[CANCER RESEARCH 42, 2552–2555, July 1982]
0008-5472/82/0042-0000$02.00

Inhibition of 7,12-Dimethylbenz(a)anthracene-induced Tumors in Syrian Hamsters by Prior Infection with H-1 Parvovirus[1]

Helene W. Toolan,[2] Solon L. Rhode, III, and John F. Gierthy

Institute for Medical Research of Bennington, Bennington, Vermont 05201

ABSTRACT

Hamsters, given injections s.c. at birth of H-1 parvovirus and 1 month later given a single injection of 7,12-dimethylbenz-(a)anthracene, had a 38% tumor incidence compared with a 95% incidence in animals receiving 7,12-dimethylbenz-(a)anthracene alone. Thus, H-1 which, it has already been shown, invokes a resistance to the incidence of spontaneous and adenovirus-induced neoplasms in hamsters also produces a suppression of a carcinogen-induced tumor in these animals; this suggests that the H-1-induced barrier to successful oncogenesis by these diverse agents has a common mechanism which, present experiments indicate, is not related to a positive or negative H-1 serology. The pathology of the 7,12-dimethylbenz(a)anthracene-induced tumors was similar for both control and H-1-infected hamsters. Although all but one of the primary neoplasms were anaplastic fibrosarcomas as reported previously by others, 25% of the affected females had, in addition, mammary adenocarcinomas, an extremely rare tumor in hamsters.

INTRODUCTION

Syrian hamsters given injections at birth of 1 to 6 PFU[3] of H-1 parvovirus develop a particular deformity characterized by small size, flattened foreface, lack of teeth, and protruding tongue (20); due to their appearance, they have become known as "funny faces" (FF). Such animals, allowed to live out their life span (which is similar to that of normal hamsters), were found to have a very low incidence of spontaneous tumors compared with that of control hamsters [4 tumors in 1729 FF, an incidence of 0.0023% or approximately one-twentieth of the incidence of 0.05% seen in the 259 control animals where 13 tumors occurred (19)]. Subsequent experiments showed that the FF state also appeared to inhibit the incidence of adenovirus-induced tumors, since adenovirus alone injected into newborn hamsters produced a 67% incidence of tumors, whereas adenovirus plus H-1 injected into babies shortly after birth resulted in an incidence of 28% tumors (21). It was the purpose of our present studies to determine if neonatal infection with H-1 parvovirus also produced resistance in the hamster to the induction of a carcinogen-induced tumor. For this purpose, we chose DMBA since Homburger (8) noted that this agent was the most potent tumor inducer of the polycyclic hydrocarbons tested in the hamster.

MATERIALS AND METHODS

Animals. Animals used in these experiments were of the outbred LAK Syrian hamster line of the Lakeview Hamster Colony, obtained from Charles River Laboratories, Inc., Wilmington, Mass. Proven females were time bred for their second litter and shipped 3 days after breeding to arrive at our laboratory on Day 4 or 5 and to give birth on the early morning of Day 16. Babies were counted in the forenoon and given injections of H-1 parvovirus in the late afternoon of the day of birth in order that no babies would be born after injections were finished.

Virus. The H-1 parvovirus used was plaque-purified virus (13) passaged in newborn hamsters from which a liver filtrate of 6-day-old infected babies was obtained. The PFU of the filtrate were determined, and a dilution was made in phosphate-buffered saline [0.02 M sodium phosphate (pH 7.4)–0.14 M $NaCl_2$] so that each newborn hamster given s.c. injection in the nape of the neck received approximately 2 PFU of virus (0.05 ml of 40 PFU/ml).

Carcinogen. Twenty mg of DMBA obtained from Sigma Chemical Co. (St. Louis, Mo.) were dissolved in 4 ml of tricaprylin (Sigma) as described by Homburger and Hseuh (10). A single injection (0.1 ml) of this material (0.5 mg DMBA) was made s.c. in the left groin area of each 1-month-old hamster in all groups while under light anesthesia.

