FURTHER OBSERVATIONS ON THE INABILITY TO TRANSMIT A RABBIT NEOPLASM BY CELL-FREE MATERIALS.

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The study of a malignant neoplasm of the rabbit which has been in progress in these laboratories for several years, included experiments in 1921 and 1922 in which it was found that propagation could not be accomplished by the use of filtrates of the tumor or of desiccated tumor tissue (1).

As serial transplantation progressed, adaptation to passage has been associated with an increase in the energy of cell growth as shown by alterations in the character of growth of the primary tumor and metastases which have resulted in a more rapid progress of the disease toward death or recovery (2). Under these circumstances, a repetition of the filtration and desiccation experiments was desirable for the purpose of checking the earlier results with material of a more favorable character as far as capacity for growth is concerned. A few experiments were also carried out with fluid media which had been in contact with tumor tissue and subsequently filtered or centrifuged in order to ascertain whether the tumor could be propagated by a cell-free agent obtained by diffusion. The present paper contains the results of these several experiments in the order named.

EXPERIMENTAL.

Materials and Method.

Tumors of the 56th, 65th and 68th generations were used in this study which was carried out in April and October, 1926, and in January, 1927. The material employed was obtained from primary testicular tumors, the growth activity of which was controlled by

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intratesticular inoculations of fresh cell emulsions not subjected to any manipulation other than emulsification with normal saline; sand was not used. This procedure and route of inoculation are those used in the majority of experiments with this tumor. In 2 preliminary experiments with Hartley's broth, as described below, a control inoculation of the fresh tumor was not made; the condition of the tissue was such, however, that its transplantation as ordinarily carried out would undoubtedly have resulted in tumor growth.

Filtration.—Immediately after removal of the tumor, portions of it were pulped, pressed through a fine meshed sieve and ground with sand. Ringer's solution was added in the proportion of 50 cc. to approximately 5 gm. of tumor. The mixture was shaken for 20 minutes, centrifuged for 10 minutes at a speed of 1500 revolutions per minute and the supernatant fluid filtered through Berkefeld V candles. The cell-free state of the filtrate was controlled by the addition of a suspension of *B. prodigiosus* to the fluid prior to filtration.

A thin suspension of Kieselguhr was thoroughly mixed with the filtrate in the proportion of 0.5 or 1.0 cc. to 25 cc. of the filtrate and 1.0 cc. of the mixture was injected intratesticularly in normal rabbits.

Desiccation.—Small pieces of the tumor were pressed through a fine sieve and a thin layer of the pulp was spread on the bottom of large Petri dishes. The dishes were placed over concentrated sulfuric acid in desiccator jars and the air evacuated by a Geryk pump to a pressure of 3 mm. The jars were then kept in a freezing box at a temperatue of − 1°C. for 5 days. The material was pulverized and taken up in a small amount of normal saline or of Ringer's solution; 1.0 cc. of this suspension was injected intratesticularly in normal rabbits.

Supernatant Fluid of Tumor "Cultures."—Cubes of tumor tissue measuring approximately 0.5 cm. along each side were placed in test-tubes containing 5 cc. of Hartley's KCl glucose broth and 1 cc. of fresh rabbit serum. The tubes were put in jars from which the air was evacuated, and the jars were kept in the ice box for 48 hours. At the end of this time, the supernatant fluid was centrifuged for 10 minutes at a speed of 1500 revolutions per minute, and filtered through a Berkefeld V candle, *B. prodigiosus* having been added before filtration. Inoculations of the filtrate were made into 1 or both testicles of normal rabbits, 0.5 cc. or 1.0 cc. being used, and in one experiment 0.2 cc. was also injected intracutaneously on the ventral surface of the sheath.

In one experiment, the supernatant fluid of the cultures was centrifuged twice but not filtered. Both intratesticular and intracutaneous injections were carried out with this material.

