Comparison of Cytocidal and Noncytotoxic Strains of Shope Rabbit Fibroma Virus

HARRY C. HINZE AND DUARD L. WALKER

Department of Medical Microbiology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Received for publication 16 February 1971

Seven strains of Shope fibroma virus were compared for their effect on rabbit cells in vitro. All but one of the naturally occurring strains examined in this study produced a similar response in the infected cultures. This consisted of continued cell multiplication together with changes in cell morphology and growth pattern. In contrast, a recently isolated strain of fibroma virus, the M1 strain, was found to produce a gradual cell destruction under the same cultural conditions. A comparison of the cytotoxic M1 strain with a representative noncytotoxic strain in vitro showed no differences in the rate of multiplication, plaque type, antigenic composition, or heat lability. Only minor differences were found in the tumors produced in rabbits by these strains.

Previous reports from this laboratory have described the multiplication and morphological alterations that occur in cultured rabbit kidney cells 3 to 4 days after infection with the Patuxent strain of rabbit fibroma virus (2, 8; Hinze and Walker, *Federation Proc.* 22:557, 1963). These changes are accompanied by a simultaneous loss of contact inhibition, resulting in a growth pattern distinctly different from that of the noninfected cells. In addition, such infected cells acquire an increased potential for growth in the hamster cheek pouch, a characteristic interpreted by other workers as indicative of a malignant transformation when observed with other tumor viruses (1, 7, 9). Recent studies have also shown that cultured rabbit cells sustaining a noncytotoxic fibroma virus infection acquire a remarkably increased capacity to support the replication of several unrelated ribonucleic acid (RNA) viruses (6).

The present work represents an extension of our initial studies to include a comparison of the Patuxent strain used originally with a variety of other fibroma strains. In so doing, one naturally occurring strain of virus has been found that reacts in a manner distinctly different from the other strains studied.

MATERIALS AND METHODS

Viruses. Five strains of fibroma virus were obtained from the late Richard Shope (The Rockefeller University, New York, N.Y.): Boerlage, Shope no. 1, Shope no. 289, Shope no. 384, and Shope no. 472. A strain designated "M1" (11) was obtained from T. H. Yuill, Department of Veterinary Science, University

of Wisconsin. This strain was isolated from naturally infected cottontail rabbits and identified as fibroma virus by the production of typical tumors in wild and domestic rabbits and by its close antigenic relationship to the standard fibroma strains. The Patuxent strain (3, 4) was a clonal isolate of the stock virus used in earlier studies (2). All strains of virus were obtained as rabbit passage material without prior passage in tissue culture. Each has been maintained in this laboratory by intratesticular or intradermal inoculation in domestic rabbits and by in vitro passage in primary and serially cultured rabbit kidney cells. Methods for the production and preparation of stock virus suspensions were the same as previously described for the Patuxent strain (2).

Cell cultures. The DRK line of serially cultured rabbit kidney cells (2) was used for all experiments. Cells were grown in 6-oz (ca. 180 ml) prescription bottles in a medium containing 199, 0.25% lactalbumin hydrolysate, and 10% heated calf serum. Stock cultures were maintained by routine serial transfer at weekly intervals. The DRK line has been found in this laboratory to remain diploid in character through approximately 45 serial passages. After this number of passages, there is an increase in the number of aneuploid cells together with slight variations in cultural behavior. To avoid the variables introduced by such changes in high passage cells, a supply of early passage diploid cells (14th to 19th passage) was stored in liquid nitrogen. Cultures were routinely discarded after 40 passages, and a fresh culture was started from the frozen stock cells. The experiments described in this report were performed with cultures of the 16th to 35th passage. All cultures were tested at suitable intervals for mycoplasma contamination.

Cell growth curves. The rate of destruction or proliferation of cells after infection with the various strains of virus was determined by comparative

growth curves of infected and noninfected cells. Methods for measuring cell multiplication have been described previously (2).

Virus assay. Plaque assays were carried out on monolayers of a cloned strain of rabbit kidney cells isolated from the parent DRK line. Cultures were grown in 30-ml plastic tissue culture flasks and overlaid, after a 4-hr adsorption period, with a previously described methylcellulose overlay (2). All assays were read after 7 to 10 days of incubation at 35 C.

