Transformation of Fibroma into Myxoma Virus in Tissue Culture. (23118)

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Transformation of Shope fibroma virus into that of myxoma was first reported by Berry and Dedrick(1,2) who used heat-inactivated myxoma virus as the transforming agent. Their findings were confirmed by other investigators(3,4). As summarized by Smith(4), results of experiments performed in rabbits were "troublingly irregular" and usually not repeatable at will. Experiments presented below demonstrate that the Berry-Dedrick phenomenon can take place in cultures of rabbit tissues, both testis and kidney. They also give some indication that positive results might be obtained with greater regularity *in vitro* than *in vivo*.

Materials and methods. Tissue cultures. General methods of growing explants of rabbit testis presented elsewhere(5) have been modified in all but 2 of the present experiments involving this tissue by the use of medium 199(6) containing 20% of inactivated horse serum. In remaining experiments, tissue cultures consisted of trypsinized(7) rabbit kidney cells. Nutrient medium for the latter cultures consisted of 0.5% lactalbumin hydrolysate, as described by Melnick(8). All cultures were in roller tubes, and all media contained penicillin and streptomycin. *Rabbits and precautions in handling.* Dutch rabbits were used as the source of kidneys and testes for cultures except for a single experiment performed with testis tissue from a cottontail rabbit. Immature New Zealand Whites were used for testing infected tissue culture fluids. Test rabbits were kept in 2 rooms. The one contained animals for the first 4 days after inoculation; the other, rabbits transferred from the first after this time, but before they were likely to develop systemic infection with myxoma virus. The rabbits were free from hemophagus arachnids. Insect sprays were used in both rooms. Most rabbits which developed myxoma were killed within 24 hours of the time it first became apparent. *Virus.* The Patuxent strain(9) of fibroma was employed throughout. Methods of virus titration in skin of domestic rabbits have been described(10). *Preparation of transforming agent.* TAM or Transforming Agent Myxoma was prepared from skin tumors of domestic rabbits which had developed myxomatosis 6 or 7 days after intracutaneous inoculation of myxoma virus. Tumors were cut away from the outer skin, ground with sand, made into 10% suspensions with Hanks' balanced salt solution containing 10% horse serum, then centrifuged several times at 3,000 rpm. Supernates from these preliminary centrifugations were centrifuged at 40,000 rpm for 40 minutes. Sediments from the higher speed centrifugation were suspended at 1/10 the previous volume, dispersed by a micro-blender, sealed in glass tubes, and submerged in water bath at 66-67°C for 40 minutes. Two lots of TAM were prepared differently, but with equally successful results as shown in Table I. In TAM 8 the myxomas were made in 20% suspension originally and were not submitted to high speed centrifugation. TAM 10 was prepared as first described, except that inactivation was limited to 12 minutes at 65°C. *Performance of experiments.* Live fibroma virus, contained in a 1:10 suspension of cottontail or rabbit fibroma in Hanks' solution or in undiluted tissue culture fluid from previous passage of the agent (Table I), was mixed with TAM in proportions of 0.1 ml of TAM (0.5 ml for TAM 8) to 0.2 ml of fibroma virus suspension for each roller tube (16 x 150) inoculated. Mixtures of TAM and virus were kept 15 minutes at room temperature before inoculation. Tubes receiving the mixture contained a final volume of 2.1 ml, and were incubated in a roller drum at 36°C. Nutrient fluids were harvested and pooled twice, or preferably 3 times a week. Fresh fluids, containing TAM in amounts originally inoculated, were then added. Test rabbits were inoculated intracutaneously with 0.5 ml from

TABLE I. Details of Positive Experiments in Which Fibroma-Myxoma Transformations Occurred in 11 Experiments.

Exp.	Rabbit tissue culture	Source fib. vir.	TAM† lot No.	Time of transformation,‡ days after T.C. inoc.
BD 1*	Testis	5P T.C. Cot. testis	T 1	13*
2	"	3P T.C. Rab. testis	2	7
3	"	Rab. fib.	3	10
6	"	Cot. fib.	5	6
7	"	*Idem*	4	6
32	"	"	8	9
39	"	"	8	5
37	Kidney	1P T.C. Rab. testis	10	2
38	"	1P T.C. Rab. kidney	10	11
41	"	1P T.C. Rab. testis	10	9
43	"	*Idem*	10	5

* Only exp. in which testis culture used was from cottontail and not domestic rabbit tissue.

† Transforming agent myxoma.

