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

II. Response of Normal and Immunized Chicks*

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Chick embryo or chick fibroblasts infected with Rous sarcoma virus *in vitro* acquire certain new morphologic, growth, metabolic and synthetic properties (1–5) in which they resemble malignant cells found in Rous sarcomas. These transformed cells also continue to multiply and release virus (6). It was of interest therefore to investigate the behavior of these cells transformed *in vitro* upon injection into the chick host to determine if they would produce effects in the host tissues that were not related to their continued release of virus (6), since early work of Rous (7) had indicated that the response of the host to fresh tumor implants (cells) could be differentiated from its response to dried tumor implants (virus). For this reason, the reactions of the normal and virus-immune chickens were compared after injection of virus, of tumor cells, of transformed cells, and of irradiated transformed cells which lose their capacity to multiply but continue to release the same amounts of virus (6).

Materials and Methods

Virus.—The Rous sarcoma virus was derived 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) for fraction T_2. These virus preparations were assayed by the chorioallantoic membrane inoculation method for pock-forming units (PFU) per milliliter previously described (9).

Tumor Cells.—For production of tumors for cell suspensions, the stock virus was diluted and 2000 PFU inoculated into the wing web of 2- to 4-week-old white Leghorn chicks. Tumors were removed when they were about 1 to 2 cm in diameter, the tumor tissue was minced, trypsinized, and strained, and the cells washed, sedimented, suspended in Earle's balanced salt solution, and counted with a hemocytometer before injection.

Tissue cultures. Chick Embryo Cells.—Ten-day-old chick embryos of a suitable strain of susceptible white Leghorn chickens were used to prepare primary cultures by the methods

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described (5), and these were infected with RSV as monolayer cultures in Petri dishes in a humidified 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 by the infective center assay method (5) revealed that 50 to 60 per cent of the cells were releasing virus at this time. They were harvested for injection as described above.

Irradiation.—Cell suspensions were placed in 25 ml plastic tissue culture flasks and irradiated, using a Picker x-ray machine at 52 kv and 10 ma filtered with 0.381 mm of Al. The cell suspension was placed 67 mm from the source where the dosage was 1020 r/minute. The half value layer of the beam was 1.0 mm of Al. The dosage of x-ray was determined by the time of exposure.

Tumor Induction in Chickens.—Following injection of RSV or cells into the wing web of young chickens, the animals were examined daily for tumors and the latent period for tumor production calculated by the method of Bryan (10).

RESULTS

Behavior of Virus, Tumor Cells, and Transformed Cells in Virus-Immune Chickens.—

During the course of an experiment involving the production of Rous sarcomas in chickens following infection with RSV in the wing web, 33 chickens were collected which had developed well defined sarcomas that later regressed. These birds and birds of the same age from the same flock were tested for their resistance following injection of 2000 PFU of RSV into the wing web. These resistant chickens, plus 4 from the control group who developed tumors which regressed, were then injected in the wing web with (*a*) 2000 PFU of RSV; (*b*) 5×10^5 tumor cells; (*c*) 5×10^5 tumor cells which were first exposed to 5000R; (*d*) 5×10^5 RSV transformed cells; (*e*) 5×10^5 transformed cells which were first exposed to 5000 r; and (*f*) 5×10^5 uninfected chick fibroblasts.

The results presented in Table I show that chickens with previously regressed tumors had a high degree of resistance to reinfection with 2000 PFU of RSV in comparison to the normal controls, and of the few tumors produced, all but one regressed. When the same birds were challenged again, they were still resistant to 2000 PFU of RSV, but this resistance could be overcome, in most cases, by injection of 5×10^5 tumor cells or the same number of transformed cells. In both cases irradiated cells were as effective in inducing tumors as were unirradiated cells, indicating that the tumors which appeared were a result of the very high dose of virus which accompanied the cells. The resistance of these chickens was confirmed by the fact that almost all of the tumors eventually regressed, and this suggests that immunity in these birds was directed against the cell as well as the virus, as described by Rous with tumor tissue and crude virus (7). As a result, it was not possible to use immune birds to distinguish between tumors induced by virus infection *vis-à-vis* those which resulted from cellular proliferation.

