Enhancement of Oncolytic Effect of Russian Encephalitis Virus.* (18619)

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In the study of the effect of neurotropic viruses on transplantable tumors in mice, it was found that the oncolytic action of these viruses was so closely associated with the neurotropism that the tumor bearing animals always died of the infection(1,2). If animals were immunized either actively or passively against the viruses no tumor destruction took place(3). It seemed possible that by continuous passage from tumor to tumor in cancer bearing animals, some change might be brought about in the tropism of the virus. That tumor destruction is not inevitably associated with nervous system injury was shown by Sharpless, Davies and Cox(4). They reported that both Russian and West Nile encephalitis viruses could cause the regression of the chicken tumor RPL-12 with the survival of the host.

For the past two years we have attempted to separate the neurotropic portion of the Russian encephalitis virus from its oncolytic portion. Although the objective has not been attained, certain changes have occurred in the affinity of the virus for tumor tissue. By continuous passage it can be shown that its oncolytic ability can be enhanced. As yet no marked change has occurred in its neurotropism.

Materials and methods. The methods used in trying to produce variant strains of virus from the parent or "stock virus" have been as follows: (1) Sarcoma 180 passages. Three types of passages are reported in this study. (a) Bi-weekly passage. Groups of 3 to 5 animals were implanted subcutaneously with tumors. When the growths were approximately 10 mm in diameter, 0.05 cc of "stock virus" was inoculated directly into the neoplasm. Three or 4 days later one animal from the group was killed, the tumor removed aseptically, and a 10% emulsion made in diluent. After centrifugation, 0.05 cc of the supernatant was inoculated into the tumors of another group of sarcoma 180 bearing animals. The remaining animals of each inoculated group were allowed to die of the infection and a record kept of their day of death and the condition of their tumor. (b) One- to 2-day passage. After 96 bi-weekly passages, another series was begun. The transfers were carried out in the same way except that inoculated tumors were removed and passed at one- and 2-day intervals. An attempt at daily tumor passage was abandoned because the virus was too readily lost. (c) Passage at higher dilutions. Numerous attempts have been made to make tumor to tumor passages after the virus has been diluted. A passage began with virus from the 117th bi-weekly passage diluted to 10^{-3} at the time of each passage had undergone 16 consecutive passages before testing. Continuous passage at higher levels (10^{-5}, 10^{-7}, 10^{-8}) have been difficult to maintain because of the possibility of diluting out the virus. (2) Brain Passage. The original "stock virus" was carried by transfer from brain to brain in order to have a comparison with the tumor to tumor passage material. 0.03 cc of 10% "stock virus" was inoculated intracerebrally and serial passages at that dilution were made at bi-weekly intervals. (3) Wagnar osteogenic sarcoma passage. Continuous passage was begun by inoculation of 0.1 cc of the "stock virus" directly into palpable Wagnar tumors. At intervals of 3 or 4 days, a tumor was removed

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3. Moore, A. E., and O'Connor, S., *Cancer,* 1950, v3, 886.

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aseptically, a 10% suspension made and 0.1 cc of centrifugate inoculated into the same type of neoplasm. *Virus.* The virus of Russian Far East encephalitis was used throughout these experiments.§ Whole mouse brains were kept frozen at −40°C in the dry ice chest. They were prepared from a single stock of brains so that all virus used and referred to as "stock virus," were at the same level of passage. All dilutions were made in 10% inactivated horse serum in physiological saline. *Titrations.* All titrations for virus content of brain and tumor were made with white mice of 18-22 g in weight obtained from commercial breeders. For intracerebral titrations 0.03 cc amounts were employed, and for the subcutaneous and intraperitoneal titrations 0.05 cc was inoculated. *Detection of changes in the virus.* Although virus variation is a recognized phenomenon the detection of variants, especially if present in small amounts, is very difficult. In the studies here reported if a "pure strain" oncolytic variant without effect on normal tissue appeared, it would be possible to detect it only by its activity against a transplantable tumor since intracerebral inoculation would produce no disease. It was necessary, therefore, to set up a standard procedure which would detect any increase in the oncolytic activity of the virus. The test for changes in activity of the sarcoma 180 passage viruses and the brain passage virus was done as follows: Mice bearing sarcoma 180 implanted 4 days previously, were inoculated intraperitoneally with 0.05 cc of a 10^{-3} dilution of virus. The inoculated virus was always titered intracerebrally and usually intraperitoneally and subcutaneously. The day following inoculation tumor and brain were removed and the amount of virus contained in each was determined by intracerebral titration. At the same time 2 mm cube pieces of tumor were implanted into virus immune but tumor susceptible mice to determine its viability (bioassay). The same procedure was done 3 days and occasionally 7 days after the original inoculation of virus.

