Growth in Tissue Culture of Cytopathogenic Agent from Strain of Virus which Produces Avian Lymphomatosis. (23899)

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Many attempts have been made to propagate in tissue culture, viruses which cause several neoplasms in the fowl. The majority of studies were performed with several forms of avian leukosis(1-3). Early studies were concerned with cultivation of virus from explanted neoplastic tissue. These attempts at serial passage for several generations, in tissue culture, have failed. Recent studies(4,5) indicate that the Rous sarcoma virus can be successfully cultivated in chick fibroblast cultures, often producing distinct cellular changes. Preliminary observations on growth of visceral lymphomatosis (V.L.) virus in tissue cultures have been reported(6,7). The greatest difficulty is the long latent period of disease in the host(8). Thus, any studies involving biological expressions of the virus may take as long as 9 months. Attempts to cultivate the virus in a host other than chicken have generally failed. It has been reported (9) that V.L. virus can be serially propagated in embryonating chicken egg; however, no gross or microscopic lesions are observed in the embryo. This paper describes recovery and cultivation of a cytopathogenic agent from strain RPL 12 which not only causes visceral lymphomatosis, but under certain conditions also erythroblastosis, osteopetrosis and, sporadically, still other neoplasms.

Materials and methods. Virus. Inoculum L29 was derived from pool of 5 livers from chickens of 17th serial cell-free passage of strain RPL 12. Inoculum L30 was from liver of one chicken of 15th serial passage of same strain. Inoculum L31 was from a pool of 11 livers from chickens of 16th serial passage. Recovery of virus and preparation of cell-free inoculum have been described(8). *Sera.* Antiserum against tissue culture propagated virus was prepared in adult chickens by injecting into breast muscle a virus-adjuvant mixture 4 ml/injection, in 2 vaccinations spaced 3 weeks apart. Normal chickens from isolated population were used for preparing all chicken anti-RPL 12 serum, as well as supplying a source of normal serum. Antiserum produced in rabbits with tissue cultured strain RPL 12 virus was kindly supplied by Dr. G. R. Sharpless.* All sera were treated at 56°C for 30

TABLE I. Growth and Cytopathogenicity of Virus from Strain RPL 12 Visceral Lymphomatosis.

Passage No.	Total effective dilution, negative logs			$TCID_{50}$, negative logs			Latent period of CPE		
	L29	L30	L31	L29	L30	L31	L29	L30	L31
1	2	2	2					5 da	48 hr
2	3	3	3			5.6		3 "	18–24 "
3	4	4	6	4.0		7.0	5 da	"	"
4	5	5	10		5.0	7.0	"	"	"
5	6	6		5.0			"	48 hr	
6	7	7			5.0			"	
7	8	8		8.0	6.0		48 hr	24 hr	
8	9	9					"	"	
9	10			8.0			"		
10	11			7.5			18–24 hr		
11	15			7.0			"		
12	19			7.3			"		
13	24			7.1			"		

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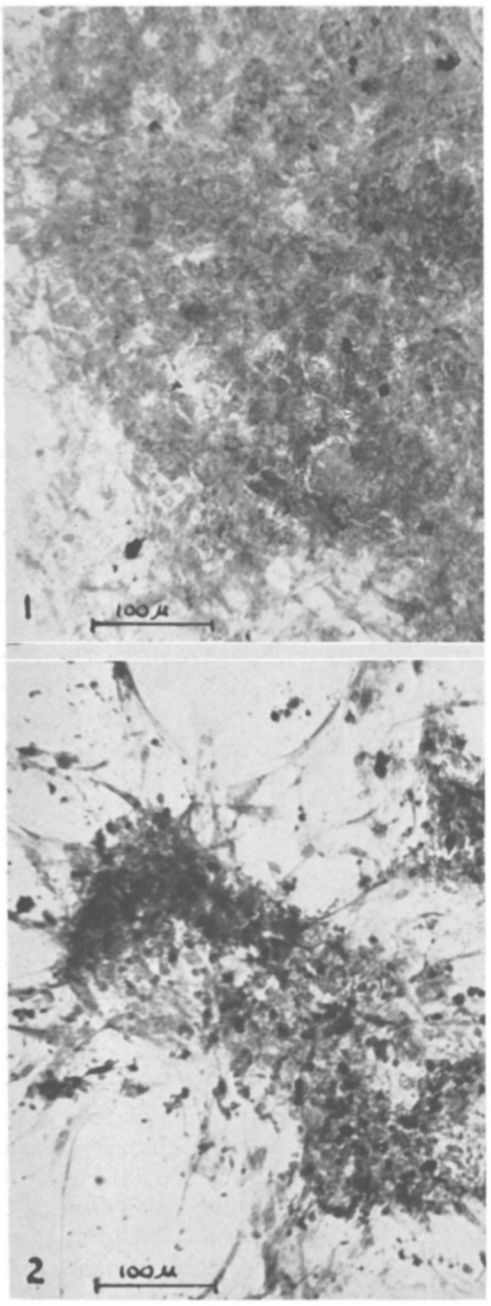

FIG. 1. Uninoculated culture of chicken embryo liver.