RESULTS

Six strains of fibroma virus were compared with the original Patuxent strain for their ability to produce similar changes in rabbit kidney cells. Serially cultured DRK cells were grown on cover slips in shell vials (21 by 70 mm). The vials were routinely seeded with a concentration of 10^5 cells and incubated overnight to permit attachment to the glass. Groups of cultures were then inoculated with the different strains of virus in a concentration of 1 to 5 plaque-forming units (PFU) per cell. This inoculum has been shown by fluorescent

antibody staining and infectious center assays to produce infection of all cells in the culture within 24 to 48 hr. All groups of cultures were incubated at 37 C and refed with growth medium at intervals of 1 to 2 days. As shown in Fig. 1, all strains of fibroma virus tested, with the exception of the M1 strain, gave results similar to those obtained with the original Patuxent virus. Growth curves of cells infected with these strains showed a typical stationary period of 3 days after which the infected cells multiplied at a rate slightly slower than that of the control cells until further increase was limited by overcrowding. In each instance, proliferation of the infected cells was accompanied by changes in cell morphology and growth pattern identical to those seen in the Patuxent-infected cultures. In contrast to this, cultures infected with the M1 strain of fibroma virus showed no increase after the virus became established in the cells. The cell number dropped progressively until the culture was completely destroyed by the 8th day after addition of virus. Cultures stained with eosin and hematoxylin during this period showed

FIG. 1. Multiplication of control and fibroma-infected DRK cells. Cultures were prepared on day 0 and infected with the indicated strains of virus on day 1. Cell counts represent the average cells per culture at each interval.

the presence of numerous cytoplasmic inclusions within 24 hr, followed by formation of clumps of round, deeply stained degenerating cells which were rapidly lost into the medium.

Comparison of cytotoxic (M1) and noncytotoxic (Patuxent) strains of Fibroma virus. Since a comparison of the characteristics of cytotoxic and noncytotoxic strains of virus first required an examination of the genetic purity of these viruses, the M1 strain of fibroma virus was plated on DRK monolayers under agar and a large number of clones were selected and grown into cultures. Examination of 25 of these clones for their cytotoxic effect in DRK monolayers, plaque formation, and production of tumors in domestic rabbits showed no difference between the clonal isolates and the parent M1 virus. One of the M1 clones was then selected for further comparison with the cloned Patuxent virus.

Cross neutralization. The plaque-neutralization test provides a sensitive technique for the measurement of neutralizing antibody and has been used by other workers to demonstrate antigenic differences between closely related myxoma and rabbit fibroma viruses (10). Antisera prepared in rabbits against cloned Patuxent and M1 strains of fibroma virus were compared for their ability to neutralize homologous and heterologous virus by this method. Serial twofold serum dilutions were mixed with 100 PFU of each virus and incubated for 1 hr at room temperature before inoculation onto DRK monolayers. Controls were inoculated with similar amounts of virus mixed with preimmunization sera. The neutralizing titer of each serum was taken as that dilution giving a reduction of approximately 50% in the number of plaques when compared with controls. Resulting plaque counts indicated no significant difference in the ability of either antiserum to neutralize its homologous or heterologous virus. Anti-Patuxent serum gave an average plaque reduction of 58% with Patuxent virus and 53% with M1 virus at a serum dilution of 1:64, whereas the anti-M1 serum (1:32 dilution) gave reductions of 60 and 56% with Patuxent and M1 viruses. Extensive comparison of the antigen makeup of the Patuxent and M1 viruses by immunodiffusion and immunoelectrophoresis has also indicated that no major antigenic differences exist between these strains (D. M. England and H. C. Hinze, unpublished data).

Comparison of plaque types. The cytotoxic character of the M1 strain of virus suggested the possibility that this agent might produce a plaque type differing from that produced by the noncytotoxic fibroma strains and similar to the lytic plaques produced by other cytotoxic poxviruses (10). Comparison of the plaques produced under

identical conditions on DRK monolayers by cloned M1 and Patuxent viruses showed no significant difference in the time of development, size, or microscopic appearance. Both viruses produced plaques of uniform size (approximately 1.0 mm in diameter at 7 days) and consisting of multilayered cell aggregates. In contrast, similar inoculation of DRK cultures with two other cytotoxic poxviruses, vaccinia and myxoma, produced lytic plaques which appeared distinctly different from the M1 plaques.

