POSSIBLE ROLE OF INTERFERON IN DETERMINING THE ONCOGENIC EFFECT OF POLYOMA VIRUS VARIANTS

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Variants of polyoma virus with differences in oncogenic properties have been investigated in several laboratories (1–4). One pair of variants consists of a virus grown in a serum containing medium (S variant) which causes thymic and other tumors in almost 100 per cent of C_3Hf/Bi mice inoculated at birth, and a virus grown in fat-free milk medium (M variant) which causes tumors in approximately 10 per cent of newborn C_3Hf/Bi mice (3). Other properties of these variants have been extensively documented (5).

A number of investigators have shown that interferon is produced by tissue cultures infected with polyoma virus (6–8), and a previous report from this laboratory indicated that mouse embryo cultures infected with the M variant produced 4-fold or greater titers of interferon than similar cultures infected with the S variant (9). It was of interest, therefore, to determine whether the same difference in interferon production could be found in *in vivo* infections of newborn mice and whether such differences could be correlated with the oncogenicity of the M and S strains. One report has shown that hamsters may be protected against the oncogenic effect of polyoma virus infection by inoculation of exogenous interferon (10).

This paper presents evidence that the previously observed *in vitro* differences in interferon production are present *in vivo* in S or M variant infected mice, and that the different levels of interferon induced by infection with polyoma virus variants are correlated with different degrees of resistance to *in vivo* infection with a heterologous agent, encephalomyocarditis virus (EMC). Growth curves of the variants *in vivo* showed that the more oncogenic variant grew to higher titers early in the course of infection. Finally, data are presented indicating that mice infected with both M and S variants together developed significantly fewer tumors than animals infected with the S variant alone. These findings suggested that the difference in oncogenic potential observed between the M and S polyoma virus variants may be due to the higher level of interferon induced by the M variant.

Materials and Methods

Animals.—C_3Hf/Bi mice were obtained from a carefully inbred National Cancer Institute colony.

Virus Strains.—The S variant of polyoma virus had undergone 10 passages in P388 D1 cells in 40 per cent human serum and 60 per cent mixture No. 199. The M variant of polyoma virus had had 15 passages in P388 D1 cells in 20 per cent autoclaved non-fat milk and 80 per cent mixture No. 199. The r and r^+ mutants (11) of encephalomyocarditis virus were originally obtained from Dr. K. K. Takemoto of the National Institute of Allergy and Infectious Diseases, Bethesda. All animal inoculations were made subcutaneously with 0.05 cc of the specified dilution.

Extraction Procedures.—In 2- and 5-day-old mice, whole extracts of decapitated animals were employed. In 10- and 20-day-old animals pools of kidney, thymus, and spleen were used. The whole animals were skinned and the limbs removed. The carcasses of at least 3 animals per point or the organ pools of several animals were then minced and the volume of the mince measured. A 20 per cent by volume suspension of the mince was placed into a glass tissue grinder and ground until a fine suspension resulted. These crude suspensions were assayed for polyoma virus in 10-fold serial dilutions.

For assay of interferon the 20 per cent suspensions were treated with concentrated HCl until pH 2 was reached. They were then left for 14 hours at 4°C. The precipitate which had formed was removed by low speed centrifugation and 5 N NaOH was added to the resulting solutions until neutral pH was reached. A newly formed precipitate was removed by low speed centrifugation. At this point the solutions were clear. Solutions were then spun in a Spinco model L preparative ultracentrifuge in a No. 50 head at a maximum force of 150,000 *g* for 3 hours. The resultant solutions were used for interferon assays.

Polyoma Virus Titers.—Polyoma virus strains were titered by limiting dilution in milk-adapted test tube cultures of P388 D1 cells, a stable mouse lymphoma cell line. Titers are reported as 50 per cent tissue culture infectious doses ($TCID_{50}$) in $\log_{10}$. End points were read 21 days after inoculation of 0.25 ml into each of 2 or 3 culture tubes per dilution.

