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# Effect of carcinogen dose and age at administration on induction of mammary carcinogenesis by 1-methyl-1-nitrosourea

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**The objective of the work reported in this paper was to determine if the tumorigenic response to 1-methyl-1-nitrosourea (MNU) in the mammary gland varied with age of administration and was dose dependent when the carcinogen was injected prior to 50 days of age. Using a recently developed method for mammary tumor induction, MNU was injected i.p. at doses ranging from 25 to 75 mg/kg at 35 days of age or 50 mg/kg at 28, 35 or 42 days of age. Treatment with MNU resulted in induction of both benign and malignant mammary tumors. The incidence of mammary gland adenocarcinomas was 100% at and above the 50 mg/kg dose of MNU, irrespective of the age at which carcinogen was administered. The number of cancers increased in proportion to carcinogen dose, whereas cancer latency decreased as the MNU dose increased. In rats injected with 50 mg MNU/kg body weight at 28, 35 or 42 days of age, differences among groups in cancer incidence, number or latency were not statistically significant. Metastases of mammary neoplasms to lung, liver and spleen were observed in rats injected with MNU at 35 or 42 days of age. These data indicate (i) the dose responsiveness of MNU-induced mammary carcinogenesis in rats initiated prior to 50 days of age; (ii) the lack of effect of age at initiation if prior to 50 days on final tumor outcome; and (iii) that the age at which MNU is injected may affect the metastatic potential of the mammary carcinomas that are induced.**

## Introduction

In a majority of experiments in which 1-methyl-1-nitrosourea (MNU*) or 7,12-dimethylbenz[*a*]anthracene (DMBA) has been used to induce mammary carcinogenesis, the carcinogen has been administered between 50–65 days of age (1). The rationale for this selection of age is based on the pioneering work of Huggins and colleagues, which indicated that the mammary gland of the virgin rat was most sensitive to carcinogenic insult with DMBA during this period of time (2–3). However, work from that laboratory and others indicates that it is feasible to inject either methylcholanthrene, DMBA or MNU prior to 50 days of age and achieve a significant tumorigenic response (4–11) (Tables I and II). Unfortunately not only is this fact not widely recognized, but from the available data it is difficult to determine either the magnitude or the kinetics of the tumorigenic response that are expected when carcinogen dose and/or age at administration of carcinogen is varied. This issue is a practical concern for investigators interested in evaluating the effects of cancer inhibitory agents since the magnitude of the carcinogenic response

*Abbreviations: MNU, 1-methyl-nitrosourea; DMBA, 7,12-dimethylbenz[*a*]anthracene; NHL, non-Hodgkins lymphoma.

**Table I.** Effect of age at administration of DMBA on induction of mammary carcinomas

| Dose | Age at administration (days) | Termination post carcinogen (weeks) | Mammary cancers | | Ref. |
|---|---|---|---|---|---|
| | | | Incidence (%) | No./rat | |
| 10 mg/100 g | 20 | 22 | 50 | 2.6 | 5 |
| body wt | 30 | 22 | 93.3 | 3.4 | |
| (i.g.) | 40 | 22 | 88.8 | 3.9 | |
| | 46 | 22 | 100.0 | 5.6 | |
| | 55 | 22 | 97.0 | 3.6 | |
| | 70 | 22 | 62.5 | 3.0 | |
| 15 mg/100 g | 25 | [a] | 47 | 1.8 ± 0.2[b] | 7 |
| body wt | 70 | [a] | 23 | 1.5 ± 0.3[b] | |
| (i.g.) | 90 | [a] | 14 | 2.2 ± 0.5[b] | |
| 3 mg/100 g | 10 | 21 | 84.6[c] | NR | 8 |
| body wt | 15 | 21 | 93.1[c] | NR | |
| (i.v.) | 20 | 21 | 88.2[c] | NR | |
| | 25 | 21 | 93.3[c] | NR | |
| | 30 | 21 | 93.3[c] | NR | |
| | 35 | 21 | 94.7[c] | NR | |
| | 55 | 21 | 97.1[c] | NR | |
| | 90 | 21 | 37.0[c] | NR | |
| | 150 | 21 | 0[c] | NR | |
| | 440 | 21 | 37.5[c] | NR | |

NR, not reported.
[a]Range between 4 and 52 weeks post carcinogen.
[b]Mean number of tumors per tumor-bearing rat.
[c]Includes benign and malignant tumors.