The time at which the sarcoma 180 is destroyed following intraperitoneal inoculation with "stock virus" at different dilutions has been determined. It is possible therefore to detect, by the method described, any increase or decrease in the oncolytic ability of the virus consequent to a procedure designed to modify that ability.

The same type of test was used for the detection of changes in the tumor effect of the Wagnar passage virus. After a number of serial passages, the virus was tested against the Wagnar tumor by inoculation of 0.05 cc of a 10^{-1} suspension of virus intraperitoneally. At the same time the "stock virus" was inoculated in the same dilution into a comparable group of tumor bearing animals. Previous experiments† had shown that in the dilutions inoculated this virus has been ineffective in causing tumor destruction. When the animals became paralyzed, usually 6 or 7 days after the inoculation, 2 or 3 from each group were killed and the viability of the tumors determined by implantation into virus immune animals. Titrations of brain and tumor were carried out at the same time. *Tissue culture.*‡ The results obtained in animals with the sarcoma 180 passage and the brain to brain passage were confirmed by tissue cul-

Per cent of Sarcoma 180 destroyed at one and three days following intraperitoneal inoculation of 0.05 c.c. of 10^{-3} dilution of Tumor to Tumor and Brain to Brain Passage.

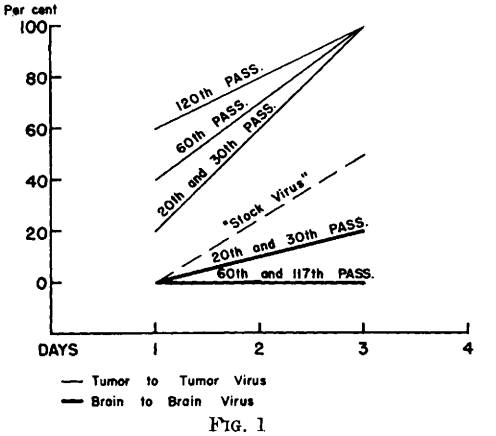

FIG. 1

§ Received through the courtesy of Dr. J. Casals of Rockefeller Institute.

† To be reported.

‡ The author wishes to thank Dr. C. Friend who performed these experiments.

TABLE I. Effect of Intraperitoneal Inoculation of Brain to Brain and Tumor to Tumor Passages on the Viability of Sarcoma 180.