Titers of Interferon.—Interferon was assayed in ME-29 cells, a stable line of mouse fibroblasts obtained in its 102nd passage from Dr. Karl Habel of the National Institute of Allergy and Infectious Diseases. These were grown and maintained in Eagle's medium No. 2 and 10 per cent fetal calf serum. The cells were incubated for 14 hours at 36°C in 30 ml plastic culture flasks with 4 ml of the dilution to be tested, the fluids decanted, and the cultures then challenged with 30 to 60 plaque-forming units (p.f.u.) of the r mutant of EMC virus. After incubation for 90 minutes at 36°C, the monolayers were washed, and an overlay containing 0.9 per cent Noble agar, 10 per cent bovine serum, and plaque medium 199 was applied. A second agar and medium overlay, containing 1:30,000 neutral red, was made at 24 hours, and final plaque counts were read at 48 hours. The results of interferon assays are reported as the reciprocal of the highest serial dilution showing approximately 50 per cent plaque reduction as compared with appropriate controls.

RESULTS

Mixed Infection with Polyoma Virus Variants.—Results of infection of newborn C_3Hf/Bi mice within 12 hours of their birth by M strain polyoma virus alone, S strain alone, and M and S strains together are summarized in Fig. 1. The results are similar to those in previously published reports (5, 12) in that inoculation at birth with $10^{8.7}$ $TCID_{50}$ of the S variant caused death due to thymic epithelial tumors in 100 per cent of the experimental animals within 102

days, while inoculation with the same number of infectious units of the M variant failed to cause any deaths within a 160 day observation period. At 160 days all 13 animals in the M variant-infected group were sacrificed and autopsied. Only 1 of 13 had a thymic tumor; no other tumors were noted.

When newborn C_3Hf/Bi mice were simultaneously infected with both $10^{3.7}$

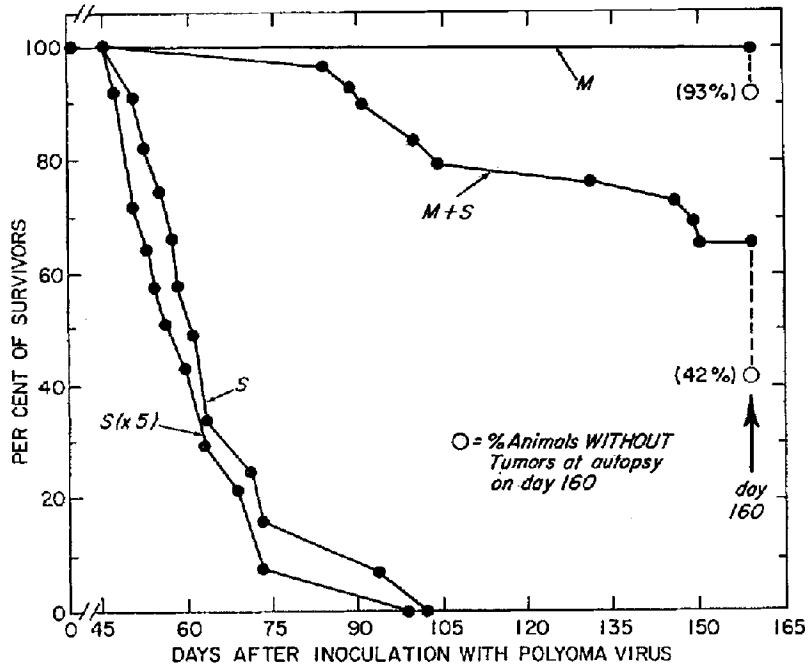

FIG. 1. Percentage of survivors following inoculation of newborn mice with polyoma virus variants. All survivors at 160 days were sacrificed and autopsied. M or S represent mice inoculated with $10^{3.7}$ $TCID_{50}$ of the M or S variants respectively. M + S represents mice inoculated with $10^{3.7}$ $TCID_{50}$ of the M variant and $10^{3.7}$ $TCID_{50}$ of the S variant. S(×5) represents mice inoculated with $5 \times 10^{3.7}$ $TCID_{50}$.

