FURTHER OBSERVATIONS ON AN EXPERIMENTALLY PRODUCED SARCOMA OF THE CHICKEN.*

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(Received for publication, November 21, 1927.)

In a series of publications from The Rockefeller Institute appearing during the period from 1910 to 1914, five transplanted chicken tumors were described, all of which proved transmissible by cell-free filtrates or desiccates of the tumor material.[1] More recently several other transplantable chicken tumors have been reported and from all so far studied it has been possible to separate an agent from the cells capable of reproducing the tumors.

Murphy and Landsteiner,[2] in the hope of gaining some information on the nature of the causative agents of this chicken tumor group, succeeded in producing typical sarcomas by the combined injection of tar and embryonic tissue in adult hens. One of these was transplantable but all attempts to transmit it by filtrates or desiccates failed in the early generations. As this tumor remained the only transplantable chicken sarcoma which could not be transmitted by an agent separable from the cells, it was considered worth while to continue the attempts under varying conditions on the later generations. In the 3 years since the original publication, the neoplasm has been repeatedly transplanted and continues to grow quite readily. In the present report we have brought together the results of all the

* This investigation was carried on by means of funds from the Rutherford Donation.

[1] Rous, P., *J. Exp. Med.*, 1911, xiii, 397. Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lix, 1793. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52. Rous, P., *J. Exp. Med.*, 1914, xix, 570. Lange, L. B., *J. Exp. Med.*, 1914, xix, 577.

[2] Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 6, 807.

numerous attempts to disassociate an agent from the cells of this tumor, which for convenience is called Chicken Tumor 9.

Filtration Experiments.

The same general methods of filtration which gave positive results with the other chicken tumors, were used in the following experiments.

Filtration Method.—About 25 gm. of fresh tumor tissue, previously trimmed of all necrotic and muscle tissues, was finely chopped in a meat grinder and thoroughly ground in a mortar with sterile sand. To the suspension was added about 400 cc. of Ringer's solution and the entire mixture was thoroughly shaken for 20 minutes. It was then centrifuged for 15 minutes to remove the sand and solid portions of tissue, and the supernatant fluid was decanted. To it a suspension of a 24 hour culture of *B. prodigiosus* was added as a means of testing the permeability of the filter and the fluid was passed through a Berkefeld V candle. A trace of kieselguhr was added to the fresh filtrate prior to injecting. Injections of varying amounts (5 to 20 cc.) of filtrate were made into the breast muscles of normal adult chickens.

TABLE I.

Experiment No.	Size filter	Tumor generation	No. of fowls	No. of regions injected	Growths +	Growths −
1	V and N	1st A	8	16	0	16
2	V and N	1st A	10	20	0	20
3	V	2nd A	8	16	0	16
4	V	2nd C	10	20	0	20
5	V	3rd B	10	20	0	20
6	V	5th A	6	12	0	12
7	V	7th A	8	16	0	16
8	V	13th B	5	10	0	10
9	V	17th D	5	10	0	10
10	V	21st F	2	4	0	4
Msl.	V		25	48	0	48
			97	192	0	192

The above table shows the condensed results of the experiments in which Berkefeld filtrate of Chicken Tumor 9 was injected into normal adult chickens. The Msl. fowls are from later experiments in which filtrate alone was injected as control in tests when filtrate was injected with other substances.

TABLE II.

Experiment No.	Age of tumors	Size of tumor		"Takes" in tumor generation
		Right	Left	
	wks.	*cm.*	*cm.*	*per cent*
1	6	5.0 × 2.3	4.9 × 3.2	10
2	7	4.2 × 2.7	4.2 × 3.1	66.6
3	10	6.0 × 5.2	5.7 × 4.6	10
4	6	6.3 × 4.7		75
5	3	6.4 × 4.3	7.0 × 4.5	80
6	6	6.0 × 3.5	6.6 × 4.5	66.6
7	5	6.0 × 4.2	4.0 × 3.2	80
8	3	4.0 × 5.8	7.2 × 3.5	62.5
9	5	6.5 × 4.5	7.0 × 4.9	50
10	7	10.0 × 5.0	10.0 × 5.0	75

The above table gives the data in regard to the tumors used in the filtration experiments summarized in Table I. It gives the size of the tumors used, the length of time since inoculation and the number of tumor "takes" in the same generation.