Induction Period of Tumors Produced by Virus and Transformed Cells in Chickens.—The latent period between infection of chickens with RSV and appearance of tumors can be measured with precision and is inversely related to

the quantity of virus used for infection (10). This induction period is related to the number of cells initially infected by virus, the time required for these cells to undergo malignant transformation and their subsequent rate of growth and release of virus. If tumors are induced *in vivo* by cells already transformed by virus *in vitro*, the time interval between infection and cell transformation should be eliminated. Therefore, if chickens were infected with identical doses of virus or with transformed cells releasing the same quantities of virus, those injected with transformed cells should show a shorter induction time, since the cells are already transformed and can initiate multiplication as well as virus release

TABLE I

Response of Immune Chickens to Virus, Tumor Cells, and Transformed Cells

Chickens	Material injected	No. tumors / No. injected	Regressed tumors / Total tumors
Birds with regressed tumors	2000 PFU of RSV	8/33	7/8
Normal birds	2000 PFU of RSV	25/28	4/25
Immune chickens	2000 PFU of RSV	0/6	
	5×10^5 tumor cells	4/5	2/4
	5×10^5 tumor cells irradiated (5000R)	3/6	3/3
	5×10^5 transformed cells	6/6	5/6
	5×10^5 transformed cells irradiated (5000R)	4/6	3/4
	5×10^5 normal chick fibroblasts	0/4	

without delay. This comparison can be carried out best by using as a source of virus, a suspension of transformed cells which have been irradiated, since it has been shown that an appropriate dose of x-rays can eliminate the capacity of such cells to divide but not their ability to produce or release virus (6). Thus, initial virus release from transformed cells or irradiated transformed cells after injection into the host is identical, but only the former can multiply to initiate tumor formation. By injecting aliquots of the same suspension of transformed cells, one of which has been irradiated, the role of the transformation of the host cell in the tumor induction period can be assessed.

Chick fibroblasts were transformed with RSV *in vitro*, prepared as suspensions and aliquots of the suspension exposed to 3000 to 5000 r in various experiments. In the first experiment (Table II), transformed cells, irradiated transformed cells, and RSV were injected into the wing webs of susceptible chickens.

It can be seen in every instance that irradiation of the transformed cells produced a substantial delay in the latent period of tumors induced by the cells, thus indicating that the transformed cell contributes directly to tumor forma-

tion through by-passing the period required for the virus released to transform host cells *in vivo*. These chicks showed a greater resistance than others used to induction of tumors by virus showing latent periods of 4.4 and 5.0 respectively following injection of 2000 PFU of RSV, as compared with a more susceptible group (Table III) which showed a latent period of 3.8 days following injection of the same quantity of virus.

TABLE II

Tumor Production with Virus, Transformed Cells, and Transformed Cells Exposed to Radiation

Material injected	Dose of cells or virus	No. tumors / No. injected	Latent period
			days
Experiment A			
Transformed cells	4×10^4	32/33	2.8
	4×10^3	33/33	3.8
	4×10^2	31/31	4.8
Irradiated transformed cells (5000 r)	4×10^4	32/32	3.4
	4×10^3	32/33	4.7
	4×10^2	31/33	6.0
RSV	2000 PFU	32/33	4.4
	200 PFU	32/33	5.0
	20 PFU	30/32	5.9
Experiment B			
Transformed cells	1×10^6	15/15	<1.0
	1×10^5	30/30	2.7
Irradiated transformed cells (3300 r)	1×10^6	30/30	3.4
	1×10^5	30/30	3.7
RSV	20,000 PFU	25/25	4.1
	2000 PFU	23/25	5.0

If transformed cells contribute significantly to tumor production after injection by multiplication, then exposing such cells to virus-neutralizing antibody in the chicken host should have little influence on the latent period, since it is known that such sera do not affect the rate of growth of virus-induced tumors once they appear (9); *i.e.*, after cells have been transformed by virus *in vivo*. However, such sera do prolong the latent period of tumors induced by virus (9) and therefore one would expect a delay in the case of tumors produced with irradiated cells, since antibody would reduce the amount of virus available to transform cells *in vivo*. An experiment was carried out to test this possibility.

Cells were transformed as before, and one-half exposed to 3740 r. One day before injection with cells or RSV, chickens were given 1 ml of potent RSV virus-neutralizing serum by intraperitoneal injection which has been shown to produce significant neutralizing antibody in the circulation 24 hours later (9). Furthermore, just before injection, the cells were mixed with the serum at a dilution of 1:10 to enhance the local concentration of antibody in the wing web.

