174. *Experimental Induction of Cancer by Feeding of Transplantable Tumors. III. Immunoserological Studies*

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(Comm. by T. SHIMIZU, M.J.A., Dec. 12, 1955)

Feeding tests with transplantable tumors have a long history, but nevertheless there has been no reported case of successful cancer production with these tests. Bürger orally administered chicken sarcoma to chicken, but the results were negative.¹⁾ He therefore concluded that intestinal infection by chicken sarcoma could not be demonstrated. However, it was noted that the test fed chicken inhibited the development of the later transplanted chicken sarcoma, and in comparison with control animals they exhibited a definite prolongation of life span. Later Nomura made a report similar to this.²⁾

Experimental. *Immunological aspects.* It has already been reported that a specific agent (HST virus)³⁾ is always capable of being isolated from the Yoshida tumor. Earlier, mice were used for the isolation. Later, however, it was found that the agent could be isolated as well by using guinea pigs, embryonated eggs, and rabbits also. In the present experiments, the agent which had been isolated and successively transmitted using guinea pigs was used.⁴⁾ Also it was always possible to isolate at will, using guinea pigs, an agent from the new tumor induced by a feeding experiment, in the same manner as in the case of the Yoshida tumor. It has already been reported that⁵⁾ when an emulsion prepared from the brain of a guinea pig which had been successively inoculated with HST virus was centrifuged, and when the supernatant fluid was repeatedly injected subcutaneously into Wistar strain albino rats, 30 per cent of the rats became completely immune to the tumor. Thus, active immunization was made on Wistar rats to establish a complete immunity to the tumor, and this complete immunity was ascertained by a real transplantation of Yoshida tumor. After one month or more, the new tumor which had been induced by a feeding experiment was transplanted. The results of these tests (35 cases) show that complete immunity to the corresponding tumor was established in the animals. Also when rats were subjected to active immunization with the agent of the new tumor,

Table I. Neutralization tests

		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	ID ₅₀
Neutralization of HST virus with the serum of rats fed with Yoshida tumor for 215 days	Lung	+	+	±	-	-	10 ^{-5.5}
	Liver	±	+	+	-	-	
	Lung	+	-	+	±	×	
	Liver	+	-	+	-	×	
	Lung	+	-	+	-	-	
	Liver	+	±	-	±	-	
Control tests with normal serum of rats	Lung	+	+	±	+	+	<10 ⁻⁷
	Liver	+	+	+	±	-	
	Lung	±	+	+	+	±	
	Liver	±	+	+	+	+	
	Lung	+	+	+	±	-	
	Liver	+	+	+	+	+	
Neutralization of the agent of new tumor with the antiserum of rats immunized with HST virus	Lung	-	+	-	-	±	10 ^{-4.5}
	Liver	+	+	-	-	±	
	Lung	×	±	×	×	-	
	Liver	×	+	×	×	-	
	Lung	+	-	-	-	-	
	Liver	±	+	±	-	-	
Control tests with normal serum of rats	Lung	+	+	±	±	+	<10 ⁻⁷
	Liver	+	+	+	±	+	
	Lung	+	×	×	×	+	
	Liver	+	×	×	×	+	
	Lung	+	+	-	+	+	
	Liver	+	-	+	+	+	
Neutralization of HST virus with the serum of Wistar rats fed with Yoshida tumor for 152 days	Lung	+	+	±	+	±	10 ^{-6.25}
	Liver	+	+	+	+	-	
	Lung	+	+	±	±	+	
	Liver	+	±	±	+	+	
	Lung	+	+	+	+	-	
	Liver	+	+	+	+	±	
Control tests with normal serum of Wistar rats	Lung	+	+	+	+	±	<10 ⁻⁷
	Liver	+	+	+	±	+	
	Lung	+	+	±	±	×	
	Liver	±	+	+	+	×	
	Lung	+	+	+	+	+	
	Liver	+	+	+	+	+	

×: Accidental death.

immunity both to the new tumor and to the Yoshida tumor was established. The test of transplanting the new tumor was also made on mix-bred rats that had accidentally been found to be naturally

immune to the Yoshida tumor, and it was found that they were immune also to the new tumor.

In the next experiment (42 cases), a mix-bred rat was inoculated subcutaneously with the new tumor and when a tumor induced by this had grown to the size of the tip of the thumb, the tumor was entirely excised; then the rat became immune to the reinoculation of the new tumor. Moreover, when inoculated with the Yoshida tumor, such a rat was found to have become completely immune to the Yoshida tumor too. The counter immune procedure between these two tumors comes also into existence.