The filtration test in this group of ten experiments (Table I) was made with ten different tumors obtained from the first to the twenty-first generations. Ninety-seven chickens received the filtrates including those of the miscellaneous group made up of controls from other experiments. The fowls were injected generally in both breasts and kept under observation from 3 to 6 months. Not a single positive result was obtained. That this failure is not due to lack of malignancy of the tumor may be judged by the growth rate and percentage of "takes" on transplantation as shown in Table II. At the time most of the tests were made this tumor was growing at a rate quite equal to that of several of the other transplantable chicken tumors which were easily transmissible by filtrates.

The Injection of the Filtrate into Growing Embryomas.

The tumor originally developed in an embryoma and the possibility that the young elements in an actively growing tissue would create a more suitable environment for its successful transmission by filtrate led us to undertake the following experiments.

Experiment.—Hashed 7 day old chick embryos were injected into both breast muscles of five adult chickens. On the 12th day following the injection, at the time when the embryonic tissue was actively growing, 10 cc. of freshly prepared tumor filtrate was injected into and around the growing embryoma. Another group of five chickens was injected into each breast muscle with 10 cc. of a mixture composed of 12 cc. of hashed 7 day old chick embryos and 90 cc. of fresh tumor filtrate; while a control group of five chickens were injected with the Berkefeld filtrate of the tumor alone.

Several embryomas continued to grow actively for a short time after the filtrate injection but microscopic sections, prepared from pieces removed at operation, showed them to be composed of the cartilage and bone usually found in typical embryoma, without any indication of malignant transformation. These nodules eventually retrogressed and finally disappeared entirely. No evidence of tumor growth was observed in any of the fifteen chickens employed in this experiment.

Injection of the Filtrate into the Developing Embryo.

It was observed by Murphy and Rous[3] that the Berkefeld filtrate of Chicken Tumor 1 rapidly gave rise to tumor nodules when the filtrate was injected into developing chick embryos. It was thought that a tumor might possibly result from the injection into the relatively unresistant chick embryo[4] of the filtrate of Chicken Tumor 9.

Experiment.—A small rectangular piece was cut from the shell of a fertile egg by means of a shortened cataract knife. Exceptional precautions are necessary to avoid cutting through the shell membrane. With a pair of sterile forceps this membrane next was torn aside exposing the chick. A syringe of 2 cc. capacity fitted with a 1 inch, 20 gauge needle was filled with freshly prepared Berkefeld filtrate of the tumor tissue, and 1 cc. injected into the embryonic membranes. The small piece of shell was carefully replaced and the edges sealed with paraffin. The age of the embryos at the time of inoculation varied from 7 to 10 days. After inoculating, the eggs were returned to the incubator until the 19th day, when they were opened for examination. Thirty-three embryos so examined in our experiments failed to reveal any evidence of tumor nodules.

The Addition of a Mucoid Fluid from a Filtrable Chicken Tumor.

The following experiment was planned with the possibility in mind that the mucoid fluid, notably present in the tissue of some of the

[3] Murphy, Jas. B., and Rous, P., *J. Exp. Med.*, 1912, xv, 119.
[4] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

filtrable chicken tumors, might have qualities of rendering the filter permeable to the causative agent of the tar tumor; for it is known that other factors beside the porosity of the filter influence the result of filtration.

