Comparison of the Effects of Inoculation Routes and Viral Preparations on Leukemogenesis[1] (36013)

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The importance of the thymus as the origin site of lymphocytic neoplasms in mice is known. Many investigators using various agents have amply demonstrated this importance (1–9).

Building on the work of Rich *et al.* (10) and Haran-Ghera *et al.* (11), experiments to test the influence of the route of inoculation on leukemogenesis in the Gross passage A (GPA) system were designed. It was expected that the results of these earlier researchers would be duplicated in the GPA system. But it was important to investigate the problem to test the leukemogenic virulence of one of the virus preparations to be used, a newly-developed transplantable GPA lymphoma in the ascites form.

The intrathymic route and intraperitoneal route of inoculation with GPA virus from leukemic tissues were compared to determine the relative effectiveness of each route in leukemogenesis. Inoculation by the intrathymic route resulted in a shorter latent period and a higher incidence of leukemia than did the intraperitoneal route. In a second series of experiments, a cell-free preparation of the new ascites-form lymphoma was used. Again the intrathymic route proved to be, as measured by the latent period and incidence parameters, more leukemogenic than the intraperitoneal route.

When the intrathymic route was used, the two virus suspensions were equally leukemogenic. No significant difference in incidence rate was observed. However, with the intraperitoneal route, malignant ascitic fluid induced a significantly higher rate of incidence than did the virus from the tissue filtrates.

Materials and Methods. Animals. Inbred mice of the C3H/Bi, DBA/2 strains, and F_1 hybrids of these strains used in this study were originally obtained from the colonies of the late Drs. John J. Bittner and Carlos Martinez. The animals have been maintained by rigorous inbreeding procedures. Both strains of mice were foster-nursed on C57BL to eliminate the mammary carcinoma milk-borne agent. The occurrence of spontaneous lymphoid malignancy in the C3H/Bi strain is relatively low. However, if newborn or suckling mice are inoculated with Gross passage A virus, lymphoid malignancy regularly develops.

The animals were given water and Purina Laboratory Chow pellets *ad libitum* throughout the experiments. After weaning, not more than 5 littermate inoculated animals were housed per cage. Development of the lymphoma was confirmed by gross and microscopic observations. The mice were examined weekly for signs of malignancy, and a detailed autopsy was performed at sacrifice or spontaneous death of the animal. Samples of organs were fixed in 10% formalin, processed routine histological methods, and stained with hemotoxylin and eosin.

Virus. The Gross passage A virus, originally obtained from Dr. L. Gross in 1962, has been maintained by serial passage in C3H/Bi strain mice. Growth and passage of

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the virus using the procedure of Gross (12) involves a latent period of several months. The viral preparations harvested usually vary in potency. To obtain the quantities of virus necessary for experimentation, a new method for growing and harvesting transplantable Gross lymphomas which grow rapidly and uniformly was found. The transplanted lymphoma has been developed into the ascites form by passage in 2–3 months old (C3H/Bi $\times$ DBA/2)F_1 hybrid mice, which are susceptible to GPA virus when injected at birth (13).

The animals were examined daily and weighed every third day for signs of developing ascites. In most mice, a significant change in body weight occurred within 7 days. A maximum development of ascites was obtained in 10–15 days, at which time the animals were sacrificed. The animals were killed with ether and the peritoneal fluid was removed under aseptic conditions. The fluid was then centrifuged at 4000 rpm (0°) for 20 min. The supernatant was transferred by sterile Pasteur pipette into sterile glass vials, which were sealed and stored at —70°. Prior to inoculation, the undiluted cell-free peritoneal fluid was filtered through a Swinney filter containing a Millipore membrane of 0.45 μ pore size.

For routine passage of ascites, each mouse was injected intraperitoneally with an average of 3.5×10^7 cells (range, $1.5–5.1 \times 10^7$ cells). Cell counts by the dye exclusion method (0.5% trypan blue) indicated 80–95% cell viability in the preparations used. When the tumor is in the ascites form, a large quantity of fluid can be obtained from the peritoneal cavity of recipients in approximately 2 weeks. Virus can be prepared from this fluid with a minimum of *in vitro* manipulation. Because of the quantity of fluid, variations in potency are avoided.

Recovery of virus from transplanted Gross lymphomas has not previously been reported, though Ioachim *et al.* (14) described the recovery of Gross passage A virus from leukemic cells grown *in vitro*.

Inoculation procedure. A sterile 1 ml syringe fitted with a 30 gauge needle was used for both intrathymic and intraperitoneal injections. The 2–5 day old mice were lightly anesthetized with ether and held on a dissecting board with ventral surface upward. The animals were inoculated with virus preparations of either 20% cell-free filtrates from leukemic tissues or undiluted cell-free peritoneal fluid. For the intraperitoneal route, the mice were injected with 0.1 ml of the virus. For the intrathymic route the needle was inserted without incision into the tissues above the manubrium, then gently moved in turn to the area of the right and left thymus lobes. Each lobe was injected with approximately 0.025 ml of the virus preparation (total, 0.05 ml).

Injections of methyelene blue dye were made in an effort to determine the actual site of inoculation. It was found that the thymus lobes were generously infiltrated by the dye, and that, with the procedure employed, the injection regularly infiltrated the thymus.

Results. As shown in Table I (Group I), injection of cell-free filtrates from leukemic tissues into 2 to 5 day old C3H/Bi mice was more efficient in the induction of leukemia when administered intrathymically than when given intraperitoneally. Even though the intrathymic inoculum was only one-half the intraperitoneal dosage, 100% of the intrathymically injected mice developed leukemia; only 82% of mice injected intraperitoneally developed leukemia.

