Propagation of St. Louis Encephalitis Virus in Cells of the Ehrlich Ascitic Tumor of Mice.* (20504)

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Koprowska and Koprowski(1) described the morphological and biological changes in the Ehrlich ascitic carcinoma of mice following infections induced with ceratin neurotropic viruses. These investigators have confirmed and extended these observations(2) with particular emphasis on the oncolytic action of the Bunyamwera virus after invasion of the malignant cells. This new experimental host-virus system has been employed by Ackerman (3) to propagate a neurotropic strain of Influenza A virus in serial passage. Price and Ginsberg(4) have demonstrated that infection of the ascitic tumor cells with Newcastle disease virus (NDV) resulted in a marked inhibition of growth of the malignant cells in the absence of demonstrable virus multiplication. In the course of their work with neurotropic viruses, Koprowska and Koprowski(2) showed that St. Louis encephalitis virus appeared in high titer in the ascitic fluid following infection although no consistent oncolytic effect was observed. The present communication reports the results of experiments dealing with the determination of the optimal conditions for the propagation of St. Louis encephalitis virus in the cells of the Ehrlich ascitic carcinoma, and the results of attempts to propagate the viral agent in these cells in serial passage. Some evidence bearing on the possible oncolytic action of SLE virus is presented.

Materials and methods. 1. *Virus.* The Webster strain of St. Louis encephalitis virus was employed in these experiments. Its LD_{50} titer approximated $10^{-7.0}$ when titrated in 3-4 week old mice by the intracerebral route of inoculation. 2. *Tissue.* The Ehrlich ascitic carcinoma(5) was obtained through the courtesy of Dr. J. C. Snyder. Prior to its use in these experiments it was carried through 15 serial mouse passages in our laboratory. For each passage ascitic fluid was removed from 3-6 mice inoculated 10-18 days previously. These fluids were pooled and diluted 10^{-2} in buffered saline or 0.2% bovine albumin broth. Each mouse received an intraperitoneal inoculum of 0.1 ml of this diluted ascitic fluid. The cell count of this inoculum (0.1 ml) approximated 100,000. 3. *Mice.* Adult Swiss white mice (Tumblebrook and CFW strains) were employed for the propagation of the ascitic tumor. Three 4 week old mice of the same strains were used in the virus titrations. No difference in the behavior of these 2 strains of mice has been noted. 4. *Determination of viral content of infected tumor cell suspensions.* Ascitic fluid was removed from at least 3 mice, pooled, quick frozen and stored in the CO_2 cabinet. After thawing, the ascitic fluid was centrifuged at 30,000 or 35,000 rpm at 4°C for 30 minutes. Since preliminary experiments had shown that the sedimented tumor cells contained approximately 10 times as much virus as the supernatant ascitic fluid, the latter was discarded. The sedimented tumor cells were washed 1-3 times in buffered saline or 0.2% bovine albumin broth and recentrifuged after each washing. After the final centrifugation the packed cells were diluted with 10 times their volume of 0.2% bovine albumin broth. The resulting suspension of tumor cells was quick-frozen and stored in the CO_2 cabinet until infectivity determinations could be carried out. At this time the tumor suspensions were thawed and after the addition of sterile alundum were ground by means of a chilled mortar and pestle. The resulting suspension was centrifuged at 8,000 rpm at 4°C for 15 minutes, and the supernatant fluid was drawn off and passed through a Seitz EK filter. The cell free filtrate was tested for infectivity by intracerebral inoculation of suitable 10-fold dilutions into 3-4 week old mice. The diluent employed was 0.2% bovine albumin broth

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TABLE I. LD_{50} Titer of SLE Virus.

Age of tumor at time of viral inoculation	Day after inoculation of SLE virus							
	1	2	4	6	8	10	12	14
0 (simultaneous)	<2.0*	3.3	4.5	4.5	<1.0	1.0	<1.0	—†
1	<1.0	<1.7	5.4	4.4	5.0	4.3	—	3.2
2	3.4	2.4	5.5	4.9	4.4	4.6	3.7	—
4	4.7	6.3	6.0	5.6	5.5	4.5	4.0	—
6	4.6	4.7	5.4	4.6	5.2	3.2	2.4	—
8	4.6	4.2	5.2	3.6	3.2	3.2	—	—
10	2.2	2.7	3.1	3.5	2.4	—	—	—
14	1.8	3.4	4.2	—	—	—	—	—

* Logarithm of reciprocal of LD_{50} dilution.
† Not tested.

and each mouse received an inoculum of 0.03 ml. Six mice were inoculated with each dilution and the LD_{50} titer was determined by the method of Reed and Muench(6). Surviving mice were observed for 12 days. 5. *Cytological technic.* Cell counts were carried out on various samples of ascitic fluid by means of a standard hemocytometer. One percent methylene blue was used as diluent. Only tumor cells were counted. Smears of viral infected and control ascitic tumor cells suspensions were prepared, fixed in formalin and stained with hematoxylin and eosin.

