**In vitro** Transformation of Primary and Continuous Rat Fibroblasts by Rous Sarcoma Virus (SCHMIDT-RUPPIN)

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**SUMMARY**

**In vitro** transformation of rat fibroblasts by the SCHMIDT–RUPPIN strain of Rous sarcoma virus is described. Primary rat embryo fibroblasts were transformed in 2 to 6 weeks after infection with the virus. The transformed cells were large, refractile and polygonal in appearance and produced multilayer colonies. Injection of the transformed cells to young and weanling Sprague-Dawley rats induced fibrosarcomas. The continuous cell line of rat fibroblasts was transformed by Rous sarcoma virus (SCHMIDT–RUPPIN) after 14 to 25 weeks. The transformation developed gradually from 1 or 2 islands of transformed cells per culture to a solid sheet of transformed cells. Cell cultures initiated tumours in young and weanling rats at the site of injection. Tumours grew slowly in weanling rats, while younger animals died from large tumours. These differences between young and old rats may reflect the immunological status of the host and/or the decrease in susceptibility of host cells to transformation by inoculated cell cultures.

**INTRODUCTION**

The induction of fibrosarcomas in rats by Rous sarcoma virus (RSV) was described by Zilber (1965) and others (Harris, Chesterman & McClelland, 1969; Svoboda, 1961). Rats were injected with the virus shortly after birth, and tumours appeared 6 to 7 weeks later. The presence of infectious RSV could not be demonstrated in most cases in tumour tissues, or in tissue cultures prepared from the tumours. Rat tumours were transplantable to syngeneic animals, and to chickens when a large number of intact cells was injected. However, cell-free extracts were inactive. *In vitro* transformation of mammalian fibroblasts by RSV has been described by Martirosyan & Shevlyagin (1968) in cells derived from guinea-pigs and from golden-hamster embryos, and by Vesely *et al.* (1968) who co-cultivated rat embryo cells with tumour cells from RSV-infected chickens. The mixed cells produced tumours in rats and the extracts of these tumours initiated tumours in chickens.

The experiments described here are concerned with the transformation of primary rat embryo cells and of a continuous rat fibroblast cell line by RSV, and the correlation between *in vitro* transformation and *in vivo* tumour production.

**METHODS**

The SCHMIDT–RUPPIN strain of RSV (SR–RSV) was used for infecting the cell cultures. Primary rat embryo cell cultures were prepared from 0.25% trypsin-dispersed cells of 15-day-old, random-bred Sprague-Dawley (SD) embryos, and propagated in Eagle's
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minimal essential medium on Earle's base, supplemented with 5% heat-inactivated calf serum, 100 μg. of streptomycin and 100 units/ml. of penicillin G (MEM). The continuous rat fibroblast cell line was derived from trypsinized SD embryos which were thereafter propagated in vitro for 56 passages in MEM. The primary and continuous rat fibroblasts, in 8 oz. prescription bottles, were inoculated with 0.4 ml. of undiluted sR-RSV (10⁶ pk.f.u./ml.) and incubated overnight at 37°C. The media were decanted, cells were washed 3 times with physiological saline, fresh MEM was added, and the cultures were thereafter incubated at 37°C. At intervals, when the sheets of cells were confluent the cells were trypsin-dispersed and transferred to new bottles with fresh MEM.

RESULTS

It was observed that primary rat embryo cells were transformed by the SCHMIDT–RUPPIN strain of Rous sarcoma virus in vitro faster than the continuous rat fibroblast line: on the second passage of the infected primary cells, small islands of larger refractile polygonal cells were detected. Characteristic piling up of cells was observed on the sixth successive weekly cell passage. This sequence of events was replicated in six experiments. Most cultures of the uninfected primary rat embryo cells did not propagate beyond three passages in vitro, although some cultures survived for 2 or 3 more passages. The number of islands of morphologically transformed cells increased very rapidly and at the tenth passage after virus infection the cultures contained only large polygonal and round cells which were organized in mounds scattered on the surface of the glass. The cells detached spontaneously from the glass within 24 hr of incubation at 37°C and were floating in the medium at the time of its increase in acidity. Cell cultures were transferred every 24 hr to keep most of the cells attached to the surface of the glass. Incubation of the transformed cell cultures at 37°C for longer periods resulted in a typical cycle of growth. The few islands of cells which remained attached to the glass surface, multiplied to form a new confluent sheet of polygonal cells arranged in piles, most of which were detached from the glass within 24 hr. This replication cycle was observed for at least 2 months in the same bottles but with frequent changes of nutrient medium.

The transformed cells were frozen in MEM with 10% glycerol and stored in liquid nitrogen for more than a year. When frozen cultures were reactivated, the cells multiplied and repeated the original growth pattern of piling and detachment from the glass surface.