Tumors. The hamsters were examined twice weekly for tumors, and any observed were allowed to develop until they reached 1 cm in diameter. At that time, anesthetized host animals were bled from the heart, killed, and autopsied. The tumors and immediately adjoining tissues were removed and fixed, and all organs were examined for the presence of metastases or other tumors. Animals without neoplasms were kept for 2 months after the last tumor was seen and then bled and autopsied.

RESULTS

Two sets of experiments were done. Experiment 1 was composed of females only. Experiment 2 contained both males and females of approximately equal numbers. Since no differential could be seen related to sex, these animals were considered as a group. Table 1 summarizes our findings for the 2 experiments which have been combined, since they were remarkably similar. As noted, there were 3 categories: the control hamsters which had been given sham-injections of phosphate-buffered saline only at birth; the deformed FF resulting from a successful neonatal infection with H-1; and the so-called "normals" which provided a second type of control. These latter animals were survivors of neonatal infection with H-1 (littermates to the FF) that had no deformities and with few exceptions were without antibodies to H-1 as determined by hemagglutination inhibition and neutralization tests. Such animals are always found where only 1 to 2 PFU of virus are presumably injected; they represent babies that failed to initiate infection due to the low dose of virus in the preparation given. Occasionally, they did have low titers of antibody to H-1, probably acquired in response to horizontal infection, since such antibodies were always 2 to 3 logs lower than those of

[1] Supported by Grant CA25866 from the National Cancer Institute, NIH.
[2] To whom requests for reprints should be addressed.
[3] The abbreviations used are: PFU, plaque-forming units; FF, hamsters deformed by neonatal *H-1* infection ("funny faces"); DMBA, 7,12-dimethylbenz-(a)anthracene; RV, rat virus; AAV, adeno-associated virus; MVM, minute virus of mice.
Received December 3, 1981; accepted March 31, 1982.

Table 1

Incidence of tumors in DMBA-treated hamsters

A single injection of 0.1 ml containing 0.5 mg DMBA in tricaprylin was made s.c. in the left groin area of 1-month-old FFs (deformed survivors of 1 to 2 PFU of H-1 injected on the day of birth), "normals" (normal-appearing survivors of the H-1 injection), and control animals (given sham injections of PBS only at birth). The animals were followed for approximately 40 weeks (2 months after the last tumor was seen). Animals with tumors were bled for sera when the tumor was about 1 cm in diameter and then sacrificed and autopsied. At the conclusion of an experiment, the remaining animals were bled, sacrificed, and examined as above. The sera were assayed by hemagglutination inhibition for the presence of H-1 antibodies.

Experiment	Group	Total no. of animals	Animals with tumors	% of tumor incidence
1 + 2	Controls	40	38	95
	Normals (total)	72	64	89
	Seronegative	58	52	90
	Seropositive	14	12	86
	FF[a]	29	11	38
	No DMBA (control)	12	0	0
	No DMBA (FF)	10	0	0

[a] The decrease in incidence of tumors for the FF animals was significant ($p < 10^{-3}$ by the χ^2 test).

the FF and similar to the antibody titer found in hamsters immunized as weanlings or adults with H-1.

The incidence of tumors in the control hamsters (given injections of DMBA only) of Experiments 1 and 2 was the same, a high 95%, while that of the normal animals was also remarkably similar in the 2 sets and, at 89 and 88% (average, 89%), not significantly different from that of the controls. Whether or not the normal animals were seronegative or seropositive did not appear to be of moment as the relative incidence of tumors was alike in both groups. In contrast, only 43% of the FF in Experiment 1 and 33% of those in Experiment 2, an overall incidence of 38%, developed neoplasms. Two additional control groups, a set of 12 normal animals that had not been given injections and a group of 10 FF which did not receive carcinogen, did not develop tumors during the experimental period.

A time study of tumor incidence and of percentage of survival (Chart 1) shows that neoplasms initially reached 1 cm in diameter, at which time host animals were killed, at about 10 weeks after injection of the DMBA. Neoplasms first appeared in all groups at approximately the same time and maintained a relatively similar rate for 3 to 5 weeks. After that period, a divergence occurred. The incidence of survival among the controls and normals rapidly declined, while that of the FF stabilized and remained high. We conclude from these studies that neonatal infection of hamsters with H-1 virus causes them to resist oncogenesis whether carcinogen-induced as here documented or spontaneous or virus induced as reported previously.

