119. Cultivation of Malignant Tumor Cells on the Chorioallantoic Membrane of Embryonated Chicken Eggs^{*)}

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Since the classic work by Murphy (1912), cultivation of tumor cells from various animal species in embryonated eggs has been studied by a number of investigators (Oshima, 1954). In these studies, however, *fragments* of tumor tissues were used as inocula. Berman et al. (1957), Moore (1958), and Leighton and Kalla (1958) inoculated *cell suspensions* onto the chorioallantoic membrane (CAM), but the rates of tumor formation were not necessarily high and much has been left to be desired. In the present paper, experiments are reported in which quantitatively controlled suspensions of malignant tumor cells, including those of human origin, were inoculated onto CAM and tumors were formed at high and constant rates.

Materials and method. Four kinds of cells representing certain characteristic types were used.

(1) *Human epithelial cells (strain HeLa).* Following incubation at 37°C for 3 to 6 days with YLA culture medium (20% ox serum, 0.5% lactalbumin hydrolysate and 0.1% yeast extract in Earle's salt solution with 0.15% dextrose), the cells were treated with trypsin. The trypsinized cell suspension was washed by centrifugations, and the final sediment was resuspended in YLA medium.

(2) *Mouse fibroblasts (strain L).* The cells were treated in the same manner as with the HeLa cells.

(3) *Chicken sarcoma cells (strain Chiba).*^{**)} This sarcoma has been passed through chickens by a subcutaneous inoculation (Chiba, 1951). Tumors, usually grown to 2 to 3 cm in diameter 1 to 2 weeks after the inoculation, were excised aseptically and emulsified by adding 10 times volume of Hanks' salt solution.

(4) *Human uterus cancer cells.* Cervical cancer tissues excised by an operation were trypsinized by a method slightly modified from those by Dulbecco and Vogt (1954) and Youngner (1954) (Hotta, 1959). The cells in the final stage were suspended in YLA medium.

In each experiment, portions of the cell suspensions were subjected

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to nuclear counting by the procedure of Sanford et al. (1951).

Embryonated chicken eggs were treated by the method of Burnet (1936). Two-tenth ml of the cell suspensions was inoculated onto CAM of each of eggs which were then incubated at 37°C. Eggs which died within 2 days after the cell inoculation were not included in the results. Apparently death of egg caused directly by inoculation of cells was not frequent. The observation period ranged from 4 to 10 days, depending on the purpose of experiment. At the suitable periods, each egg was opened and CAM was examined macroscopically and histologically.

Results. HeLa and L cell suspensions of an approximately same concentration were inoculated into eggs of various ages. The results obtained are tabulated in Table I, which also includes results with the chicken sarcoma cells as a comparison. It was shown that eggs 9-day-old or older produced tumors at rates higher than those obtained with eggs of the younger ages. Even a 100% formation of tumor was apparently possible. The chicken sarcoma cells were capable of producing tumor without great difficulty.

Table I. Correlation between age of egg and tumor formation rate

Cell	Age of egg, in days	Incubation time, in days, after cell inoculation	Rate of tumor formation
HeLa ¹⁾	6	8	0/1
	8	10	0/2
	9	10	2/2
	10	10	4/4
L ¹⁾	9	10	1/2
	10	10	1/1
	13	5	2/2
Chicken ²⁾ sarcoma	10	7	5/8
	11	6	4/5
	11	6	4/8
	11	7	4/4
	12	7	4/4

1) Two million cells in 0.2 ml were inoculated into each egg.

2) Cellular concentration was not determined because of the mucilaginous nature of tumor.

Cell suspensions of various concentrations were inoculated into 9 to 13-day old eggs, and the rates of tumor formation for each cellular concentration were determined. A comparative list is shown in Table II, in which results from several separate experiments are summarized. It was apparent that number of cells inoculated was a factor affecting the tumor formation. In the present data, practically all eggs receiving $5-6 \times 10^5$ or more cells produced definitive tumors.

Table II. Correlation between cell inoculum size and tumor formation rate

Cell strain	Number of cells ($\times 10^4$)		Age of egg, in days	Incubation time, in days, after cell inoculation	Rate of tumor formation
	per ml	per egg			
HeLa	2240	448	9	9	4/4
	1300	260	10	8	4/4
	650	130	10	8	2/2
	560	120	9	9	2/2
	300	60	12	7	3/3
	100	20	11	7	2/3
	100	10	11	7	0/2
L	500	100	13	7	2/2
	500	50	13	7	2/2

In eggs inoculated with HeLa or L cells of the sufficient number, tumors became definitely visible usually 3 or 4 days after the inoculation. Within 2 days after the cell inoculation, no macroscopic change has been found except for a slightly opaque area around the original inoculating point, which was regarded to represent, at least partly, a distribution of the inoculated cells. The tumors once formed increased in diameter and thickness during the succeeding days. Seven to 10 days after the cell inoculation, it was not rare to see a tumor $10 \times 15 \times 5$ mm in size. Nodules 1 or 2 mm in diameter were also noticed.