Passage No.	Titer of inoculum i.c.	i.p.	subq.	1 day after inoculation Tumor*	Brain*	% tumor destroyed	3 days after inoculation Tumor	Brain	% tumor destroyed
Original "stock"	9.5§	7.5	7.5	4.5	1.5	0	9.0	4.0	50
B to B† 10th	>9.0	8.0	7.5	>5.0	3.0	20	8.5	4.5	20
T to T‡ 10	9.5	7.5	6.0	3.5	1.0	0	8.25	3.5	60
B to B 20	8.5	—	—	2.0	4.0	0	8.0	3.5	20
T to T 20	8.0	—	—	6.5	1.5	20	8.0	>3.0	100
B to B 30	9.5	—	—	2.0	<1.0	0	8.0	3.5	20
T to T 30	8.0	—	—	4.5	3.0	20	8.0	4.0	100
B to B 40	9.5	—	—	<1.0	4.0	0	7.5	2.0	0
T to T 40	7.75	—	—	<1.0	2.5	20	8.0	4.0	60
B to B 60	8.0	—	—	4.0	<1.0	20	8.0	2.0	0
T to T 60	7.5	—	—	>5.0	1.75	40	6.5	2.0	100
B to B 77	9.5	6.5	7.5	4.0	<1.0	0	7.5	2.0	60
T to T 77	7.5	5.5	6.0	4.0	<1.0	20	8.0	4.0	100
B to B 100	8.0	6.0	4.0	2.5	<1.0	0	7.5	2.5	0
T to T 97	7.5	6.5	4.5	4.0	<1.0	75	8.0	2.0	100
B to B 111	7.5	5.5	5.5	1.5	<1.0	0	6.5	1.5	0
T to T 108	8.5	6.5	7.5	6.0	1.75	25	8.5	4.0	100
1 to 2 day T to T 60	7.5	4.0	4.0	4.5	1.25	40	>7.0	4.0	100
B to B 117	8.5	6.5	5.5	>7.0	>3.0	0	>7.0	2.5	0
T to T 120	8.0	6.0	6.0	>7.0	3.0	60	>7.0	4.0	100
1 to 2 day T to T 84	6.0	6.5	5.5	4.0	>3.0	60	7.0	>5.0	100

* Amt of virus determined by intracerebral titration.
† B to B indicates brain to brain.
‡ T to T indicates tumor to tumor.
§ All figures represent the reciprocal of the logarithm of the LD_{50}.

ture technics. One flask containing a simple Maitland type medium consisting of 4.5 cc Tyrode's solution, 0.5 cc normal horse serum and minced tumor was inoculated with 0.5 cc brain passage virus at a dilution of 10^{-3}. A comparable flask was inoculated with the tumor passage virus. Twenty-four, 48 and 72 hours later 10 tumor pieces along with tumor from a control flask were removed and implanted into immunized mice to determine their viability. Titrations of the supernatant were also carried out.

Results. Sarcoma 180 *passage compared with brain passage.* After 20 passages it was apparent that the bi-weekly tumor to tumor passage virus was destroying the tumor to a greater extent than the brain to brain passage, and further passage has resulted in an increase in the oncolytic effect of this virus while the brain passage has lost its ability to attack the tumor in the 3 day period. In fact bioassay experiments with the 128th brain to brain passage showed that only 30% of the sarcoma 180 was destroyed 7 days after inoculation, whereas inoculation of "stock virus" invariably destroys 100% of the tumor in the same length of time. The results are shown graphically in Fig. 1. Table I gives the detailed information obtained from the bioassay experiments. It can be seen that in each test but one, the inoculated brain to brain passage virus had a higher intracerebral titer than the tumor to tumor passage so that the tumor destructive ability of the latter virus can not be due to an increase in the number of infective virus particles. The titers of brain and tumor from animals killed one and 3 days after inoculation show no great consistency. In general the tumor passage virus appears in larger amounts in the tumor than does brain passage virus (5 of 8 instances) one day after inoculation, whereas 3 days after inoculation both passages are more apt to be present in nearly equal titer (5 of 8 instances). Obviously the intracerebral titer has little bearing on whether a tumor is destroyed since tumors of animals inoculated 3 days previously with brain to brain

TABLE II. Effect of Inoculation of Brain to Brain Passage Virus and Tumor to Tumor Passage Virus on the Viability of Sarcoma 180 in Tissue Culture.

		24 hr*		48 hr*		72 hr*		96 hr*	
	Titer of virus inoc.	Titer	% tumor destruction	Titer	% tumor destruction	Titer	% tumor destruction	Titer	% tumor destruction
48th B to B†	8.0	3.0	0	6.5	60	6.0	70	7.0	87
48th T to T‡	8.0	1.5	33	5.0	50	6.0	70	7.0	100
107th B to B	9.5	5.0	50	3.0	20	2.5	25	—	—
107th T to T	8.5	3.5	100	5.0	100	1.0	100	—	—
1-2 day 52nd T to T	6.5	3.0	62	3.0	80	71.0	100	—	—

* Incubated at 37°C. Titrations and bioassay carried out at 24, 48, 72, and 96 hr.
† B to B indicates brain to brain.
‡ T to T indicates tumor to tumor.
Control tumor pieces exposed for the same length of time in the same medium grew in 100% of the instances.

passage have high titers and yet the virus has no ability to destroy the tumor tissue.