Tissue of "Cultures."—Tumor tissue which had been kept in Hartley's broth in the ice box for 48 hours was emulsified with normal saline and 0.4 cc. of the emulsion was injected into the testicles of normal rabbits.

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"Stored" Tissue.—Pieces of the same tumor used for the above culture experiment were placed in small Petri dishes with bits of gauze soaked in normal saline, care being taken that the tissue did not come in contact with the gauze. The dishes were sealed with adhesive tape and placed in the ice box for 48 hours. Each piece of tumor was then emulsified with normal saline and 0.4 cc. of each emulsion was injected into both testicles of 5 normal rabbits.

TABLE I.

Results of Filtration Experiments.

Experiment	Generation of tumor	Tumor filtrate					Controls—fresh tumor				
		No. of rabbits	Inoculation			Growth	No. of rabbits	Inoculation			Growth
			Route	Number	Amount	Positive / Negative		Route	Number	Amount	Positive / Negative
					cc.					cc.	
I	65	10	R. testicle	10	1.0	0 / 10	10	R. testicle	10	0.3	10 / 0
			L. "	5	1.0	0 / 5					
II	68	5	R. "	5	1.0	0 / 5	5	" "	5	0.3	5 / 0
			L. "	5	1.0	0 / 5					

TABLE II.

Results of Desiccation Experiments.

Experiment	Generation of tumor	Desiccated tumor						Controls—fresh tumor				
		No. of rabbits	Duration of desiccation	Inoculation			Growth	No. of rabbits	Inoculation			Growth
				Route	Number	Amount	Positive / Negative		Route	Number	Amount	Positive / Negative
			days			cc.					cc.	
I	65	5	5	R. testicle	5	1.0	0 / 5	10	R. testicle	10	0.3	10 / 0
II	68	5	5	" "	5	1.0	0 / 5	5	" "	5	0.3	5 / 0
				L. "	5	1.0	0 / 5					

The rabbits were examined frequently in order to determine any reaction at the site of inoculation which could be diagnosed as tumor growth by inspection or palpation. The period of observation varied from 5 weeks to 3 months. With the intratesticular route of inoculation, the usual incubation period of this tumor at present is 5 to 8 days, at the end of which time there is no doubt of the active character of the growth.

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In the experiments in which tumor filtrates or desiccates were employed, the rabbits were observed for 2 and 3 months, since it was probable that growth, if any occurred, would be greatly delayed. In the experiment in which centrifuged

TABLE III.

Results of Diffusion Experiments.
Anaerobic Cultures in Hartley's Broth 48 Hours—Ice Box Temperature.

Experiment	Generation of tumor	Supernatant fluid	Inoculation					Controls—fresh tumor				
			No. of rabbits	Site Number	Amount	Growth		No. of rabbits	Site Number	Amount	Growth	
						Positive	Negative				Positive	Negative
					cc.					*cc.*		
I	56	Centrifuged	5	Testicle 5	0.5 in 3 1.0 " 2	0	5					
				Sheath 5	0.2	0	5					
II	56	" and filtered	5	Testicle 5	0.5 in 3 1.0 " 2	0 0	5					
				Sheath 5	0.2	0	5					
III	68	Centrifuged and filtered	5	Testicle 10	1.0	0	10	5	Testicle 5	0.3	5	0

TABLE IV.

Results of Experiments with Stored Tissue.

Procedure		Inoculation				Growth	
		No. of rabbits	Site	Number	Amount	Positive	Negative
					cc.		
Hartley's broth (anaerobic)	ice box 48 hrs.	5	Testicle	10	0.4	0	10
Stored (moist condition)	" " 48 "	5	"	10	0.4	9	1?

supernatant fluid of the tumor cultures in Hartley's broth was used, and in one of the experiments in which this fluid was filtered, the observation period was 34 days; in the remainder it was 2 months. In a few instances, the testicle or the skin

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of the inoculation area was removed during the experiment in order to obtain additional evidence of the presence or absence of tumor growth from gross inspection or from the histological picture.