$TCID_{50}$ of the M variant *and* $10^{3.7}$ $TCID_{50}$ of the S variant, only 6 of 31 (19 per cent) animals died of thymic tumors within the first 102 days. Between 102 and 160 days, 4 additional animals died, 1 with a thymic tumor and 3 with salivary gland tumors. All 21 remaining mice were sacrificed and autopsied when 160 days old. Of these, 13 had no tumors, 3 had thymic tumors only, 2 had both thymic and salivary gland tumors, 2 had salivary gland tumors only, and 1 animal had a tumor of the orbit. Therefore, at 160 days 68 per cent of the mice infected with both strains were still alive, and 42 per cent had no tumors. The oncogenic effect of the dual infection was intermediary between the effect of in-

fection with each strain separately. Of the animals which did develop tumors, ⅓ (6 of 18) did not have thymic tumors.

That these findings were not due to doubling the infecting dose of virus (since the double infection was with a total of $2 \times 10^{3.7}$ $TCID_{50}$) is shown by the data from infection with $5 \times 10^{3.7}$ $TCID_{50}$ of the S variant alone. The results, also shown in Fig. 1, are similar to those seen when $10^{3.7}$ $TCID_{50}$ of S alone were employed.

These results suggested that infection with the M variant resulted in some protection against the oncogenic action of simultaneously inoculated S variant.

TABLE I

*Effect of Infection with Polyoma Virus Variants on Infection with EMC Virus**

Animal group	Dilution of EMC virus pool used (in $\log_{10}$)							
	−2.7	−3.0	−4.0	−5.0	−6.0	−6.5	−7.0	LD_{50}
Controls	ND‡	0/15§	0/4	0/13	1/17	10/24	7/13	−6.8
Infected with M variant	0/17	8/16	4/6	4/4	6/6	13/13	ND	−3.0
Infected with S variant	0/6	4/13	4/6	7/7	7/7	ND	ND	−3.5

* Infected with S or M polyoma virus variant on day of birth and with EMC virus when 5 days old.

‡ No experiment performed on this group.

§ Reported as number of survivors/total in group.

Resistance to Infection with a Heterologus Virus.—

Newborn C_3Hf/Bi mice were inoculated subcutaneously with $10^{3.7}$ $TCID_{50}$ of either M or S polyoma variant, and, when 5 days old, infected subcutaneously with various dilutions of an r^+ variant of EMC virus. Animals were observed for 13 days and the number of deaths in each group recorded. The results are tabulated in Table I where they are compared to those in controls which had not been infected with one of the polyoma virus variants at birth.

The LD_{50} in the control group by the method of Reed and Muench (13) was −6.8. In the group preinfected with the M variant, it was −3.0, and in the group preinfected with the S variant, −3.5. These results showed an increase in the resistance to EMC virus in mice preinfected with polyoma virus, and suggested a difference in the degree of resistance induced by the variants.

The results of infection with a $10^{-3.0}$ dilution of EMC virus are presented in detail in Fig. 2. These data indicate a significant difference in the degree of resistance induced by each of the polyoma variants. In the group of 13 animals inoculated at birth with the S variant, all but 1 of 9 deaths occurred by the end of the 5th day after infection with EMC virus; in the group of 16 animals inoculated at birth with the M variant, however, only 1 of 8 deaths occurred before the 6th day after EMC virus infection. At 5 days after EMC infection, there-

fore, the difference between the M and S variant-infected groups was highly significant ($p < .01$). The induced resistance in the M variant group was transient and appeared to wane after the 5th day following infection.

These results indicated that infection at birth with polyoma virus induced resistance to infection with a heterologous virus, EMC. They also indicated