The results of this experiment are presented in Table III. The data show that antibody produced a marked delay in the latent period of tumors produced by irradiated cells, but had no effect on the tumors induced with unirradiated cells. Irradiation alone had no effect because the susceptibility of this lot of chickens to RSV was so great, as indicated by their rapid response to 2000 PFU of virus (*i.e.* 3.8 days), that the birds responded to challenge with cells or virus within

TABLE III

Effect of Antibody on Tumor Production with Transformed Cells and Transformed Cells Exposed to Radiation

Material injected	Dose of cells or virus	Antibody	No. tumors / No. injected	Latent period
				days
Experiment A				
Transformed cells	7.5×10^4	0	30/30	2.3
	7.5×10^4	+	30/30	2.3
Irradiated transformed cells (3740 r)	7.5×10^4	0	28/30	2.3
	7.5×10^4	+	30/30	4.2
RSV	2000 PFU	0	29/29	3.8
	200 PFU	0	28/28	4.4

2 days and, with this rapid response, no differences between irradiated and non-irradiated cells could be measured; however, when tumor sizes were measured in both groups, it was noted that tumors in the group receiving unirradiated transformed cells reached a volume of 0.1 ml one-half day sooner than the irradiated group, suggesting that the former had a head start. The delay caused by presence of antibody in the chickens receiving irradiated transformed cells indicates again that the release of virus alone is not sufficient to account for the rapid development of tumors in the birds receiving unirradiated transformed cells and that these cells *per se* contribute to the formation of tumors independent of their capacity to release virus.

Discussion.—The data support the premise that transformed cells injected into susceptible chickens contribute more to tumor formation than just their release of virus. With chickens that are highly resistant to infection with RSV, these transformed cells induce tumors as efficiently as the same number of tumor cells and thus appear to possess similar properties. In normal chickens,

it appears likely that the transformed cells multiply, contribute to the tumor mass, and thus shorten the time required for tumor induction by equal numbers of irradiated transformed cells where the virus released must have time to infect and transform cells *in vivo* before tumor growth can be initiated. Therefore, transformed cells behave like tumor cells when introduced *in vivo* because Rous (7) had 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 extension of the tumor growth. If the process *in vivo* is similar to that *in vitro*, it would require at least 12 hours for the first cells to undergo transformation and begin to grow as tumor cells (6). The role of the transformed cell in tumor formation is particularly clear when large doses of cells are used (*i.e.* 10^6) where the production of tumors by the transformed cells occurs in less than 24 hours, whereas the same dose of irradiated cells requires 81 hours, even though these cells might release up to 4×10^7 PFU into the tissues in the first 24 hours after injection (6). Thus, large numbers of transformed cells which have the capacity to multiply produce tumors almost immediately, while cells incapable of multiplication but releasing the same quantity of virus require an induction period to allow for *in vivo* transformation of cells to the malignant state for multiplication and tumor production. The delay in latent period varied from 0.6 to 2.4 days depending on the number of transformed cells injected and the resistance of the group of chickens used. This difference in tumor induction time of unirradiated and irradiated cells was most striking when the resistance of the chicks employed for tumor induction by virus was high (*e.g.* Table II), whereas very susceptible chicks (Table III) responded to the virus released from irradiated cells so rapidly that no differences in appearance of tumors could be detected with the numbers of cells used (*i.e.* 7.5×10^4). This could also account for the failure, in early investigations, to observe any effect of x-rays on the capacity of tumor tissue implants to cause tumors, since the virus content of such tumors might have been high (7).

It appears that normal chick fibroblasts infected by RSV *in vitro* not only develop morphologic, metabolic, and synthetic properties characteristic of the cells of Rous sarcomas (1–4) and growth patterns suggestive of malignancy *in vitro* (3), but that such cells appear to possess properties of malignancy *in vivo* independent of their release of the tumor virus. Thus, the *in vitro* transformation of normal chick cells by RSV appears to be an accurate model for the cellular reactions involved in the induction of tumors by this virus *in vivo* as the transformed cell appears to be malignant when introduced into the chick host. Further experiments are under way to investigate the malignant properties of the *in vitro* transformed cell after injection into the susceptible host using sex chromatin and sex chromosome analyses of the injected transformed cells, the host cells and cells of the induced tumor to assess the contribution of injected transformed cells and host cells to the formation of the tumor. Preliminary

results support the observations presented here which indicate that the *in vitro* transformed cell is malignant (**11**).

SUMMARY

Chick embryo fibroblasts infected *in vitro* with Rous sarcoma virus have properties similar to tumor cells when injected into virus-immune chickens. When such virus-transformed fibroblasts are injected into normal chickens, they apparently participate in the production of tumors independent of their release of virus and are thus apparently malignant *in vivo*.

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