If the neurotropism of the tumor to tumor passage virus were decreased one would expect to get some survivors among the passage mice. This has happened in scattered instances but the total number is not very impressive—10 in 349 animals injected. However, when one realizes that 0.05 cc of a 10% suspension of virus is inoculated (approximately 1,000,000,000 LD_{50}'s) any survivors at all have special interest. None were observed in the brain to brain passage. The animals which survived with regressions of tumor were immune to challenge by fully virulent virus and, in most instances, reimplantation of sarcoma 180 resulted in failure of the tumor to grow. The results of testing 2 of the one to 2 day sarcoma 180 passages are shown in the last 2 experiments in Table I. So far these appear to be of the same order as the biweekly tumor passage and about the same number of survivors (14 out of 438) have been obtained. It was hoped that in this manner the particles of virus having strong tumor affinities could be selectively passed. One test of the 10^{-3} passage after 16 passages showed it to be similar to the other tumor to tumor passages.

Tissue culture experiments. Effective destruction of tumor by the Russian virus can also take place in tissue culture. Therefore the tumor to tumor passage and the brain to brain passages were tested by this means to see if the differences observed in animals could be confirmed. The set up of the test is given under *Methods*. The results obtained are given in Table II. The *in vitro* experiments showed no great difference between the brain to brain and tumor to tumor passages on the growth of the sarcoma 180 at the level of the 48th passage. However the 107th passage showed a difference between them. In this instance the tumor to tumor passages tested showed a much greater ability to destroy the tumor than did the brain to brain passage whose ability in this direction appeared to have decreased.

Wagnar passage virus compared to "stock virus." At regular intervals during serial passage, the virus was tested by bioassay experiments in the manner previously described to ascertain if it had acquired the ability to cause tumor destruction. "Stock virus" was tested in exactly the same manner each time for comparison. A summary of these experiments is given in Table III. It is clear that the Russian encephalitis virus, by continuous growth in a non-susceptible tumor, has been changed so that it is now capable of causing its destruction.

Discussion. From the results presented two conclusions can be drawn: (1) the oncolytic activity of the virus of Russian encephalitis for a susceptible tumor can be increased or decreased quantitatively by suitable procedures, (2) the activity can be modified, qualitatively so that it will cause destruc-

TABLE III. Effect of Intraperitoneal Inoculation of Wagnar Passage Virus on Viability of Wagnar Tumor Compared to the Parent Strain.

Passage No.	Titration			Titer		% tumor destroyed
	i.c.*	i.p.†	subq.‡	Tumor	Brain	
"Stock virus"	9.75§	7.5	6.5	6.5	8.0	0
41st passage	7.5 §	6.0	3.5	6.0	6.0	20
"Stock virus"	8.5	6.0	6.0	8.0	7.5	8
55th passage	9.0	7.5	3.5	8.0	8.5	64
"Stock virus"	8.5	6.0	6.5	6.5	8.0	0
79th passage	9.5	6.0	7.5	8.5	8.5	100
"Stock virus"	10.0	8.0	3.0	6.5	8.0	0
90th passage	10.0	6.5	3.0	7.5	8.5	100

* Intracerebral.
† Intraperitoneal.
‡ Subcutaneous.
§ Expressed as the reciprocal of the logarithm of the LD_{50}.

tion of a cytologic type of tumor which was originally resistant to the parent virus. These results were obtained by continuous passage of the virus in tumor or in brain tissue for relatively long periods of time. The changes in viral activity which occurred with passage were gradual. They show little indication of a discontinuous variation (mutation). Rather the product viruses can be looked upon as adaptive variants of the parent strain. They have retained their ability to attack nervous tissue and have changed only in their destructive capacity for tumor.

