Parasitization of Mouse Sarcoma 180 by Vaccine Virus and Its Effect on Tumor Growth*

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In the design of these experiments the governing idea has been that microorganisms might selectively infect neoplastic cells and then operate competitively to restrict tumor growth. The decision to try first to establish such a relation for some member of the class of filtrable viruses was made because these agents are intracellular and could, therefore, be capable of interference with cell metabolism at fundamental levels perhaps otherwise inaccessible.

Levaditi (5-9), Rivers and Pearce (10-12) and Findlay and MacCallum (2) established that viruses may infect tumors of rodents and, in addition, be carried there for such unusually long periods of time as to suggest that perhaps malignant cells cannot develop an immunity to virus (12). The behavior of the neoplastic cells appears for the most part to have been unaffected. Levaditi observed necrosis of tumor, but evidently only as part of a generalized infection of the host which was usually fatal (5, 7). However, the more recent experience of Andrewes (1) indicates at least the possibility that as the result of the presence of a virus the proliferation of neoplastic cells may be modified in some way without the complication of host injury. He found that an infectious fibroma of rabbits, itself caused by a filtrable virus, can support Virus III. The fibroma ordinarily regressed spontaneously in an average period of 33 days, but the carriage of Virus III was associated with a reduction of the time for this process to about 19 days. The suggestion was made that the change perhaps represented an example of the interference phenomenon.

In these reports little attention has been paid to the amount of virus involved, a consideration that could be supposed to be of importance for any effect produced, whatever its mechanism. Quantitative relationships have, therefore, been examined in the experiments now to be described, in which a particular effort has been made to introduce large amounts of virus into a tumor.

METHODS

Sarcoma 180 was the 180th spontaneous mouse tumor to be studied in the Crocker Institute for Cancer Research, Columbia University: It has now been transplanted for more than 30 years, and has shown exceptionally vigorous growth. Analysis of the results of grafting 21,663 mice over the period 1914 to 1934 has been made by Haagensen and Prime (3). The tumor grew progressively in 98.4 per cent of the animals. “Cure” by ulceration, necrosis, and slough occurred in 1.08 per cent, and spontaneous regression in only 0.33 per cent. Thus, fewer than 1 animal in 20 can be expected to survive inoculation of Sarcoma 180. Moreover, the behavior of this tumor has been remarkably constant from year to year. Appreciable fluctuations in percentage of "takes" and regressions are infrequent, though they may be encountered.

Grafts of sarcoma 180 were made routinely into the subcutaneous tissues of the right flank of mice at about the level of the costal border. The amount implanted was standardized as one piece of about 1.5 mm. in all diameters. The fragments were introduced by a large needle and trocar in the customary way.

The strain of vaccine virus employed was neurotropic. To begin with, a specimen of commercial calf-lymph was mixed with an equal amount of a solution of penicillin and streptomycin (2,500 units of each per cc.) and 0.3 cc. was then injected intracerebrally into a rabbit. Four days later there were signs of encephalitis. The brain was removed, and, having been found bacteriologically sterile, was macerated and suspended in 4 parts of broth. Inoculations of 0.025 cc. of this suspension were now made into the brains of mice under light ether anesthesia. After a few blind passages, the animals regularly developed fatal encephalitis. For the remainder of the work intracerebral passages in mice were made at frequent intervals.

While adapted to mouse-brain, the virus has been found to have the wide scope of tissue affinities that characterizes most strains of vaccinia. Thus, it retains a dermotropism and produces typical lesions in the skin of the rabbit. It gives rise

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to discrete pox on the chorioallantoic membrane of the embryonated hen's egg. For the mouse it is lethal if given intracerebrally. Moderate doses, however, may be given subcutaneously or intravenously without the development of any gross lesions or impairment of health.

For routine tests for virus a piece of the brain, or tumor, or other tissue to be examined, was cut up with scissors in a mortar and macerated thoroughly. A suspension was then made by adding about 4 parts of broth containing per cc. 1,000 units both of penicillin and of streptomycin. It may be noted that this standard antibiotic broth was used routinely as a vehicle throughout these experiments and appeared to influence the results only in providing complete control of bacterial contamination. Titrations were performed by making serial ten-fold dilutions from 0.2 cc. of the 1:5 suspensions. The precaution of changing pipettes for each tube in the titration was observed routinely.

Tests for the presence of virus were carried out in rabbits. The shaved skin of the back of grey chinchillas weighing 2,500 to 3,500 gms. was marked off into squares and about 25 tests carried out in each animal; 0.05 cc. of suspension was inoculated intradermally and reactions were examined after 48 hours. Final readings were taken on the sixth day. Positive reactions usually appeared between 48 and 72 hours after inoculation.

The mice employed were for the most part of a Swiss strain bred in the Medical Department laboratories for some 15 years and proven to be satisfactory for investigations concerning a number of respiratory viruses. C57 black mice, obtained from the Jackson Memorial Laboratory, Maine, were also used for a few experiments, as indicated below. All mice were between 4 and 8 weeks old at the beginning of each experiment.