‡ Refers to time that myxoma virus was first detectable by testing supernatant fluids from tissue cultures in the skin of a rabbit.

each pool of infected fluid. The same rabbit was usually inoculated with fluid from a number of experiments. If the animal developed only local lesions after 12 days of observation, all fluids were considered negative, *i.e.*, no transformation of fibroma to myxoma had occurred. However, if the test rabbit developed a systemic infection in 6 or 7, but occasionally to 9 days after inoculation, samples of the original supernatant fluids, stored in dry ice, were again tested, each in a single rabbit. Generalized myxomatosis was first indicated by appearance of white pus at corners of the eyes, followed by swelling of eyelids, tracheal rattles, and enlargement of regional lymph nodes within 24 hours. At this time most rabbits were killed and a specimen taken of the swollen, jelly-like conjunctival tissues. These specimens were fixed in 10% formalin. A few rabbits, not killed, succumbed within 3 or 4 days of the first appearance of pus in the eyes.

Results. TAM. Each lot was tested for possible presence of infective virus particles by injection of 1 ml into several sites in the skin of rabbits. Some rabbits were likewise inoculated intraperitoneally. All rabbits remained healthy for the 2-week period of observation, without development of local lesions. Lots 4 through 10 were carried in tissue cultures, in the same manner as in actual transformation experiments, only without the presence of live fibroma. Fluids harvested from these passages produced neither local nor systemic reactions in rabbits inoculated with them, indicating that inactivated particles had not been restored to activity. TAM represented myxoma virus heated 40 minutes, but it was found that no infective virus was recoverable after 3 minutes exposure to the temperature used. The effectiveness of 65°C and above for inactivating myxoma virus has been attested by others (2,3,4). Seven of 10 lots of TAM prepared were effective in at least one successful experiment (Table I).

Transformations in tissue culture. Table I demonstrates that transformation of fibroma virus, as indicated by appearance of infectious myxoma virus in supernatant fluids, took place in 2 to 14 days of inoculation of the tissue cultures. In the group of 4 experiments performed in cultures of rabbit kidney, fibroma virus inoculated at the start had already grown in the presence of TAM for 5 to 6 days, since fluids used as a source of the agent were from previous experiments in which no transformation had occurred. Experiments usually continued for about 2 weeks. At the end of this time cultures might show degenerative changes whether they had been inoculated or not. Once fluids became positive for myxoma virus they remained so until termination of the experiment, with one exception. In experiment BD 32 the testis cultures used were usually good, so that the experiment was carried on for 32 days, at which time the cultures were 38 days old. As shown in Table II, fluids harvested 9, 11, 14, and 16 days after inoculation were positive for myxoma virus and had virus titers of from 10^{-4} to 10^{-5}. Fluids harvested before and after the 9-16 day period had lower titers and contained fibroma virus only.

TABLE II. Results of Tests and Titrations on Supernatant Fluids Taken Serially from Tissue Cultures of Two Transformation Experiments.

		Results of tests and titrations in rabbit skin								
		Days after inoculation of tissue culture								
Exp.	TAM added	4	7	9	11	14	16	18	25	32
BD 32	With fibroma virus	FIB* 10^{-2}†	FIB 10^{-3}	MYX 10^{-4}	MYX 10^{-4}	MYX 10^{-5}	MYX 10^{-5}	FIB 10^{-2}	FIB 10^{-1}	FIB 10^{-1}
35	4 days after fibroma virus	FIB 10^{-1}	FIB 10^{-2}	FIB 10^{-3}	FIB 10^{-2}	FIB 10^{-2}	FIB 10^{-2}	FIB 10^{-2}	FIB 10^{-1}	FIB 10^{-1}

* Type of disease produced in test rabbit. † Virus infectivity titers of the fluids tested.

Results of another experiment, BD 35, are also given in Table II. This experiment was set up in parallel with BD 32, using the same tissue cultures and virus preparation, the only difference being that TAM was not added until 4 days after the inoculation of fibroma virus. The tissue cultures grew equally well in both experiments. Myxoma virus was never recovered from fluids of the BD 35 experiment and virus titers never rose above 10^{-3}. It thus appeared that when transformation did take place, virus titers became elevated. One might explain that fibroma and myxoma give similar type tumors on titration in the skin of a rabbit.

Discussion. Present experiments indicate that transformation of fibroma into myxoma virus may be accomplished with some variation in the type of tissue culture, preparation of transforming agent, and in the source of fibroma used in an individual experiment. Two conditions appeared to be essential. One was addition of the transforming agent (TAM) at the same time as the live virus and secondly, that the tissue cultures have a good growth of cells to support a vigorous proliferation of virus. Negative experiments have not been described; a feature common to the majority, however, was a poor growth of supporting cells.