Virus multiplication and release. The rates of multiplication, maximum production, and virus release of the Patuxent and M1 strains in DRK cells were compared by using a one-step growth cycle. Groups of cultures grown in shell vials were inoculated with approximately 20 PFU per cell of either Patuxent or M1 virus and centrifuged for 15 min at $1,000 \times g$ to aid virus adsorption (5). The inoculated cultures were then incubated at 37 C for 1 hr after which they were washed three times with balanced salt solution, refed, and returned to the incubator at 37 C. At the times indicated, three cultures from each infected group were centrifuged at slow speed ($100 \times g$) for 3 min to sediment floating cells and cell debris. The supernatant medium was carefully removed, and the virus content of the cells and medium was determined by plaque assay. The results presented in Fig. 2 show an identical pattern of intracellular development for both viruses. Each demonstrated a latent period of 8 hr, a period of logarithmic increase during the next 7 hr (from the 8th to the 15th hr after infection), and a gradually developing plateau thereafter.

The maximum yield of cell-associated virus was similar for both viruses. Under the conditions provided in this experiment, both the Patuxent and M1 strains of fibroma virus reached a peak of 60 PFU per cell at 24 hr after infection. Release of virus into the medium was minimal in both groups of infected cultures. As indicated in Fig. 2, the concentration of Patuxent virus present in the medium remained at a constant low level during the period of incubation and most probably represents residual virus from the inoculum. The M1-infected cultures showed a gradually increasing amount of virus in the medium as a reflection of the greater degree of cell destruction produced by this virus, although at 48 hr after infection released virus was still only a minute fraction of the total amount produced.

Thermal inactivation. A comparison of the rate of inactivation of M1 and Patuxent viruses at 56 C is presented in Fig. 3. One-milliliter samples of each virus were distributed into ampoules, sealed, and submerged in a water bath at 56 C. At 5-min intervals, samples of each virus were re-

FIG. 2. Comparison of multiplication of Patuxent and M1 viruses in DRK cells. Virus titer represents total cell-associated or free virus per culture vial.

FIG. 3. Inactivation of Patuxent and M1 viruses at 56 C. Each point represents titer of infectious virus remaining after heating at 56 C for the indicated intervals.

moved, chilled, and assayed for surviving infectious virus. It is apparent from Fig. 3 that the viruses were inactivated at an identical rate under these conditions.

Tumor production in rabbits. To compare the tumor-producing ability of cytotoxic and non-cytotoxic strains of fibroma virus in rabbits, the following experiment was done. Eight adult New Zealand white rabbits were inoculated intradermally on the shaved skin of the back with serial 10-fold dilutions (10^{-1} to 10^{-6}) of the stock Patuxent and M1 strains of virus. Six animals were inoculated with dilutions of both viruses, whereas the remaining animals were each given a single virus strain. The inoculum consisted of 0.1 ml of virus diluted in tissue culture growth medium. Both virus stocks had a titer of approximately 4×10^6 PFU per ml before dilution. Tumors were compared for time of onset, maximum size, general appearance, and time of regression.

Although there was some variation in susceptibility to the viruses, all of the rabbits showed an essentially similar response. Tumors appeared uniformly with both viruses at 2 to 3 days after inoculation. Tumors produced by the Patuxent strain of virus reached a maximum diameter of 15 to 20 mm in 12 days, whereas those produced by the M1 strain reached a maximum diameter of 15 to 18 mm in 8 days. By 12 days after infection, tumors produced by the cytotoxic M1 virus

showed beginning signs of regression and were completely resolved in about 3 weeks. Tumors produced by the noncytotoxic Patuxent strain regressed at a considerably slower rate and remained visible until 4 to 5 weeks after inoculation of the virus. Tumors produced by either virus did not differ significantly in gross appearance during their development. Since previous work with the M1 strain of virus (11) has indicated that tumors induced by this virus have a microscopic appearance identical to those induced by other strains of rabbit fibroma virus, a similar comparison was not made in this study.

