**Determination of the Number of Events Required for Mammary Carcinogenesis in the Sprague-Dawley Female Rat**

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**ABSTRACT**

Female Sprague-Dawley rats were exposed to a single, graded dose of either of two highly effective mammary chemical carcinogens, 7,12-dimethylbenzanthracene (DMBA) or N-methylnitrosourea, in order to determine the number of mammary cancers per rat induced by a range of carcinogenic doses. These data were then used to separately construct dose-response curves characteristic for DMBA- and N-methylnitrosourea-induced mammary carcinogenesis. Analysis of these characteristic dose-response curves demonstrated that, following a single exposure to either DMBA or N-methylnitrosourea, the number of mammary cancers per rat increased not linearly but as the second power of dose of carcinogen used. These results are clearly incompatible with mammary carcinogenesis being a single step process in the female Sprague-Dawley rat. In direct contrast these results are entirely consistent with a malignant process requiring two transformation events. When female Sprague-Dawley animals are exposed multiple times to a suboptimal dose of DMBA, the number of mammary cancers induced per rat increases synergistically, not merely additively, as compared to a single dose exposure. Again this result is consistent only with mammary carcinogenesis requiring at least two transformation events.

**INTRODUCTION**

Experimental and epidemiological studies have provided strong support for the belief that the probability of occurrence, \( P \), of a relevant cellular event required in the process of malignant transformation of a normal cell into a cancer is a function of both the dose and time of application of the carcinogen (1-8). Based upon this starting assumption if an animal is given a single pulse exposure to a carcinogen, then the time of application is fixed at a constant and \( P \) will now be a function (\( f \)) of only the dose (\( D \)) of carcinogen used. Thus

\[
P_r = f(D) = kD
\]

If \( N_0 \) is the total number of cells at risk within the target tissue of interest, then the number of cells (\( N_1 \)) within this tissue in which a relevant cellular event would be expected to occur following a single dose of carcinogen is given by the equation

\[
N_1 = P_1N_0 = k_1DN_0
\]

If only a single event is required for complete malignant transformation, then each \( N_1 \) cell should produce an individual cancer; therefore the number of cancers per animal should follow Equation \( B \) and increase linearly with the dose of carcinogen. As pointed out by Wollman (1), however, if more than a single event is required for complete transformation of an individual cell (i.e., multistep), then the number of cancers per animal should increase not linearly but as a higher power of dose. For example the number of cells (\( N_1 \)) having undergone a single step in the carcinogenic process is equal to

\[
N_1 = P_1N_0 = k_1DN_0
\]

where \( P_1 \) is the probability for the specific transformation event. A subset of \( N_1 \) cells can then undergo a second event in the process and the number of such cells (\( N_2 \)) would be equal to the product of the probability for the second event, \( P_2 \), times the number of \( N_1 \) cells or

\[
N_2 = P_2N_1 = P_2P_1N_0 = k_2k_1DN_0 = k_2k_1D^2N_0
\]

If \( n \) number of events are required for a cell to be completely transformed, then the number of fully transformed cells (\( N_n \)) is equal to

\[
N_n = P_n ... P_2P_1N_0 = k_2 ... k_1DN_0 = k_n ... k_1D^nN_0
\]

When \( n \) steps are involved the number of cancers per animal therefore should increase not linearly but as the \( n \) power of dose. Therefore based upon the assumption that the probability of occurrence for each of the individual steps required for complete carcinogenesis is a function of the dose of carcinogenic exposure (i.e., Equation \( A \)), it should be possible to evaluate if the malignant transformation of cells of a particular target tissue involves a single or multiple events by means of determining how the number of such cancers per animal varies with the dose of carcinogenic exposure. To make this distinction groups of animals should be exposed at a single time to varying doses of an effective carcinogen and then the mean number of cancers per animal which subsequently develop could be determined to evaluate how this cancer number varies with dose. To do this Equation \( E \) can be logarithmically transformed to the equation

\[
\log_{e} \text{Log of number of cancers per animal} = n \log D + \log (K)
\]

where \( K \) is equal to the product of the multiplication of the various proportionality constants (i.e., \( k_1 ... k_n \)) and \( N_0 \). If one plots the log of the number of cancers per animal versus log of dose of carcinogen and a straight line relationship is obtained (i.e., \( y = mx + b \)), then the value \( n \), the number of events required for carcinogenesis in the particular tissue, can be mathematically estimated from the slope (i.e., \( m \)) of the line.

