Influence of Age at Inoculation on Avian Oncornavirus-induced Brain Tumor Incidence, Tumor Morphology, and Postinoculation Survival in F344 Rats

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SUMMARY

Intracranial neoplasms were induced by intracerebral inoculation of a standardized, cell-free inoculum of the Bratislava-77 strain of avian sarcoma virus in F344 rats at 1, 9, 97 to 99, and 528 days of age. Deaths from diseases that occur spontaneously in aged F344 rats complicated assessment of tumor incidence in rats inoculated at 528 days; 20 of 30 rats inoculated at this age developed brain tumors. All rats inoculated at age 1 day (47 rats), at age 9 days (37 rats), and at 97 to 99 days of age (41 rats) developed brain tumors. The incidence of animals developing tumors was 100% in these three groups, but the incidence of multiple tumors declined with increasing age at inoculation. The mean and variance of postinoculation survival increased from 83.8 ± 21.5 days for rats inoculated at 1 day of age to 284.6 ± 151.5 days for rats inoculated at 97 to 99 days of age. Poorly differentiated astrocytomas and astrocytomas of mixed morphology were common among rats inoculated as neonates. Solitary, pilocytic astrocytomas were the most common tumors among rats inoculated as adults.

INTRODUCTION

It is commonly claimed that with few exceptions only neonatal animals are susceptible to tumor induction by oncogenic viruses (4). Nevertheless, suspensions of tumor cells derived from AVS4-induced chicken sarcomas have been shown to be tumorigenic after s.c. inoculation of adult hamsters (2), adult guinea pig (1), and adult marmoses (18), and after i.c. inoculation of adult mice, cats, and rats (15), and adult monkeys (16). Suspensions of avian tumor cells cannot be standardized or quantified with respect to oncogen dose (7), and comparison of susceptibility of adults and neonates to AVS oncogenesis must be based on inoculation of cell-free virus. We have recently shown that i.c. inoculation of adult F344 rats with cell-free B-77-ASV induces brain tumors in some adult animals (5-7). In a 5-month study the incidence of animals developing brain tumors declined from 100% among rats inoculated on the 1st postnatal day to 50% among rats inoculated at 100 days of age (7). There was an inverse linear relationship (correlation coefficient = 0.96; p < 0.01) between the mean number of tumors per animal inoculated and the logarithm of the age of inoculation (7). The boldest and most parsimonious hypothesis would be that this relationship holds for all ages. Extrapolation would then predict that inoculation of F344 rats older than 400 days of age should induce no brain tumors. The hypotheses formulated on the results of this initial 5-month study have now been tested in a lifetime study of B-77-ASV-induced brain tumors in F344 rats. The results of the lifetime study are reported here.

MATERIALS AND METHODS

Virus. Cell-free, homogeneous Subgroup C B-77-ASV was obtained, grown, concentrated, and titrated by previously described methods (3).

Rats. Pregnant F344 rats, CDF strain, were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. Litters born to these females were standardized to 8 pups/female. Experimental animals were drawn from these litters at ages calculated by counting the day of birth as the 1st postnatal day.

Inoculation. A viral inoculum of 0.005 ml (8.7 x 10⁵ focus-forming units in 0.05 M sodium citrate buffer, pH 6.7) was delivered from a gastight microliter syringe in an automatic mechanical dispenser. Needles of different lengths (1/16 inch for neonates, 1/32 inch for 9 days of age, and 1/4 inch for 97 to 99 and 528 days of age) were used for the different age groups so that the inoculum would be introduced into the region of the basal ganglia of the right cerebral hemisphere. Forty-seven rats were inoculated on the 1st postnatal day. Thirty-seven rats were inoculated on the 9th postnatal day. Forty-one rats were inoculated between 97 and 99 days of age. Thirty rats were inoculated at 528 days of age. Details of inoculation of both neonatal and adult animals have been previously described (6, 7). Twenty-six 9-day-old rats were inoculated in the same fashion with a 10⁻¹ dilution of virus (8.7 x 10⁵ focus-forming units) in 0.005 ml of 0.05 M sodium citrate buffer.