Preparation of virus-infected sarcomas.—For the introduction of virus into tumors a major consideration was that the infective dose should be large, and an experiment was designed with this end in view. First, sarcoma was grafted routinely in the flank of several animals. Six days later a certain number of the tumors, which had reached the size of a pea, were injected directly with 0.1 cc. of a 1:5 suspension of vaccinia-infected mouse brain. At the same time an equal number of control tumors were prepared by injection of suspensions of normal mouse brain or of heat-inactivated (100° C.) virus. Four days later both control and infected sarcomas were removed and pieces of them were implanted into new hosts for a study of their comparative development.

RESULTS

The proliferation of vaccine virus in sarcoma 180.—Established grafts of tumor that had been injected on the sixth day of growth with stock 1:5 suspensions of infected mouse brain were tested at intervals thereafter for virus. At 24 hours the virus was recovered only in small quantities, i.e., in titer of $10^{-1}$ or less. By the second day, however, the titer was found to be as high as $10^{-7}$, and it reached a maximum soon after, running out sometimes to $10^{-9}$ by the fourth to sixth day after injection.

If fragments of sarcoma were exposed in vitro to suspensions of vaccinia and then implanted in mice, the virus was taken up and proliferated in the growing tumor. Pieces of untreated sarcoma were prepared as for routine grafting. They were placed in a test tube containing a 1:5 broth suspension of infected mouse brain and allowed to remain there for 2 hours at room temperature. They were then washed several times with normal saline and grafted into 7 mice. Two weeks later the animals were sacrificed. Tumors had grown in 6. Vaccinia was recovered from all of these; for 2 a titration was performed and the titer were found to be $10^{-2}$.

Following almost complete inactivation by heat this strain of vaccinia appeared to multiply in sarcoma 180. A 1:5 suspension of infected mouse brain was placed in a 56° water-bath for 2 hours. Mice inoculated intracerebrally with the heated suspension died only after 13 days, and the mortality rate was then 50 per cent. An unheated suspension of this concentration may be expected to kill all mice injected intracerebrally within 4 days. Two sarcomas were injected with the same heated suspension. Four days later the tumors were removed and ground with 4 parts of broth, and 2 mice were inoculated intracerebrally with 0.025 cc. of each preparation. All of the mice died on the third or fourth day. Evidently the virus had been almost completely inactivated by heat and upon intracerebral injection could kill only slowly and irregularly. Passage through the sarcoma, however, rapidly restored high pathogenicity, presumably through growth of virus and local increase of its concentration in the tumor.

Comparative development of grafts of vaccinia-infected and control tumors.—The rate of growth of grafts of tumors prepared as described above has been examined in several ways: (a) by noting at intervals after transplantation the number of animals bearing distinctly palpable nodules, (b) by sacrificing the hosts after some days and weighing the tumors, (c) by an examination of the fatality rate.
In 5 different experiments 99 mice have received vaccinia-infected grafts of sarcoma 180, while parallel controls numbered 106. By the 7th day after implantation 97 (91 per cent), and by the 10th day 100 (94 per cent), of control grafts could be appreciated as distinct nodules giving unequivocal evidence of growth of the transplant. Meanwhile on the seventh day, only 38 (38 per cent) of the infected implants could be felt and on the tenth day this number had increased to no more than 59 (60 per cent). There is thus a pronounced inhibition of virus-infected grafts during the first week or 10 days of growth.

If the tumors were removed and weighed 11 days after implantation, similar findings were encountered. In one such experiment the mean weight of 20 control tumors was 0.77 gms., almost 3 times the mean weight, 0.27 gms., of 18 tumors containing virus. Moreover, while there was considerable variation in weights of individual tumors in both groups, the largest infected tumor (0.5 gm.) weighed less than the mean of the controls (0.77 gm.), and not half as much as the largest single control sarcoma (1.3 gm.). Again, within the first 2 weeks there is evidence of a clear difference in rate of growth between vaccinia-infected and control tumors.

Table I indicates the results of an experiment in which the animals were left for a longer period of time to submit to the natural course of events. On the tenth day after grafting the first control had died of tumor, and by the end of the third week 90 per cent of these animals had died in the same way. Meanwhile, relative slowness of growth of infected sarcomas was noted during the first week, and was further demonstrated in a delay in the occurrence of deaths of the hosts. Four transplants did not grow at all. Of the 24 (86 per cent) that did grow, no less than 7 regressed, while one was cured by slough. Thus, in all, 43 per cent of the mice survived implantation of virus-infected grafts.

The main point is once more that the growth of the virus-infected tumors was slower and ultimately less successful than that of the controls. The number of animals is small, yet perhaps sufficient to suggest an increased incidence of regression for infected sarcomas.