Plan of experiment and results. In the first experiment 200 adult mice were inoculated intraperitoneally with 0.1 ml of a 10^{-2} dilution of ascitic fluid and then divided into several groups. Mice of the first group received an immediate intraperitoneal injection of 0.1 ml of a 10^{-2} dilution of SLE virus. Other groups of mice were similarly injected with virus 1, 2, 4, 6, 8, 10 and 14 days after the inoculation of ascitic fluid, respectively. At regular intervals thereafter, mice from each group were sacrificed, the ascitic fluid removed and pooled, and the viral content of the centrifuged cells determined. In the event that no ascitic fluid could be obtained the peritoneal cavity was washed out with 3 ml of 0.2% bovine albumin broth. The results (Table I) show that in the case of mice inoculated simultaneously with tumor cells and virus an increase in the viral activity of the peritoneal washings or ascitic fluid could be demonstrated over a period of 4 days. Similar results were obtained in the case of mice inoculated with virus 1, 2, and 14 days after the implantation of tumor cells. Mice inoculated with virus 4, 6, 8 and 10 days after implantation of the tumor cells showed high titers 24 hours after inoculation, and no further significant increase could be demonstrated. Mice implanted with tumor cells 4 days before inoculation of a virus yielded the highest titers. As a general rule there was a decrease in viral titer after the 8th or 10th day following inoculation.

In the second experiment, 6 mice injected 6 days previously with the ascitic tumor were inoculated intraperitoneally with 0.1 ml of a 10^{-2} suspension of SLE virus which had an LD_{50} titer of approximately 10^{-7} when tested in mice by the intracerebral route of inoculation. Four days later all the mice were sacrificed and the ascitic fluid was removed. These fluids were pooled and an aliquot was removed and diluted to prepare a 10^{-3} suspension. Six mice inoculated 6 days previously with the tumor were injected with 0.1 ml of this diluted suspension. The remaining pooled fluid was ampuled and shell frozen for subsequent determination of infectivity titer. This procedure of continuous serial passage at 4 day intervals was repeated through 14 passages. The tumor cells of passages 1-5, 10 and 14, respectively, were washed, ground and filtered and the infectivity of the resulting filtrates determined. The results are summarized in Table II. Passages 1-5 revealed an LD_{50} titer of approximately $10^{-5.5}$. In passages 10 and 14 the infectivity titer had increased by one log to approximately $10^{-6.5}$.

In the third experiment, 35 adult mice were each injected with 0.1 ml of a 10^{-2} dilution of ascitic fluid. Four days later 17 of these mice received an intraperitoneal injection of

TABLE II. LD_{50} Titer of SLE Virus Propagated in Serial Passage.

Passage No.	LD_{50}
1	5.5*
2	5.3
3	5.7
4	5.6
5	5.7
10	6.6
14	6.6

* Logarithm of reciprocal of LD_{50} dilution.

a 10^{-2} dilution of SLE virus. The remaining 18 mice were left as controls. Both groups of mice were observed for signs of encephalitis and for the development of ascitic tumors. No deaths were observed which could be ascribed to viral infection. All mice of both groups gradually developed abdominal distention, and all died from the effect of the ascitic tumor. The mean survival time of the control group was 20 days after inoculation of the original ascitic fluids, as compared to 25 days in the group of mice injected with virus 4 days after the inoculation of tumor. This difference in mean survival time suggested the possibility that the virus might be exerting some oncolytic effect.

In the fourth experiment 23 adult mice were injected with 0.1 ml of a 10^{-2} dilution of ascitic fluid. Four days later 12 of the mice were injected intraperitoneally with 0.1 ml of a 10^{-2} dilution of 14th passage SLE infected ascitic fluid. The mean survival time of the two groups of mice was identical—viz.: 19 days after the inoculation of the original ascitic fluid. Five of the mice inoculated with 14th passage SLE infected ascitic fluid died with encephalitic signs within a period of 9-11 days after this inoculation. If these animals are eliminated the mean survival time of the 7 mice which died due to the effects of the tumor was 25 days, somewhat longer as compared to the control group. Eleven additional mice were injected with 0.1 ml of the 10^{-2} dilution of the 14th passage SLE infected ascitic fluid alone. Of these, 3 died with encephalitic signs presumably due to the effect of the virus on the 8th, 9th and 11th days after injection respectively; 3 gradually developed abdominal distention and ultimately died as a result of the tumor on the 25th, 25th and 47th day, respectively, while 5 survived throughout the 90-day observation period without developing any sign of tumor formation.