**Table II.** Effect of age at administration of MNU on induction of mammary carcinomas

| Dose | Age at administration (days) | Termination post carcinogen (weeks) | Mammary cancers | | Ref. |
|---|---|---|---|---|---|
| | | | incidence (%) | No/rat | |
| 50 mg/kg i.v. | 35 and 42 | 29 | 100 | 8.9 | 9 |
| (2 injections | 50 and 57 | 29 | 94 | 6.0 | |
| 1 week apart) | 80 and 87 | 29 | 59 | 1.4 | |
| | 140 and 147 | 29 | 30 | 0.3 | |
| | 200 and 207 | 29 | 22 | 0.2 | |
| 50 | 50 | 63 | 100 | 6.5 | 10 |
| 45 | 50 | 63 | 100 | 4.8 | |
| 40 | 50 | 63 | 100 | 3.2 | |
| 35 | 50 | 63 | 90 | 3.2 | |
| 30 | 50 | 63 | 95 | 2.8 | |
| 25 | 50 | 63 | 70 | 1.9 | |
| 20 | 50 | 63 | 65 | 2.6 | |
| 15 | 50 | 63 | 55 | 1.1 | |
| 10 | 50 | 63 | 45 | 1.1 | |
| 0 | 50 | 63 | 20 | 0.3 | |

**Table III.** Effect of dose and age of administration of MNU on induction of mammary carcinomas

| MNU dose[a] (mg/kg) | Age MNU injected (days) | Mammary carcinomas | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Incidence (%) | Cancer-free time (weeks) | | No. of cancers | | | |
| | | | Median | Mean | Total | Mean | Median | Range |
| 25 | 35 | 63[b] | 25.7[b] | 22.3 | 29 | 1.0 ± 0.2[e] | 1 | 0–4 |
| 50 | 35 | 100[c] | 8.3[c] | 10.4 | 154 | 5.1 ± 0.6[f] | 5 | 1–19 |
| 75 | 35 | 100[c] | 5.9[d] | 6.4 | 249 | 8.6 ± 0.6[g] | 8 | 2–16 |
| 50 | 28 | 100[c] | 7.9[c] | 9.4 | 147 | 4.9 ± 0.5[f] | 4 | 1–11 |
| 50 | 42 | 100[c] | 8.6[c] | 8.7 | 161 | 5.4 ± 0.5[f] | 6 | 1–10 |

[a]Thirty rats were assigned to each treatment group and given an i.p. injection of MNU at the specified age.
[b–d]The median and mean cancer-free times were computed via life-table analyses. The latency curves from which the cancer-free times were computed were statistically different ($P < 0.01$) for treatment groups bearing different superscripts.
[e–g]Values are mean ± SEM. Values within this column having different superscripts are statistically different ($P < 0.05$), based on *post hoc* comparisons by the method of Tukey (14).

that is anticipated from a particular carcinogen dose regime is important to know in designing an experiment. The objective of this study was to evaluate the dose responsiveness of the tumorigenic response in the mammary gland and the specificity of tumor induction to this target tissue in rats injected at different ages prior to 50 days and with different doses of MNU. MNU was chosen as the carcinogen based on work reported in (9) in order to avoid concerns about age-related differences in carcinogen metabolism.

## Materials and methods

*Female Sprague–Dawley rats, with specified date of birth, were obtained from* Taconic Farms, Germantown, NY, at 21 days of age. Rats were housed three per cage in solid-bottomed polycarbonate cages bedded with hardwood chips. The animal facility was environmentally controlled and maintained at 19°C with 50% relative humidity and a 12 h light, 12 h dark cycle. All rats were fed Agway RMH 3200 rodent chow and distilled water *ad libitum* throughout the experiment. At 21 days of age, rats were randomized into one of five groups of 30 rats each. The design of the experiment to which these rats were assigned is shown in Table III. Rats were assigned cage rack positions so that differences in light intensity due to vertical positions on the cage rack were equally represented in each experimental group. Carcinogen was prepared and administered as previously described (13). Following carcinogen administration, rats were weighed weekly and palpated twice per week for the detection of mammary tumors. The location of each tumor and date of its detection were recorded. The experiment was terminated 32 weeks following carcinogen administration. At necropsy, rats were killed with gaseous carbon dioxide and then skinned. The skin was examined through translucent light and the location of all grossly observed tumors was noted and correlated with the tumor palpation record. All tumors and suspect areas were excised and fixed in 10% buffered formalin. Tissues were then processed for routine histologic evaluation. Five micron paraffin sections of all tissues excised at necropsy were prepared and stained with H&E. Mammary tumors were classified histologically as recommended by Young and Hallowes (12).