FIG. 2. Cytologic changes in embryo liver culture 24 hr after inoculation with strain RPL 12 virus. Note degeneration in epithelial-like cells.

minutes prior to use. *Tissue cultures.* Livers from 14-day chick embryos were minced and trypsinized by method similar to that of Youngner(10). Tubes were seeded with 0.8 ml of 1:200 dilution of cells in Mixture 199 (11) containing 0.14% sodium bicarbonate and 30% horse serum. The tubes were incubated at 37°C. A confluent monolayer was formed in about 3 days and growth medium was replaced with 199 alone or 199 plus 25% Difco Tryptose Broth and 2% horse serum. Passage and bioassay were made by replacing maintenance media with 0.8 ml virus at appropriate dilutions.

Results. 1. *Recovery and passage of virus.* Cultures were observed daily for 7 days, during which time no cytopathogenicity was observed. A 2nd blind passage was made and cytopathogenic effect (CPE) was again inapparent at 7th day. In passage 3 CPE became apparent on the 5th day in 5 of 12 cultures. Fluids from these 5 tubes were pooled for the next passage. The virus was carried through 10 consecutive passages. Beginning with passage 11, cultures showing CPE at the terminal endpoint dilution were used for the next passage. At passage 13 the original virus inoculum would have been theoretically diluted to 10^{-24}. Data relative to the serial passage of the different preparations of RPL 12 are given in Table I. In general, cellular changes followed a definite sequence; refractile granules appeared in the cytoplasm of the polygonal epithelial-like cells; the nucleus became pyknotic, and this was followed by shrinkage of cytoplasm and rounding up of the cells, together with a tendency toward formation of grape-like clusters. At approximately 2 days after inoculation, the clusters began to slough off the glass. The spindle shaped cells remained viable up to the 5th or 6th day when a CPE became evident manifested by margination of chromatin and karyorrhexis. Comparison of normal and infected chicken embryo liver tissue cultured cells can be seen in Fig. 1, 2, and 3.

2. *Pathogenicity of tissue culture virus for chicks.* Three groups of chicks were inoculated intravenously at 2 weeks of age with 0.2 ml tissue culture virus. Of 22 chicks inoculated with fluids from the 2nd passage, 13 died showing typical lesions of visceral lymphomatosis. Of 25 chicks inoculated with fluids from the 3rd passage, 13 died of visceral lymphomatosis, and 2 of erythroblastosis.

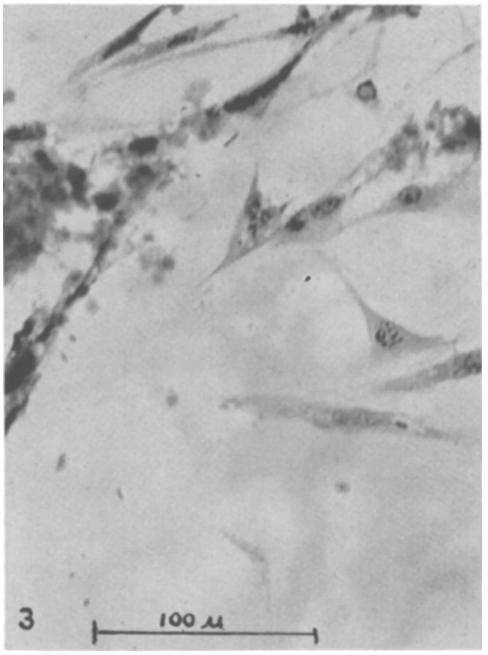

FIG. 3. Cytologic changes in embryo liver culture 96 hr after inoculation. Note clumping of chromatin in spindle-shaped cells.

Chicks inoculated with fluids from passage 10 responded with only 6 out of 50 birds developing visceral lymphomatosis. In 3 groups of chickens death occurred between 117 to 260 days postinoculation. These results are typical of response in chickens to virus freshly isolated from lymphomatous livers (8), where a proportion of chickens are always refractive.

3. *Serum neutralization tests.* Serum or serum dilutions were mixed with equal volumes of tissue culture fluids in final concentrations of 100 or 1000 $TCID_{50}$ of virus. The serum-virus mixtures were incubated 2 hours at 20°C and 0.8 ml inoculated into each of 10 tubes. Tests were concluded when cells in the virus control tubes showed complete CPE. Serum from a chicken which had been hyperimmunized with virus from the 10th tissue culture passage, diluted 1:256, neutralized 1000 $TCID_{50}$ of virus, while serum from the same chicken taken before immunization, diluted 1:16, failed to neutralize the virus. Rabbit anti-tissue culture-virus serum at 1:256 dilution also neutralized 1000 $TCID_{50}$ of virus. These results show that the virus was capable of stimulating specific neutralizing

TABLE II. Neutralization of Tissue Culture RPL 12 Virus by V. L. Antisera from Various Sources.

