Development of a Neonatal and Metastatic Murine Neuroblastoma Model

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ABSTRACT

A neonatal murine neuroblastoma (NB) model has been developed in addition to a murine NB model for metastatic involvement. The S2OY NB cell line used was cloned from the A/Jax murine C1300 NB and was a gift from Dr. M. Nirenberg. Dose-response studies on adult male and female mice given s.c. injections showed no meaningful sex-related differences in either the appearance or the growth rate of the tumors. No significant difference in tumor appearance or growth rate was observed between 10-day-old pups and adults. However, 24- to 48-hr-old pups (neonates) inoculated with the same number of cells as the adults demonstrated a marked delay in tumor appearance. Neonates challenged with 5 × 10⁶ cells showed a 75% reduction in tumor appearance when compared with their adult counterparts. An inoculum of 2 × 10⁶ S2OY cells resulted in 100% of the adult mice developing tumors within 26 days, while none of the 24- to 48-hr-old pups had developed tumors more than 90 days postinjection. Comparison of the s.c. and intradermal routes of injection of NB cells was made using 6- to 8-week-old male mice. Tumors developed at approximately the same rate. The intradermal tumors were removed at various stages of growth. Over 90% of the mice which underwent surgery and a similar percentage of the mice with intradermal tumors which did not have surgery subsequently developed metastases to the lymph nodes, liver, lungs, adrenals, heart, kidney, spleen, and abdominal area, sites commonly found to harbor metastatic lesions in cases of human NB. Metastases at these sites were not observed with s.c. inoculations.

INTRODUCTION

In humans, NB is one of the most common malignant solid tumors of childhood (3, 13). NB is usually diagnosed in patients less than 5 years old, with a peak incidence at the age of 2 (11). It is a tumor which, while it sometimes can undergo spontaneous regression (5), has remained refractory to various chemotherapeutic approaches (9).

The murine C1300 NB isolated by Cloudman (6) has been used as an in vivo model system. Most studies have been done using adult A/Jax strain mice (2). However, in humans NB is a tumor of the young; therefore, a more appropriate model might be one in which young mice are inoculated with murine NB cells. This paper presents the results obtained for neonates (24- to 48-hr-old mice), and 6- to 8-week-old mice given injections of murine NB cells. The results suggest that there is a definite age-dependent response as well as a correlation between the response observed and the route of injection of the NB cells.

MATERIALS AND METHODS

Syngeneic A/Jax mice were obtained at 6 to 8 weeks of age from The Jackson Laboratory, Bar Harbor, Maine. Ten-day-old and 24- to 48-hr-old pups (neonates) were obtained from a breeding colony maintained at this institution. Pups were allowed to remain with their mother for at least 35 days; those bearing tumors were allowed to remain with their mother until their death. Mice were fed ad libitum on standard mouse laboratory chow.

S2OY cells, cloned from the A/Jax mouse C1300 NB, were a gift from Dr. M. Nirenberg. The cells were grown in Dulbecco's medium containing 10% fetal calf serum and 0.225% sodium bicarbonate. The cells were grown in an atmosphere of 5% CO₂/95% air at 37°. The medium was changed every second day. Cells used for tumor induction were harvested (by rapping the bottles sharply to detach the cells) when confluent. Cell viability was determined by trypan blue exclusion.

To induce tumor formation, mice were given injections of the appropriate number of cells suspended in 0.05 ml of growth medium, via a dorsal s.c. inoculation slightly posterior to the right foreleg. In each study, a minimum of 5 mice were treated with the specified number of cells. In studies relating age and tumor development, direct comparisons were made between adults and pups or adults and neonates treated with the same cell preparation on the same day. Six mice to eight weeks old given i.d. injections were inoculated on the dorsal surface posterior to the transverse plane. In studies comparing adults and pups or neonates, s.c. injections were made using a 27-gauge needle. In studies in which tumor development in adult male and female mice was compared, a 25-gauge needle was used. Inoculations i.d. of adult mice were routinely done with a 25-gauge needle. Mice were checked at least twice a week for tumor appearance. Tumor development was measured in 2 dimensions every second day.

Tumors i.d. were excised at various stages (5 to 10 and 15 to 20 mm in diameter) of growth. Mice were anesthetized with 0.7% w/v Nembutal in 10% ethanol (0.01 ml/g body weight). After removal of the tumors, the incisions were closed with autoclips, and the mice were allowed to recover in a dry, warm area. These mice were maintained as above and the development of metastases was noted.

Statistical evaluations were done using the Fisher exact test or the χ² test (12).
RESULTS

Male and female mice (6 to 8 weeks old) responded similarly to s.c. inoculation with $10^5$ S2OY cells. Inoculation with $10^5$ cells also resulted in all of the mice developing tumors at about the same rate. When $10^4$ S2OY cells were injected, 3 of 5 male and 5 of 5 female mice developed tumors. The average time from inoculation until the tumors were palpable was 17 and 23 days, respectively. Inoculating the mice with $10^4$ cells resulted in none of the 6 males and only 1 of the 5 females developing tumors. The observed differences were not statistically significant. Therefore, subsequent studies using 10-day-old pups and neonates were done without regard to sex.