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Rats that developed symptoms of intracranial mass lesions and became moribund were anesthetized with methoxyflurane and perfused through the left ventricle of the heart with buffered formaldehyde (4% formaldehyde, 0.135 M sodium phosphate monobasic, and 0.10 M sodium hydroxide). Rats that were found dead were necropsied. Brains were removed and fixed by immersion in phosphate-buffered formaldehyde (4% formaldehyde, 0.029 M sodium phosphate monobasic, and 0.046 M sodium phosphate dibasic) (6). The presence or absence of brain tumors was determined by examination of serial horizontal sections of whole brain. Tumors were assigned to the following categories according to previously described criteria (6): poorly differentiated astrocytoma, pilocytic astrocytoma, gemistocytic astrocytoma, polymorphic astrocytoma, sarcoma, gliosarcoma, and unclassified.

RESULTS

Aged Rats. The necropsy results for the 30 rats inoculated at 528 days of age are shown in Table 1. Twenty of these rats developed 21 parenchymal brain tumors for a mean of 0.70 ± 0.53 tumor/animal inoculated. Pilocytic astrocytomas accounted for 13 (62%) of these tumors, while polymorphic astrocytomas accounted for 3 (14%). There were 3 gliosarcomas (14%) and 2 sarcomas (10%). The mean survival in this group of rats was 262.20 ± 107.19 days postinoculation. Eighteen of the 20 rats that developed brain tumors were judged to have died from intracranial mass effect. The mean survival among these 18 animals was 299.94 ± 82.02 days postinoculation with a range of 157 to 415 days. The remaining 12 rats died of diseases that commonly occur in aged F344 rats, including neoplasms: pituitary adenoma, myelogenous leukemia and lymphoma, and chronic interstitial nephritis (Table 1).

Adult and Neonatal Rats. All of the rats inoculated at 1, 9, and 97 to 99 days of age developed brain tumors (Table 2). Although the incidence of animals developing tumors was 100% in each group, the mean survival and variance in survival after inoculation increased with increasing age at inoculation (Table 2). This effect is seen in the mortality curves for the 3 groups in Chart 1. Multiple tumors were the rule among rats inoculated at 1 day of age: 41 of 47 (87%) had more than 1 intracranial tumor. Multiple tumors were less common among rats inoculated at 9 days of age [13 of 37 (35%)] and were rare among rats inoculated at 97 to 99 days of age [3 of 41 (7%)]. The effect of declining tumor multiplicity is also seen in the values for mean tumors per animal inoculated, which declined from 3.09 among rats inoculated on the 1st postnatal day to 1.01 among rats inoculated between 97 and 99 days of age (Table 2).

The distribution of tumor types was noteworthy for the greater frequency of sarcomas and gliosarcomas among rats inoculated at 9 days of age. In both the group inoculated at 1 day of age and in the group inoculated at 97 to 99 days of age 80% of the tumors were astrocytomas. However, among the rats inoculated as adults, most of the astrocytomas were pilocytic astrocytomas (30 of 35), whereas pilocytic astrocytomas were rare (3 of 116) among rats inoculated at 1 day of age (Table 3). Poorly differentiated astrocytomas and astrocytomas of mixed morphology (polymorphic astrocytomas) were common among rats inoculated at 1 day of age (92 of 116) but were rare among rats inoculated at 97 to 99 days of age (5 of 35).