While there are a series of chemical carcinogens which are known to be highly effective in inducing the development of mammary cancers in animals following a single exposure (9, 10), such a dose-response analysis has not been reported for mammary cancer. Analysis of the epidemiological data on human...
breast cancer has suggested strongly, however, that at least two steps are involved in mammary carcinogenesis (8). Therefore female Sprague-Dawley rats were exposed to a single, graded dose of either of two different, highly effective mammary chemical carcinogens, DMBA* or MNU, in order to perform the dose-response analysis described.

MATERIALS AND METHODS

Four hundred fifty random-bred virgin Sprague-Dawley female rats were obtained from Harlan Sprague-Dawley, Inc. (Madison, WI) and individually ear tagged for identification. At various ages as noted cohorts of these animals were divided into groups of 25 rats each. For the DMBA experiments groups of these animals were given either a single or multiple doses of sesame oil containing either none or varying amounts of DMBA by means of gastric intubation according to the method of Huggins et al. (9). For the MNU experiments groups of rats were given a single injection, via the femoral vein, of physiological saline (i.e., 0.85% NaCl solution) adjusted to pH 5.0 with 3% acetic acid and containing either none or varying amounts of MNU according to the method of Gullino et al. (10). DMBA and MNU were both obtained from Sigma Chemical Co. (St. Louis, MO). Following exposure to carcinogen all animals were housed in a Vickers Total Containment Isolation Unit (London, United Kingdom) for 1 month before being returned to normal animal rooms. Animals were housed in a room artificially lighted 12 h/day and maintained at a temperature of 23°C. All rats were palpated for mammary tumors twice weekly with the time of detection post-carcinogen exposure being individually recorded for each tumor which developed in each rat. Once palpable each tumor was measured weekly with calipers to determine its volume by methods described previously (11). Since the tumor volume-doubling time varies with the absolute size of any cancer (12), the accumulated tumor volume data were used to mathematically generate the appropriate Gompertz-fitted growth equation for each individual cancer according to the technique of Simpson-Herren and Lloyd (13). From its respective growth equation the individual doubling time, in days, for each cancer was calculated at a standard tumor size of 1 cm³. The rats in each dose group underwent a complete autopsy at the time of spontaneous death or at 200 days post-carcinogen exposure. The 200-day observation period was chosen for the following reasons. If a valid determination is to be obtained of the total number of mammary cells per rat which are fully transformed by any single dose of carcinogen, then a sufficiently long observation period postexposure must be allowed in order for each of these cells to produce a palpable tumor. In order for a cancer to be detected routinely by palpation, it must be at least 5 mm in diameter. These dimensions translate into a volume of approximately 0.06 cm³ which, assuming 10⁸ cancer cells/cm³ means that 6 x 10⁸ mammary cancer cells must be present for a tumor to be palpable. This cell number requires approximately 25 population doublings. Simpson-Herren and Lloyd (13) have demonstrated that DMBA-induced mammary cancers have a doubling time of 8 days at the time of initial tumor detection. Since it is known (12) that tumor-doubling time increases with tumor size (i.e., Gompertzian effect), a faster doubling time of less than 8 days is probably more realistic for the DMBA-induced mammary cancers during the period when they are undetectable by palpation. Therefore to be conservative, 200 days (i.e., 25 doubling times x 8 days/doubling = 200 days) post-carcinogen exposure were allowed before the animals were killed to allow any mammary cell which was completely transformed to grow to a size large enough to be detected by palpation. Just as important as allowing enough time for maximal cancer detection, the observation period should not be excessively long for two reasons: (a) if too long a period of observation is allowed, animals will begin to die from their cancer burden thus producing a dose-dependent difference in the overall survival percentage for each of the varying dose groups; (b) if too long a time period is allowed, cancers will develop not only from cells which were completely transformed at the time of single exposure to the carcinogen but also from cells which were only partially transformed at the time of exposure and which spontaneously undergo the additional steps needed for complete malignant transformation at a later time following exposure. Since the cancers produced from these partially transformed cells do not result solely from the single carcinogenic exposure, the number of these cancers per rat will not be a function of as high a power of dose as those cancers due solely to the single carcinogenic exposure (i.e., from fully transformed cells). The development of mammary cancers from partially transformed cells will thus produce a change in slope of the log of mammary cancers per rat versus the log of carcinogenic dose. Therefore as described in detail by Scherer and Emmelot (14), a constant time period must be chosen following exposure to a single dose of carcinogen which results in a log-log plot with only a single slope. In preliminary experiments it was found that greater than 80% of female SD rats exposed to the highest dose of DMBA or MNU used in the present studies were still alive at day 200 post-carcinogen exposure, even though each rat had multiple mammary cancers. If a longer observation period was used, however, there was a rapid decline in the percentage of the total exposure group surviving due to continuous growth of the mammary cancers. Therefore the maximum observation period which could be used following carcinogen exposure without having to make major statistical adjustments for a differential survival between the various dose groups was 200 days. In addition analysis of the data at this time point produced log-log plots which had no obvious break in their slopes.