RESULTS.

These experiments which are summarized in Tables I to IV turned out entirely negative. No growth was obtained from any of the 25 injections (15 rabbits) of filtrates of tumor emulsions nor from the 15 inoculations (10 rabbits) of desiccated tumor. In like manner, no growth resulted from the inoculation of filtered or centrifuged Hartley's broth which had been in contact with tumor tissue in the ice box for 48 hours. There were 20 testicular and 10 intracutaneous injections carried out with these materials (15 rabbits). Furthermore, the tumor tissue which had been "cultured" in Hartley's broth failed to grow when injected into the testicles of 5 rabbits. On the other hand, the same tumor used in a cultivation experiment was still capable of active growth after being kept in the ice box for 48 hours under moist conditions. Primary tumors developed from 9 of the 10 inoculations made with this material.

These results therefore contrast sharply with those obtained in the control series of rabbits in which the material used for inoculation was not subjected to any manipulation other than emulsification. Primary growths were obtained in every instance from the inoculation of the same tumors which had been used for filtration, desiccation or cultivation in Hartley's broth.

DISCUSSION.

The results of the present experiments confirm the earlier observations in that it has not been possible to propagate this malignant neoplasm of the rabbit with Berkefeld filtrates of the tumor or with desiccated tumor tissue. Furthermore, no success attended the attempts to demonstrate an agent capable of growth which could be separated from the tumor cells by diffusion into a fluid nutrient medium as is the case with a filtrable chicken tumor.*

It would appear therefore that as far as this neoplasm is concerned,

* Unpublished experiments of Jas. B. Murphy.

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it is reasonably certain that living cells are essential for its propagation. This deduction is supported by the results of the experiment in which active growths were obtained with tissue which had been kept in a moist condition in the ice box for 48 hours as contrasted with the failure to obtain growth from the inoculation of the same tissue which had been kept in Hartley's broth in the ice box for the same time. The probable explanation of this failure is the rapid autolysis of cells which occurs under the latter conditions.

It is significant in this connection to recall that the cells of this tumor resist supposedly deleterious influences to a remarkable degree. Repeated freezing and thawing, for instance, destroys most cells, judging from dark-field examination, but a few apparently intact cells may be recognized and intratesticular inoculation of tumor tissue subjected to these procedures is followed by tumor growth (1).

The first filtration and desiccation experiments were carried out with the 4th, 10th and 12th generations of tumor transplants, while growths of the 56th, 65th and 68th generations were used in the work now reported. Whatever changes have occurred in the growth capacity of the tumor cells incident to long continued transplantation there has evidently been no alteration in a hypothetical cell-free agent by which this agent would be more readily demonstrable with the procedures employed.

It is evident from the present experiments as well as from those previously reported, that there is an essential biological difference between this neoplasm of the rabbit and certain tumors of fowls which can be propagated with tissue filtrates or desiccates. This difference may possibly be a matter of animal species since the satisfactory demonstration of the filtrability of a mammalian tumor has yet to be made. It is not unlikely that the biological differences between such species as birds and mammals may extend to the occurrence of tumor agents distinct from tumor cells. On the other hand, the significant factor may be the type of cell involved since the fowl tumors are classified as sarcomata, while the rabbit neoplasm is considered to be of epithelial origin.

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CONCLUSIONS.

It has not been possible to propagate a malignant neoplasm of the rabbit with cell-free filtrates, or desiccated tumor tissue or by the use of fluid media kept in contact with tumor tissue. These findings confirm the results of previous experiments carried out with early generations of the tumor.

The existence of an agent distinct from the tumor cell which could initiate growth has not been demonstrated.

The experiments bring out an essential biological difference between this mammalian neoplasm which is considered to be of epithelial origin and certain filtrable tumors of fowls which have been classified as sarcomata.

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