

Further Investigation on the Transmission of Induced Tumors in Fowls

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The possibility that the fowl tumor agents are of endogenous origin has been frequently suggested but no definite evidence for this idea has been produced. The demonstration of transmitting agents in induced tumors while not absolutely proof might be considered as giving support for such a conclusion. In 1925 Murphy and Landsteiner (9) reported the induction of two typical sarcomas in chickens by the injection of a high boiling point fraction of coal tar. One of these proved to be transplantable. It grew actively in new hosts, was highly invasive and metastasized freely. Repeated attempts to transmit this tumor by Berkefeld filtrates or desiccates gave negative results. In 1928 Sturm and Murphy (14) reported further tests on transmission of this tumor in which modification of methods of filtration were used, and in which the products of filtration or desiccation were brought in contact with a variety of cell types, both in adult fowls and chick embryos. Again no evidence for an agent separable from the cell could be found. Peacock (12) failed to demonstrate transmissible agents in 7 strains of transplantable induced tumors or in 8 others which were not transplantable. Mellanby (6), and Rothbard and Herman (13) among others have reported similar results. In contrast to these negative findings McIntosh (4) in 1933 reported that 3 out of his 4 transplantable induced chicken tumors were transmissible by filtrates. In 1939 with Selbie (5) he added a second series in which 2 out of 4 induced chicken tumors could be transmitted by cell-free extracts. While there has been some criticism of McIntosh's experiments, there is no immediate explanation of this divergence from the results obtained by a group of other competent investigators. The methods of induction, the system used in transplantation and filtration did not vary essentially from those in general use. The point as to whether the induced fowl tumors do contain agents separable from the cell is so important, that we consider it essential for McIntosh's observations to be confirmed by others before it is accepted. The present report is an attempt in this direction.

TRANSPLANTATION

The methods of induction and description of tumors have been given in a previous paper (11). Dibenzan-

thracene was used as the inciting agent and this was injected either in repeated small doses in lard or in a single large dose in benzol. There were some 62 progressive tumors induced in the fowls of the group injected and from these was selected the material used for transplantation and transmission studies. All of the tumors were spindle cell sarcomas with only slight variations.

The usual procedure was to remove a piece of the tumor as soon as growth was evident; a portion was used for an autograph and the remainder for histological study. The reinoculations resulted in progressive tumors in 22 of the 23 chickens in which this procedure was carried out. These tumors or the primary ones, depending on which showed most active growth, were used for grafting into other fowls. In all, 23 of the induced tumors were used for transplantation and they were inoculated into 124 fowls. Eleven of the tumors failed to take in the first generation with a total of 40 animals inoculated. The remaining 12 tumors inoculated into 84 fowls gave positive growth in 22 (27.4 per cent). From the 12 strains in the 1st generation 6 inoculated into 19 fowls resulted in no growth. Of the remaining 6 strains inoculated into 71 chickens, 25 were positive (35.2 per cent). The 3rd generation of 66 gave 31 takes (46.9 per cent). The 4th transfer in which 48 chickens were inoculated 25 (52.1 per cent) gave progressive tumors. Some of the strains continued to give increasing numbers of takes so that by the 8th generation progressive growth took place in from 75 per cent to 100 per cent of the groups of fowls inoculated.

TRANSMISSION EXPERIMENTS

The methods used for the preparation of cell-free tumor material have been Berkefeld filtrates, extracts of desiccated tumor material, and differential sedimentation of tumor extracts.

Filtration.—The tumor material freed of necrotic areas was finely minced and then thoroughly ground with sterile sand. Water in the amount of 100 cc. for each gram of tissue was added. A pH of 7.0-7.2 was maintained throughout the process by the adding of N/25 NaOH. The suspension was shaken for 20 minutes and then centrifuged at 4,000 r.p.m. to remove

the larger particles. The supernatant fluid was passed through a Berkefeld V candle and the filtrate was concentrated at 1/20 of its original volume in aluminum thimbles lined with a 5 per cent collodion membrane. Kieselguhr was added to the concentrate and 1 cc. injected into each breast of normal young Plymouth Rock fowls.