Two other experiments were done relative to our primary finding. It might be hypothesized that the heightened tumor surveillance seen in the FF could be due to a persistent infection in these animals and that virus itself might kill potential or actual neoplastic cells at a stage prior to formation of palpable tumors. If this hypothesis were correct, cells from tumors of the control group should be more sensitive to infection by H-1 than those from the FF. This was tested by culturing cells from some of the tumors of each group and determining their sensitivity to infection by H-1 and 3 other parvoviruses by immunofluorescent staining. Nearly all of the DMBA sarcomas, however derived, were quite resistant to infection with H-1 as

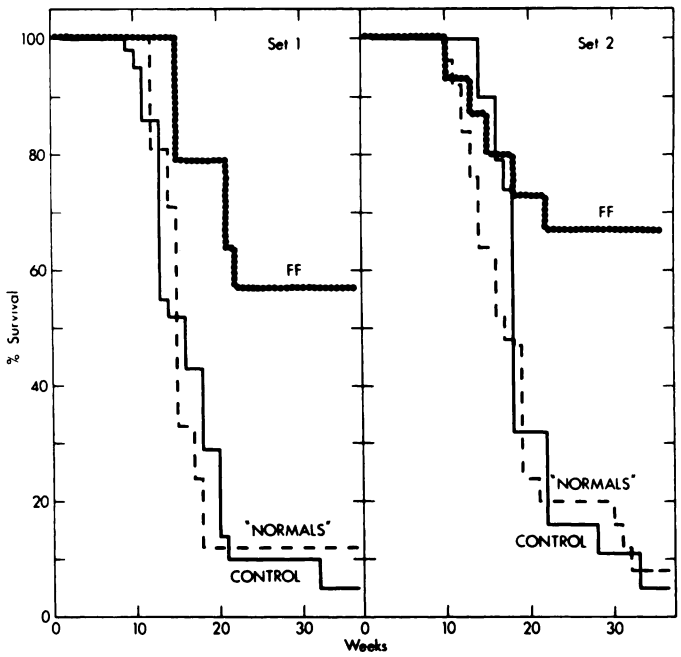

Chart 1. Survival of hamsters given injections of DMBA as a function of time. The experimental design is summarized in Table 1. The FF were deformed survivors of neonatal H-1 infection. Normals were survivors of H-1 infection which were largely seronegative as a result of a virus dose too low to initiate infection. The controls received no H-1 but PBS only.

well as to the other viruses tested. There was no evidence for an increased resistance in tumors from FF animals. Therefore, a virolytic tumor surveillance of the type postulated is unlikely. A second supplemental experiment was conducted to determine if FF animals were protected from SV40-induced neoplasms. In this study, the inbred line of LHC cream hamsters (Lakeview) was used as recommended by Diamandopoulos (3), a procedure which required various changes in the injection schedules since these animals proved to be highly sensitive to H-1. No distinctive protective effect was found. It may be that any differences between the controls and FF were masked by the severity of the SV40 challenge, since 100% of the control hamsters and 95% of the FF rapidly developed tumors. Or, it is possible that the change in injection schedule was important and of note, for, in order to keep down mortality to H-1 and to obtain FF, it was necessary to inject the H-1 virus not at birth but 7 to 8 days later. Perhaps, the factor associated with resistance in the hamsters occurs, or is vulnerable, during that crucial first neonatal week. Our findings in this study indicate as well that the FF state, *per se*, does not preclude successful growth of tumors.[4]

The pathology of the tumors seen in the experimental animals was most interesting. With one exception, all the primary neoplasms observed while the animals were alive proved to be fibrosarcomas, usually of undifferentiated or anaplastic type, at or near the site of the s.c. injection of DMBA. This finding has been reported previously by others (7, 10, 14). The exception was in one FF male hamster of Experiment 2 with a melanoma in the middle of his back; no fibrosarcoma was present in this animal. In addition, a most surprising finding at