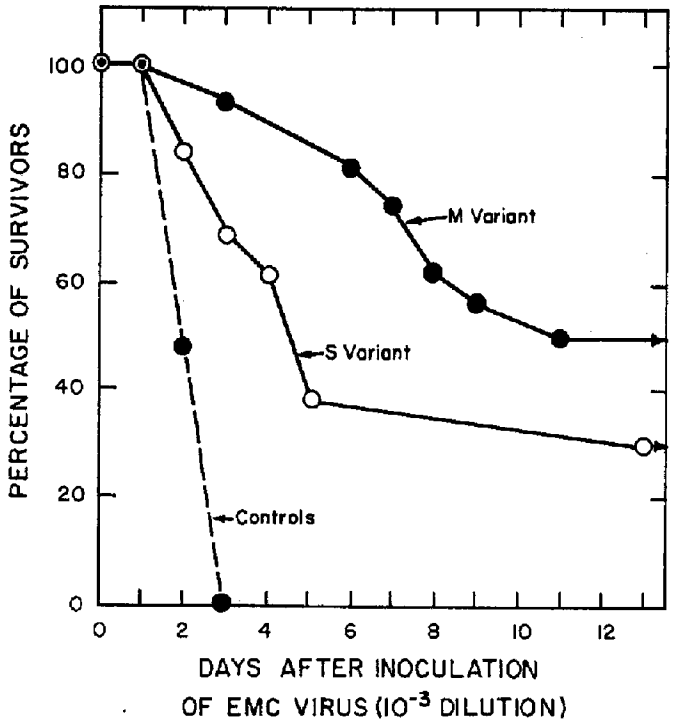

FIG. 2. Percentage of survivors following inoculation of a 10^{-3} dilution of an EMC virus pool into 5-day-old control mice or mice which had been infected with M or S polyoma virus variants at birth.

that a somewhat greater degree of resistance is induced by infection with the M variant than with the S variant. The resistance to infection by a heterologous virus suggested that the differing oncogenicity of the polyoma virus strains might be related to variation in *in vivo* production of interferon.

In Vivo Production of Interferon Induced by Polyoma Virus Variants.—

Extracts of whole mice or organ pools taken from 2-, 5-, 10-, or 20-day-old mice after infection with $10^{3.7}$ $TCID_{50}$ of either the M or the S variant on the day of birth were tested for interferon activity. The results are plotted in Fig. 3.

While less than 4 units of interferon was present in all of the pools taken from animals infected with the S variant, significant titers were found in some of the

pools from M variant-infected animals. A peak titer of 20 units was reached on the 5th day after infection with the M variant, a fall to 4 units was noted on the 10th day, and to less than 4 units on the 20th day. These results agree with the previously mentioned *in vitro* studies of interferon production by polyoma virus

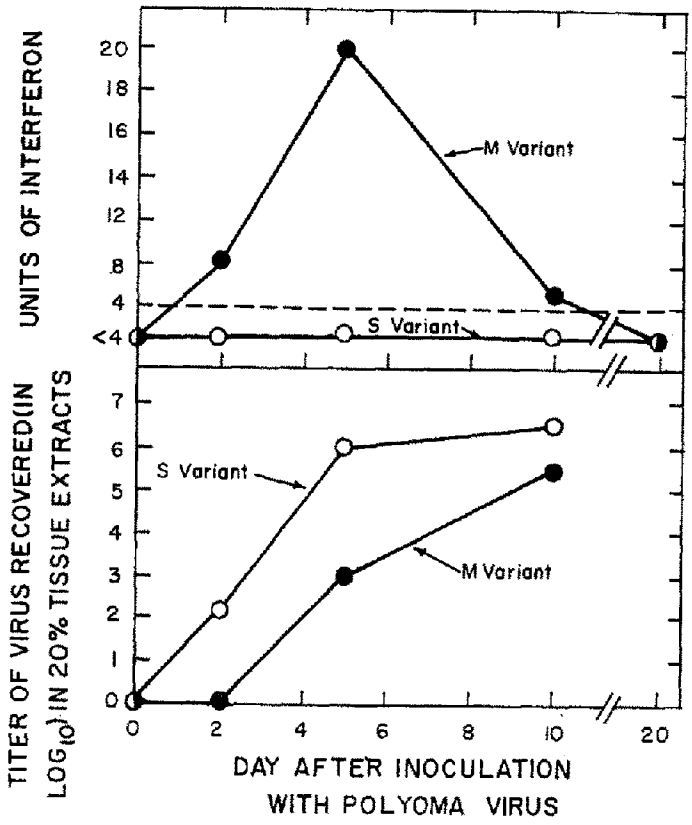

FIG. 3. Top, titers of interferon recovered from tissue extracts after infection of newborn mice with M or S polyoma virus variants. Bottom, growth of polyoma virus variants in tissues of mice infected at birth.

variants in tissue culture in that higher titers of interferon were induced by infection with the less oncogenic M variant (9).