Desiccation.—Fresh tumor tissue was finely minced, spread in a relatively thin layer and placed in a vacuum jar over sulfuric acid. The chamber was evacuated and put immediately into the freezing box where it was kept till desiccation was completed.¹ The dry material was finely powdered and taken up in water in the ratio of 12 cc. to each gram of dry tissue. From 0.5 to 1 cc. of this emulsion was injected into each breast of normal young fowls.

Sedimentation.—The method used here was that developed by Claude (1, 2) for the purification and concentration of the agent of chicken tumor I. Extracts of tumor tissue were prepared as described above using 25 gm. of tissue to 250 cc. of water. The supernatant fluid after centrifugation for 20 minutes at 4,000 r.p.m. was strained through sterile cheese cloth to remove the fatty material which floats on the surface. A pH of 7.0-7.2 was maintained throughout the procedure. The solution was then spun at 18,000 r.p.m. for 1½ hours. The sediment from all the tubes was collected and resuspended in 8 cc. of distilled water. The pH was again adjusted to 7.0 and this extract sedimented at 18,000 r.p.m. for 4 minutes. The supernatant was put aside and the sediment resuspended in 8 cc. of water and again spun for 4 minutes. This process was repeated twice more, giving 4 spinings of 4 minutes each which resulted in the removal of the larger particles with a minimum loss of the smaller ones. The supernatant fluids from these four centrifugations were pooled and the total amount subjected to 1½ hours of centrifugation at 18,000 r.p.m. The sediment from this was resuspended in 8 cc. of distilled water. This formed the material for tests and 0.5 cc. was injected into each breast of normal young chickens.

The only variation in the above procedure in the different tests was that in some the extracts were prepared from tumor tissue freshly removed from the animal while in others, the tissue was kept from 16 hours to 6 days in a frozen state. The reason for this is that chicken tumor I kept frozen for a time gives a larger yield of the transmitted agent than when fresh tissue is used.

¹It was demonstrated by Murphy in 1911 that the chicken tumor I agent withstood drying. Later he demonstrated that if the tumor material was kept frozen during the desiccation process, the activity of the agent was little impaired and could be kept over long periods. In 1929, Hawkins in this laboratory reported that several viruses, dried in the frozen state, showed no appreciable loss in virulence after a year in storage. (Hawkins, J. A., Proc. Soc. Exper. Biol. & Med., 26:479-480. 1929.)

With any of the above methods the agent of chicken tumor I may be secured in highly active form. The most satisfactory of these methods in transmitting this tumor is by sediments. This material diluted up to the volume of the original extract has a greater tumor producing activity than the full unfiltered extract from which it is prepared. This increase in activity is undoubtedly due to the fact that the method of preparation eliminates the inhibitors which are known to be present in crude extracts and filtrates (10). The concentration of this factor may be so great as to neutralize the tumor agents. This was true in one chicken tumor which was not transmissible by filtrates or desiccates prepared in the usual way but when the inhibitor was eliminated by sedimentation, transmission was easily accomplished (3).

A total of 36 tests was made on different generations of 8 strains of transplantable induced tumors. In all 150 chickens were inoculated with the cell-free products divided as follows: 118 chickens with the sedimented material, 18 with concentrated Berkefeld filtrates, and 14 with the suspension of desiccated tumors. In the grand total of 150 each having two sites of inoculation, totaling 300 tests, not even a single suspicious tumor arose. The details of the tests are given in Table I.

In addition to the above experiments, sediments from two actively growing tumors of strain 870 were tested on the chorio-allantoic membranes of chick embryos. This has been shown by one of us to be a delicate indicator for the agents of the filterable tumors.