The tumors produced either by HeLa or L cells were of the same macroscopic type. However, their histological architectures were markedly different each other. HeLa cells in the tumor were arranged in aciniform grouping, and the acini gathered together into larger conglomerates. Rounded concentric masses which reminded an observer of the so-called pearly bodies found in certain kinds of tumor tissues were also noticed. In the L cell tumors, round or a little elongated cells infiltrated diffusely without any particular arrangement; the general pattern resembled that of the chicken sarcoma which was more infiltrative and typically 'sarcomatous'. Figs. 2 and 3 depict examples of the findings observed.

The tumors were removed and treated with trypsin by the method cited above. The trypsinized cells were either dispensed in glass tubes or inoculated into eggs. Cells characteristic of the original HeLa or L strain proliferated in the tubes; and the tumor formation was reproducible in eggs of the succeeding passages.

In 10-day old eggs inoculated with suspensions of cells from human uterus cancer tissues, tumors were formed on CAM. The rates of tumor formation were fairly high (for example, 2/2 in an experiment). Average size of the tumors was approximately $5 \times 5 \times 2$ mm 7 days

after the cell inoculation. Histological observations of the tumors revealed cell clusters of a particular type: the cells in the clusters were of the undifferentiated characters, and apparently different from the elements of chicken origin; the general appearance of the cellular

Fig. 1. Histological findings of tumor formed by HeLa cells

One million cells were inoculated onto CAM of a 9-day old egg which was incubated for additional 10 days.—Hematoxylin-eosin stain, $\times 350$.—The cells are arranged in aciniform clusters which further form larger conglomerates. Rounded and concentric masses resembling the so-called 'pearly bodies' are seen.

Fig. 2. Histological findings of tumor formed by L cells

One million cells were inoculated onto CAM of a 13-day old egg which was incubated for additional 5 days.—Hematoxylin-eosin stain, $\times 350$.—Round or a little elongated cells infiltrate diffusely. No specific arrangement of cells is seen.

Fig. 3. Histological findings of tumor formed by cells from human uterus cancer tissues

Cell suspensions prepared by trypsinization of human uterus cancer tissues were inoculated into 10-day old eggs which were incubated for additional 7 days.—Hematoxylin-eosin stain, $\times 350$.—Cells with undifferentiated characters form acinous clusters. (A) and (B) represent different sections of the same material.

grouping resembled the acinous arrangement seen in the HeLa cell tumors, on the one hand, and to the parakeratosis found in the uterus cancer tissues, on the other hand. Fig. 3 shows examples of the typical findings.

Discussion and conclusion. (1) HeLa and L cells were shown to form tumors when inoculated onto the chorioallantoic membrane of embryonated chicken eggs. The cells dispersed with trypsin and suspended in culture medium were used as the inocula. Some of factors affecting the formation of tumor were: age of eggs and number of cells inoculated. In the present experiments, in which 9 to 13-day old eggs were used and each egg received $5-6 \times 10^5$ or more cells, the rate of tumor formation was practically 100%. The data are compatible with those by Moore (1958) and even show higher rate of formation. Hamamoto et al. (1959) have confirmed our results.

(2) The tumors became visible 3 or 4 days after the cell inoculation and increased in size during the additional 3 to 5 days. The maximum size was about $15 \times 15 \times 5$ mm. A possible sequence of the tumor formation was investigated in the present study.

(3) Although the tumors produced either by HeLa or L cells were of the similar macroscopic appearance, histological features exhibited by each of the cell strains were different: (a) HeLa cells were arranged in acinous clusters which further formed larger conglomerates. This may be correlated with the concepts which have been proposed

regarding the growth of cancer cells—'aggregate replication' of Leighton (1959) or 'islands formation' of Yoshida (1956, 1959). Rounded concentric masses resembling the so-called 'pearly bodies' were also seen. (b) The L cell tumors were characterized by diffuse infiltration of round or a little elongated cells without showing any specific arrangement. This resembled, although to a less extent, the pattern of tumors produced by the chicken sarcoma cells, and suggested a sarcomatous feature of L cells.

(4) Cell suspensions prepared by the trypsinization of the produced tumors were either cultivated in glass tubes or inoculated into eggs of the succeeding generation. The *in vitro* restoration or egg passage of the original cell strains was possible.

(5) Human uterus cancer tissues were trypsinized, and the homogeneous cell suspensions prepared were inoculated onto the chorioallantois of eggs. The inoculated membranes produced tumors at a fairly high rate. It was shown histologically that the tumors included acinous clusters of cells with undifferentiated characters; the type of cellular grouping had a resemblance to the specific arrangement of HeLa cells as described above, on the one hand, and to that of the cells found in the original uterus cancer tissues, on the other hand. A potentiality of this technique for studying human cancer cells was suggested.

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