*Statistical analysis*

Tumor count data have a Poisson distribution. Therefore, in addition to analyzing these data by analysis of variance following square root transformation as recommended in (14), we also evaluated tumor multiplicity by the Kruskall–Wallis procedure (15). Tumor latency data were assessed by the Mantel–Haenszel procedure as discussed in (16) and implemented in the Survival Module of Systat statistical analysis system (Evanston, IL). Differences among groups in final cancer incidence were evaluated as recommended by Peto (17).

## Results

Intraperitoneal administration of MNU at doses ranging from 25 to 75 mg/kg at 35 days of age or 50 mg MNU/kg at 28 or 42 days of age induced no acute toxicity in treated animals. Administration of MNU resulted in the induction of both benign and malignant mammary tumors. Mammary cancers were defined to include both adenocarcinomas and papillary carcinomas; a significant number of lesions showed a mixed adenopapillary pattern. Benign mammary tumors were defined to include fibroadenomas, fibromas and adenomas; mammary cysts were not included.

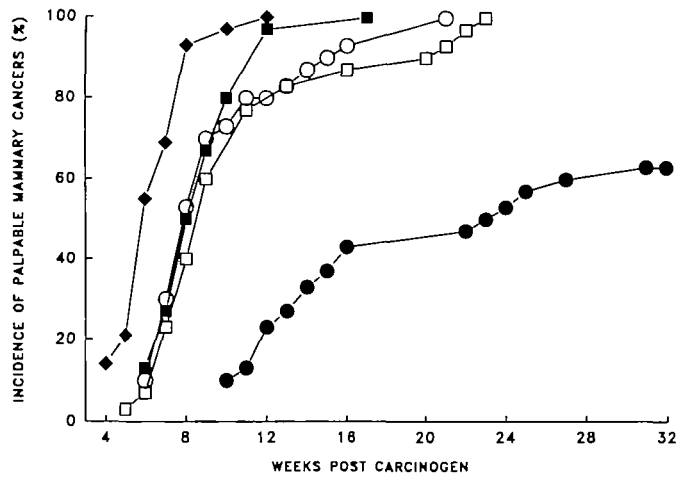


**Fig. 1.** Incidence of palpable mammary cancers (%) as a function of time post carcinogen. Thirty-five day old rats were given a single i.p. injection of 25 (●), 50 (■) or 75 (◆) mg MNU/kg body wt; 28 day old rats (○) and 42 day old rats (□) were given a single i.p. injection of 50 mg MNU/kg body wt.

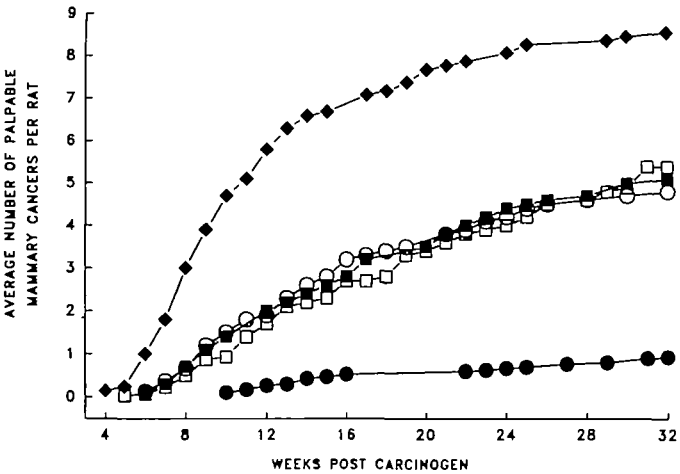


**Fig. 2.** Average number of palpable mammary cancers per rat as a function of time post carcinogen. Thirty-five day old rats were given a single i.p. injection of 25 (●), 50 (■) or 75 (◆) mg MNU/kg body wt; 28 day old rats (○) and 42 day old rats (□) were given a single i.p. injection of 50 mg MNU/kg body wt.