Experiment.—The mucoid fluid was aspirated from a large Chicken Tumor 1 and filtered through filter paper to remove any lumps of tissue. It was then sealed in a glass tube and immersed in a water bath for 30 minutes, the temperature of which was kept at 55°C. in order to kill any tumor cells present and to render inactive the tumor-producing agent. Thirty cc. of this fluid was added to 30 gm. of finely chopped tar tumor tissue and ground with sterile sand in a mortar. The remainder of the filtering process was carried out in a manner similar to that of the previous experiments.

Six adult chickens were injected into each breast muscle with 10 cc. of this filtrate and as controls two chickens were injected with 10 cc. of the inactivated mucoid fluid. Another control group of four chickens were injected with 10 cc. of filtrate freshly prepared from the tumor tissue alone. All of the fowls remained negative for tumor growth during 2 months of observation.

Experiments with Desiccated Material from the Tar Sarcoma 9.

The failure to obtain any positive results by filtration led us to attempt a series of experiments in which desiccated Chicken Tumor 9 tissue was used in place of the filtrate. If this tumor was found resistant to drying, a partial analogy to the previously described transplantable chicken tumors could probably be established.

Experiments.—Large, actively growing tumors were removed under aseptic precautions, trimmed of all adhering muscle and necrotic tissue and ground in a meat grinder. The mashed tissue was then evenly spread over the bottom of a glass dish and placed in a desiccating jar containing a layer of sulfuric acid. The jar was evacuated to 4 mm. pressure and immediately placed in a freezing box where the temperature was several degrees below 0°C. In 3 to 4 days, or when the tissue was thoroughly dry, the scaly substance was pulverized in a mortar and about 2 gm. of this material was emulsified in 20 cc. of either sterile distilled water or Ringer's solution. From 2 to 5 cc. of this emulsion was injected into the breast muscles of normal chickens.

Five experiments were conducted with the desiccated material obtained from tumors in the first, second, third and sixth generations and in all forty-two chickens were injected into eighty-four regions. The fowls were kept under observation for from 3 to 6 months. No tumors developed. A summary is given in Table III.

TABLE III.

Summary of the Desiccation Experiments.

Experiment No.	Tumor generation	Size of tumor	Age of tumor	No. of chickens injected	No. of regions injected	Results +	Results −
		cm.	*wks.*				
1	1st A	5.0 × 3.3 4.9 × 3.2	6	11	22	0	22
2	1st F	4.2 × 3.6 3.0 × 2.4	6	5	10	0	10
3	2nd C	4.2 × 2.6 4.2 × 3.1	7	10	20	0	20
4	3rd B	6.3 × 4.7	6	11	22	0	22
5	6th B	6.0 × 3.5 6.6 × 4.5	6	5	10	0	10
5				42	84	0	84

The Addition of Embryonic Tissue to the Desiccated Tumor Tissue.

As the original tumor was obtained by the injection of embryonic tissue and tar, as noted above, it seemed possible that the addition of some fresh, living embryonic tissue, to an emulsion of the desiccated tumor material, might produce the necessary stimulus for a positive growth.

Experiment.—A mixture was prepared consisting of equal portions of 7 day old chick embryonic tissue, and desiccated tumor tissue emulsified in Ringer's solution. Two cc. of this combination was injected into thirteen normal hens, and weekly observations were recorded for several months. As in all of the previous experiments, these animals remained negative, without suggestion of tumor formation.

Inoculation of the Developing Embryo with Desiccated Tumor Tissue.

In a series of eight experiments we injected into the chick embryo small portions of freshly prepared desiccate of tumors from the sixth to the sixteenth generations. Out of ninty-three living embryos injected between the 6th and 8th days and examined on the 18th day

of incubation, not one had developed any suggestion of tumor-like nodules.

From the results of the foregoing experiments it seems certain that Chicken Sarcoma 9 cannot be propagated from the cell-free filtrate of the tumor or the desiccated tumor tissue by any of the usual methods.

Further Attempts to Transmit Chicken Tumor 9 by the Addition of "Cultures" of This Tumor and Normal Tissues to Filtrates.