Viruses are well known for their capacity to vary with respect to the type of tissue which they can invade and injure. An understanding of the mechanism by which this takes place awaits further work on virus-host relationships but we can speculate on some of the possibilities, with reference to the present results with the strains.

If one thinks of a virus population as containing a number of variant particles, and there appears to be much evidence for this concept(5), it is conceivable that the above results were the result of selection. According to this interpretation the particles responsible for the neurotropism of the virus would have remained constant in number while the ones responsible for the tumor effect would have increased or decreased.

Another possibility resides in the make-up of the virus itself. At one point virus reproduces itself within the tumor without injury to its host cells. After long passage in that tissue the ability to destroy it is acquired by the virus. The transition is a gradual one. The process is reminiscent of the acquisition, by other forms, of the capacity to metabolize substrates after many generations of contact with them(6). It is conceivable that there exist in certain neoplastic cells specific components required for the anabolic processes of their reproduction. These may well be of the nature of nucleic acid precursors(7), and may be similar to those required by viruses(8). By continuous growth in a medium containing these components the virus may achieve the capacity for metabolizing one of them which is essential for the growth of the tumor cell. In this fashion a minor adaptive variation could endow a virus with oncolytic properties.

Still another possible explanation for the observed results may be offered. It has been shown that variant viruses may have different rates of growth(5) and it may be that the tumor trophic viruses become more rapidly adsorbed to the susceptible cell or that they may multiply more rapidly within the cell.

Summary. By continuous passage in the transplantable mouse tumor sarcoma 180 it has been possible to increase the ability of the virus of Russian encephalitis to destroy

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this tumor. Conversely, continuous passage in the mouse brain resulted in a loss of oncolytic activity. In other experiments the virus of Russian encephalitis was changed by continuous passage in the Wagnar osteogenic sarcoma so that it caused destruction of this hitherto resistant tumor.

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Delay of the Early Inflammatory Response by Cortisone.* (18620)

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Experimental and clinical observations(1) have shown that cortisone and ACTH alter the course of bacterial infections, usually having an adverse effect on the host. The inhibitory effect of these substances on the development of granulation tissue(2) has suggested to some that disruption of this phase of tissue reaction might explain these observations. However, since granulation tissue containing its new blood vessels, fibroblasts, and ground substance develops late in the sequence of body defense against infection, it was thought advisable to explore the effects of cortisone on the earliest tissue phases of the acute inflammatory reaction. Croton oil was used as an irritant to incite a cutaneous inflammatory reaction similar to that produced by many bacteria.

Materials and methods. White New Zealand rabbits weighing 2 kg were used. The skin of the abdomen was prepared with fine-tooth, animal clippers 24 hours before the experiment was commenced. Cortisone was given in single daily doses of 12.5 mg intramuscularly, beginning 24 hours before the inflammation was produced. Two drops of croton oil were placed on each of 6 separate sites on the shaved skin of the abdomen. Four longitudinal and 4 horizontal scratch marks, each approximately 2 cm in length, were made with a cutting needle through the oil; the scarified area was then rubbed with a glass rod. Paired biopsies of control and of cortisone-treated animals were made at frequent intervals under intravenous nembutal anesthesia. The sections were fixed in Zenker's solution and stained with phloxine methylene blue. The observations reported here are based on four lesions on each of 16 animals, 8 of which received cortisone.

Results. Because of the difficulty in producing uniform trauma at each site of scarification, the degree of the inflammatory response in the multiple areas of trauma of the same animal was slightly variable. There was also slight variation in response in different animals similarly treated. However, the inflammatory reaction in animals given cortisone was significantly and uniformly less intense than that in the controls.

Gross changes. In animals not given cortisone, the lesions became faintly erythematous within 20 minutes after application of the trauma. By 4 hours these had assumed an intense purplish coloration. Edema was marked by 8 hours. By 24 hours necrosis was obvious, and eschar formation had begun. On the other hand, in the animals given cortisone, the erythema could not be detected until at least 2 hours following scarification through croton oil. In these animals, the purplish discoloration appeared only after 12 to 18 hours and then was confined to the scratch marks. Edema was less than in the controls,

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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