Experiments have been presented as if transformations took place entirely in tissue culture. They probably do. However, one must consider that final stages of the process could take place in the test rabbit, since the rabbit inoculum contains both live fibroma virus and residual TAM. Several facts suggest that transformations originated in the course of virus proliferation within the roller tubes. For example, once supernatant fluids became positive in an experiment, successive fluids were generally positive, and repeated tests on each fluid were also positive. Test rabbits given tissue culture fluids before this time did not develop myxoma, although such fluid contained TAM and fibroma virus. Transformations in rabbits alone have usually not been as consistent as judged by the experience of other investigators(4). Supernatant fluids from successful experiments presumably contained a mixture of fibroma and myxoma viruses. A small amount of the latter agent may be sufficient to outgrow an excess of the former, especially in a rabbit. Test animals usually developed myxoma within 6 days of inoculation. This represents nearly a minimum incubation period for the strains of virus and of rabbit employed. The shortness of the time suggests that the infective myxoma particles were in the tissue culture fluids inoculated and that the rabbits had not acquired accidental infections. So far there has been no evidence of accidental infections and control rabbits, in the same animal rooms, have remained free of myxoma. It is likely that myxoma is transmissible directly from rabbit to rabbit only by the intervention of blood-sucking arachnids. Potential vectors of this type were not encountered on continued inspections of the animals used.

Experiment BD 32 was an exception to other experiments in that the testis tissue cultures were maintained for 38 days. Myxoma virus was recoverable from fluids for only a week of this period, from 9-16 days after inoculation. One may speculate that the myxoma transformation had taken place in only a few cell areas. These areas may have been lost with the general deterioration of the tissue cultures which was observable after 2

weeks. Thus cells remaining would continue to produce fibroma virus as they had originally. Fibroma virus, and presumably myxoma, may grow in local areas surrounded by uninfected cells. Distribution of cytoplasmic inclusions in stained preparations of previous testis tissue cultures(5) give support to this hypothesis. The objective of continuing experiments on the Berry-Dedrick phenomenon is to increase the predictability with which it can be performed in tissue culture and to effect a chemical separation of the transforming factor contained in myxoma virus.

Summary. 1. Eleven experiments are described in which fibroma was transformed into myxoma virus in cultures of rabbit tissues, both of testes and of kidneys. 2. The transforming agent (TAM) was myxoma virus inactivated by heating at 65-67°C. 3. Live fibroma virus and TAM were inoculated into tissue cultures simultaneously and TAM was re-added at times of fluid change. 4. A good growth of supporting cells appeared essential to successful transformations.

1. Berry, G. P., and Dedrick, H. M., *J. Bact.*, 1936, v31, 50.

2. ———, *ibid.*, 1936, v31, 356.

3. Gardner, R. E., and Hyde, R. R., *J. Infect. Dis.*, 1942, v71, 47.

4. Smith, M. H. D., *Ann. N. Y. Acad. Sci.*, 1952, v54, 1141.

5. Kilham, L., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 739.

6. Morgan, J. F., Morton, H. J., and Parker, R. C., *ibid.*, 1950, v73, 1.

7. Youngner, J. S., *ibid.*, 1954, v85, 202.

8. Melnick, J. L., *Ann. N. Y. Acad. Sci.*, 1955, v61, 754.

9. Kilham, L., and Woke, P. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 296.

10. Kilham, L., and Fisher, E. R., *Am. J. Hyg.*, 1954, v59, 104.

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Effect of Insulin on Thyroid Gland Microhistometric Studies.* (23119)

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Several reports indicate that insulin treatment may affect the structure of the thyroid gland. The results of these investigations are somewhat contradictory: for instance, Watrin and Florentin(1) observed in guinea pigs, and Raiha and Uotila(2) in rabbits, a hypertrophy of the thyroid after administration of large doses of insulin. Raiha(3) and Schulze (4) produced in rabbits and in rats an involution of the thyroid by prolonged administration of insulin; but Schulze(5) himself found no changes in the thyroid of rats when one unit of insulin was given daily for 4 days and Borgorello(6) observed no histological changes in the thyroid of the rabbit, even after prolonged administration of insulin.

With these findings in view we used microhistometric methods to investigate the effect of insulin on structure of thyroid gland of rats exposed to room temperature (24-26°) and to cold (3°C).

Method and materials. Male rats of the Wistar strain, weighing 90 to 125 g were used. The diet, fed *ad libitum,* consisted of ground Purina laboratory chow, which contains according to our determinations, 200 μg of iodine/100 g. The rats received distilled water for drinking. Regular insulin (Lilly) was administered subcutaneously. The animals were kept in individual cages. One-half unit of insulin was given twice daily for 8 days. The controls received 0.1 cc saline subcutaneously at the same intervals. Higher doses of insulin were not used because the cold exposed animals succumbed on injection of slightly increased doses. At the end of the experiments the rats were sacrificed under ether anesthesia by exsanguination from the dorsal aorta. The thyroid glands were removed, dissected free of fat and connective

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