Adult male mice and 10-day-old pups also responded similarly to s.c. inoculation of equivalent numbers of S2OY cells over the range of $10^3$ to $10^6$ cells. The average results obtained for the 4 cell concentrations tested are shown in Table 1. No statistically significant difference in tumor development was observed for these mice.

Comparison of the rate of NB development in adult and neonatal mice showed a statistically significant reduction in the rate of tumor appearance in the neonatal mice given injections of $5 \times 10^4$ or $2 \times 10^4$ cells. The average results obtained for the 4 cell concentrations tested are shown in Table 2. No significant differences in tumor development were observed at cell concentrations of $10^6$ or $10^5$.

Approximately 90% of adult male mice inoculated i.d. with $2 \times 10^5$ cells developed tumors at the site of injection. Over 90% of these mice subsequently developed metastases to the lymph nodes, liver, lung, adrenal, heart, kidney, spleen, and abdominal areas, whether or not the primary tumor had been surgically excised or allowed to grow. Multiple metastases were frequently found in the same mouse, with the most frequently affected organs being the lymph, liver, and lungs. No gross metastases were visible in the brains of these animals. The incidence of metastases to different sites is shown in Table 3. Inoculation i.d. of 12 adult male mice with $2 \times 10^5$ cells and of a second group of 11 adult male mice with $5 \times 10^5$ cells resulted in 11 of 12 of the first group developing tumors and all of the second group developing tumors at the primary site of injection. All of the mice autopsied had metastases. No metastases were observed in any of the more than 100 adult mice inoculated s.c. with $5 \times 10^5$ cells or in the adult mice used for the dose-response studies.

DISCUSSION

It has been reported that in humans the occurrence of NB is not sex related (10). The results presented here on the development of NB in adult male and female mice concur. There was no statistically significant difference in the rate of NB development in adult male and female mice. Therefore, male mice were used as the adult control for subsequent studies since they were more readily available. In studies done with neonates and 10-day-old pups, all members of each litter were used without regard to sex.

Studies comparing the rate of tumor appearance and growth in 10-day-old pups with that in adults showed no statistically significant differences. However, of the 6 pups inoculated with $2 \times 10^5$ S2OY cells, 3 did not develop tumors, and in the mice which did develop tumors, the rate of tumor appearance appeared to be slower. In contrast to the results obtained for 10-day-old pups, the neonates reacted quite differently from the adults when inoculated with either $2 \times 10^5$ or $5 \times 10^5$ S2OY cells. These mice either
did not develop tumors or showed a reduction in the rate of tumor appearance.

It is difficult to make extrapolations from an animal model to the human situation. There are the obvious differences in such parameters as the proportional rate of change in body weight (1), central nervous system development (8), and immunological competence at birth (7) which must be considered. Then, there are the experimental differences introduced in developing the animal model. For the neonatal murine NB model described, some of these differences are as follows. Neonatal mice were inoculated with NB cells, while in humans the etiology of NB is unknown. In the neonatal mouse, failure to obtain tumor growth may reflect an immunological rejection of the inoculated cells or lack of a factor or factors needed for growth, while in the young child it is not a failure of the tumor to grow but is a spontaneous regression which occurs. There can be no carry over of a maternal response developed to fetal NB in the murine system. The s.c. location of the tumor in this model is different from that for the human, and cholinergic cells were used in this study, while most NB's are adrenergic in nature. However, the neonatal model does show that the 24- to 48-hr-old mouse reacts differently than the adult does to inoculation with NB cells, just as the young child with NB has been shown to have a better prognosis than does the older one.

The development of a metastatic NB model using the i.d. route of injection provides a system that might be used for further studies on possible treatment. In the clinical staging system proposed by Evans et al. (4) for children with NB, the spread of the disease indicates an increasingly poor prognosis. The progression is defined as: (a) Stage I (tumor confined to the organ or structure of origin); (b) Stage II (tumors continuing beyond the organ or structure of origin but not crossing the midline, with possible involvement of regional lymph nodes on the ipsilateral side); (c) Stage III (tumors continue across the midline, and the regional lymph nodes may be involved bilaterally); and (d) Stage IV (remote disease involving the skeleton, organs, soft tissues, or distant lymph node groups). The appearance of NB metastases in the mice was similar to that expected for an increasingly poor prognosis for the human. Usually, the inguinal lymph node on the ipsilateral side was the first observable metastatic site. Subsequently, the ipsilateral axillary and brachial and contralateral inguinal lymph nodes were involved and, finally, the contralateral axillary and brachial nodes. Upon autopsy, NB metastases to the liver, lung, adrenals, heart, kidneys, spleen, and abdominal area were also found. The development of NB after i.d. inoculation of S2OY cells appears to resemble more closely the pattern of NB in the older child than does the s.c. route of inoculation, in which death is apparently due to growth of the tumor at the primary site.

The models described provide a system for studying the effects of NB therapy on a younger animal (pups) as well as on the metastatic system. Since these systems seem to resemble the pattern of NB development in children, it may be possible to apply the knowledge obtained in treating murine NB to human NB treatment.

REFERENCES

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