Gye[5] has shown that it is possible to obtain growths of Chicken Tumor 1, after the filtrate of this tumor has been inactivated by chloroform, providing there is added to the filtrate an equal amount of fluid obtained from "cultures" of malignant tissue. More recently Murphy[6] and Flu[7] have demonstrated that not only malignant tissue but normal tissue "cultures" as well, will bring about this reactivation. With the idea that some essential factor might be removed by filtration or destroyed by drying Chicken Tumor 9, or that the agent is naturally feeble, we have attempted to supply the factor or augment the activity of this agent by the substances which activate the chloroform filtrate of Chicken Tumor 1.

Experiments.—The base of the medium used throughout these experiments was Hartley's broth to which had been added .2 per cent KCl, .7 per cent dextrose and 1 cc. of fresh rabbit serum. Pieces of tumor or embryonic tissue were introduced and the "cultures" were incubated under strict anaerobic conditions at 37.5°C.

To portions of freshly prepared Berkefeld filtrate of Chicken Tumor 9 were added equal amounts of supernatant fluid obtained from 3 day anaerobic "cultures" of rat placenta and chicken embryos. Five cc. of each of these combinations was injected into two groups of four normal hens, the experiment being controlled by injecting 10 cc. of the chicken tumor extract alone into two normal chickens. The animals were observed for a period of 2 months after which they were discarded as no tumors had developed.

In a second experiment, we mixed together equal portions of Chicken Tumor 9 filtrate and the supernatant fluid from 5 day "cultures" of the same tumor. Ten cc. of this mixture was injected into both breasts of three adult chickens. Another group of chickens were injected with 20 cc. of the extract alone to serve as controls. Not a single tumor developed from any of these injections.

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[5] Gye, W. E., *Lancet*, 1925, ii, 109.

[6] Murphy, Jas. B., *J. Am. Med. Assn.*, 1926, lxxxvi, 1270.

[7] Flu, P. C., *Centr. Bakt., I. Abt., Orig.*, 1926, cix, 332.

In a third experiment we attempted to activate the Chicken Tumor 9 filtrate by adding to it 7 day "cultures" of Chicken Tumor 1, but without results.

TABLE IV.

Materials	No. of fowls	No. of injections	Results Positive	Results Negative
Filtrate alone in adult	97	192	0	192
Filtrate in growing embryoma	10	20	0	20
Filtrate in developing embryo	33	33	0	33
Filtrate and mucoid fluid from C. T. 1	6	12	0	12
Desiccate alone in adult	42	84	0	84
Desiccate and embryo tissue	13	26	0	26
Desiccate in developing embryo	93	93	0	93
Filtrate and "culture" fluid	13	13	0	13
Total	307	473	0	473

A summary of the various experiments with filtration and desiccation of Chicken Tumor 9 is given in Table IV. There is no indication that a substance exists separable from the cells by these methods, capable of reproducing the tumor.

Attempts to Demonstrate a Diffusible Substance from "Cultures" of Chicken Tumor 9.

While attempting to discover some method by which the hypothetical agent of the tar tumor could be separated from the cells, it was observed that the fluid from the "cultures" of this tumor sometimes produced tumors when the cultivation was made in sterile Ringer's solution and the tubes allowed to stand in the ice chest under anaerobic conditions for a period of 5 days or longer. These observations indicated the possibility that an active substance had diffused from the tumor fragments. In order to test the matter, the following experiments were planned.

Experiment.—1. To the filtrate of Chicken Tumor 9 was added an equal amount of supernatant fluid from "cultures" in Ringer's solution of Chicken Tumor 9 which had been kept in the ice chest for 5 days under strict anaerobic conditions. Two chickens were injected with 10 cc. of this mixture. One of these chickens

subsequently developed a tumor typical of Chicken Tumor 9. However, it was observed that one of the two control chickens, previously injected with 5 cc. of "cultural" fluid alone had also developed a fair sized nodule which eventually grew extensively and resembled Chicken Tumor 9.

