COMPARATIVE STUDIES IN ROUS SARCOMA WITH VIRUS, TUMOR CELLS AND CHICK EMBRYO CELLS TRANSFORMED IN VITRO BY VIRUS

III. Malignancy in Vivo of Cells Transformed in Vitro by Virus*

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Previous investigations have shown that chick embryo fibroblasts infected with Rous sarcoma virus (RSV) *in vitro* acquire certain new morphologic, growth, metabolic and synthetic properties (1–5) in which they resemble the malignant cells found in Rous sarcomas and it has been assumed that this transformation of cells by virus *in vitro* was analogous to the infection of cells *in vivo* with the subsequent development of tumors. However, proof that cells infected *in vitro* are actually malignant is lacking since such cells have not been shown to be capable of producing tumors. In previous experiments (6), it was shown that the fate of cells cultivated *in vitro* could be followed after their injection *in vivo* by using sex chromatin as an index of the identity of cells and preliminary evidence was presented that cells infected with RSV *in vitro* were capable of producing tumors when injected into young chicks (6, 7).

The present studies confirm and extend these preliminary observations.

Materials and Methods

Virus.—The Rous sarcoma virus employed was prepared from the standard strain (batch CT776) of Dr. W. R. Bryan of the National Cancer Institute and partially purified virus stock (RSV) was prepared according to the method of Moloney (8). These virus preparations were assayed by the method of Temin and Rubin (4) employing chick embryo cell monolayers and the results recorded as focus forming units (FFU).

Standard Medium.—A medium composed of 8 parts Eagle's medium with double the concentration amino acids and vitamins, 1 part Difco bacto-tryptose phosphate and 1 part chicken or other serum was used (4).

Tissue Cultures. Tumor Cells.—Tumors induced with 20,000 FFU of RSV injected into the wing webs of white Leghorn chicks 2 to 4 weeks old were removed when they were 1 to 2 cm in diameter and tissue cultures prepared as previously described (3).

Chick Embryo Cells.—Ten-day-old chick embryos of a suitable strain of susceptible white

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Leghorn chickens were used to prepare primary cultures in Petri dishes by the methods described (4). Later these cultures were infected with RSV in the medium containing 10 per cent calf serum in a CO_2-air flow incubator. The cells were subcultured twice and examined to determine that cells gave visible evidence of being transformed by the virus. Determination of the number of cells transformed by the virus was carried out by the infective center assay method (4).

Cell Suspensions.—Tumor or transformed cells were removed from the Petri dishes with 0.05 per cent trypsin in tris buffered saline, sedimented in a centrifuge, and suspended in medium without serum for counting in a hemocytometer prior to use.

Irradiation.—The cells were irradiated as described previously (7) using a Picker x-ray machine and were exposed to 5000 r.

Tumor Induction in Chickens.—RSV or a cell suspension in a volume of 0.1 ml was injected into the wing web of 2-week-old white Leghorn chicks and birds were examined daily for tumors which were removed when they reached 1 to 2 cm in size (usually in 7 to 10 days).

Sex Determination of Tumors, Tumor Cells or Transformed Cells.—Cultures of cells from the tumors were prepared as previously described (3). For sex determination of cells, they were removed from the Petri dish tissue cultures with a 0.05 per cent trypsin and 600,000 cells were inoculated into each of several tubes containing coverslips and culture medium. When the cells had formed a good sheet, the coverslips were removed, fixed, and stained as described (6) and the sex determined by counting the number of cells containing sex chromatin using the criteria of Kosin and Ishizaki (9) who demonstrated that male cells in young chicks show sex chromatin in 1.0 to 6 per cent of cells whereas cells from females exhibit sex chromatin in 35 to 52 per cent of cells.

Tumor Transplantation.—At the time that tumors were removed from the chickens with cell-induced tumors, fragments were prepared weighing about 0.01 gm and these were implanted in the wing web of chicks. These birds were examined daily for tumors and when they reached 1 to 2 cm in size (usually in 5 to 7 days) were removed, tissue cultures prepared, and the sex of the tumor determined.

Tumor Induction in Hamsters.—Young Syrian hamsters were injected with 3.75 mg of cortisone (Merck) every 3 days for 9 days before injection of cells and once weekly after implantation. The animals were lightly anesthetized and 0.1 ml of the culture medium containing the cells implanted into the upper layer of the pouch epithelium as previously described (10). After 2 weeks, the animals were sacrificed, the tumors dissected free, and weighed on a torsion balance.

RESULTS

Origin of Tumors in Chickens Injected with Transformed Cells.—Transformed cells from a single chick embryo showing sex chromatin characteristic of a male were injected in dosage of 1×10^6 and 1×10^3 transformed cells into the wing web of 2-week-old female chicks and the sex of the tumors appearing determined. As a control for the validity of the sex determination of tumors 200,000 FFU of virus was injected into 4 male and 4 female birds of the same age. A control group of 26 birds was injected with 10^6 cells and examined daily until their death in 10 to 14 days to follow the natural evolution of the tumors.