Diluted Inocula. Twenty-six of 26 rats inoculated with 10⁻¹ dilution of virus developed brain tumors (Table 2). The mean number of tumors per animal inoculated was slightly less in the group receiving diluted virus (1.23 tumors/animal inoculated) than in the group receiving undiluted virus (1.51
Table 2

Tumor incidence and survival in F344 rats inoculated with B-77-ASV

F344 rats were inoculated i.c. with B-77-ASV in 0.005 ml of 0.05 m sodium citrate buffer at the dose and ages indicated. The inoculum was delivered into the periventricular region of the basal ganglia of the right cerebral hemisphere. The occurrence of multiple tumors in the same animal followed a Poisson distribution. Hence, the Freeman-Tukey transformation for Poisson counts (20) was utilized, and the data were analyzed by analysis of variance procedures. For groups inoculated with $8.7 \times 10^4$ focus-forming units, the likelihood of an animal having multiple tumors was greater if it was inoculated at age 1 day than if inoculated at 9 days and greater if inoculated at 9 days than if inoculated at 97 to 99 days. Each difference is significant at the 0.01 level. No significant difference was detected between the 2 groups inoculated at age 9 days with different doses of virus.

Variance in postinoculation survival was analyzed by F tests. There was no significant difference among rats inoculated at 9 days of age with different doses of virus. For rats inoculated with $8.7 \times 10^4$ focus-forming units, comparison of sample standard deviations indicated that: 1 day < 9 day < 97 to 99 day, with each difference significant at the 0.01 level. Because of the differences in variability, nonparametric procedures were used to assess group differences in mean survival; pairwise comparisons were made by Mann-Whitney U tests. It was found that for mean survival:

$1 \text{ day}^b < 9 \text{ day}^b < \left[ 9 \text{ day}^d, 97-99 \text{ day}^a \right]$  

Each difference is significant at the 0.01 level.

<table>
<thead>
<tr>
<th>Age at inoculation (days)*</th>
<th>Animals with tumors/animals inoculated</th>
<th>Tumors/animals inoculated</th>
<th>Mean tumors/animal</th>
<th>Mean survival in days postinoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>47/47</td>
<td>145/47</td>
<td>3.09 ± 1.25*</td>
<td>83.8 ± 21.5*</td>
</tr>
<tr>
<td>9*</td>
<td>37/37</td>
<td>56/37</td>
<td>1.51 ± 0.84</td>
<td>173.5 ± 68.2</td>
</tr>
<tr>
<td>97-99</td>
<td>26/26</td>
<td>32/26</td>
<td>1.23 ± 0.51</td>
<td>308.0 ± 88.6</td>
</tr>
<tr>
<td></td>
<td>41/41</td>
<td>44/41</td>
<td>1.07 ± 0.26</td>
<td>290.7 ± 158.6</td>
</tr>
</tbody>
</table>

* Animals inoculated at 528 days of age are not included since death from spontaneously occurring diseases obscures the relationship between brain tumor induction and postinoculation survival.

b Inoculated i.c. with $8.7 \times 10^4$ focus-forming units of B-77-ASV.

c ±S.D.

d Inoculated i.c. with $8.7 \times 10^4$ focus-forming units of B-77-ASV.

distribution of tumor types for the rats receiving diluted virus was similar to that of the group of rats inoculated at 9 days of age with undiluted virus (Table 3).

DISCUSSION

When neonatal or adult animals are inoculated with oncogenic DNA (9, 11-14, 17, 26) or RNA viruses (8, 14), tumor incidence and tumor latency depend on the age of the animal at virus inoculation. As age increases, tumor latency increases and tumor incidence decreases. Intracranial neoplasms have been induced in adult mice, rats, cats, and monkeys by inoculation of whole-cell suspensions of viable Rous chicken sarcoma cells (15). The mechanism of neoplastic transformation after inoculation of whole chicken sarcoma cells in mammals is unclear, and the dose of oncogen cannot be quantified and standardized in the case of chicken sarcoma cell suspensions (7). Comparison of relative susceptibility of adults and neonates must be made on the basis of inoculation of cell-free virus.