That the antiviral activity tested was due to interferon was shown by its properties. It was stable to pH 2, destroyed by exposure to 95°C for 5 minutes, effective against a heterologous virus (EMC), and was not sedimented by 150,000 *g* for 3 hours (14).

These findings suggested that the *in vivo* differences observed in oncogenicity

and in protection against EMC virus infection, and the decrease in tumors noted in the mixed infection with M and S variants when compared to infection with S alone, might have been due to the at least 10-fold greater interferon production induced at 5 days after infection by the M variant when compared to the S variant. It was of interest to compare the *in vivo* growth curves of the two variants to find out whether a depression in growth of the M variant was correlated with the production of interferon.

In Vivo Growth Curves of Polyoma Virus Variants.—The growth curves of the M and S polyoma virus variants over the 10 day period following infection on the day of birth also appear in Fig. 3. The 5 day tissue suspensions from mice inoculated with the S variant titered 10^6 $TCID_{50}$, the 10 day suspensions, $10^{6.5}$ $TCID_{50}$. These figures generally agreed with those observed in similarly conducted experiments (15). In contrast, in mice inoculated with the M variant, less than 1 $TCID_{50}$ could be recovered from tissue suspensions after 2 days, while 5 day suspensions titered $10^{3.0}$ $TCID_{50}$. By the 10th day, however, a titer of $10^{5.5}$ $TCID_{50}$ was reached.

These results showed that the S variant grew to a high titer by 5 days after inoculation. At 2 days there was at least a 100-fold difference in titers between the variants, while by 5 days there was a 1000-fold difference. At 10 days, however, the difference was down to 10-fold. The differences noted varied directly with the interferon titers produced, the greatest difference in virus titers being present after 5 days when the highest titer of interferon produced by the M variant was found.

DISCUSSION

The findings presented showed that infection with both M and S variants together produced significantly fewer tumors than infection with the S variant alone. Infection with the M or S variant was associated with resistance to infection with EMC virus; the M variant, however, caused a transient greater degree of resistance, although growth curves of the variants revealed a 1000-fold greater titer of the S variant on the 5th day after infection at birth. By the 10th day, the titer of the S variant was only 10-fold greater. Finally, tissue extracts from animals infected with the M variant had measurable levels of interferon, reaching a peak on the 5th day after infection on the day of birth, but no interferon could be found in comparable extracts from mice infected with the M variant.

The growth curves of the variants during the first 5 days after infection were directly correlated with the number of tumors seen later in each group, the more oncogenic S variant at 5 days giving rise to a 1000-fold greater virus titer than the less oncogenic M variant. Also, the polyoma virus growth during the first 5 days after infection on the day of birth was inversely correlated with the level of interferon produced by infection with each of the variants respectively.

Since newborn mice become progressively resistant to the oncogenic effect of polyoma virus over the first few days of life (16), it is possible that the level of interferon produced by infection with the M variant was enough to depress that variant's growth (or the growth of a simultaneously inoculated dose of the S variant) over the first few days after infection. This decrease in M polyoma virus titer, occurring when it did, in turn might cause a decrease in the number of animals later noted to have tumors, possibly because a high enough titer of virus to induce tumors was reached in the tissues of M variant-infected mice only after a degree of immunologic maturity had developed (17).

Findings in previously published reports have provided strong evidence that differences in antibody production by mice infected with polyoma virus variants could not be an important factor in determining the oncogenic effect of these variants. Antipolyoma virus antibody must be inoculated into newborn hamsters or mice before polyoma virus infection in order to protect against tumors induced by the virus. When antibody inoculation was delayed for 1 hour, however, no protection was observed, even in mice in which daily antibody inoculations were kept up for 17 days after infection (18, 19). Moreover, active antipolyoma virus antibody production in the newborn mouse has not been demonstrated until 10 days following infection at birth (15, 5). This suggests that no antibody at all is present during the period of tumor induction by polyoma virus infection in the newborn mouse (17). Since antibody, in order to be effective, must be present at or very close to the time of polyoma virus inoculation, and since the onset of production of antipolyoma virus antibody does not seem to occur in the newborn mouse until several days after infection, differences in antigenicity or in levels of antibody induced by polyoma virus strains could not account for their differing oncogenicity.