The data in Table I indicate that when male cells are infected with RSV in tissue culture and are injected into female chicks, the tumors which develop are predominantly male indicating that they are primarily a consequence of the multiplication of the cells injected. In contrast, the injection of RSV into male chicks results in formation of male tumors and into females of

female tumors thus providing evidence for the validity of the method. The birds which were followed until death developed large and necrotic tumors before dying thus demonstrating that the cell-induced tumors were not rejected but were capable of causing death of the recipients.

Histological examination of the tumors revealed that they were typical spindle-cell sarcomas with a matrix of mucopolysaccharide (3).

Comparison of Origin of Tumors Induced in Chickens with Transformed Cells, Tumor Cells and Irradiated Cells.—Since transformed cells produced tumors in the wing web of chicks, it was desirable to compare their characteristics with those of tumor cells and to examine the properties of irradiated cells of both

TABLE I

Sex of Tumors Produced by Injection of Transformed Cells

Donor cells			Recipients	
Type	No.	Sex chromatin	Sex	Sex chromatin of tumors
		per cent		*per cent*
Transformed cells*	1×10^6	6	Female	3, 1, 6, 13
	1×10^3	6	Female	6, 2, 4
Virus—200,000 FFU			Male	3, 4, 6, 9
			Female	32, 44, 39, 43

* By assay tests, 66 per cent of these cells were infected with RSV.

types since such cells would be incapable of multiplication but are known to continue to release virus (5).

Transformed cells from a single female chick and from a tumor in a female bird were separated into two aliquots. One lot of cells was exposed to 5000 r of x-rays. Then all cells were injected into male chicks. The tumors produced were excised and the sex determined.

As in the first experiment, Table II shows that the transformed cells caused tumors since such tumors were predominantly of the sex of the donor cells though the consistently lower sex chromatin values obtained for cells from the tumors as compared with donor cells indicate content of some recipient cells. These transformed cells however showed no difference in behavior from the donor tumor cells. Following exposure to x-rays, the cells did not multiply when injected into the chickens and the tumors were caused by the virus released since they were of the same sex as the recipient bird. When six tumors of Experiment A caused by transformed cells were sampled from the center and the edge for sex chromatin determination, the center was always clearly of the sex of the donor cells but cells from the edge of three tumors showed sex

chromatin values in the range of 20 to 30 per cent indicating that some of the host cells were being infected with the virus and thus the sex chromatin incidence of the mixed donor and recipient cell tumor elements at the periphery was reduced. However, the bulk of the tumor was clearly of donor cell origin.

Transplantation of Tumors Induced by Transformed Cells.—Since the initial tumor induced by donor transformed cells was clearly mainly of donor cell origin, it was of interest to determine if such tumors could be transplanted and retain their capacity to grow.

TABLE II

Sex of Tumors Produced by Untreated and Irradiated Transformed and Tumor Cells

Donor cells				Recipients	
Type	No.	Sex chromatin	Sex	Non-irradiated cells: sex chromatin of tumors	Irradiated (5,000R) cells: sex chromatin of tumors
		per cent		*per cent*	*per cent*
Experiment A					
Transformed cells*	9.4×10^5	64	Male	39, 56, 39, 41, 48	9, 9, 10, 13, 7
	9.4×10^3			41, 44, 49, 32	5, 7, 14
Tumor cells	8.4×10^5	61	Male	38, 54, 49, 60, 66	4, 11, 11, 9, 5
	8.4×10^3			48, 52, 51, 51	16, 9, 7, 9
Experiment B					
Transformed cells‡	5.1×10^5	55	Male	33, 40, 41, 48, 50	8, 14, 11, 6
	5.1×10^3			44, 30, 37, 46, 39	13, 15, 12, 11, 9
Tumor cells	4.3×10^5	3	Female	9, 14, 17, 11	38, 36, 30, 39
	4.3×10^3			9, 11, 13	36, 39, 38

* By assay tests, 67 per cent of these cells were infected with RSV.

‡ By assay tests, 80 per cent of these cells were infected with RSV.

A population of male and of female transformed cells was used to induce tumors in chicks. Fragments of these tumors (0.01 gm) were then implanted into chicks of the opposite sex and the sex of the tumors induced in the first and second transplant generations determined.

It is clear from the data in Table III, that most of the tumors induced by transformed cells retained their sex in the first transplant but that in two instances the first transplant showed a sex chromatin pattern intermediate between that of donor and recipient. In the second transplant this was even more striking in several of the chicks. Thus recipient cells made up a significant element of these transplanted tumors.

Induction of Tumors in Hamster Cheek Pouch Following Injection of Tumor Cells and of Transformed Cells.—Neoplastic cells exhibit the property of extended survival in heterologous hosts in comparison with normal cells and the hamster cheek pouch (10) has been found one of the most useful sites for injection of heterologous cells for such studies.