The tumor appears less able than normal tissues to destroy or eliminate virus. Perhaps it would not be surprising to find small traces of vaccinia persisting in sarcomas. But large amounts of virus, titering out to $10^{-3}$ to $10^{-7}$ were recoverable from many of the tumors, including those removed even in the phase of regression. The sarcoma is thus differentiated from susceptible normal tissues, in which it would be unusual to find virus at all after 2 weeks from the time of invasion.

There is no evidence that the changes observed were the consequence of ill-health of the animals or of generalized vaccinial infection. In all experiments weights were taken every two days. No significant differences from normal controls were encountered. The mice did not look sick, and virus could not be recovered from the viscera of those bearing infected grafts, though in several separate experiments this point was examined at intervals after transplantation.

It might be that a pox virus would be capable of producing inflammation which could interfere with the proliferation of a tumor in the neighborhood. This has not been found. Moreover, attempts to produce subcutaneous lesions by the injection of concentrated suspensions of this strain of vaccinia into the flank have failed. Tumor nodules have been examined microscopically at intervals after grafting, and there is no evidence of inflammation around the infected grafts, either in the connective tissues or the overlying epithelium. It also seems unlikely that there is sufficient alteration in the vascular supply or the bed of the implant to account for the degree of retardation of growth. The detailed histological findings will be presented elsewhere (4).

Only a partial answer can be given to the question as to whether immune mechanisms of the host may contribute to the effect. The inhibition of growth is seen even during the first few days after transplantation and it is not likely that antibodies to the tumor would develop within this time in sufficient quantity to exert such a pronounced action. The influence of active immunity to vaccinia has been tested. Mice were immunized by subcutaneous injection of 0.1 cc. of suspension of vaccinia-infected brain. Two weeks later they withstood intracerebral challenge. In these mice, vaccinia-infected grafts were again found to grow more slowly than control grafts and at about the same rate as in non-immunized hosts. For the
early retardation of growth of vaccinia-infected sarcomas, therefore, no evidence has yet been uncovered to suggest that an immune mechanism is responsible. Analysis of subsequent development of the tumors and of their regression is incomplete, and it may well be that antibodies of some sort contribute appreciably to the changes seen in the later stages of growth.

DISCUSSION

While the findings have been left temporarily incomplete in several respects, including the histological changes, two principal points may be made. First, vaccinia virus can be shown to possess considerable affinity for a malignant tumor and within the tumor it may attain high concentration which can persist for an unusually long time without causing obvious injury to the host. Second, after transplantation the tumor carrying large amounts of virus does not grow as rapidly as it otherwise would.

As for the proliferation of virus in the sarcomas, it seems fair to assume that this occurs within the neoplastic cells rather than in the stroma. A rigid proof can hardly be given in the absence of visible inclusion bodies, and these are not likely to form in cells derived from mesoderm. However, the well-known predilection of viruses for rapidly growing normal tissues, adult and embryonic, makes an infection of vigorously growing tumor cells reasonably probable. Moreover, the virus persisted in the sarcomas for a longer period of time and in higher concentrations than could be expected from simple invasion of the supporting connective tissues.

The retardation of growth of infected tumors has been demonstrated by a method which is not unobjectionable. In the past, the direct injection of various chemical agents into neoplasms has been followed at times by restriction or cessation of growth, yet introduction of the same agents in other ways, e.g., intravenously, has proved quite without effect. The method has been employed here, nevertheless, largely in order to secure concentrations of virus that it may or may not be possible to attain otherwise. Moreover, the behavior of the tumors has been studied after their subsequent transplantation into new hosts, so that an injurious action of the initial injection upon the vascular supply and bed of the implant cannot be held accountable for the results. Therefore, whatever the mechanism involved may be, it probably concerns primarily the neoplastic cells rather than the supporting tissues.

While it seems likely that the inhibition of growth of the tumor is due largely to a direct action of virus on the tumor cells, further detailed analysis of the phenomenon must depend on the accumulation of quantitative data. It would appear that large amounts of virus are necessary for the effect, and it may be that varying concentrations of virus can be shown to exert correspondingly different degrees of growth restriction. Without such evidence the hypothesis of competitive antagonism remains unsupported. Other explanations may be offered, for example, it is possible that the virus elaborates a toxin capable of producing severe damage to cells.

In any case, there are certain aspects of the problem that would appear to deserve further investigation. Among them may be included the possibility of influencing the growth of an established tumor by intravenous injection of virus, and the extension of the observations to other tumors and other viruses.

SUMMARY

A strain of vaccinia that was adapted to mice has been shown to be capable of infecting sarcoma 180. Within the tumor the virus may attain high concentrations (titres 10 to 10⁻⁹) which can persist for long periods of time without causing obvious injury to the host. Upon transplantation tumors carrying large amounts of virus grow more slowly and less successfully than they otherwise would. Infected grafts have been found to weigh less than half as much as controls after 11 days of growth, and they have been observed to regress more frequently.

The explanation is offered that the virus acts directly on the neoplastic cells. The mechanism of action is not clear, and may involve the operation of a viral toxin or a competitive antagonism between virus and the infected neoplastic cells.

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