Several findings in this work are similar to those in previously published reports suggesting interferon effect *in vivo*. Hitchcock and Isaacs showed protection of mice against intraperitoneal inoculation of Bunyamwera virus by intranasal inoculation of an avirulent strain of influenza virus (20). Protection against hemorrhagic encephalopathy by intravenous inoculation of NWS strain of influenza virus was noted in chick embryos with established allantoic infections by other influenza strains (21). In neither of these cases was circulating interferon found, but attempts at recovery of interferon in *in vivo* infections, as in the newborn M variant infected mice in the present report, have been successful in the case of localized vaccinia infection in rabbits (22) and guinea pigs (23), in the lungs of influenza-infected mice (24), and in the blood of mice with viremia (25). In all of these studies, the recovery of the infected animals could more reasonably be attributed to interferon than to other factors. In the instances in which interferon has not been recovered, and in the case in this report of the S variant-infected mice which resisted infection with EMC virus, the resistance to a second infection could have been due to levels of interferon below those which are presently capable of being measured (20, 21). The inability to find significant titers of interferon does not necessarily exclude interferon as a basis for resistance which is imparted by infection with a live virus.

Law and Rabson (5) in experiments somewhat similar to those herein reported showed that mice infected at birth with the M variant resisted the oncogenic effect

of inoculation with the S variant 10 days later. They concluded that the resistance noted was probably due to the development of antibody since, following inoculation of the M variant at birth, hemagglutinin-inhibiting antibody was present after 10 days, and neutralizing antibody, after 15 days. They also suggested interferon production by the M variant as a possible additional mechanism to explain their observations, and this is consistent with the finding of interferon on the 10th day after infection at birth with the M variant. It may be that the early developing resistance to the S variant infection noted in the present study was due to interferon, whereas the later demonstrated resistance in the report of Law and Rabson was due to antibody. This was apparently the case in a double infection with both pantropic and neurotropic strains of Rift Valley Fever virus (26). When mice were systemically infected with the neurotropic strain first, a very early protection against the pantropic strain was felt to be due to interferon and a later developing resistance, to antibody.

An additional point of interest, discovered previously and confirmed by this study, is the apparent cessation of interferon production in animal tissues despite the persistence of high titers of an infecting virus. Baron *et al.* (27) have noted that mice with an influenza virus pneumonia cease making detectable levels of interferon at a time when the virus is still present in the lungs. The significant drop in interferon recovered from M strain-infected mice after the 5th day, despite rising polyoma virus titers, is another instance of this puzzling observation. A possible explanation may be that cells can make only a certain amount of interferon before they become "exhausted" for this function, but several other explanations are not unlikely.

The production of interferon and sensitivity to its antiviral action may be important general phenomena among tumor viruses since both DNA tumor viruses, such as polyoma virus (6–9), and RNA tumor viruses, such as Rous sarcoma virus (28, 29), and avian lymphomatosis virus (29) have these properties. In mouse polyoma virus infection with relatively low doses of virus, where multiplication of the infecting agent to high titers in tissues is probably important to the induction of tumors, production of interferon may be decisive in determining the final outcome of the system.

SUMMARY

1. Infection of newborn C_3Hf/Bi mice with both the highly oncogenic S variant and the poorly oncogenic M variant of polyoma virus caused significantly fewer tumors than infection with the S variant alone.

2. Infection of newborn C_3Hf/Bi mice at birth with either M or S variant caused resistance to infection with a heterologous agent, encephalomyocarditis virus, but the M variant caused a somewhat greater degree of resistance.

3. Extracts of tissues of animals infected at birth with the M variant had measurable levels of interferon, reaching a peak on the 5th day after infection with the virus. Extracts of animals infected with the S variant showed no such activity.

4. The growth curves of the two variants in newborn C_3Hf/Bi mice showed

that the S variant grew to a 1000-fold greater titer than did the M variant at 5 days after infection. Samples tested at 10 days showed a 10-fold difference.

5. These findings suggested that the difference between the variants in oncogenic potential might have been due to the greater interferon production induced by infection with the M variant than by infection with the S variant.

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