Thus, it is sure that complete cross immunity was established between the new and the original tumors.

Next, tests on neutralizing antibodies were performed (Table I). That tumor formation after 120 days from the start of the feeding test was exceptional, as stated before. A test of neutralizing HST virus with the serum of an albino rat which had not produced a tumor even after 215 days yielded an ID_{50} of $10^{-5.5}$, whereas the control tests with normal rat serum gave a value smaller than 10^{-7} . A second test, neutralizing the agent isolated from the new tumor with the serum of Wistar rat that had been actively immunized with HST virus, yielded an ID_{50} value of $10^{-4.5}$ in contrast to a value smaller than 10^{-7} in control. A third test, neutralizing HST virus with the serum of a Wistar albino rat which had failed to induce a tumor even after 152 days gave an ID_{50} value of $10^{-6.25}$ (the control value being smaller than 10^{-7}), a result different from those of the above two tests, showing that the production of neutralizing antibodies was markedly less than in the first two experiments.

Cross neutralization tests between the HST virus and the agent isolated from the new tumor. Neutralization test of the new tumor agent and the HST virus with the anti-HST virus serum (Table II): For antigen the HST virus isolated from the Yoshida tumor and fixed on a guinea pig brain, was used. The virus emulsion used in these tests was made by homogenizing the lungs of guinea pigs which were inoculated with the virus 7–10 days prior to the sacrifice. The supernatant of the centrifuged emulsion was intravenously injected into rabbits repeatedly; the antiserum of rabbits was thus obtained.

The emulsion of the new tumor agent was prepared with the lungs of guinea pigs which were inoculated with the agent isolated from the new tumor through guinea pigs' brains. The supernatant of the centrifuged emulsion was decimal diluted with the range of 10^{-1} to 10^{-7} ; the same amount of the antiserum of the HST virus was added to each dilution. Thus neutralized supernatant was in-

Table II. Accumulated "percent morbidity" in cross neutralization tests of the new tumor agent and the HST virus
(For each dilution range four mice were used.)

		10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	ID ₅₀
Anti HST virus serum	Heterologous agent	100	60	40	0	0	0			10 ^{-3.5}
	Homologous agent	50	20	0	0	0	0			10 ⁻²
Anti new tumor agent	Heterologous agent	92.31	90	85.33	16.67	12.50	0			10 ^{-4.5}
	Homologous agent	100	100	100	0	0	0			10 ^{-4.5}
Control tests with normal rabbit serum										
	HST virus	100	100	100	100	100	100	0	0	10 ^{-7.5}
	New tumor agent	100	100	100	100	100	100	100	33.33	10 ^{-8.74}

jected into mice brains and sacrificed after 3 weeks; the morbidity of the test animals was histologically determined.

The test of the neutralization by the heterologous system yielded an ID₅₀ in value of 10^{-3.5}, whereas the test by the homologous system of the procedure gave a value of 10⁻².

The neutralization tests of the HST virus and the new tumor agent with the antiserum of the new tumor agent:

The neutralization procedure was performed in the same manner as mentioned above. The ID₅₀ of the neutralization test by the heterologous system was estimated to be 10^{-4.5}, and that of by homologous system also gave the same value.

The dilution tests of the HST virus and the agent of the new tumor with normal rabbit serum were also pursued; ID₅₀ were figured out as 10^{-7.5} and 10^{-8.74} respectively.

Judging from the above results of cross neutralization tests, the new tumor agent can be identified with the HST virus.

Discussion. The virulence of the subcellular agents of tumors is generally so weak that it can not directly cause a test animal to die. In studying the tumor agents used in the various tests which were mentioned in the previous reports,⁶⁾ one finds two types; that is, a type which forms tumors through inoculation of the homologous host animal (chicken sarcoma virus and infectious rabbit myxoma virus), and a type which does not form tumors through routine inoculation (HST virus, nn chicken sarcoma virus,⁷⁾ Brown-Pearce agent,⁸⁾ Takeda sarcoma agent, Hirosaki sarcoma agent, and Ehrlich

carcinoma agent⁹⁾). In the latter group it can be considered that the HST virus is a neoplastic virus, in view of the results of virological and seroimmunological studies and the aforementioned extensive feeding tests. It may also be considered that the filtrable agent of the Hirosaki sarcoma belongs to a neoplastic agent, in view of the studies made with the HST virus and in light of the facts that the isolation and stabilization of this agent were successful, and especially that tumors were developed by feeding tests at a high rate. (Its virological studies are now in progress.) As the identification of nm (non-neoplastic) chicken sarcoma virus¹⁰⁾ with ordinary chicken sarcoma virus (Rous) has been confirmed, there is no problem.