At the time of autopsy all mammary tumors were removed and fixed in 10% buffered formalin, and then paraffin sections were prepared and stained with hematoxylin and eosin. Each mammary tumor was individually classified histopathologically according to the criteria of Van Zwieten (15). This histological evaluation thus allowed the number of mammary cancers per each rat to be individually assigned; therefore the mean number ± SD of mammary cancers per rat for each experimental treatment group could be determined at various times post-carcinogen exposure. The mean values for the mammary cancers per animal for each dose of carcinogen were determined at 10-day intervals from 0 to 200 days post-carcinogen exposure based upon the total animals per group still alive at each time point; this calculation thus takes into consideration intercurrent mortality.

Statistical Analysis. All group data are presented as the mean ± SE. Statistical analysis of the data was performed by a one-way analysis of variance (ref. 16, pp. 215-233) followed by Duncan’s multiple range test of the difference between group means (17). Linear regression, tests of null hypothesis, and 95% confidence intervals were performed by the method of Snedecor and Cochrane (Ref. 18, pp. 135-171).

RESULTS

Dose-Response Analysis Following a Single Exposure to DMBA. Six groups of 50-day-old female SD rats, containing 25 rats/group, were fed a single dose of sesame oil containing either 20, 15, 10, 5, 2.5, or 0 mg of DMBA per animal. Following DMBA dosing each of the rats was palpated routinely to determine the time of appearance of any mammary tumor which developed during the 200-day observation period. In addition once recorded as being palpable each tumor was measured at weekly intervals to determine its individual volume-doubling time. Since the female rat has 6 pairs of mammary glands at risk following DMBA exposure (i.e., 3 thoracic, 1 abdominal, and 2 inguinal on each side, for a total of 12 different glands), each rat has the possibility of developing multiple mammary cancers. Therefore the cancer induction data are expressed, not on the...
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basis of whether an individual rat did or did not develop mammary cancer, but as to the total number of mammary cancers per rat which developed. This was determined by killing all animals still alive at 200 days post-DMBA exposure and carefully examining each of the 12 different mammary glands individually for the presence of mammary tumors. Each tumor was then examined histologically to determine the number of mammary cancers per rat, Table 1. Using these histological data and assuming that a tumor found to be a mammary cancer at 200 days post-DMBA exposure was also a mammary cancer at the time of its initial tumor palpation, the temporal pattern of mammary cancer appearance was constructed (Chart 1A). These data demonstrate that the number of mammary cancers per rat increases with increasing doses of DMBA. In contrast the mean time (Table 1) as well as the actual distribution of times to mammary cancer detection (Chart 2A) are, however, very similar in each of the different dose groups. These results suggest that while the mean number of mammary cancers per rat does differ widely with dose, the individual mammary cancers induced by these different doses of DMBA are very similar from the standpoint of both histology and initial growth kinetics (i.e., the time required for an individual cancer to become initially palpable). In addition while the analysis of the subsequent growth rates of the individual mammary cancers once palpable revealed a substantial variability, as demonstrated by the more than 2-fold range of volume-doubling times postdetection is not statistically different between any dose group (Table 1). These results suggest that the same basic process of carcinogenesis occurs when DMBA is able to successfully induce a mammary cancer in a treated rat regardless of the actual dose of DMBA used; only the frequency of this induction is dose related.