[4] This has been ascertained also by the growth of transplantable hamster tumors in FF, of either LAK or LHC breeding, at a rate and size compatible with those seen in control hamsters. H. W. Toolan, unpublished observations.

microscopic examination was the hitherto unrecorded presence of small mammary adenocarcinomas adjacent to or mixed with the fibrosarcomas in 25% of the tumor-bearing female hamsters (23 mammary tumors among the 91 fibrosarcomas). Such mammary carcinomas are extremely rare, either spontaneous or induced, in the hamster (6, 7, 9, 19, 22, 23). In our present experiment, 5 secondary, miscellaneous tumors were also found at autopsy along with the primary fibrosarcomas in the females: an adrenal carcinoma; a carcinoma of the hepatobiliary tract; a squamous cell carcinoma of the skin; an adenocarcinoma of the uterus; and a chondrosarcoma. A secondary tumor was not found where a mammary carcinoma was present. Among the males with tumors in Experiment 2, there were 3 miscellaneous neoplasms: the primary melanoma that was unassociated with a fibrosarcoma; and also 2 secondary tumors seen at autopsy, a cavernous hemangioma of the liver and an extensive adenoma of the sebaceous glands associated with hair follicles. Only one metastasis of any of the tumors was found; this consisted of numerous pin dots of the melanoma. Whereas the incidence of mammary adenocarcinomas was the same for tumor-bearing females of all groups, none of the miscellaneous tumors occurred in the control animals. Five were found in the normal hamsters, and 3 were in the FF. Sections of the tumors, whether derived from FF or control animals, are providing a provocative display of "invading" lymphocytes. Further in-depth study of this material is planned so that the type of lymphocyte involved can be determined.

DISCUSSION

In vivo protection against carcinogen-induced or spontaneous tumors by infection with viruses, particularly C-type agents, has been documented previously, particularly for mice and/or rats (11, 24–26), and it has been suggested by Lindemann and Klein (15) and Gillette and Boone (4) that increased immunogenicity of tumor cell membranes might be produced due to the cell surface-budding nature of these viruses. In contrast to C-type agents, the parvoviruses are small, single-stranded DNA agents that do not bud from cell membranes (20). Of these, H-1 was the first reported as inhibiting both spontaneous (19) and adenovirus-induced tumors (21) in hamsters. We have now learned that it inhibits a carcinogen-induced tumor as well, making it the first virus recorded as suppressing all 3 types of carcinogenesis, thus indicating that a common mechanism is responsible for this inhibitory effect. H-1 is not the only parvovirus that suppresses oncogenesis. Bergs (1) has noted that strain 9HV-B of RV, another parvovirus, could inhibit the leukemia produced by Moloney leukemia virus in Sprague-Dawley rats, since he found that the incidence of leukemia in rats receiving an i.p. mixture of Moloney virus and RV was approximately one-third of that observed in control animals given injections of Moloney virus alone. Although Kirschstein *et al.* (12) as well as Mayor *et al.* (16) have reported that an AAV, one of the defective parvoviruses, can inhibit the formation of adenovirus 12-induced tumors in hamsters, this finding may not be entirely analogous to the suppression seen with H-1 and RV, since the AAV enters into complex relationships with its helper virus, adenovirus 12, in order to replicate. However, the degree of inhibition is the same as reported for H-1 and RV, since the incidence of tumors in treated animals (19%) was again one-third of that observed in control hamsters (60%). Ostrove *et al.* (18) postulate that the molecular basis of the AAV inhibitory action is a reduction in the production of M_r 58,000 tumor antigen as determined by fluorescent antibody and immunoprecipitation studies.