It goes without saying that not all viral tumors form tumors by feeding tests. This is clearly exemplified in the case of chicken sarcoma.

The isolation and stabilization of the Brown-Pearce agent, Takeda sarcoma agent, and Ehrlich carcinoma agent can be done using guinea pigs, but the fixation of these agents is rather difficult. Moreover, tumors can not be formed by feeding tests with these tumors. It is necessary to discriminate between these agents and the virus in the strict sense of the word. What then are these agents? It has been reported that when certain cell granulas (mitochondria and microsoma) are inoculated on a heterogeneous host, they assume a virus-like attitude and exhibit a neoplastic effect in a certain case.¹¹⁾ Thus, they can not be offhand called viruses, but it is felt that many gradients exist among the cell granula, filtrable agent, and the virus. The three tumor agents that we have mentioned above perhaps make up part of these gradients. In chicken sarcoma also, there is a well-known variable filtrability of the agents; one is easily filtrable, while the other is not so easy or very difficult or impossible to filtrate at all.¹²⁾ A kind of complement-fixing antibody is obtained by ultra-centrifuging the Brown-Pearce tumor, which is considered to be the agent of this tumor, but with this agent tumors can not be formed.¹³⁾ It is known that both a morphological and chemical distinctions can not be made between the Rous virus and the microsome obtained from the chicken embryo extract.¹⁴⁾ Isolation of the filtrable agent is possible with tumors, assuming ascites form, whose transplantability rate is high, but it is impossible with benign tumors, whose transplantability rate is low. Although sufficient studies have not yet been done on the agents of benign tumors, a filtrable agent in the fibrosarcoma of rat produced by methylcholanthren could not be demonstrated.

Summary. 1. In the serum of mix-bred rats that had failed

to develop tumors more than 215 days after the start of the feeding test, neutralizing antibodies against the HST virus (agent of the Yoshida tumor) have clearly been demonstrated. With such albino rats, transplantation of the Yoshida tumor was not successful.

2. From the new tumor that was induced by the feeding test a filtrable agent has been isolated by the same procedure as that used in isolating the agent of the Yoshida tumor.

3. The identity of both agents has been established by cross immunization tests: When a rabbit was subjected to active immunization with one of the two agents, neutralizing antibodies against the other agent were produced in the serum. In the case of albino rats of the Wistar strain treated in a similar manner, the same extents of immunity against both tumors were acquired simultaneously.

Between the new and the original tumors a complete cross immunity experimentally comes into being though they morphologically show a different character to some extent.

References

- 1) Bürger, M.: *Krebsforsch.*, **14**, 526-542 (1914).
- 2) Nomura, H.: *Byorigaku-Kiyo*, **3**, 523-774 (1926).
- 3) Hamazaki, Y., et al.: *Cancer Res.*, **13**, 119-124 (1953).
- 4) Masutani, K.: In press.
- 5) Hamazaki, Y., et al.: *Gann*, **45**, 344-346 (1954).
- 6) Hamazaki, Y., et al.: *Proc. Japan Acad.*, **31**, 480-491 (1955).
- 7) Hamazaki, Y., et al.: *Trans. Soc. Path. Jap.*, **42**, 1-2 (1953).
- 8) Hamazaki, Y., et al.: *Gann*, **45**, 379-381 (1954).
- 9) Shimomura, I.: *J. Karyopatholog.*, **3**, No. 1 (1955).
- 10) Hamazaki, Y., et al.: *Gann*, **45**, 379-381 (1954).
- 11) Lettre, H.: *Krebsforsch.*, **57**, 345-352 (1951).
- 12) Carr, J. G.: *Virus Multiplication*, 284 (1953).
- 13) Kidd, J. G.: *J. Exp. Med.*, **71**, 351-371 (1940).
- 14) Claude, A.: *Proc. Soc. Exp. Biol. Med.*, **39**, 398-403 (1938).