Mixed tumors with areas of carcinoma and fibroadenoma were scored as cancers. Other malignant neoplasms found in the mammary chain included eight non-Hodgkins lymphomas, one

**Table IV.** Effect of dose and age of administration of MNU on induction of benign mammary tumors

| MNU dose[a] (mg/kg) | Age MNU injected (days) | No. of tumor-bearing rats | No. of tumors | | | |
|---|---|---|---|---|---|---|
| | | | Fibroadenoma | Adenoma | Fibroma | Total |
| 25 | 35 | 3 | 2 | 1 | – | 3 |
| 50 | 35 | 12 | 22 | – | – | 22 |
| 75 | 35 | 8 | 9 | 2 | 1 | 12 |
| 50 | 28 | 7 | 10 | 1 | 1 | 12 |
| 50 | 42 | 6 | 7 | – | – | 7 |

[a]Thirty rats were assigned to each treatment group and given an i.p. injection of MNU at the specified age. The experiment was terminated 32 weeks post carcinogen.

malignant fibrous histiocytoma, one carcinosarcoma and two sarcomas.

The final incidence and number of malignant mammary tumors found at necropsy is shown in Table III. The number of cancers increased with dose of MNU, whereas cancer incidence was 100% at and above 50 mg MNU/kg body wt. Differences in incidence and number of mammary cancers per rat among MNU treatment groups injected at 35 days of age were dose dependent ($P < 0.05$). However, the injection of 50 mg MNU/kg body wt at 28, 35 or 42 days of age had no significant effect on the final incidence or number of mammary cancers detected at necropsy. The temporal pattern of appearance of palpable mammary tumors confirmed histologically to be carcinomas is illustrated in Figure 1 for incidence and Figure 2 for tumor number. Median cancer-free time in weeks post-carcinogen

**Table V.** Effect of dose and of age of administration of MNU on the induction of non-mammary tumors

| MNU dose (mg/kg) | Age MNU injected (days) | No. of tumors | Carcinomas | Sarcomas | Non-Hodgkins lymphoma | Other |
|---|---|---|---|---|---|---|
| 25 | 35 | 2 (2)[a] | 1 transitional cell carcinoma, urinary bladder<br>1 adenosquamous carcinoma, lung | | | |
| 50 | 28 | 7 (6) | 1 undifferentiated carcinoma, abdomen | 2 fibrosarcomas, spleen/liver<br>1 undifferentiated sarcoma, spleen<br>2 mesenchymal tumors, kidney | | 1 theca cell tumor, ovary |
| 50 | 35 | 10 (10) | 2 squamous cell carcinoma, skin<br>1 undifferentiated carcinoma, salivary gland<br>1 adenocarcinoma, lung<br>1 hepatocellular carcinoma | 1 low-grade sarcoma, mammary chain<br>1 myxosarcoma, mammary chain<br>1 hemangiosarcoma, small intestine | 1 NHL in salivary gland | 1 theca cell tumor, ovary |
| 50 | 42 | 6 (5) | 2 adenocarcinoma, lung | 2 mesenchymal tumors, kidney<br>1 malignant fibrous histiocytoma in mammary chain area with lung metastasis[b] | 1 NHL in mammary chain | |
| 75 | 35 | 8 (7) | 1 adenocarcinoma, lung<br>1 undifferentiated carcinoma, liver | 3 mesenchymal tumors, kidney<br>1 angiosarcoma, spleen<br>1 fibrosarcoma, thoracic tumor<br>1 undifferentiated sarcoma, cecum | | |

[a]Numbers in parentheses indicate the number of tumor-bearing animals.
[b]Also included in Table VI.

**Table VI.** Metastases induced by MNU

| MNU dose (mg/kg) | Age MNU injected (days) | No. of tumors | Primary tumors | | Metastases |
|---|---|---|---|---|---|
| | | | site | histology | |
| 50 | 35 | 1 | mammary gland | non-Hodgkins lymphoma | liver, spleen, abdomen |
| | | 1 | liver | hepatocellular carcinoma | embolus in a pulmonary blood vessel |
| | | 2[a] | mammary gland | adenocarcinoma | lung |
| 50 | 42 | 1 | mammary chain area | malignant fibrous histiocytoma | lung |
| | | 1 | mammary gland | adenocarcinoma | lung |
| | | 1 | mammary gland | non-Hodgkins lymphoma | NHL involving spleen |
| 75 | 35 | 1 | mammary gland | adenocarcinoma | spleen |

[a]In a repeat experiment in which 30 rats were injected with this MNU dose regime, 2/30 rats were observed to have mammary gland adenocarcinomas that metastasized to the lung.