Ten prescription bottles with the 56th passage of continuous cell line of rat fibroblasts infected with sR–RSV did not show any evidence of transformation until the tenth passage after virus infection. On this passage small islands of transformed cells appeared, which increased slowly until the 15th passage. After the latter passage, cells in one of the culture bottles suddenly showed fast transformation and repeated the cycle shown by primary rat fibroblasts infected with sR–RSV and described above. Cells in the other culture bottles continued a gradual increase in number of centres of transformed cells. At the 25th passage and almost 25 weeks, all of the continuous infected cell cultures were transformed and resembled the pattern of the fast replicating cycle. At various passages cells were frozen and stored in liquid nitrogen and, when reactivated, all the surviving cultures resumed the cyclic propagation pattern of fast replication.

There was a direct correlation between the transformation of cells in vitro and their ability to produce tumours in vivo. When 10 x 10⁶ transformed cells from primary (after ten passages in vitro) or continuous cell lines (after 25 passages in vitro) were injected subcutaneously into 10 and 7 random-bred SD rats of 4 to 5 days old and into 10 and 9
Transformation of rat fibroblasts by RSV

Weanlings all developed localized fibrosarcomas within a week. The tumours grew rapidly to very large size and the younger rats died after 2 to 4 weeks. Metastatic tumours were occasionally found in the lungs. In weanling rats the growth of tumours was arrested and death did not occur. All tumours were transplantable. When early passages (from the 10th to 14th) of the continuous cell line infected with sr-RSV (with only few visible islands of transformed cells) were injected, small tumours appeared in the 3 of 10 rats at 4 to 5 days old but not in the 10 weanling rats. The tumours were very small, appeared 3 months after inoculation of the cells and regressed within 2 months. Earlier passages (up to the 10th) of the virus infected continuous fibroblast line, which did not show in vitro cell transformation, did not initiate tumours in vivo. Cell-free extracts of fast-replicating transformed cells induced no tumours in 16 young and 9 weanling rats and no lesions on the chorioallantoic membranes of 10-day-old embryonated chicken eggs. Rous sarcoma virus was rescued from the transformed cells following fusion with chick embryo cell cultures by β-propiolactone-inactivated Sendai virus: for this purpose the method used was that described by Neff & Enders (1968) for replication of poliovirus. Transformed chick embryo cells were observed to produce lesions on the chorioallantoic membranes of 10-day-old embryonated chicken eggs.

DISCUSSION

Rous sarcoma virus transformed rat fibroblast cells in vitro into refractile polygonal tumour cells. The transformed cells, when injected into very young or weanling SD rats, initiated fibrosarcomas similar to the tumours induced in rats following neonatal injections of infectious sr-RSV (Pollard, 1970). The latent period for the appearance of the tumours was related inversely to the numbers of polygonal, transformed, cells observed in the inoculum. Increase in the number of transformed cells shortened the latent period. Primary rat embryo cells ('young' cells) were transformed faster than a continuous line of rat fibroblasts ('old' cells) and observable tumours were initiated in rats after a short latent period of one week. Gotlieb-Stematsky, Yaniv & Gazith (1966) described an increase in the number of colonies transformed in vitro in hamster embryo cells, as a result of trypsinization of the initial tissue as compared to mechanically dispersed cells. However, the difference in the culture described above could not be trypsin-related since both sr-RSV-transformed cultures were passaged by trypsinization. It is unlikely that an immune system, very important in in vivo experiments, would have a role in an in vitro transformation of cell cultures. Therefore, it seems possible that the age of the cells was the only significant factor influencing the rate of the in vitro transformation by sr-RSV. The 'young' cells were more sensitive to the onco- genic effect of the virus than the 'old' cells. Initiation of tumours by the cells transformed in vitro can be divided into two phases: (a) the original transformation of the normal fibroblasts by the oncogenic virus and (b) the multiplication of the inoculated transformed cells in vivo. The rate of transformation in the first phase was independent of the immunological status of the host but was related to the age of the cells. The rate of transformation in the second phase depended on the number of transformed cells in the inoculum and on the age of the host, which reflected its immunological status, and on the sensitivity of its cells to transformation. The transformed cells produced large tumours in 4- to 5-day-old and weanling rats. These tumours killed only the younger animals. In the older rats the tumours ceased to grow, in contrast to their continued growth in the younger rats. If in vitro results can be extrapolated to in vivo situations, it appears that the arrested growth and non-rejection of tumours in older rats may be due not only to the immune mechanism but possibly also to the low host cell sensitivity to transformation. Tumours were rejected only when cultures
with few islands of transformed cells were injected to young or weanling rats, indicating that a threshold dose of transformed cells was required to overcome innate host resistance. Transformation of cell cultures by SR-SRV and subsequent inoculation into experimental animals reduced the latent period for tumour appearance from 6 to 7 weeks to 1 week, and increased the age of susceptible animals from newborn to weanling. This method of in vitro and in vivo combination may be useful for rapid and precise assessment of oncogenic effects of viruses in alien species. Since the rat tissues appear to be virus-free and susceptible to a wide range of oncogenic agents (Pollard, 1971), they could serve as a sensitive detection system for oncogenic biological agents.

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REFERENCES


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