How can we explain the *in vivo* action of H-1 and RV? The high H-1 virus antibody titer which the FF possess cannot be a definitive factor, since it did not appear to matter whether our second group of controls, the normal hamsters, were seronegative or seropositive; nor did the serum titer of the FF change during oncogenesis. It is possible, however, that the FF exist in a state of heightened immunoresponsiveness, since their antibody titer remains at the same extremely high level throughout life with only the stimulation of the initial neonatal injection (20); their B-cells must be permanently active. If this should be so, it is likely that one or more of the T-cell subsets are also constantly stimulated. Gorczynski (5) in a study of irradiated mice given injections of the tumor-inducing Moloney sarcoma virus found that animals given T-cells from animals where Moloney tumors had regressed were protected from tumor induction. T-cells from age-matched control mice were not effective. Pretreatment of the immune T-cells with anti-θ antibody and complement, but not anti-immunoglobulin antibody, abolished this effect. Of special interest to this discussion is a recent paper by McMaster *et al.* (17) based on previous work by Bonnard *et al.* (2) describing a newly isolated parvovirus (MVM-i, also known as MVM-B) related to the prototype MVM. The outstanding characteristic of this MVM variant is that it inhibits the generation of cytolytic T-lymphocytes in mixed-leukocyte cultures of mice which MVM does not. The authors speculate that small differences in the viral genome must account for this biological difference as the DNAs of MVM and MVM-B are similar although not identical. They suggest that isolation of additional variants of MVM with altered specificity to host cell differentiation may allow them to pinpoint the area suppressing cytolytic T-cell proliferation. In a preliminary experiment using a small number of hamsters and various concentrations of MVM-B, the present authors found an inhibiting effect of MVM-B on the number of tumors derived from a DMBA injection, providing a high titer of the virus was used. Should this finding be verified by the same results in a more comprehensive experiment, it would appear that cytolytic T-cells are not concerned with the inhibition observed. It is also possible that T-cell sensitivity and tropism are different in hamsters than in mice. Perhaps determination of the area in the MVM-B gene which is responsible for the immunosuppression of the cytolytic T-cell proliferation will provide a clue to the regions of H-1 and/or RV genes that are associated with a stimulation of a cellular or other response causing oncogenic inhibition, possibly a T-cell subset other than that concerned with cytolysis. Certainly, lymphocytes were very prominent in the sections of almost all DMBA-induced tumors examined, and a comparative investigation of the status of the various component cells of the immune system of FF, normal, and control animals is indicated for the future. A special focus of interest might be the cells of those FF that develop tumors as early as the other groups compared with those evincing a long latent period or complete resistance to oncogenesis. Also, on the basis of our experience with the simian virus-induced tumors, it would be interesting to determine if a delay in injecting H-1 parvovirus (from shortly after birth to 1 or 2 weeks) changes the inhibitory effect on

DMBA-induced neoplasms, *i.e.*, whether the effect of the parvovirus is determined only in the immediate neonatal period.

ACKNOWLEDGMENTS

The authors wish to thank Dr. K. A. O. Ellem for initial discussions, M. S. Hopkins for assistance in performing serology tests, Becky Smith for histological preparations, and Virginia Haas for secretarial expertise.

