Studies of malignancies in animals have been carried out to gain information of possible use in human cancer. The work of Eddy and Stewart on the polyoma virus raises basic questions in this area and is a matter of great interest for public health workers. The two papers that follow give a good view of the developments so far.

CHARACTERISTICS OF THE SE POLYOMA VIRUS

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Source of the Virus

THE PURPOSE OF THIS PAPER is to present in some detail information derived from our studies on the characteristics of the SE polyoma virus. The virus was obtained from AKR mice from three sources: from a mouse with spontaneous leukemia from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.; from a mouse with spontaneous leukemia from Dr. L. Laws' colony, National Cancer Institute, Bethesda, Md.; and from a mouse with transplanted leukemia from Dr. L. Gross' Laboratory, Veterans Hospital, Bronx, N. Y.

Propagation of the Virus

The first isolations of the virus were made by inoculating monkey kidney cell cultures that had grown out in sheets with either minced enlarged lymph glands, liver, and spleen or extracts of these tissues. The tissue extracts were prepared by grinding the weighed tissues with alundum, diluting with Earle's balanced salt solution[1] to make a 10 per cent suspension, and centrifuging to remove the sediment. The tissue cultures were nourished with synthetic medium 199[2] containing 1 per cent heated calf serum and incubated at 35°-36° C. The nutrient fluids were changed at weekly intervals. Fluids removed after one, two, and often three weeks' incubation were transferred to new cell cultures or used to inject newborn $(C_3H \text{ f } X \text{ AKR})F_1$ hybrid mice. When it was learned that fluids from one-week old cultures rarely induced tumors in newborn mice, these fluids were discarded and only fluid from cultures incubated for two weeks or longer was used to transfer to new cell cultures or to inject into animals.[3,4] Virus was recovered from mice with parotid gland or other tumors in the same way. The tumor tissue was minced or ground and an extract prepared.

Since uninoculated control monkey kidney cell cultures kept for two or three weeks often degenerated, monkey kidney cultures were not ideal for propagating the SE polyoma virus. Swiss mouse embryo cell cultures were prepared and these not only supported growth of the virus better than monkey kidney cell cultures,[3] but the cultures could be maintained for long periods of time.[5] The method of preparing cultures has been modified from time to time. Some lots of calf serum con-

tained inhibitors, so rabbit serum and later horse serum was substituted. At present, cell growth is initiated with 2 per cent inactivated calf serum in synthetic medium and maintained in 2 per cent inactivated horse serum in synthetic medium.

Mouse embryo cell cultures contain many kinds of cells, some of which appear to grow well and others that do not. The dark degenerated appearing cells may be eliminated by preparing cell cultures in 32-ounce bottles, allowing a sheet of cells to develop and retrypsinizing. The retrypsinized cells are suspended in medium containing 5 per cent inactivated calf serum and dispensed in 2-ounce prescription bottles or tubes. Clear uniform cells develop that soon cover the glass surface. Such retrypsinized mouse embryo cells are now the choice cultures for propagating the SE polyoma virus.

Chorioallantoic membrane cell cultures from 10- or 11-day-old developing chick embryos also supported growth of the virus[6] and work currently in progress indicates that the virus multiplies in mouse kidney, mouse heart, and mouse thymus cultures.

Cytopathogenicity and Hemagglutination

In early experiments virus infected tissue cultures appeared to be identical with control uninoculated cultures when examined under a low-power lens of the microscope. Occasionally, but not regularly, patches of small dark cells were noted in the virus infected cultures. As the virus was transferred from tissue culture to tissue culture, and particularly after mouse embryo cell cultures were substituted for monkey kidney cell cultures, and after maintenance medium containing rabbit or horse serum rather than calf serum was used, cytopathogenic changes were seen regularly.[7] In most cultures the changes in the cells occurred slowly, progressing as the cells became older. Cytopathogenic changes have been noted as early as four days and as late as 21 days.

More recently variation in the amount of sodium bicarbonate in the medium has been shown to have a striking effect on cytopathogenicity[5] and on propagation of the virus. This is shown in Table 1. When a low concentration

Table 1—Virus Content of Fluids from Cell Cultures of the SE Polyoma Virus Nourished with Medium Containing Different Amounts of Sodium Bicarbonate as Measured by Cytopathogenicity and Hemagglutination

5 Per cent Sodium Bicarbonate/ 100 ml of Medium*	Cytopathogenic Titers† at: 7 days	14 days	Hemagglutinating Titers
0.5 ml	1.0‡	2.0	<1:10
1.0 ml	2.0	4.7	<1:10
2.0 ml	3.6	5.3	1:20
2.5 ml	4.5	5.5	1:80
3.5 ml	4.6	5.6	1:80

* Control cultures without virus but with different amounts of sodium bicarbonate in the medium showed no degenerative changes.