Cells from these induced strains grow readily when placed on the chicken membranes but in three tests involving 30 embryos, neither the extract nor the sedimented fraction caused tumor formation.

EFFECT OF X-RAY ON INDUCED TUMORS²

It has been established that the transmitting agents of the filterable chicken tumors are remarkably resistant to x-ray, while the tumor cells can be easily destroyed by irradiation. The following experiments were carried out in the Department of Pathology, Cornell University Medical College, by Messrs. H. C. Miles, R. G. Marquardt, and E. H. Tuttle under the Staff of the Leukemia Funds.

The tumors to be investigated were cut up into fine particles and half of these were irradiated by x-rays using the following factors: 140 kv., 5 ma., 9 cm. distance, and no filter. This combination gave 887 r per minute, with 23 minutes required to deliver 20,000 r units to the tumor particles. During this time the material was kept on an iced pan. The chicks for the tests varied in age from 1 to 4 weeks, but in each

²Through the kindness of Dr. Jacob Furth we are able to include these interesting results in the present publication.

experiment those used were of the same age. As previous experiments have shown that x-ray increases susceptibility to inoculated tumors, all of the chicks were irradiated with 250 to 400 r units before inoculation. Each one was injected in both breasts and legs.

two strains 11 and 13 of spontaneous origin from the series of Drs. Furth and Stubbs.

This extensive material gives still further evidence of the absence of transmitting agents in the induced chicken tumors. The amount of x-ray given is shown

TABLE I: TRANSMISSION EXPERIMENTS*

Tumor strain number	Generation	Age of tumor	Number injected	Condition tumor tissue	Method	
474	2nd	6 wks.	5	Frozen 16 hrs.	Sedimentation	
	3rd	8 wks.	3	Fresh	Sedimentation	
475	2nd	10 wks.	5	Fresh	Sedimentation	
	3rd	10 wks.	3	Frozen 18 hrs.	Sedimentation	
491	2nd	10 wks.	4	Frozen 48 hrs.	Sedimentation	
753	2nd	4 wks.	4	Frozen 16 days	Sedimentation	
	6th	16 days	5	Fresh	Sedimentation	
	7th	3 wks.	5	Frozen 18 hrs.	Sedimentation	
	8th	3 wks.	5	Frozen 16 hrs.	Sedimentation	
	10th	3 wks.	5	Fresh	Sedimentation	
808	5th	16 days	5	Fresh	Sedimentation	
	8th	3 wks.	3	Frozen 24 hrs.	Sedimentation	
281	4th	4 wks.	5	Frozen 48 hrs.	Sedimentation	
	6th	3 wks.	5	Frozen 24 hrs.	Sedimentation	
	8th	3 wks.	5	Frozen 48 hrs.	Sedimentation	
	9th	4 wks.	5	Fresh	Sedimentation	
	9th	3 wks.	5	Frozen 48 hrs.	Sedimentation	
	10th	3 wks.	2	Fresh	Sedimentation	
870	5th	4 wks.	5	Fresh	Sedimentation	
	5th	4 wks.	5	Fresh	Sedimentation	
	6th	3 wks.	5	Fresh	Sedimentation	
	7th	3 wks.	3	Frozen 5 days	Sedimentation	
	8th	3 wks.	3	Frozen 4 days	Sedimentation	
	9th	3 wks.	4	Fresh	Sedimentation	
	10th	3 wks.	4	Frozen 6 days	Sedimentation	
	10th	3 wks.	5	Frozen 3 days	Sedimentation	
	287	2nd	4 wks.	5	Frozen 3 days	Sedimentation
753	8th	3 wks.	5	Frozen 16 hrs.	Conc. Berk. filt.	
281	9th	4 wks.	4	Fresh	Conc. Berk. filt.	
	10th	3 wks.	2	Fresh	Conc. Berk. filt.	
870	7th	3 wks.	4	Fresh	Conc. Berk. filt.	
808	8th	3 wks.	3	Frozen 1 day	Conc. Berk. filt.	
870	12th	3 wks.	4	Desiccated	Emulsion of desiccate	
474	2nd	6 wks.	4	Desiccated	Emulsion of desiccate	
	2nd	4 wks.	2	Desiccated	Emulsion of desiccate	
475	3rd	10 wks.	4	Desiccated	Emulsion of desiccate	

* Total—8 strains; 150 chickens inoculated: 118 with sediment; 18 with Berkefeld filtrates; 14 with desiccate.