Experiment.—2. A large number of "cultures" of Chicken Tumor 9 tissue in both Hartley's medium and Ringer's solution were prepared. The "cultures" were anaerobically sealed and placed in the ice chest for a period of 6 days. One half of each group of "cultures" were united and filtered through a Berkefeld V filter, while the fluid from the other half was decanted and centrifuged several times at high speed. Both the filtrate and the centrifuged cultures were injected into individual groups of two normal chickens each in measured amounts of 5 cc. No tumors developed in chickens injected with the filtered "cultures" from Hartley's medium, the supernatant fluid from Hartley's medium or from the injection of the filtered Ringer's solution "cultures." However, a typical Chicken Tumor 9 was observed in one of the two chickens injected with the centrifuged supernatant fluid from the Ringer's solution "cultures."

Experiment.—3. The general procedure of this experiment was identical with that of the preceding experiment. Here again it was observed that all three of the chickens injected with the filtrate were negative for tumor growth, whereas both of those injected with supernatant fluid from the Ringer's solution "cultures" developed tumors.

Experiment.—4. In this experiment we substituted sterile distilled water for Ringer's solution in one set of tubes while in another Hartley's medium was used. A long period of observation of the ten fowls used failed to show any tumors resulting from the injections.

As the filtrates of these "cultures" always failed to give tumors it was concluded that the occasional tumors resulting from the injection of the centrifuged material were due to the presence of living cells. This supposition was strengthened by the fact that the sediment contained large numbers of unquestionably living cells. The result then cannot be considered as giving evidence of the presence of an agent separable from the tumor tissue.

DISCUSSION.

The experiments reported here represent an extension of the original study of a tar tumor reported by Murphy and Landsteiner. In its general features the growth is a typical neoplasm with minor histological differences from other chicken tumors studied but it differs no more from these tumors than the individual tumors in the group differ from each other. Yet it appears to differ from all other transplant-

able chicken tumors having for their origin a spontaneous growth, in that despite many efforts no causative agent has been separated from the living cell. It is, of course, possible that some new method or change in technique may lead to a positive result, yet considering the very wide range of conditions resorted to in this study, its negative result would appear significant. The possibility that the agent might be highly susceptible to oxidation has not been completely tested but the negative results obtained in this laboratory with extracts of rat and mouse tumors filtered under anaerobic conditions indicate that this possibility is not of importance in explaining the failure in filtrability. That the agent might require contact with cells of the type from which the tumor presumably arose has been well covered by injecting the filtrate and desiccate into growing embryoma. That the failure is not due to natural resistance in the chicken is shown by the fact that the developing embryo, an organism without resistance,[4] failed to yield growths on the injection of filtrate or desiccate.

While Chicken Tumor 9 is not so rapid in its growth as Chicken Tumor 1, yet it is more rapid than several of the other transplantable spontaneous tumors[8] which have been easily transmitted by filtrates. It would seem, therefore, that the failure of filtrability in its case is not explainable on the basis of lack of malignancy.

For the present this tumor must stand as an exception in the chicken tumor group, in that it resembles the mammalian tumors in the failure to be transmitted by an agent separable from the living cell.

SUMMARY.

Numerous attempts have been made by us to separate from the cells of a tar sarcoma of the chicken (Chicken Tumor 9) a causative agent for the growth. Experiments with filtrates and desiccates injected as such or in combinations with embryonic tissues have all failed to give positive results. So too have injections of filtrates and desiccates into developing chick embryos failed to yield a response. The results confirm those of previous work with the tumor in this laboratory. The growth would appear to differ in a fundamental respect from all tumors of the fowl previously studied.

[8] Tytler, W. H., *J. Exp. Med.*, 1913, xvii, 466. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651. Rous, P., *J. Exp. Med.*, 1914, xix, 570.