To determine if DMBA-induced mammary carcinogenesis is a single or multistep process, the data in Table 1 were plotted, as discussed in the "Introduction," as the log of the mean number of mammary cancers per rat at 200 days post-DMBA exposure versus the log of DMBA dose (Chart 3A). Based upon a regression analysis of these data the log of the number of mammary cancers per rat is highly correlated with the log of DMBA dose (correlation coefficient, 0.99, which is significantly different from zero at $P < 0.001$). The slope $±$SE of this regression analysis is 1.97 $±$ 0.04 (95% confidence intervals, 1.84 to 2.11), which is a slope significantly greater than one at $P < 0.001$. From the slope of this linear relationship the number of events required for DMBA induction of mammary cancer in female SD rats is estimated to be 2.

Dose-Response Analysis Following a Single Exposure to MNU. In order to determine the generality of the requirement for two events in mammary carcinogenesis in female SD rats as demonstrated using DMBA, similar dose-response studies were performed using MNU as the carcinogen. As shown by McCormick et al. (18), the mammary glands of 50-day-old female SD rats are extremely susceptible to the carcinogenic effects of MNU. The importance of using MNU for these second dose-response studies is that, unlike DMBA which must be metabolically activated, MNU is a direct acting mammary carcinogen which requires no such activation (19). In addition MNU differs widely chemically from DMBA. Therefore 6 groups of 50-day-old female SD rats containing 25 rats/group were given a single i.v. injection of saline containing either 7.5, 6.0, 4.5, 3.0, 1.5, or 0 mg MNU per animal. At 200 days post-MNU exposure, all rats still alive were killed and processed as described previously for the DMBA experiments, and the number of mammary cancers per rat was determined (Table 1). The temporal pattern of mammary cancer appearance is presented in Chart 1B. These data demonstrate that the number of mammary cancers per rat increases with increasing doses of MNU. The mean time to mammary cancer detection (Table 2) as well as the overall distribution of the time to detection for each mammary cancer (Chart 2B), however, are very similar for each of the MNU dose groups. These results suggest that while the mean number of mammary cancers per rat does differ widely with MNU dose, the individual mammary cancers induced by these different doses are very similar both by histological criteria and from a growth kinetics standpoint (i.e., the time required for a cancer to become palpable). In addition while the analysis of the subsequent growth rates of individual mammary cancers once palpable revealed substantial variability, as demonstrated by the more than 2-fold range of volume-doubling time within each MNU dose group, the mean tumor volume-doubling times are not statistically different between any dose group (Table 2). These results suggest that the same basic process of carcinogenesis occurs when MNU is able to successfully induce a mammary cancer in a treated rat regardless of the actual dose of MNU used; only the frequency

<table>
<thead>
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<th>Dose of DMBA (mg)</th>
<th>No. of rats/group on Day 0</th>
<th>Mammary cancers/rat at Day 200</th>
<th>Mean time to mammary cancer detection (days)</th>
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</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
<td>2.50 ± 0.28</td>
<td>103 ± 4</td>
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<tr>
<td>15</td>
<td>25</td>
<td>1.48 ± 0.50</td>
<td>104 ± 4</td>
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<td>10</td>
<td>25</td>
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<td>5</td>
<td>25</td>
<td>0.16 ± 0.07</td>
<td>125 ± 10</td>
</tr>
<tr>
<td>2.5</td>
<td>25</td>
<td>0.04 ± 0.03</td>
<td>140 ± 2</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
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</tbody>
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* Statistically significant difference ($P < 0.05$) between the dose group immediately lower in amount of DMBA given (e.g., 5.0-mg group versus 2.5-mg group, etc.).