Although cell counts made on pools of ascitic fluid drawn from viral infected and control animals have shown consistently fewer cells in the former group the differences noted have not been dramatic. Furthermore, no significant differences have been noted between normal and viral infected tumor cells upon microscopic examination.

Discussion. The results of these experiments confirm the original observation of Koprowska and Koprowski(2) that the virus of St. Louis encephalitis multiplies in the cells of the Ehrlich mouse carcinoma. In addition, the SLE virus has been propagated without difficulty in serial passage in this ascitic tumor, and consistently high yields of virus have been obtained. Within broad limits the age of the tumor at the time of viral inoculation has not affected the final viral titer obtained to any significant degree although mice implanted with tumor cells 4 days before inoculation of the agent yielded the greatest amount of virus over the longest period of time (8-10 days after inoculation of the virus).

In the description of their experiments on the effect of certain neurotropic viruses upon Ehrlich mouse carcinoma Koprowska and Koprowski(2) described the results with SLE virus as equivocal in that a marked and consistent oncolytic effect could not be demonstrated although the virus was present in the ascitic fluid in high concentration. The results of the experiments reported in this paper are substantially in accord with these observations. Although no direct microscopic evidence of damage to the tumor cells as a result of viral infection was obtained, the fact that tumor bearing mice subsequently inoculated with virus tended to survive longer than control animals suggested that the virus was exerting some oncolytic effect. Additional evidence on this point was furnished by the lower tumor cell counts in viral infected animals, and by the fact that some mice inoculated with viral bearing tumor cells failed to develop ascitic tumors during the period of observation although all control animals did.†

The SLE virus appears to be one which parasitizes the tumor cells and proliferates readily while exerting no more than a slight oncolytic effect at best on the malignant cells in the process.

Summary. The virus of St. Louis encephalitis has been propagated in serial passage in the Ehrlich carcinoma of mice. The optimal conditions for the multiplication of the agent have been determined and relatively high viral titers have been obtained in the ascitic fluid. Some evidence suggesting a slight oncolytic effect has been obtained.

† Preliminary comparative Warburg studies carried out by Dr. Robert E. Olson on normal and viral infected tumor cells have shown no significant differences in endogenous metabolism, or in exogenous metabolism when the following substrates were added: pyruvate, lactate, succinate or glucose.

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Consideration of Dose-Weight Relationships. (20505)

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Susceptibility of individual animals to the action of a drug is frequently expressed in terms of the dose required to produce a given effect. With some drugs this dose varies in a systematic fashion with the weight of the animal. It has become common practice to express dose as a linear function of body weight (mg/kg) in an effort to remove this source of systematic variation. That such a correction implies a direct proportionality between dose and weight of animal is not generally recognized, in spite of the fact that the inadequacies of this correction have been emphasized(1-5). Moreover, application of this correction to relationships other than a direct proportionality serves only to trade one type of systematic variation for another. Occasionally, this correction term has been applied when none was indicated, and erroneous interpretations of the influence of weight on susceptibility have resulted.

An example of the inappropriate use of the linear correction factor may be found in the susceptibility of wild Norway rats to alpha-naphthyl-thiourea (ANTU). Adequate data have been presented on this(6) to allow investigation of the dose/weight relationships.

On examining these data (Table I) it is apparent that the LD_{50} in mg/kg varies with weight, and in a systematic fashion. In the last column is found the LD_{50} in mg/animal for each weight group which we have calculated as the product of mean weight and LD_{50} in mg/kg. These values, in contrast to the LD_{50}'s in mg/kg, are relatively constant and show no systematic variation related to body weight. The mean LD_{50} is 2.63 mg/animal with a coefficient of variation of 15.6%. The comparable index of variation for the LD_{50}'s in mg/kg is 83.3%. The slope constant of the regression of LD_{50} in mg/animal on body

TABLE I.
Toxicity of ANTU for Wild Norway Rats.

No. rats*	Mean wt* (kg)	$LD_{50} \pm$ S.E.* (mg/kg)	LD_{50}† (mg/animal)
24	.039	58 ± 4.4	2.3
40	.081	43 ± 5.7	3.5
35	.112	22 ± 3.2	2.5
22	.140	18 ± 2.7	2.5
29	.170	16 ± .9	2.7
36	.263	8.1 ± .9	2.1
82	.348	7.7 ± 1.0	2.7
56	.448	6.2 ± .6	2.8

* From Dieke and Richter.
† Calculated as product of mean wt and LD_{50} in mg/kg.