In the second series of experiments (Table I, Group II) in which cell-free ascitic fluid from animals carrying transplantable Gross lymphoma was used, all the animals which received the virus intrathymically developed leukemia. Ninety-six percent of the mice injected intraperitoneally became leukemic.

A statistically significant difference was observed between the mean latent periods of tissue filtrates injected intrathymically and intraperitoneally (87 days versus 141 days, respectively; Fig. 1). Significance was established by Student's t test. Similarly, a significant difference was found between the mean latent periods for cell-free peritoneal fluid inoculated intrathymically and intraperitoneally (94 days versus 119 days, respectively; Fig. 1). The difference between the mean latent periods of the groups inoculated with

TABLE I. Incidence of Leukemia, Latent Period, and Thymus Enlargement in 2- to 5-day-old C3H/Bi Inbred Mice by Intrathymic and Intraperitoneal Inoculation of Gross Passage A Virus.

	Intrathymic route				Intraperitoneal route			
		Latent period (days)		Thymus enlargement		Latent period (days)		Thymus enlargement
Group	Leukemia (%)	Range	Mean	Per total no. of animals (%)	Leukemia (%)	Range	Mean	Per total no. of animals (%)
I[a]	100	66–179	87	19/20 95	82	94–171	141	9/17 53
II[b]	100	75–144	94	27/29 93	96	77–143	119	21/26 84

[a] C3H/Bi mice which received virus preparation from leukemic tissues—20% cell-free filtrate.
[b] C3H/Bi mice which received virus preparation from ascitic fluid—undiluted cell-free fluid.

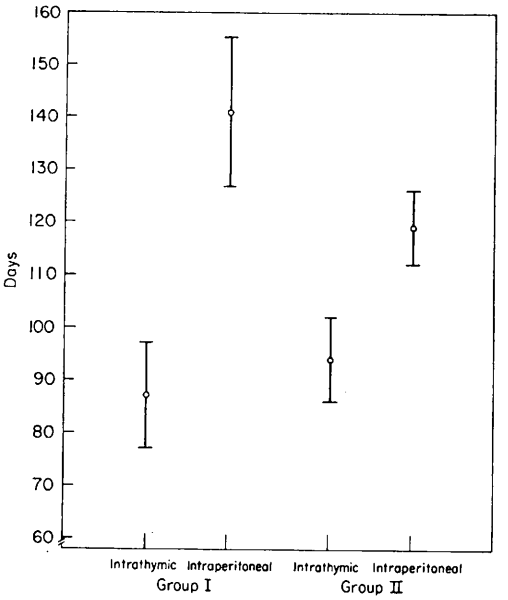

FIG. 1. Mean latent periods in leukemogenesis: Effect of Gross passage A virus inoculation of 2 to 5 day old C3H/Bi mice using cell-free filtrates from leukemic tissue (Group I) and cell-free filtrates from peritoneal fluid (Group II).

either preparation intrathymically was not statistically significant. However, the difference between the mean latent periods following intraperitoneal injection of the two suspensions was significant suggesting that virus in ascitic fluid is more leukemogenic than virus from leukemic tissues. Since the mean latent period significantly reduced following intrathymic injection of the virus, irrespective of the source, virus preparations are now routinely administered by this method.

Using either virus preparation, bilateral thymus enlargement occurred in more than 90% of the animals injected intrathymically (Table I). Following intraperitoneal injections, bilateral thymus enlargement was observed in 53% of the animals which received leukemic tissue-derived virus and in 84% of the recipients of virus suspended in ascitic fluid. This difference is in accord with the earlier observation that the percentage of mice which developed leukemia was greater when the virus used was derived from ascitic fluid than when it was obtained from leukemic tissues.

Discussion. As expected, this study showed

that the intrathymic route of GPA virus inoculation induced leukemia in a larger percentage of animals than did the intraperitoneal route of virus inoculation. It also confirmed and extended previous findings (10, 11) that intrathymic injection of virus decreases the latent period in leukemogenesis. The two findings together add further support to the hypothesis that the thymus is a site for the primary virus–cell interaction during leukemogenesis.

That intraperitoneal injection of GPA virus from peritoneal fluid is more effective in leukemogenesis than intraperitoneally administered virus derived from leukemic tissues is a provocative finding. The most obvious explanation would be that the peritoneal fluid from animals bearing ascites tumor perhaps contains more virus than an homogenate of the virus-induced tumor. Alternatively, the virus may be present in a more infective form, or it may be combined with helper virus or tissue component that fosters infectivity. That a higher rate of development of leukemia was observed after intraperitoneal inoculation of the virus from ascitic fluid than after thymic inoculation of the virus suggests that some kind of virus transport factor ought to be implicated in the explanation of these observations. Such a factor could involve the physical state of the virus, or its association with a tissue factor or some other agent. Factors such as the recognition phenomena involved in receptor mechanisms could also, of course, account for these differences. Further investigation is needed to clarify this phenomenon and to make possible an evaluation of these various postulates.

Summary. Two preparations of Gross passage A virus (one from leukemic tissues and the other from cell-free peritoneal fluid from mice bearing transplantable Gross lymphoma in the ascites form) were used to compare the effectiveness of the intrathymic route of injection with that of the intraperitoneal route. With both preparations, the intrathymic route of inoculation produced a shorter latent period and a higher incidence of leukemia than did the intraperitoneal route. These findings further support the hypothesis that the thymus is a site of primary virus–cell interaction during leukemogenesis.

Intraperitoneal injection of virus from peritoneal fluid was more effective in leukemogenesis than similarly administered virus derived from leukemic tssues. Several hypotheses are suggested to explain this phenomenon. It could be the result of the virus titer or a heightened infectivity of the virus in the ascites form. It might also result from the combination of the virus with a helper virus or tissue components that foster infectivity. Or, perhaps, some transport mechanism reflecting a recognition phenomenon is involved.

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