Inheritance of a Genetic Factor from the Copenhagen Rat and the Suppression of Chemically Induced Mammary Adenocarcinogenesis¹

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ABSTRACT

Female rats of ten different inbred strains were tested for their susceptibility to 7, 12-dimethylbenz[a]anthracene (DMBA)-induced mammary adenocarcinogenesis. Five of these strains (OM, NSD, WF, LEWIS, BUFF) were demonstrated to be highly susceptible to DMBA exposure development of >2 continuously growing, macroscopically detectable mammary adenocarcinomas/rat following gastric intubation with DMBA. In contrast, the COP female rat is completely resistant. Cross-breeding COP to any of the highly susceptible strains produced F₁ hybrids which are resistant to DMBA-induced mammary adenocarcinogenesis. Cross-breeding the same highly susceptible strains to the Fischer strain (i.e., a strain only intermediately susceptible to DMBA) produced F₁ hybrids which were just as highly inducible as their highly susceptible non-Fischer parental strain. The resistance of the F₁ hybrids produced by cross-breeding these highly susceptible strains to the COP strain therefore is not due to a recessive lack of susceptibility but to an active process of suppression of the high susceptibility of these F₁ hybrids to DMBA-induced mammary adenocarcinogenesis.

To determine if the site of action of the COP gene responsible for this suppression is in the mammary gland itself or at the host systemic level, a series of factors (i.e., host growth rate, mammary gland growth rate, systemic hormone level during the estrus cycle, serum and tissue levels of DMBA and its metabolites) were compared between female rats of the highly susceptible NSD versus the resistant COP strain. These results suggested that host systemic factors are not involved. To test this directly, donor mammary glands from the highly susceptible NSD and the resistant COP strains were transplanted into F₁ hybrids produced by cross-breeding these two strains. The resultant donor glands were then directly exposed to DMBA, the animals were followed, and the incidence of mammary adenocarcinomas was determined. Seventy % of the NSD donor glands developed continuously growing cancers while only 10% of COP donor glands did the same. These results are clearly incompatible with host systemic factors being the major determinant in the resistance of NSD x COP F₁ hybrids to DMBA-induced mammary adenocarcinogenesis. Instead, these results directly demonstrate that it is the genetic makeup of the donor mammary gland itself which determines its response to DMBA exposure.

INTRODUCTION

Previous studies by a variety of investigators have demonstrated that there is a definitive gradation between the various strains of rats and their susceptibility to mammary adenocarcinogenesis induction by either of two highly effective mammary chemical carcinogens, DMBA or MNU. Genetic analysis has demonstrated that the resistance of the mammary epithelium of the female COP rat to either DMBA or MNU is due to the Mendelian inheritance of a single dominant, autosomal genetic allele. The inheritance of a single copy of this resistance allele is able to suppress both the DMBA- and MNU-induced development of mammary adenocarcinomas in F₁ hybrids produced by cross-breeding COP to the highly susceptible NSD or WF animals (1).

In order to test further the generality of the ability of the COP resistance allele to suppress DMBA-induced mammary adenocarcinogenesis, a series of additional strains of inbred rats was cross-bred to the COP rat and the resulting F₁ hybrids were tested for their response to DMBA exposure. In addition, a series of factors was compared between 50-day-old female rats of the highly susceptible NSD versus the resistant COP strain in order to determine if the genetically controlled resistance of the female COP rat to DMBA-induced mammary adenocarcinogenesis is due to an intrinsic resistance of the mammary epithelial cells themselves or to some general host systemic factor (e.g., systemic hormonal environment, etc.) which secondarily allows the mammary epithelial cells to become resistant.

MATERIALS AND METHODS

Animals. Inbred ACI/Seg (ACI/SegHsdBr), inbred Fischer (F344/NHsdBr), inbred Wistar-Furth (WF/NHsdBr), inbred COP (COP/NHsdBr), inbred Lewis (LEW/NHsdBr), and inbred Buffalo (BUFF/NHsdBr) female rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The inbred Sprague-Dawley (NSD/N), the inbred Osborne-Mendel (OM/N), and the inbred Wistar (WN/N) animals were derived from an in-house colony established from breeding nuclei of animals obtained through the generosity of the Veterinary Resources Branch of the NIH Genetic Resources. The inbred August (AUG/Har) was generously supplied by Dr. G. M. Harrington, University of Northern Iowa, Department of Psychology. Following exposure to carcinogen, as described below, all animals were housed in a Vickers Total Containment Isolation Unit (London, UK) for 1 mo before being returned to normal animal rooms. Animals were housed in a room lighted 12 h/day and maintained at a temperature of 20°C. All animals were palpated for mammary tumors twice weekly with the time of detection post-carcinogenic exposure being individually recorded for each tumor which developed in each rat.