† Four roller tubes cell cultures were used for each tenfold virus dilution and the medium on the roller tube cultures contained 2.5 ml of 5 per cent sodium bicarbonate per 100 ml of medium.

‡ Reciprocal of negative log 10.

of sodium bicarbonate (0.5 ml of 5 per cent sodium bicarbonate per 100 ml of medium) was present little virus was present in the tissue culture fluids and cytopathogenic changes were not pronounced. The same virus inoculum on the same cells but nourished with medium containing a higher concentration (1.0-3.5 ml of 5 per cent sodium bicarbonate per 100 ml of medium) gave rise to increasing cytopathogenic changes and higher concentrations of virus.

The virus has the property of causing hemagglutination of guinea pig, hamster, or human 0 erythrocytes in the cold.[8] Both cytopathogenicity and hemagglutination are useful tools for studies on the SE polyoma virus. Table 1 illustrates how the two tests have been used to titrate virus propagated in cell cultures with varying amounts of sodium bicarbonate in the maintenance medium.

Infectivity of the SE Polyoma Virus in Animals

Newborn mice,[3] hamsters,[4] and rats[9] when injected with the virus subcutaneously react by the development of malignant neoplasms that in time kill the animals. Almost all mice develop parotid gland tumors and in addition tumors in other tissues. Twenty-six histologically different tumors have been observed. The virus induces multiple sarcomas and angiomatous lesions in the hamster and the commonest sites are the wall of the heart, liver, lungs, kidneys, and subcutaneous tissues. The most common tumors induced in the rat are sarcomas of the kidneys and subcutaneous tissues.

Unlike these rodents, newborn rabbits injected with the virus developed nonmalignant multiple subcutaneous nodules at 13-64 days of age and these nodules regressed when the rabbits were 93-134 days old. Virus has been isolated in tissue cultures from a nodule excised from a rabbit 56 days old. This virus injected into newborn rabbits again induced multiple subcutaneous nodules. Newborn hamsters injected with virus recovered from the rabbit developed malignant neoplasms in the heart and liver.

Routes of Injection of the SE Polyoma Virus

Tumors are routinely induced in animals by the introduction of virus subcutaneously in newborn animals. Work in progress indicates that tumors are induced in hamsters when virus is given either subcutaneously, intramuscularly, intraperitoneally, or intracerebrally. The dose of virus given intracerebrally was 0.03 ml as compared to 0.2 ml doses by other routes; yet many of the animals developed tumors in about the same period of time. The route was less satisfactory for tests for tumor induction than other routes because some animals died and no tumors could be detected macroscopically.

Passage of the SE Polyoma Virus Through Animals and Tissue Cultures

The SE polyoma virus has been passed from animal to animal and from one species to another with tissue culture passage between each animal passage as shown in Table 2. All of the animals injected with the tissue culture-grown virus developed tumors with the exception of the two dwarfed mice that had received the virus at birth. These were sacrificed and the parotid glands, liver, and spleen were minced and used to inoculate mouse embryo cell cultures. Virus was recovered from the remaining animals by inoculation of cell cultures either with minced tumor tissue or with an extract of ground tumor tissue. In the case of the rat, the kidney tumor and normal appearing spleen were minced and then used to

inoculate mouse embryo cell cultures.

The virus after 39 consecutive passages in mouse embryo cell culture was still capable of inducing tumors in hamsters, whether given subcutaneously, intramuscularly, intraperitoneally, or intracerebrally.

Effect of Dilution on Propagation of the Virus

In transferring 2 ml of the undiluted tissue culture-grown virus to succeeding tissue cultures in two-ounce bottles and testing all fluids harvested after a two-week period for hemagglutination, it was noted that the virus titers became progressively lower. Plaques propagated from such low titered virus diluted and grown under an agar overlay and then recultured in mouse embryo cell cultures with fluid medium gave higher hemagglutination titers. Later it was determined that an agar overlay was unnecessary; if the virus was diluted when transferred to new cell cultures the hemagglutinating titers remained high. At present, virus pools are prepared by diluting the virus used as the inoculum 10^{-3} or 10^{-4}. Cytopathogenicity does not appear as quickly, but the eventual titers are higher whether measured by cytopathic changes or by hemagglutination. This phenomenon is not an exclusive characteristic of the SE polyoma virus. Cantell[11] has reported that dilution of the mumps virus for transfer has a similar effect and the advantage of diluting throat washings from influenza patients for the recovery of the influenza virus has been known[12] for a number of years.