We have recently shown a decrease in tumor incidence and an increase in tumor latency with increasing age at inoculation for brain tumors induced in F344 rats by i.c. inoculation of B-77-ASV (7). In that study, in which animals were observed for only 5 months after inoculation, brain
The classification of B-77-ASV-induced neoplasms in F344 rats

F344 rats were inoculated i.c. with B-77-ASV in 0.005 ml of 0.05 M sodium citrate buffer at the dose and age indicated in the table. The inoculum was delivered into the periventricular regions of the basal ganglia of the right cerebral hemisphere. Tumors were classified according to a scheme based on light and electron microscopic studies of B-77-ASV brain tumors in F344 rats (6). Unclassified tumors were those tumors which by light microscopy fell into categories that are rare in ASV-induced brain tumors and unsubstantiated by fine structure studies, e.g., ependymoma and oligodendroglioma.

The data were analyzed by $\chi^2$ procedures. There was no significant difference in distribution of tumor types between groups inoculated at 9 days of age with different doses of virus. The distribution of types of astrocytoma among rats inoculated with $8.7 \times 10^8$ focus-forming units differed significantly at the 0.01 level among groups inoculated at 1, 9, and 97 to 99 days of age. The difference was due to the decrease in frequency of poorly differentiated astrocytomas and increase in frequency of pilocytic astrocytomas with increasing age at inoculation. Comparison of all astrocytomas with nonastrocytomas (sarcomas and gliosarcomas) demonstrates a significant ($p < 0.05$) increase in frequency of sarcomas and gliosarcomas among rats inoculated at 9 days of age.

<table>
<thead>
<tr>
<th>Age at inoculation (days)</th>
<th>1$^a$</th>
<th>9$^a$</th>
<th>97-99$^a$</th>
<th>528$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poorly differentiated astrocytoma</td>
<td>45/145 (31)$^c$</td>
<td>12/56 (21)</td>
<td>4/32 (12)</td>
<td>0/44</td>
</tr>
<tr>
<td>Pilocytic astrocytoma</td>
<td>3/145 (2)</td>
<td>12/56 (21)</td>
<td>6/32 (19)</td>
<td>30/44 (68)</td>
</tr>
<tr>
<td>Gemistocytic astrocytoma</td>
<td>21/145 (15)</td>
<td>0/56</td>
<td>2/32 (6)</td>
<td>0/44</td>
</tr>
<tr>
<td>Polymorphic astrocytoma</td>
<td>47/145 (32)</td>
<td>9/56 (16)</td>
<td>8/32 (25)</td>
<td>5/44 (12)</td>
</tr>
<tr>
<td>All astrocytomas</td>
<td>116/145 (80)</td>
<td>33/56 (59)</td>
<td>20/32 (62)</td>
<td>35/44 (80)</td>
</tr>
<tr>
<td>Gliosarcoma</td>
<td>10/145 (7)</td>
<td>7/56 (12)</td>
<td>5/32 (16)</td>
<td>3/44 (7)</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>18/145 (12)</td>
<td>14/56 (25)</td>
<td>6/32 (19)</td>
<td>5/44 (11)</td>
</tr>
<tr>
<td>Unclassified</td>
<td>1/145 (1)</td>
<td>2/56 (4)</td>
<td>3/32 (6)</td>
<td>1/44 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>145/145 (100)</td>
<td>56/56 (100)</td>
<td>32/32 (100)</td>
<td>44/44 (100)</td>
</tr>
</tbody>
</table>

$^a$ Inoculated i.c. with $8.7 \times 10^8$ focus-forming units of B-77-ASV.  
$^b$ Inoculated i.c. with $8.7 \times 10^9$ focus-forming units of B-77-ASV.  
$^c$ Numbers in parentheses, percentage.

The incidence of spontaneously occurring parenchymal brain tumors in aged F344 rats is less than 2% (22). This difference, which is significant, falsifies the hypothesis of an inverse linear relationship between tumor incidence and age at inoculation and suggests that F344 rats do not become resistant to the oncogenic effects of ASV at any age.