The rats in each group underwent a complete autopsy at the time of spontaneous death or at 365 days post-carcinogen exposure. At the time of autopsy all grossly detectable mammary tumors were removed and fixed in 10% buffered formalin and paraffin sections were prepared and stained with hematoxylin and eosin. Each mammary tumor was individually classified histopathologically according to the criteria of Van Zwieten (10). This histological evaluation allows the number of detectable mammary adenocarcinomas, sarcomas, and fibroadenomas for each rat to be individually assigned; therefore, the mean number of mammary adenocarcinomas per rat for each experimental treatment group could be determined.

Carcinogenic Exposure. For the systemic exposure to DMBA, groups
of animals were given a single dose of sesame oil containing 20 mg of DMBA by means of gastric intubation according to the method of Huggins et al. (5). For the direct in vivo exposure of mammary glands to DMBA, the technique of Sinha and Dao (11) was used. This involves anesthetizing rats with Metapana (Pittman-Moore, Inc., Washington Crossing, NJ), exposing the mammary gland, dusting 3 mg of a 2:1 mixture of cholesterol and DMBA (i.e., 1 mg DMBA total) directly onto the exposed gland, and then closing the skin incisions with stainless steel autoclips.

Mammary Gland Growth Rate Determination. The growth rate of the mammary gland epithelium from 20 to 200 days of life in NSD, COP, and their F1 hybrids was estimated from the slope of the plot of the log of the mean volume (mm³) of the right inguinal abdominal mammary gland ductal network versus the respective host age. The volume of the right inguinal-abdominal mammary gland ductal network was determined by the method of Cowie and Folley (12). This entails removal of the entire inguinal-abdominal mammary fat pad from the right side of each rat. This tissue is then fixed for 24 h in methanol:acetic acid (3:1) followed by defatting in aceton for an additional 24 h. The tissue is then stained with Harris’ hematoxylin and a wholemount preparation prepared by placing the stained tissue between two large glass slides. The thickness of the tissue wholemount is determined by caliper measurement and, using a dissecting microscope, the outline of mammary ductal network is traced with a marking pen onto the top glass slide. The outline of the marked slide is then traced on a Hewlett-Packard Model 9844A digitization pad (Palo Alto, CA) which is connected to a Hewlett-Packard Model 9825B computer programmed to compute the surface area (mm²) of the ductal network from the tracing. By multiplying the thickness of the tissue wholemount by the surface area, the total mm³ of ductal epithelium was calculated for each right inguinal-abdominal mammary gland complex.

Biochemical Assays. The serum levels of estradiol, progesterone, and prolactin were determined through the normal estrus cycle of NSD and COP female rats. Vaginal smears were performed on groups of these rats daily to determine the individual position of each rat in the estrus cycle. Each animal was followed for at least two cycles to determine the uniformity of its cycling behavior. At 7 a.m. and 7 p.m. on diestrous, proestrus, estrus, and metestrus day of the cycle, five rats were killed and blood was collected. The resultant serum was assayed for its estradiol content using a solid-phase 3H-estradiol radioimmunoassay kit (catalog no. 1024) obtained from Radioassay Systems Laboratories (Carson, CA). Serum progesterone was assayed using a liquid phase 3H-progesterone radioimmunoassay kit (catalog no. 1024) obtained from Radioassay Systems Laboratories (Carson, CA). Serum prolactin was assayed using the reagents and protocol provided by the National Institutes of Arthritis, Diabetes, Digestive Diseases, and Kidney. This assay was performed on 200 µl of whole serum in duplicate using National Institutes of Arthritis, Diabetes, Digestive Diseases, and Kidney rat prolactin (PR3) as standard. The 3H-labeled prolactin used for the assay was purchased from New England Nuclear (Boston, MA) (catalog no. NEN-108). This material was further purified before use by passage over a Sephadex G-75 column.