** Mean ± SD.

Only a single mammary cancer was detected in one animal of the entire group.
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Carcinogenic Effect of Multiple versus a Single Exposure to DMBA. The DMBA and MNU dose-response data both suggest that mammary carcinogenesis in the female SD rat is a multi-step process involving two transformation events. An additional method to check the validity of these data involves the comparison of the effectiveness of multiple, low dose carcinogenic exposure to induce mammary cancers. The rationale of this method is as follows. If only a single transformation event is required for mammary carcinogenesis, then each time an animal is exposed to a suboptimal dose of carcinogen capable of inducing only a low number of fully transformed cells (i.e., cancers) per single exposure, the same number of normal cells should be fully transformed per exposure. Therefore multiple exposures to this same suboptimal dose of carcinogen should produce only an additive effect on the total number of mammary cancers induced per rat (i.e., if x exposure, then x times the effect of a single exposure). Conversely if two or more events are required for mammary carcinogenesis it should be possible to give a dose of carcinogen, which given only once is too low to induce all of the transformation events in high frequency but which is still high enough to induce at least one of the events with a substantial frequency. Under this latter condition multiple exposures to the same dose of carcinogen should produce not merely an additive, but a synergistic effect (i.e., if x exposure, then >x times the effect of a single exposure). This is because the first exposure, besides fully transforming an occasional normal cell to become a cancer, also will create a pool of partially transformed cells which have undergone only one of the needed steps for complete transformation. These partially transformed cells will require only one additional event for complete transformation; therefore these cells will be at a higher risk to complete their conversion during the subsequent exposures to the carcinogen. Each subsequent low dose carcinogen exposure therefore would be expected to do three things: (a) induce both transformation events in an occasional completely normal cell; (b) induce the second transformation in some of the partially transformed cells created by the previous carcinogen exposure; and (c) further increase the pool of partially transformed cells. Therefore each subsequent exposure to the suboptimal dose of carcinogen would be expected to synergistically, not additively, increase the number of fully transformed mammary cells (i.e., cancers) per animal.

To determine whether multiple exposures to a suboptimal dose of carcinogen produce additive or synergistic effects on the number of mammary cancers per animal, two preconditions must be established: (a) a dose of carcinogen must be chosen which is suboptimal for high cancer yields but which still induces some mammary cancers even when given as a single exposure. Table 1 demonstrates that a 5-mg dose of DMBA meets this first condition; (b) since multiple exposures to the 5-mg dose of DMBA are to be given this requires dosing the animals over a range of host ages. It is therefore critical to establish as a control that a 5-mg dose of DMBA produces an equal effect when given at any host ages. Therefore female SD rats were given a single p.o. dose of 5 mg of DMBA at 36, 43, 50, or 57 days of age (Table 1). The results demonstrated that there is no difference between the number of mammary cancers induced per rat by a single exposure to 5 mg of DMBA during this 4-week period, the average of the four means being 0.19 mammary cancer/rat.

To determine if MNU-induced mammary carcinogenesis is a single or multistep process, the data in Table 2 were plotted as the log of the number of mammary cancers per rat. Based upon regression analysis of the data the log of the number of mammary cancers per rat is highly correlated with the log of MNU dose, at a correlation coefficient of 0.99, which is significantly different from zero at P < 0.001. The slope ±SE of this regression analysis is 2.15 ± 0.14 (95% confidence intervals, 1.70 to 2.60), which is a slope significantly greater than one at P < 0.01. From the slope of this linear relationship the number of events required for MNU induction of mammary cancer in female SD rats is estimated to be 2.
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At 50 days of age virgin female Sprague-Dawley rats received a single i.v. injection of physiological saline containing either no or varying doses of MNU. Animals were palpated biweekly between 0 and 200 days post-injection to document the development of all mammary tumors. Upon detection the volume of each tumor was individually measured to determine its growth rate. All tumors were evaluated histologically.

Table 2
Relationship between dose of MNU and induction of mammary cancer

At 50 days of age virgin female Sprague-Dawley rats received a single i.v. injection of physiological saline containing either no or varying doses of MNU. Animals were palpated biweekly between 0 and 200 days post-injection to document the development of all mammary tumors. Upon detection the volume of each tumor was individually measured to determine its growth rate. All tumors were evaluated histologically.

Table 3
Relationship of multiple versus single dose of DMBA on the induction of mammary cancer

At various ages as indicated groups of 25 virgin female Sprague-Dawley rats each received a single or multiple p.o. feeding of sesame oil containing various doses of DMBA. Animals were palpated biweekly between 0 and 200 days post-initial DMBA exposure to detect mammary tumor development. All tumors were individually classified histologically.