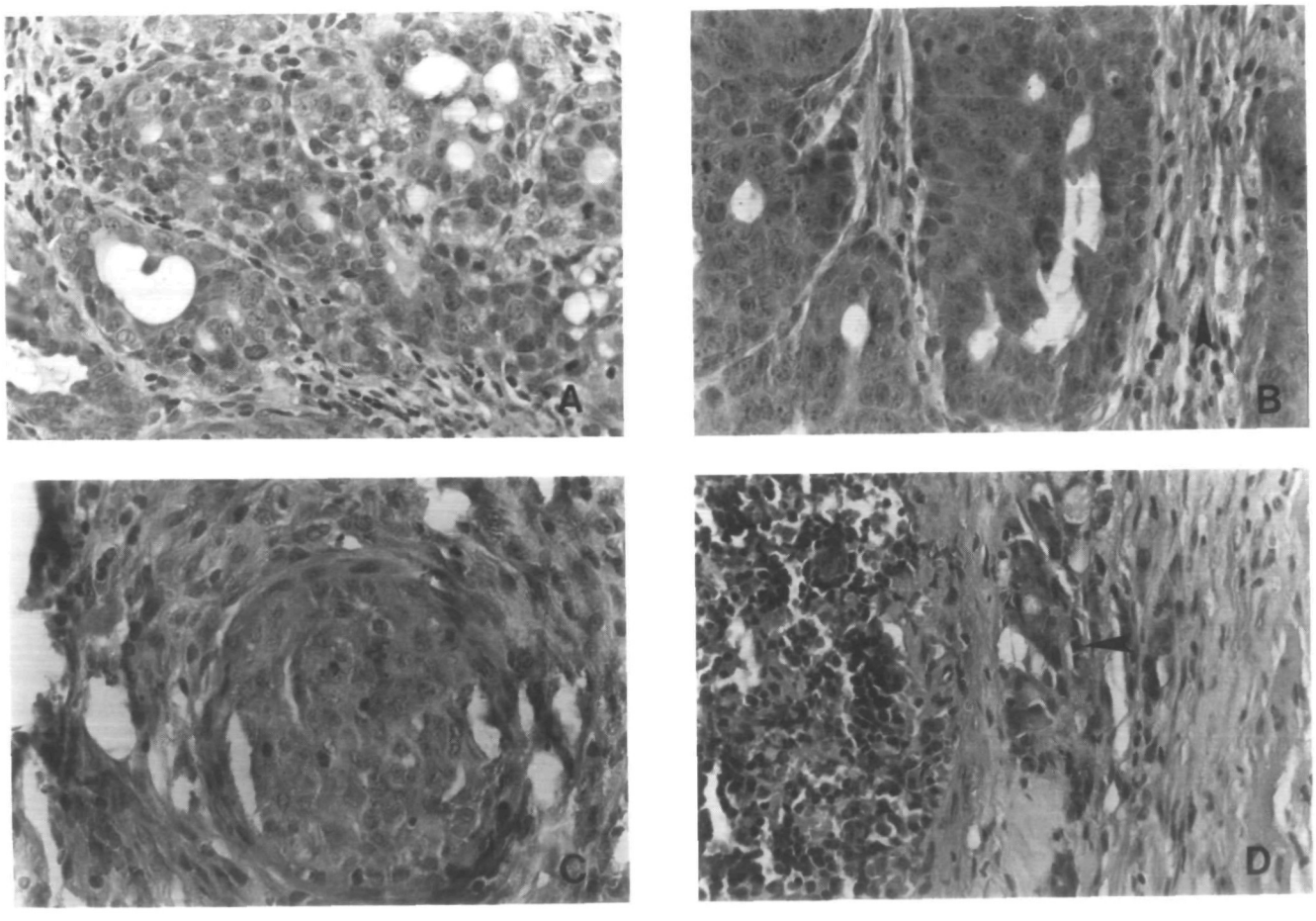


**Fig. 3.** (A) Adenocarcinoma, mammary gland. Magnification ×400. (B) Metastatic adenocarcinoma, lung. Cell morphology and pattern similar to carcinoma shown in (A); compressed lung parenchyma seen between metastatic deposits (arrow). Magnification ×400. (C) Tumor embolus within a blood vessel, narrowing its lumen to a slit. Collapsed lung alveoli at the periphery. Magnification ×400. (D) Mammary gland adenocarcinoma metastasis to splenic capsule (arrow). Magnification ×400.