TABLE III

Sex of Original Tumors Produced by Injection of Transformed Cells or Tumor Cells and of Primary and Secondary Transplants

Donor cells			Recipients			
Type	No.	Sex chromatin	Sex	Original: sex chromatin of tumors	First transplant: sex chromatin of tumors	Second transplant: sex chromatin of tumors
		per cent		*per cent*	*per cent*	*per cent*
Experiment C						
Transformed cells	1×10^6	7	Female	3	5	—
				8	—	10
				5	3	12
				7	—	16
				4	13	—
				7	—	12
				3	6	18
				8	9	15
Experiment D						
Transformed cells	5.1×10^3	55	Male	53	56	23
				43	30	14
				52	21	—

—, Cells failed to grow in tissue culture.

TABLE IV

Tumors Produced in Hamster Cheek Pouch by Injection of RSV Infected Cells

Cell inoculum		Weight of tumors in 2 wks.	Average weight in 2 wks.
Type	No.		
		gm	*gm*
Tumor cells	10^6	0.03, 0.04, 0.05, 0.10, 0.15	0.074
	10^5	0.01, 0.01, 0.015, 0.015, 0.015	0.013
	10^4	<0.01, <0.01, <0.01, <0.01, <0.01	<0.011
	10^3	No lesions visible	
Transformed cells	10^6	0.06, 0.058, 0.14, 0.12, 0.17	0.109
	10^5	0.015, 0.025, 0.04, 0.015, 0.02	0.023
	10^4	<0.01, <0.01, <0.01 neg. <0.01	<0.01
	10^3	No lesions visible	
Normal cells	3×10^6	No lesions visible	

Populations of tumor cells, transformed cells, and normal chick embryo cells were prepared and varying doses injected into the cheek pouches of 5 hamsters, each of which had been conditioned with cortisone. The tumors produced were examined and weighed to compare growth.

The results presented in Table IV indicate that normal cells did not survive

for 2 weeks in the hamster cheek pouch but that a dosage of 10^4 cells or greater with transformed or tumor cells caused an easily visible tumor and this persisted for at least 2 weeks.

In another experiment, 10^6 transformed, tumor and normal cells were injected into the anterior chamber of eyes of young guinea pigs. The transformed and tumor cells were still visible after 1 week but no normal cells were seen.

DISCUSSION

The early observations of Rous (11) presented evidence that in some instances implanted tumor cells produce tumors mainly by growth of the implanted cells though in others the virus may aid in the extension of the tumor growth, and the previous studies (7) clearly showed that chick embryo fibroblasts infected *in vitro* with RSV participate in the production of tumors independent of their release of virus. In these experiments it has been conclusively shown that the normal chick embryo fibroblast transformed to a new cell entity by infection with virus *in vitro* (1–5) is in fact malignant for it has the capacity to produce typical Rous sarcomas in the chick which are composed principally of the multiplying donor cells. These cells exhibit the same properties of malignant cells obtained from tumors induced by virus infection *in vivo*. The sex chromatin marker has proven to be a reliable index for the identification of the origin of tumor cells (6) since when such cells are exposed to x-rays which prevent their multiplication, the tumors induced are the result of the virus released from the cells since they have the sex chromatin pattern of the recipient.

Furthermore, the tumors induced by donor transformed cells are transplantable, though by the second generation of transplantation these tumors give evidence of containing significant numbers of recipient cells due to the addition of cells to the tumor by virus infection *in vivo*. These tumors are not therefore indefinitely transplantable.

In the experiments conducted here, there was no evidence of an effective transplantation rejection reaction to the donor cell–induced tumors but the maximum period that they were studied was 14 days since the birds died at this time with large, often necrotic tumors, and it is conceivable that some of the necrotic process was the result of a rejection reaction.

The malignant character of the transformed cells was also indicated by their survival in heterologous hosts (hamster and guinea pig) to the same degree as tumor cells under conditions which did not permit survival of normal cells (10).

Thus following infection with RSV *in vitro* the normal chick embryo fibroblasts not only acquire certain new morphologic, growth, metabolic, synthetic properties (1–5) in which they resemble the malignant cell of Rous sarcoma tumors, but are truly analogous in their neoplastic nature since they produce characteristic Rous sarcomas when injected into a susceptible host.

SUMMARY

Chick embryo fibroblasts infected with Rous sarcoma virus *in vitro* are rendered malignant for such cells produce typical Rous sarcomas when injected into susceptible chicks since the tumors produced predominantly retain the sex chromatin patterns of the donor cells when such cells are injected into a recipient of the opposite sex. However, examination of the sex chromatin of cells at the periphery of the tumor shows presence of recipient cells though the bulk of the tumor is clearly of donor cell origin. Such tumors grow and cause death of the recipient. Injection of RSV induces tumors of the sex of the recipient as also does the injection of transformed cells rendered incapable of multiplication by x-rays. Following their injection into susceptible chicks, the cells transformed *in vitro* by virus behave in the same manner as tumor cells obtained from tumors induced by virus *in vivo* and cultivated in the same conditions *in vitro*.

When such tumors induced by transformed cells are serially transferred in recipients of the opposite sex, they gradually convert to the sex of the recipient indicating that the tumors are not indefinitely transplantable.

These chick embryo fibroblasts transformed *in vitro* show the same neoplastic properties as tumor cells when they are introduced into the cheek pouch of the hamster or the eye of the guinea pig.

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