other rapidly growing lymphoid tumor strains. Because propagation of viruses in various cell-medium systems is now common, it was thought that a system might be found in which the agent of avian visceral lymphomatosis would multiply and show its presence by induction of cytological damage. Previous attempts to do this have been only partially successful. Doljanski and Pikovski(4) have reviewed previous work and reported that they were able to propagate the agent of fowl leucosis in cultures of common mesenchyme cells but were unable to produce cytopathological changes.

The present communication is a preliminary report on successful propagation from avian lymphomatosis liver filtrates of an agent producing focal lesions in tissue culture, serial passage of the agent, infection of embryos and chicks with the cultured agent, and recovery of the agent from the diseased embryos and chicks.

Materials and methods. Virus. The virus was contained in a filtrate of lymphomatous liver obtained from Dr. B. R. Burmester, Regional Poultry Research Laboratory, East Lansing, Mich. The filtrate was designated RPL-12-L28 and represented the 16th serial filtrate passage in White Leghorn chicks. *Tissue culture.* Chick embryo liver cultures were prepared as follows: Sixteen-day-old chick embryo livers were minced and mildly agitated with 0.85% trypsin solution. Gross particles that settled rapidly were discarded. The remaining cells were then sedimented by low-speed centrifugation and washed with balanced salt solution. Tubes were seeded with approximately 500,000 cells in 1 ml of medium consisting of 30% horse serum and 70% medium 199(5), and incubated 2 days at 37°C. The medium was then replaced with 100% medium 199, after which the tubes were inoculated with tissue filtrates or infective tissue culture fluid. Cultures of whole chick embryo were prepared in a similar manner from 10- to 12-day-old embryos. A slightly modified Eagle(6) medium containing 10% horse serum was used. After 24 hours' incubation at 37°C, the medium was changed to 100% 199, and the tubes were inoculated. *Neutralization tests.* Serum neutralization tests in tissue culture were done by inoculating tubes with 0.1 ml of serum-virus mixture. A constant amount of virus (1,000 to 10,000 tissue culture infectious doses) was mixed with serial 2-fold dilutions of serum, and the mixture remained at room temperature for at least 2 hours. The neutralization titer of the serum was the highest dilution of serum in the incubated inoculum which prevented cellular destruction for 7 days.

Results. Tissue culture. Chick embryo liver cells grew on glass as discrete islands of hepatic cells surrounded by large strands of fibroblasts. On low power microscopic examination of living preparations, the first noticeable effect of the virus was swelling of he-

patic cells, some of which appeared to wander out of the islands. The cells showed increasing granularity and density of nucleus until cellular contours were lost. At this stage the cells often detached from the glass. Pathological changes were also observed at a later stage in the fibroblasts. These were essentially characterized by a diffuse granulation of cytoplasm giving the impression of sand scattered on the monolayer. That these changes were caused by the agent and not by toxic products from destroyed hepatic cells was shown by experiments with cultures of whole chick embryo cells. In such cultures the cell type is principally fibroblast. The agent induced pathological changes in these cultures which were similar, both in time of appearance and in microscopic characteristics, to those observed in fibroblasts of embryo liver cultures. When the inoculum was undiluted, very few cellular changes were evident in the first passage, but after the second or third passage the parenchymal cells showed characteristic changes within 48 hours. With diluted inocula, cytological lesions were not evident before the sixth day (144 hours). In the early blind passages, cultures were kept 10 or 12 days, or as long as cells of control tubes remained healthy. Incubation temperature was 37°C after adaptation, but cytological lesions occurred earlier at 39°C, and this temperature may be best for the first 2 or 3 passages. The infectious titer of culture fluid varied but $TC_{50}(7)$ titers as high as $10^{9.4}$/ml have been obtained.

Other chick and chick embryo cells such as lung and kidney, and whole chick embryo cells (mostly fibroblasts) are attacked by the virus, but the most satisfactory results were obtained with embryonic liver preparations. Duck embryo cells were also susceptible but neither primary cultures nor established cell lines of mammalian cells would support growth of the virus.