The details of the experiments with the results are given in Table II.

The following tumors were used in the tests: Strain 16, originally induced by injections of methylcholanthrene and transplanted in Dr. Furth's laboratory; strain 870 induced by dibenzanthracene in our laboratory and is included in the group reported above; and

to be without perceptible effect on the agents of the two filterable tumors, included in the experiments. From other studies it is known that the filterable agents of tumors will withstand far greater doses of x-ray without damage to their tumor producing activity. If transmitting agents existed in the induced tumors it would be expected that some evidence of

TABLE II: EFFECT OF X-RAY ON TRANSPLANTS OF INDUCED TUMORS

Tumor used	Untreated tumor tissue			X-rayed tumor tissue				Cell-free filtrate		
	No. inoc.	No. positive	Per cent	Dose	No. inoc.	No. positive	Per cent	No. inoc.	No. positive	Per cent
Strain 16										
Exp. 1	8	8	100.0	10,000 r	20	11	55.5	24	0	0.0
Exp. 2	24	20	83.3	10,000 r	24	0	0.0			
Exp. 3	24	20	83.3	20,000 r	16	0	0.0			
Exp. 4	16	11	68.8	20,000 r	28	0	0.0	32	0	0.0
Exp. 5	24	17	70.8	20,000 r	32	0	0.0			
Exp. 6	20	12	60.0	20,000 r	20	1	5.0	40	0	0.0
Strain R. I. 870										
Exp. 1	16	16	100.0	20,000 r	20	0	0.0			
Exp. 2	20	20	100.0	20,000 r	24	0	0.0	40	0	0.0
Controls										
Sarc. 11 . . .	24	24	100.0	20,000 r	24	24	100.0			
Sarc. 13 . . .	24	24	100.0	20,000 r	24	24	100.0			

their presence would manifest itself after this comparatively mild treatment which is barely sufficient to inhibit the growth of the tumor cells. It is of interest to note that strain 870 gives quite regularly 100 per cent takes and is more rapid in its growth than many of the transplanted tumors of spontaneous origin which are easily transmitted by filtrates.

DISCUSSION

The importance of the question as to whether induced fowl tumors contain a filterable agent justifies a continued investigation. As noted above numerous tests by several workers have failed to demonstrate transmission of induced fowl tumors except by living tumor cells. On the other hand McIntosh (4, 5) has reported 5 induced tumors which he claims are transmissible by filtrates. It was difficult to estimate the first of McIntosh's results, for leukemia appeared in his strains and at least one of the tumors arose at a distance from the tar injection. These complications do not appear to come into his second series. In this present report there is added another series of transplanted induced tumors, some 8 strains, in which repeated attempts to transmit by cell-free materials have given only negative results.

The methods used by McIntosh seem to vary in no essential particular either in the manner of induction or method of filtration from those used by other investigators. Nor do the tumors he studied appear to have been of a higher grade of malignancy or to have been more easily transplanted.