Table 3

The classification of B-77-ASV-induced neoplasms in F344 rats

F344 rats were inoculated i.c. with B-77-ASV in 0.005 ml of 0.05 M sodium citrate buffer at the dose and age indicated in the table. The inoculum was delivered into the periventricular regions of the basal ganglia of the right cerebral hemisphere. All animals in each group died with a brain tumor of sufficient size to account for death. The onset of mortality was delayed in the group inoculated at 9 days of age with a 0.1 dilution of virus, but the slope of the mortality curve corresponds to the slope of the mortality curve for the group inoculated at 9 days of age with undiluted virus in contrast to the precipitous slope for the group inoculated at 1 day of age.

Tumor incidence fell from 100% in rats inoculated on the 1st postnatal day to 50% in rats inoculated at 100 days of age. There was a strong inverse linear relationship between mean tumors per animal inoculated and the logarithm of the age of the animal at inoculation. Application of Ockham's razor favors this inverse linear relationship over alternate hypotheses for the effect of age at inoculation on tumor incidence. The hypothesis is boldest and most readily falsifiable when extrapolation of the linear relation predicts that i.c. inoculation of F344 rats with B-77-ASV after 400 days of age should induce no brain tumors (7). We tested this prediction in the present study by inoculation of B-77-ASV in rats 528 days of age. Two-thirds (20 of 30) of the animals developed characteristic ASV-induced brain tumors, and 18 of these 20 animals died of an ASV-induced brain tumor. The incidence of spontaneously occurring parenchymal brain tumors in aged F344 rats is less than 2% (22). This difference, which is significant, falsifies the hypothesis of an inverse linear relationship between tumor incidence and age at inoculation and suggests that F344 rats do not become resistant to the oncogenic effects of ASV at any age.

Among rats inoculated at 1, 9, and 97 to 99 days of age, we found a 100% incidence of brain tumors. Although the incidence of brain tumors did not decrease with age, the
frequency of multiple tumors decreased, and the latency from inoculation to tumor-related death increased. Our initial study followed F344 rats for only 150 days after inoculation (7). Since, in the present study, half of the animals inoculated at 9 days of age and three-quarters of the animals inoculated at 97 to 99 days of age died of brain tumors after 150 days postinoculation, the true values for tumor incidence among adult animals were underestimated in the initial study. A 100% tumor incidence after long latency in rats inoculated with B-77-ASV as adults underscores the need for prolonged follow-up in studies of relative susceptibility of neonates and adults to viral oncogenesis.

Brain tumors induced by i.c. inoculation of B-77-ASV in F344 rats are either astrocytic, mesenchymal, or mixed astrocytic-mesenchymal regardless of the age at inoculation. However, the morphology of brain tumors induced in adults and in neonates differs. The most significant difference was a decrease in the incidence of poorly differentiated astrocytomas among rats inoculated as adults and a corresponding increase in the incidence of pilocytic astrocytomas. The difference could be attributed either to events which take place at the time of inoculation or, since tumors develop later in rats inoculated as adults, to differential selection factors in the older animals. Inoculation of 9-day-old rats with a diluted inoculum increased latency so that tumors developed at ages corresponding to rats inoculated at 97 to 99 days. Nevertheless, the tumor types that developed were similar to those in rats inoculated at 9 days of age with undiluted virus, suggesting that tumor morphology is determined by events which take place at, or shortly after, inoculation and not by the in vivo milieu in which the tumor grows.

The cells of the pilocytic astrocytomas are morphologically better differentiated than those of the poorly differentiated astrocytoma (6). The increase in frequency of pilocytic astrocytomas and decrease in frequency of poorly differentiated astrocytomas with increasing age at inoculation is consistent with a shift in the maturity of cells transformed from the poorly differentiated subependymal cell in the neonate to a more mature cortical astrocyte in the adult. This hypothesis is further supported by observations of incipient tumor formation in the subependymal region in neonatally inoculated dogs (23-25) and neonatally inoculated rats and incipient tumor formation in the cortex of F344 rats inoculated with B-77-ASV as adults (5).

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