Metabolism of DMBA in Vivo. 3H]DMBA of high specific radioactivity (47 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). The 3H]DMBA was diluted with carrier DMBA obtained from Sigma (St. Louis, MO) to give a final specific activity of 20 µCi/µmol. Nine 50-day-old female rats of both inbred NSD and COP strains were given 10 mg of this 3H]DMBA mixture in 1 ml of sesame oil via gastric intubation. At 1 and 3 h post-DMBA feeding, blood was collected via retroorbital puncture from 3 different rats of each of the NSD and COP groups. At 6, 16, and 24 h post-DMBA feeding, 3 rats had blood collected via cardiac puncture, and then these animals were killed and a 1-g portion of liver and the entire right and left inguinal-abdominal mammary gland complex were removed separately. For the blood determinations, 100 µl of serum were completely digested by adding 1 ml of Soluene 350 tissue solubilizer (Packard Instrument Corp., Downers Grove, IL). The mixture was heated at 50°C for 3 h, then 10 ml of Dimilume-30 scintillation cocktail (Packard Instrument Corp.) was added, and the mixture was counted. The radioactive counts per minute were converted to disintegrations per minute per sample by correcting for quenching using the external standard ratio method. These disintegrations per minute values were then converted based on the starting specific activity of the [3H]DMBA fed to pmol of DMBA-related compounds and the results expressed as the serum concentration (µM) of total DMBA compounds. Additional aliquots of serum (10 µl) were applied to individual lanes of Whatman LK63 silica gel TLC plates, 20 cm in length (Whatman Labsales Inc., Hillsboro, OR). These silica gel plates have a preadsorbtion sample area composed of diatomaceous earth to which the unextracted serum sample is directly applied. Due to this unique feature of these TLC plates, no prior extraction of the serum is required. In addition, 20 µl of a 0.25 mg/ml standard solution of DMBA in ethanol were added as carrier to each lane of the TLC plates. The TLC plates were developed using a 95:5 benzene:ethanol solvent system to separate unmetabolized DMBA from its polar metabolites. After the TLC separation, the plate was examined with a fluorescence lamp to localize the exact position of the carrier DMBA standard (i.e., RF = 0.90) which was marked. Each lane was then divided into 1-cm fractions which were individually counted for their radioactivity. The radioactivity of each fraction was then added to determine the total radioactivity per entire lane. This total was divided into the radioactivity found isopolar with the DMBA standard to determine the percentage of DMBA unmetabolized in the serum. This relative percentage was then multiplied by the concentration of total DMBA compounds, determined on the digested serum, to determine the concentration of unmetabolized DMBA in the serum. By subtracting the concentration of unmetabolized DMBA from the concentration of total DMBA compounds, the concentration of polar DMBA metabolites was calculated.

For the tissue determinations, a 1-g aliquot of liver, previously perfused to eliminate blood, and the combined right and left inguinal abdominal mammary gland tissue (i.e., approximately 2 g) from each rat were separately homogenized in 10 ml of water with an all-glass conical homogenizer. The separate homogenates were placed in a 50-ml screw top centrifuge tube and 0.5 ml of the homogenate was saved for total DNA determination by the method of Coffey et al. (13) and 0.1 ml of the homogenate was completely digested by adding 1 ml of Soluene 350 tissue solubilizer and heating at 50°C for 3 h. This mixture was then added to scintillation cocktail and counted to determine the total nanomoles of DMBA-related compounds (i.e., DMBA plus its metabolites) in the total 10 ml of starting homogenate. From the DNA determination, the total number of cells present in the original homogenate was determined using a value of 10 pg of DNA/cell (determined by measuring the amount of total DNA assayed on autoradiographs of prometaphase mammary gland nuclei). By dividing the total nanomoles of DMBA-related compounds by the total number of cells present, the nanomoles of total DMBA-related compounds (i.e., macromolecular-bound and unbound) per 10⁹ cells was determined. To the remaining 9.4 ml of original homogenate in the 50-ml tube, 20 ml of ethyl-ether were added and the mixture was shaken for 10 min and then centrifuged at 50 g for 10 min. The tube was then placed in a dry ice-acetone bath to freeze the lower aqueous phase. The ether phase was decanted and then taken to dryness and counted with 10 ml of scintillation fluid (i.e., unbound ether-soluble fraction). The aqueous phase (i.e., 9.4 ml) was thawed and divided into a 3-ml and a 6.4-ml aliquot. To the 3-ml aliquot was added 0.5 ml of 1.6 M PCA and to the 6.4-ml aliquot was added 1.0 ml of 1.6 M PCA to give a final PCA concentration of 0.22 M to quantitatively precipitate the macromolecules present (i.e., RNA, DNA, protein) (14). The 3.5-ml and 7.4-ml mixtures (mixtures A and B, respectively) were centrifuged at 10,000 X g 10 min to pellet the macromolecules. The supernatant from mixture A was decanted and an aliquot counted with 10 ml of scintillation fluid (i.e., unbound water-soluble). The pellet from mixture A was solubilized with Soluene 350 and heating and then added to 10 ml of scintillation fluid and counted (i.e., macromolecular-bound fraction). The total counts in the three separate fractions (i.e., unbound ether-soluble, unbound water-soluble, and molecular-bound fractions) were then summed and this value was used to calculate the relative percentage of the total DMBA-associated radioactivity obtained in each of the three separate fractions. These respective relative percentages were then multiplied by the nanomoles of total DMBA-related compound per 10⁹ cells previously determined.
SUPPRESSION OF MAMMARY CANCER

for the total starting homogenate to calculate the nanomoles of DMBA-related compound in each of the unbound ether-soluble, unbound water-soluble, and molecular-bound fractions per $10^6$ cells.