A definite answer in either direction would open up some interesting grounds for speculations. If we could accept the results of the majority of tests as the correct answer and accept the induced tumors as true neoplasms, it might throw some doubt on the classification of the filterable fowl tumors which still occupy a unique place in the tumor group. Could it be that cells may become malignant in two ways as has been suggested by one of us (7, 8) *i.e.* through a mutation,

to have lost a controlling factor or to have acquired the ability to produce an excess of a growth factor? Why should there be this sharp difference between the induced and spontaneous fowl tumors? All of the tumors of the latter class, if properly investigated, have given evidence of containing a transmissible agent. Failure in at least one strain, a very slow growing tumor, was due to the presence of a strong inhibiting factor and when this was eliminated the active agent was found in abundance. The methods used in the present experiments eliminate the inhibitor so that the failure in transmission can not be attributed to this factor. It is unfortunate for the better understanding of the chicken tumor group that McIntosh's observation can not be confirmed in other laboratories. It is our opinion that it is necessary to reserve the acceptance of his results in as much as at least three groups of competent workers with a large number of tests have failed to confirm them.

SUMMARY

Tests on 8 strains of chicken tumors induced by carcinogenic chemicals have failed to give any evidence of transmissible agents separable from the tumor cells. The methods used for preparing the material included desiccation, filtration, and high speed sedimentation, all of which methods have proved successful in securing the active agents from fowl tumor strains of spontaneous origin. The cell-free products of the induced tumors were tested by 300 inoculations in 150 young fowls of the same breed as that in which the tumors were induced. A report is also included which shows that x-ray given in sufficient amount to damage the tumor cells, but insufficient to inactivate the filterable agents, destroys the transplantability of the induced tumors. This more extensive study confirms results of our earlier experiments and those of others, showing that the induced fowl tumors like the tumors in mammals can be transmitted only by intact living tumor cells.

REFERENCES

1. CLAUDE, A. Concentration and Purification of Chicken Tumor I Agent. *Science*, **87**:467-468. 1938.
2. CLAUDE, A. A Fraction from Normal Chick Embryo Similar to the Tumor Producing Fraction of Chicken Tumor I. *Proc. Soc. Exper. Biol. & Med.*, **39**:398-403. 1938.
3. CLAUDE, A. Preparation of an Active Agent from Inactive Tumor Extracts. *Science*, **85**:294-295. 1937.
4. McINTOSH, J. On the Nature of Tumors Induced in Fowls by Injections of Tar. *Brit. J. Exper. Path.*, **14**:422-434. 1933.
5. McINTOSH, J., and F. R. SELBIE. Further Observations on Filterable Tumors Induced in Fowls by Injections of Tar. *Brit. J. Exper. Path.*, **20**:49-63. 1939.
6. MELLANBY, E. 11th Annual Rep. Brit. Empire Cancer Campaign. 1934. pp. 81-82.
7. MURPHY, J. B. Factors Involved in the Malignant Process. *Acta, union internat. contre cancer*, **1**:352-357. 1936.
8. MURPHY, J. B. A Discussion of the Etiology of Cancer Based on Present Knowledge. *Trans. Coll. of Physicians of Philadelphia* **4**:21-24. 1936.
9. MURPHY, J. B., and K. LANDSTEINER. Experimental Production and Transmission of Tar Sarcomas in Chickens. *J. Exper. Med.*, **41**:807-816. 1925.
10. MURPHY, J. B., and E. STURM. Properties of the Causative Agent of a Chicken Tumor. IV. Association of an Inhibitor with the Active Principle. *J. Exper. Med.*, **56**:107-116. 1932.
11. MURPHY, J. B., and E. STURM. Further Investigation of Induced Tumors in Fowls. *Cancer Research*, **1**:477-483. 1941.
12. PEACOCK, P. R. Studies of Fowl Tumors induced by Carcinogenic Agents II Attempted Transmission by Cell-Free Material. *Am. J. Cancer*, **25**:49-65. 1935.
13. ROTHARD, S., and J. R. HERMAN. Attempts to Propagate Fowl Tumors Produced by Benzpyrene and by a Virus. *Arch. Path.*, **28**:212-222. 1939.
14. STURM, E., and J. B. MURPHY. Further Observations on an Experimentally Produced Sarcoma of the Chicken. *J. Exper. Med.*, **47**:493-502. 1928.