DISCUSSION

The infection of cultured cells with the virus of rabbit fibromatosis offers a convenient system for the study of a virus-host cell relationship in which infection allows continued cell survival and proliferation rather than causing immediate cell death. The term "noncytotoxic" is used here to distinguish this type of relationship from that seen with more destructive (cytotoxic) viruses in which cell death occurs rapidly without further cell division. The results presented in this paper serve to substantiate the original observation of cell proliferation with altered morphology and loss of contact inhibition following infection with the Patuxent strain of virus. Since this type of response has now been observed with five additional

strains of fibroma virus, it would appear to be representative of most strains under the conditions presented in these experiments. Although the Patuxent strain of virus used in this work and in our original studies was adapted to growth in tissue culture by repeated serial passage, the other virus strains described above were used after only one or two in vitro passages. This adds emphasis to the concept that the noncytotoxic response produced by fibroma virus does not depend on selection of an atypical strain of virus by the process of laboratory manipulation.

The finding that the M1 strain of fibroma virus produces a distinct and rapid cell destruction serves as a distinctive feature setting apart this strain from the others. In addition, it provides a system in which a detailed comparison can be made between cytotoxic and noncytotoxic infections by closely related viruses. As a preliminary step in such a study, it was necessary to determine the genetic purity and stability of each virus. This was done by the demonstration that clonal isolates of both the M1 (cytotoxic) and Patuxent (noncytotoxic) viruses retained all of the characteristics of the parent strains through many generations. Despite the contrasting host cell response to these two viruses, they appear to be remarkably similar in their multiplication cycle, major antigenic properties, and heat lability. Although both viruses induce tumors with equal facility when inoculated into rabbits, the more rapid regression of the M1 tumors possibly reflects the greater potential for cell destruction possessed by this virus.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service research grants CA-05682 and CA-10395 from the National Cancer Institute.

LITERATURE CITED

1. Black, P. H., and W. P. Rowe. 1963. Transformation in hamster kidney monolayers by vacuolating virus, SV40. *Virology* 19:107-109.
2. Hinze, H. C., and D. L. Walker. 1964. Response of cultured rabbit cells to infection with the Shope fibroma virus. I. Proliferation and morphological alteration of the infected cells. *J. Bacteriol.* 88:1185-1194.
3. Kilham, L., and E. R. Fisher. 1954. Pathogenesis of fibromas in cottontail rabbits. *Amer. J. Hyg.* 59:104-112.
4. Kilham, L., and P. A. Woke. 1953. Laboratory transmission of fibroma (Shope) in cottontail rabbits by means of fleas and mosquitoes. *Proc. Soc. Exp. Biol. Med.* 83:296-301.
5. Padgett, B. L., and D. L. Walker. 1962. Use of centrifugal force to promote adsorption of myxoma virus to cell monolayers. *Proc. Soc. Exp. Biol. Med.* 111:364-367.
6. Padgett, B. L., and D. L. Walker. 1970. Effect of persistent fibroma virus infection on susceptibility of cells to other viruses. *J. Virol.* 5:199-204.
7. Shein, H. M., J. F. Enders, J. D. Levinthal, and A. E. Burkett. 1963. Transformation induced by simian virus 40 in newborn Syrian hamster renal cell cultures. *Proc. Nat. Acad. Sci. U.S.A.* 49:28-34.
8. Tompkins, W. A. F., D. L. Walker, and H. C. Hinze. 1969. Cellular deoxyribonucleic acid synthesis and loss of contact inhibition in irradiated and contact-inhibited cell cultures infected with fibroma virus. *J. Virol.* 4:603-609.
9. Vogt, M., and R. Dulbecco. 1962. Properties of cells transformed by polyoma virus. *Cold Spring Harbor Symp. Quant. Biol.* 27:367-374.
10. Woodroffe, G. M., and F. Fenner. 1965. Viruses of the myxoma-fibroma subgroup of the poxviruses. I. Plaque production in cultured cells, plaque reduction tests, and cross-protection tests in rabbits. *Aust. J. Exp. Biol. Med. Sci.* 43:123-142.
11. Yuill, T. M., and R. P. Hanson. 1964. Infection of suckling cottontail rabbits with Shope's fibroma virus. *Proc. Soc. Exp. Biol. Med.* 117:376-380.