Table 2—Animal Passage of the SE Polyoma Virus with Passage in Tissue Cultures Between Successive Animal Passages

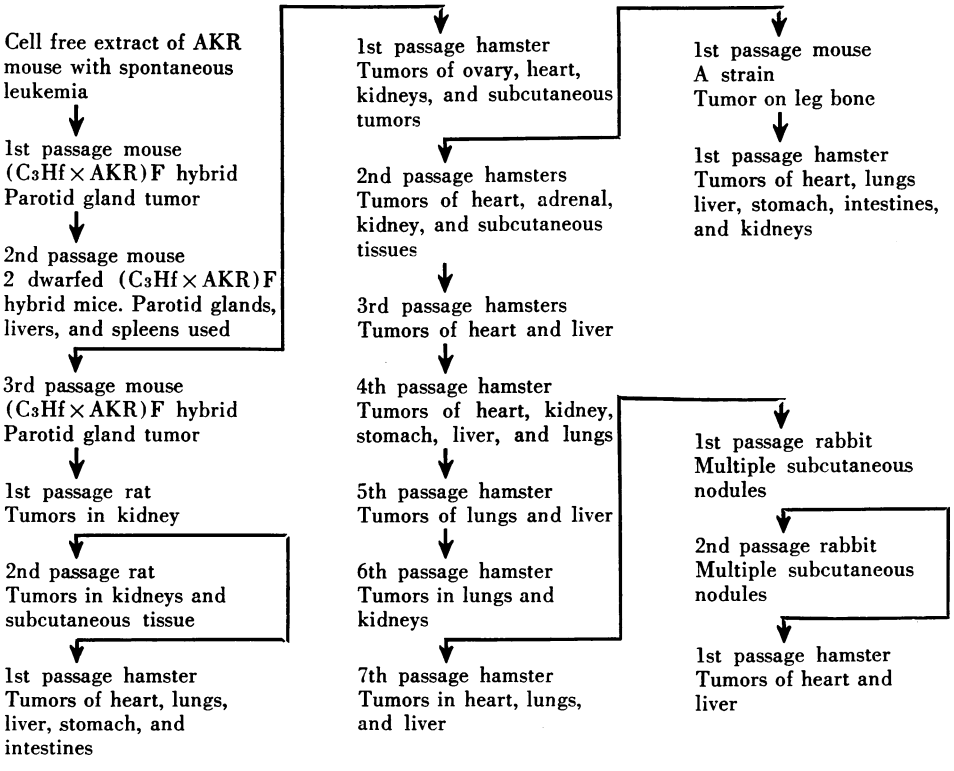

Table 3—Tumors Induced in Hamsters by Virus Propagated in Monolayer Cell Cultures from Different Organs of the Mouse

Tissue Culture Source	Injected	Lost	With Tumors	Latency Period	Sites of Tumors	Surviving
Mouse embryo	4	1	3	18–21 days	3 liver, 2 heart, 2 lungs, 1 kidney	0
Mouse kidney	9	0	5	49 days	5 subcutaneous, 1 heart, 1 liver, 1 lung, 1 kidney 1 intenstine	3
Mouse heart	9	0	4	49–67 days	4 subcutaneous	5
Mouse thymus	10	2	8	28–44 days	5 heart, 3 lungs, 8 liver, 1 kidney, 1 intestine 1 ovary	0

Stability and Preservation

The virus is insensitive to many physical and chemical agents that destroy other microorganisms.[5,13] Heating to 60° C for 30 minutes did not affect the tumor-inducing capacity of the virus, 60° C for one hour delayed time of induction of tumors in hamsters, and the virus heating to 70° C for one-half hour induced tumors in only four of 19 hamsters with a latent period of 63-157 days.

Viability was retained when tissue culture-grown virus was mixed with an equal volume of anesthetic ether and held at room temperature for 14 days, treated with trypsin at 36° C for 18 hours, stored at 4° C for 169 days, or kept at −70° C for 404 days.

The virus has been freeze-dried with an equal volume of sterile skimmed milk. Dried virus cultures kept for 244 days then dissolved in tryptose broth caused cytopathic changes in mouse embryo cell cultures and induced tumors in hamsters.

Filtration and Size

The SE polyoma virus passes through filters, such as millipore, gradocol membranes of 120 millimicrons or greater pore size, and Seitz S3. The Seitz pads absorb much of the virus and induction of tumors in hamsters with Seitz filtrates was often delayed. No evidence has been obtained that any of the virus passes through a gradocol membrane of 43 mμ pore size and most of it was held back by membranes of 77 or 79 mμ pore size. Only two of 51 hamsters that were injected with filtrates passed through membranes of 77 or 79 mμ pore diameter or fluid from cell cultures incubated with such filtrates developed tumors.[5,7] These filtration experiments would place the size of the tumor virus between 26 and 39 mμ according to Elford's empiric formula.