REFERENCES

1. Bergs, V. V. Rat virus-mediated suppression of leukemia induction by Moloney virus in rats. Cancer Res., *29:* 1669–1672, 1969.
2. Bonnard, G. D., Manders, E. K., Campbell, D. A., Herberman, R. B., and Collins, M. J. Immunosuppressive activity of a subline of the mouse EL-4 lymphoma: evidence for minute virus of mice causing the inhibition. J. Exp. Med., *143:* 187–205, 1976.
3. Diamandopoulos, G. T. Incidence, latency, and morphologic types of neoplasms induced by Simian Virus 40 inoculated intravenously into hamsters of three inbred strains and one outbred stock. J. Natl. Cancer Inst., *60:* 445–449, 1978.
4. Gillette, R. W., and Boone, C. W. Augmented immunogenicity of tumor cell membranes produced by surface budding viruses: parameters of optimal immunization. Int. J. Cancer, *18:* 216–222, 1976.
5. Gorczynski, R. M. Evidence for *in vivo* protection against murine sarcoma virus-induced tumors by T lymphocytes from immune animals. J. Immunol., *112:* 533–539, 1974.
6. Handler, A. Spontaneous lesions of the hamster. *In:* W. E. Ribilein and J. R. McCoy (eds.), Pathology of Laboratory Animals, pp. 210–240. Springfield, Il.: Charles C Thomas, Publisher, 1965.
7. Homburger, F. Chemical carcinogenesis in the Syrian golden hamster. Cancer (Phila.), *23:* 313–339, 1969.
8. Homburger, F. Chemical carcinogenesis in Syrian hamsters. Prog. Exp. Tumor Res. *16:* 152–175, 1972.
9. Homburger, F. Chemical carcinogenesis in Syrian hamsters: a review. Prog. Exp. Tumor Res. *23:* 100–179, 1979.
10. Homburger, F., and Hseuh, S. S. Rapid induction of subcutaneous fibrosarcoma by 7,12-dimethylbenzanthracene in an inbred line of Syrian hamsters. Cancer Res., *30:* 1449–1452, 1970.
11. Kelloff, G. J., Peters, R. L., Donahoe, R. M., Ghazzouli, I., Sass, B., Nims, R. M., and Heubner, R. J. An approach to C-type virus immunoprevention of spontaneously occurring tumors in laboratory mice. Cancer Res., *36:* 622–630, 1976.
12. Kirschstein, R. L., Smith, K. O., and Peters, E. A. Inhibition of adenovirus-12 oncogenicity by adeno-associated virus. Proc. Soc. Exp. Biol. Med., *128:* 670–673, 1968.
13. Ledinko, N. Plaque assay of the effects of cytosine arabinoside and 5-iodo-2′-deoxyuridine on the synthesis of H-1 virus particles. Nature (Lond.), *2:* 1346–1347, 1967.
14. Lee, K. Y., Toth, B., and Shubik, P. Carcinogenic response of the Syrian golden hamster treated at birth with 7,12-dimethylbenz(a)anthracene. Proc. Soc. Exp. Biol. Med., *114:* 579–582, 1963.
15. Lindemann, J., and Klein, P. A. Viral oncolysis: increased immunogenicity of host cell antigen associated with influenza virus. J. Exp. Med., *126:* 93–108, 1967.
16. Mayor, H. D., Houlditch, G. S., and Mumford, D. M. Influence of adeno-associated satellite virus on adenovirus-induced tumors in hamsters. Nat. New Biol., *241:* 44–46, 1973.
17. McMaster, G. K., Beard, P., Engers, H. D., and Hirt, B. Characterization of an immunosuppressive parvovirus related to the minute virus of mice. J. Virol., *38:* 317–326, 1981.
18. Ostrove, J. M., Duckworth, D. H., and Berns, K. I. Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. Virology, *113:* 521–533, 1981.
19. Toolan, H. W. Lack of oncogenic effect of the H-viruses. Nature (Lond.), *214:* 1036, 1967.
20. Toolan, H. W. The parvoviruses. Prog. Exp. Tumor Res. *16:* 410–425, 1972.
21. Toolan, H. W., and Ledinko, N. Inhibition by H-1 virus of the incidence of tumors produced by adenovirus 12 in hamsters. Virology, *35:* 475–478, 1968.
22. Van Hoosier, G. L., and Trentin, J. J. Spontaneous and induced tumors of the hamster. *In:* P. Altman and D. Katz (eds.), Inbred and Genetically Defined Strains of Laboratory Animals, Vol. II, pp. 480–495. Bethesda, Md.:FASEB, 1979.
23. Van Hoosier, G. L., and Trentin, J. J. Naturally occurring tumors of the Syrian hamster. Prog. Exp. Tumor Res. *23:* 1–12, 1979.
24. Weislow, O. S., Allen, P. T., Shepherd, R. E., Twardzik, D. R., Fowler, A. K., and Hellman, A. Protection against 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinoma by injection with mouse xenotropic type C virus. J. Natl. Cancer Inst., *61:* 123–129, 1978.
25. Whitmire, C. E. Virus-chemical carcinogenesis. A possible viral immunologic influence on 3-methylcholanthrene sarcoma induction. J. Natl. Cancer Inst., *51:* 473–478, 1973.
26. Whitmire, C. E. Influence of pre-infection of C57BL-L mice with Graffi leukemia virus on 3-methylcholanthrene-induced subcutaneous sarcoma. Proc. Soc. Exp. Biol. Med., *144:* 674–679, 1973.