administration decreased with increasing dose of MNU injected at 35 days of age (Figure 1, Table III), but was not affected by the age at which the 50 mg/kg dose of MNU was administered. Cumulative tumor number increased linearly with increasing dose of MNU, except at the 75 mg/kg dose in which the response was curvilinear. The rate of multiple tumor appearances in rats injected with 50 mg MNU/kg was essentially identical irrespective of the age at which MNU was injected (Figure 2). As shown in Table IV, induction of benign mammary tumors tended to increase with increasing dose of MNU, but no consistent patterns were associated with occurrence of benign tumors. Table V provides a summary of non-mammary tumors that were observed in each treatment group. The number of non-mammary tumors increased with increasing dose of MNU but was not significantly affected by the age at which MNU was injected.

As shown in Figure 3 and summarized in Table VI, injection of MNU at 35 or 42 days of age resulted in the induction of primary mammary neoplasms that metastasized to lung, spleen or liver. These lesions were detected by gross examination at necropsy and two of the mammary carcinomas that metastasized to the lung were detected within the first 90 days following carcinogen administration.

## Discussion

The data shown in Table III and Figures 1 and 2 are consistent with other reports that the mammary gland of the rat can be initiated with a chemical carcinogen between birth and 50 days of age and give rise to a significant tumorigenic response (4–11). However, the data presented in this study extend those observations. First, in none of the studies cited was a carcinogen dose response performed in rats younger than 50 days of age. In the present study, 35 days of age was chosen for the dose response since this was an age at carcinogen injection commonly used in earlier work (Tables I and II). The data presented in Table III and Figures 1 and 2 clearly show the tumorigenic response to be dose dependent. A second finding that is contrary to earlier reports is that the mammary gland was not significantly more responsive to carcinogen administration at earlier ages (6,9). Rather in terms of tumor latency, the response to injection of 50 mg MNU/kg was essentially identical when the carcinogen was injected at 28, 35 or 42 days of age. A third observation of importance was that a dose of 75 mg MNU/kg was well tolerated by rats injected at 35 days of age and resulted in a proportional increase in the number of tumors induced in comparison to the lower doses of carcinogen administered at the same ages. Other work has raised the question about what doses of MNU are tolerated (9). These data provide a definitive basis for those wishing to design experiments in which MNU is injected prior to 50 days of age. This information may be of particular value for investigators who want to set risk for mammary tumor development via choice of carcinogen dose prior to sexual maturation of the rat in order to evaluate the effect of changes in age of sexual maturation on the tumorigenic response in the mammary

gland. Furthermore, if stage of estrous cycle at which carcinogen is administered is a consideration in determining tumor outcome as suggested in several papers (18,19), this situation can be avoided by injecting MNU prior to the onset of vaginal opening and estrous cycling. The ability to inject MNU at an early age should also offer new opportunities for those interested in studying effects on mammary tumor development that require rats to be assigned to treatment immediately after weaning, e.g. when it is necessary to initiate induction of a nutrient deficiency at weaning. Finally, the ability to begin experiments at an earlier age would reduce the cost of conducting such studies, since young animals are less expensive to purchase and experiments could be completed over a shorter time frame. The data shown in Table V indicate that there is some loss of specificity in tumor induction for the mammary gland when MNU is injected prior to 50 days of age. However, the effect is relatively small and did not significantly alter intercurrent mortality.

Another potentially significant finding is shown in Table VI and Figure 3. Injection of MNU at 35 or 42 days of age resulted in the induction of mammary carcinomas, a small percentage of which metastasized within the 8 month course of the experiment. Of particular note is that some metastases were detected as early as 3 months after the injection of MNU. This unexpected observation, which has been confirmed in subsequent experiments, is not without precedent. Guillino *et al.* (11) reported that MNU-induced mammary tumors readily metastasize, though others have failed to confirm that observation (10,20). Our laboratory has injected large numbers of animals with 50 mg MNU/kg body wt at 50 days of age for over a decade and never have we observed metastases from primary mammary tumors within the time frame of the present experiment. The fact that we have observed metastases reproducibly in three sequential experiments when carcinogen was injected between 35 and 42 days of age suggests that these observations are unlikely to be due to chance alone. Given that a significant deficiency of both the MNU- and the DMBA-induced mammary tumor systems is that mammary carcinomas rarely metastasize (13,20), the findings reported in Table VI suggest a possible approach for the development of a mammary tumor model that has metastasis as a reproducible facet of its natural history.

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