Attempts to correlate the organ used to prepare cell cultures for propagating the SE polyoma virus and the sites of tumors in hamsters—The results of some experiments along this line are shown in Table 3. Virus grown in cell sheet cultures prepared from a single organ does not induce tumors only in that organ when injected into the hamster. Whether long-continued cultivation of the virus in cell cultures prepared from a single organ would increase the number of tumors induced in that organ cannot be answered yet.

One or More Viruses

No evidence has been obtained that more than one virus is responsible for the tumors described in different animals. The diluted virus produces plaques under an agar overlay that can be observed under a low-power lens. Virus from such a plaque when diluted again forms plaques.[5] Hamsters one to three days of age injected with virus recovered from a second plaque developed tumors in the same sites as before.

Virus subjected to treatment with heat, glycerol, or ether, stored at different temperatures or filtered through gradocol membranes of sizes that permitted virus to pass, all induced tumors in hamsters and mice in the same way. If two viruses were present their resistance to physical and chemical agents were the same.

Concentration

The virus has been concentrated by adsorption or elution on guinea pig erythrocytes, high-speed centrifugation, and precipitation by alcohol in the cold.[5]

Concentration by adsorption and elution on erythrocytes was accomplished by mixing 0.1 ml of 30 per cent washed guinea pig erythrocytes with 10 ml of cooled tissue culture grown virus and allowing the cells to settle at 4° C. The suspension was centrifuged at 4,000 rpm for 10 minutes, the supernatant fluid decanted, and the fluid replaced with a reduced volume of warm saline solution.

Centrifugation was carried out by adding normal rabbit serum to tissue culture-grown virus to make 1 per cent, distributing the mixture in stainless steel cups in 10 ml volumes, and centrifuging in a No. 40 rotor in a Spinco Model L centrifuge at 40,000 rpm (85,700-142,900 g) for three hours. The fluid was decanted and the sediment containing the virus was taken up in 1 ml of tryptose broth.

The virus was precipitated with alcohol by distributing tissue culture-grown virus containing 1 per cent rabbit serum in test tubes in 10 ml amounts, chilling to 0° to −5° C in a refrigerated methanol bath and adding 5 ml of chilled absolute ethyl alcohol. A precipitate soon formed. This precipitate which contained most of the virus was removed by centrifugation in a chilled angle centrifuge and taken up in 0.5 ml of tryptose broth (Difco).

Antigenicity

High titer humoral antibodies are elicited in mature guinea pigs or rabbits by inoculation of the animals with one dose of Freund's adjuvant mixed with tissue culture-grown virus followed by two or three doses of virus without adjuvant. Humoral antibodies are also found in the sera of mice or hamsters that develop tumors as a result of being inoculated with virus when newborn.[5]

Antibodies may be determined by serum-virus neutralization tests in hamsters or mice, in mouse embryo roller tube cultures, or by hemagglutination inhibition. Details have been given in other publications.[7,8,14-16]

Comment

Studies of malignancies in animals have been carried out by many investigators over a long period of time in order to gain information that may be of use in the problem of human cancers. Beard[17] states that: "There are no a priori reasons to suspect that the etiology of cancer in man differs in principal from that in animals." But can the SE polyoma virus be used as a model for studies of human cancer? Does man become infected with tumor viruses at a very early age or are such problematic viruses antigenic and highly resistant to physical and chemical

agents? The answers to these questions must await the future.

Summary

The SE polyoma virus recovered in tissue cultures from AKR mice from three sources has been propagated in monkey kidney, chick embryo, mouse embryo, mouse kidney, mouse thymus, and mouse heart cell cultures. It induces malignant neoplasms that eventually cause death in different strains of mice, hamsters, and rats. The same virus induces nonmalignant subcutaneous nodules in the rabbit that regress as the animal gets older. This virus that causes proliferation of cellular growth in vivo causes degenerative changes in monolayer mouse embryo cell cultures and the cytopathogenic effects have been utilized for titrating the virus and its antibody. The virus can be absorbed onto guinea pig, hamster, or human 0 erythrocytes in the cold-causing hemagglutination. Hemagglutination can also be used to titrate the virus and its antibody. Concentration methods that have been used for other viruses are effective for concentrating the SE polyoma virus. The virus is resistant to physical and chemical agents that destroy many viruses. Among these are heating to 60° C for an hour, treatment with ether at room temperature, the action of trypsin at 36° C for 18 hours, and storage at 4° C for 169 days.

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