Serum	Source	Serum dilution neutralizing 1000 $TCID_{50}$
Chicken anti-TCP	Individual hen	1: 256
Idem	Pooled	"
Rabbit anti-TCP	Individual rabbit	"
Chicken anti-CP	Pooled	1: 128
Idem	Individual hen	1:1024
"	Pool of progeny from vaccinated hens	1: 64
Normal	Pool of progeny from unvaccinated hens	0
"	Pool from isolated hens	0

TCP = Tissue culture virus. CP = Chicken virus.

antibodies. Antiserum to strain RPL 12 chicken tumor produced in chickens, likewise showed neutralizing properties against tissue culture passed virus, as did serum from progeny of RPL 12 immunized hens. Details for this immunization procedure have been described(12). Serum pools from an isolated population, at 1:4 dilution, failed to neutralize 100 $TCID_{50}$ of virus. Results of these neutralization tests shown in Table II indicate that the cytopathogenic agent for chick embryo liver cell culture is related, at least antigenically, to chicken propagated RPL 12 virus.

Discussion. Evidence has been presented which indicates that a cytopathogenic agent for chick embryo liver tissue culture was recovered from filtrates of leukotic livers of chickens inoculated with strain RPL 12 virus. Fluids from serial passages of the virus have induced typical visceral lymphomatosis in inoculated chickens. In tissue culture the virus has shown titers up to 10^{-8} $TCID_{50}$. It is interesting to note that there was a decrease in virulence for the chicken after continuous *in vitro* passage. This is not a peculiar event, since it is well known that viruses isolated from various passages vary in their pathogenicity. Furthermore, viruses grown under different conditions often become modified in their pathogenicity. This can be manifested in partial to complete loss in virulence. The most practical use of this phenomenon has been in the development of modified vaccines.

Additional evidence for relationship of this

cytopathogenic agent to strain RPL 12 virus is provided by results of serum neutralization tests. Antibodies produced in chickens with RPL 12 virus readily neutralized the tissue cultured virus in the same range as did the specific antibodies to tissue cultured virus. A significant fact is that antiserum to a virus of similar origin and biological characteristics, but propagated *in vitro* at another laboratory, neutralized our tissue cultured virus at high dilutions.

Summary. A cytopathogenic agent isolated from RPL 12 in chicken embryo liver tissue cultures is reported. The virus produces a marked CPE on liver parenchymal cells. Virus serially passed in tissue culture, when inoculated into susceptible chicks, produced visceral lymphomatosis. Neutralization tests with antiserum against RPL 12 indicate a relationship of tissue culture virus with RPL 12; however, positive identity of the cytopathogenic agent with the virus causing visceral lymphomatosis has not been established.

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Received February 6, 1958. P.S.E.B.M., 1958, v97.

Structural Analogues of Puromycin in Production of Experimental Nephrosis in Rats.* (23900)

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Sherman, Taylor, and Bond(1) first demonstrated that the antibiotic 6-dimethylamino-9 - (3′-p-methoxy-L-phenylalanylamino-B-d-ribofuransoyl) purine (Puromycin) obtained from *Streptomyces alboniger,* has a nephrotoxic effect in rats. Subsequently, it was shown by Frenk and Antonowicz(2), that the derivative of Puromycin, 6-dimethyl-aminopurine-3′-amino - d - riboside (Aminonucleoside), produced a typical nephrotic syndrome. Feigelson, Drake, and Recant(3) described the syndrome in detail. The present work was undertaken to determine the structural configuration of these compounds essential for production of the nephrotic syndrome. To achieve this end, a variety of structural analogues of Puromycin was studied.

Materials and methods. Fifty-five male Sprague-Dawley rats, weighing 80-100 g, were divided into 10 experimental groups of 4 animals, and one control group of 15 animals. Each group was treated with daily subcutaneous injections of 0.3 ml of 0.017 M solution of one of the following: Aminonucleoside, Puromycin, 3-amino - d - ribose, 6-dimethyl-amino-purine, and 6-dimethyl-adenosine. 3′-amino-adenosine was administered in dosages of 0.3 ml of 0.017 M, 0.0085 M, 0.0034 M, and 0.0017 M solutions.† An additional group re-

* Supported by research grant from St. Louis Heart Assn.

† We are indebted to Lederle Laboratories, who generously supplied these compounds.