DISCUSSION

The pathogenesis of mammary cancer development following a single exposure of 50-day-old female SD rats to DMBA has been studied in detail by Russo et al. (20). These studies have demonstrated that the earliest histological lesion detectable in the mammary glands of treated animals is a hyperplasia of the terminal end bud epithelium of the mammary ducts. These ductal lesions are microscopically detectable as early as 14 days post-carcinogen exposure and it is the continuous proliferation of these end bud lesions which produces the microscopically palpable intraductal carcinomas induced by DMBA (9). Likewise Guillon et al. (10) have demonstrated that the mammary carcinomas induced by MNU exposure are also of similar ductal origin. These results demonstrate that the terminal end bud epithelium of the developing mammary ducts is the same common target cell for both DMBA and MNU induced mammary carcinogenesis. In addition, DMBA and MNU exposure also induces a very similar range of histological types of mammary carcinomas (i.e., tubulopapillary, compact tubular, cribriform) (10, 15). These results suggest that the same basic mechanism is involved in mammary carcinogenesis induced by either DMBA or MNU.

Additional evidence supporting this conclusion is the demonstration that both DMBA- and MNU-induced mammary carcinogenesis involves changes in the c-rasH system. For example Sukumar et al. (21) have demonstrated that induction of mammary carcinomas by MNU involves the specific activation of the c-ras gene locus by a single point mutation. Similarly Huang and Cho-Chung (22) have demonstrated that DMBA-induced mammary cancers have enhanced expression of the same c-rasH.
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oncogene. These studies therefore suggest that one of the necessary transformation events involved in mammary carcinogenesis in the rat may involve changes in the c-rasH system. The DMBA and MNU dose-response analysis performed in the present study, however, clearly suggests that two transformation events are required for mammary carcinogenesis in the female SD rat. These findings are consistent with the growing evidence obtained with a variety of other tissues that complete malignant transformation may require two or more oncogenes acting in concert (23–25).

This suggestion is supported by the recent studies of Stewart et al. (26) using transgenic mice which had incorporated into the genome of their germ cells a synthetic fusion gene containing the normal mouse cellular myc protooncogene (i.e., c-myc) coupled to a hormonally inducible mouse mammary tumor virus promoter sequence. In this way the increased expression of the c-myc gene in female transgenic mice could be induced during pregnancy by prolactogenic hormones and the effect of this increased c-myc expression upon cancer induction could be determined. These studies demonstrated that parental female mice carrying the c-myc fusion gene, and their female F1 progeny, do develop mammary cancers but only after their second to third pregnancy. In addition in each of the affected female mice only 1 or 2 of the total of 12 different mammary glands per animal produce mammary cancers. This suggests that increased expression of the c-myc gene is a predisposing factor accelerating the development of mammary cancers but by itself not sufficient for the complete induction of mammary carcinogenesis. Taken together these molecular studies are entirely consistent with the dose-response data presented in the present paper which indicates that two transformation events are required for complete mammary carcinogenesis. Along these lines it will be critical to determine if the combined alteration in the expression of both the c-rasH and c-myc oncogenes are completely sufficient to explain the multistep nature of mammary carcinogenesis or whether additional factors are required.

An understanding of the multistep nature of mammary carcinogenesis, besides being of basic scientific importance, has several significant practical implications. One of the most important implications is that mammary carcinogenesis does not have to occur as an all or none phenomenon. Following a single exposure to a mammary carcinogen, none of the normal mammary cells might necessarily undergo all of the events required for complete transformation. A small number of these cells, however, might still undergo at least one of the events required and thereby become partially transformed. While this partial transformation would not immediately produce a mammary cancer, it would create a pool of mammary cells which have an increased probability of undergoing the additional events required for mammary cancer development. In this way multiple exposures to apparently suboptimal doses of carcinogens could eventually produce mammary cancers. The validity of this point is demonstrated by the data presented in Table 3 which illustrates that suboptimal doses of a carcinogen can synergistically induce mammary cancers when given as multiple exposures.

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