Identification. Antisera to infectious bronchitis, Newcastle disease, laryngotracheitis, fowl pox and serum from normal chicks did not neutralize the agent, and the presence of pleuro-pneumonia-like organisms was not demonstrable in the cultures. Antisera to the agent prepared by inoculating chicks with infected tissue culture fluid had titers of 1:256. When an adjuvant was used with the culture fluid in preparation of immune sera, the titer was increased to 1:2048. Immune sera having similar titers were obtained when guinea pigs or rabbits were immunized. Two antisera to the lymphomatosis agent prepared by Dr. Burmester had titers of 1:256 and 1:512 respectively. Antisera prepared against normal chick embryo tissues and against Forssman antigen failed to neutralize the agent.

Immune sera to erythroblastosis and myeloblastosis were supplied by Dr. J. Beard, Duke University, and Dr. B. R. Burmester. The relation of these viruses to the agent under study was suggested by neutralization titers of 1:32 to 1:128 obtained with myeloblastosis antiserum, and 1:64 to 1:128 with erythroblastosis antiserum.

Infectivity. Chick embryo. Infection of embryos with this isolate was obtained regularly by the intravenous route and usually by the intracranial route of injection. Other routes of inoculation did not always produce infection. Best results were obtained with intravenous inoculation of 12-day embryos and death occurred in 3 to 7 days, depending on amount of virus introduced. Two types of lesions were observed. In the first 24 hours, lesions of 1 type were characterized by appearance in hepatic cells of large swollen nuclei containing numerous and conspicuous inclusion bodies. The hepatic cells became progressively dense, forming an amorphous mass in which no particular structures could be recognized. During this period no alterations were evident in other organs. It is interesting to note that identical chronological and morphological sequence of changes was also encountered in stained preparation of chick embryo liver tissue cultures infected with this virus. The second type of lesion was observed after 36 to 48 hours. The number of hepatic cells containing inclusions decreased, and round cells with a conspicuous vesicular nucleus and strongly basophilic cytoplasm appeared in the mesenchyme of various organs. These cells were particularly evident in the portal spaces surrounding the wall of vessels, but were also seen in interstitial connective

tissue of the myocardium, mesonephros and metanephros. The spleen volume was usually 2 to 3 times that of embryos inoculated with noninfectious material, and the large round cells constituted the greatest percentage of the cell population of the organ; correspondingly there was a depression of myeloid elements. Blood, bone marrow, yolk sac and *Fabricius bursa* of infected embryos showed no relevant changes. Three to 5 days after inoculation, the mortality was very high and at autopsy, concomitant to round cell infiltration, large areas of necrosis, often hemorrhagic, were observed in the liver.

Chicks. Chicks of White Rock stock known to have a low natural incidence of lymphomatosis were inoculated by various routes when 1 day to 3 weeks of age, with virus that had been propagated in either tissue culture or chick embryo. Virus that had gone through 8 or more serial passages was used to reduce the chance that the actual infectious agent had used the observed virus as carrier without itself being propagated in the process.

While there have been no cases of lymphomatosis observed in uninoculated birds, the response to inoculation has ranged from 15 to 56 percent. The best infection rate observed was 9 of 16 birds showing visceral lymphomatosis in a 9-month observation period. With the exception of 3 chicks which showed neural lymphomatosis, all had large livers in which the type of lymphomatosis varied from diffuse to nodular form.

Recovery of virus. Virus was readily recovered from infected chick embryos in titers which ranged up to 10^8 TCD_{50}/gram of embryo. Recovery of virus from adult lymphomatous livers has been less successful. A cytopathogenic agent has been found in most livers but some isolates induced infection only in fibroblasts of the culture.

Discussion. Avian visceral lymphomatosis in this report refers to the disease in chickens characterized by a round lymphoid type cell infiltration which is seen in intravascular infiltration or in extravascular nodules in liver or other organs. The spleen is usually greatly enlarged and in some cases primitive cell types may appear in the blood. Our work is interpreted to indicate that any one agent may cause various disease manifestations.

Although our isolate infected the chick embryo only by intravenous or intracranial route, some more recent isolates regularly induce infection of the embryo by inoculation of the yolk sac, allantoic cavity or chorio-allantoic membrane. There is considerable variation in behavior of all isolates in tissue culture.

Summary. An agent has been cultivated in tissue culture from avian lymphomatous liver filtrates which can be passed serially in chick embryo liver cells and which induces cytopathological changes. The agent induces in 12-day-old chick embryos a disease histologically resembling lymphomatosis. In chicks it has induced lymphomatosis in 20% to 55% of the birds in 9 months, and an agent similar to the original agent has been recovered from some of the diseased livers.

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