In order to determine the amounts of DMBA compounds bound specifically to RNA, DNA, and protein, the pellet from mixture B was sequentially extracted using a slight modification of the procedure of Munro and Fleck (14). This entailed incubating the pellet in 4 ml of 0.3 M KOH at 37°C for 2 h to quantitatively hydrolyze the RNA. After this incubation, the mixture was cooled on ice and the protein and DNA were quantitatively precipitated by adding 2.5 ml of 1.2 M PCA. The mixture was centrifuged at 10,000 $\times g$ for 10 min, and supernatant containing the hydrolyzed RNA was counted on a scintillation counter to determine the nanomoles of DMBA metabolites bound to RNA per $10^6$ cell. The pellet was next incubated with 4 ml of 0.8 M PCA at 70°C for 20 min to quantitatively hydrolyze the DNA. After incubation, the mixture was cooled on ice and then centrifuged at 10,000 $\times g$ 10 min to pellet the protein. The supernatant containing the hydrolyzed DNA was counted on a scintillation counter to determine the nanomoles of DMBA metabolites bound to DNA per $10^6$ cells. The pellet containing these precipitated proteins was then solubilized with 1 ml of Soluene 350. The mixture was then added to Dimiluene 30 scintillation fluid and counted to determine the nanomoles of DMBA metabolites bound to protein per $10^6$ cells.

RESULTS

Susceptibility versus Resistance of Various Inbred Rat Strains to Chemically Induced Mammary Adenocarcinogenesis. Fifty-nine-day-old female rats of a series of 10 different inbred strains were tested for their susceptibility to mammary adenocarcinoma development following a single gastric intubation feeding of 20 mg of DMBA (Table 1). Examination of Table 1 reveals that there are four distinct genotypes displayed by the various inbred strains tested with regard to DMBA-induced mammary adenocarcinogenesis. These genotypes are (a) highly susceptible, developing >2 continuously growing, macroscopically detectable mammary adenocarcinomas/rat (i.e., OM, NSD, WF, LEWIS, and BUFF strains); (b) intermediately susceptible, developing 1 but <2 adenocarcinomas/rat (i.e., Fischer strain); (c) low susceptible, developing >1 but <0.1 adenocarcinomas/rat (i.e., AUG, ACI, and WN strains); and (d) resistant, developing no macroscopically detectable mammary adenocarcinomas/rat (i.e., COP strains). The fact that the COP is truly resistant to DMBA-induced mammary adeno-carcinogenesis has been demonstrated previously (1). These studies demonstrated that no matter how the DMBA is given (p.o., i.v., or direct application) or how many exposures, the female COP does not develop continuously growing, macroscopically detectable mammary adenocarcinomas.

These results demonstrate that rat strains derived from the Wistar genetic background (i.e., BUFF, LEWIS, WF, NSD) are usually highly susceptible to mammary cancer development as compared to non-Wistar-derived strains (i.e., Fischer, AUG, ACI, COP). (Note: for the detailed history and relatedness of the various rat strains, see Ref. 15.) However, this relationship is not universal since the Wistar-derived WN strain is of the low-susceptibility phenotype and the non-Wistar-derived OM strain is just as highly susceptible as the highly inducible Wistar-derived strains (i.e., SD, WF, NSD, LEWIS, and BUFF).

Table 1: Mammary adenocarcinoma development in various inbred strains of female rats following a single gastric intubation with 20 mg of DMBA

<table>
<thead>
<tr>
<th>Strain of rat</th>
<th>Mammary cancers/rat $^a$</th>
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<tbody>
<tr>
<td>OM (20)</td>
<td>5.00 ± 0.45</td>
</tr>
<tr>
<td>WF (25)</td>
<td>2.76 ± 0.26</td>
</tr>
<tr>
<td>NSD (25)</td>
<td>2.48 ± 0.30</td>
</tr>
<tr>
<td>LEW (25)</td>
<td>2.2 ± 0.35</td>
</tr>
<tr>
<td>BUFF (20)</td>
<td>2.2 ± 0.45</td>
</tr>
<tr>
<td>Fischer (25)</td>
<td>1.2 ± 0.20</td>
</tr>
<tr>
<td>AUG (10)</td>
<td>0.40 ± 0.17</td>
</tr>
<tr>
<td>ACI (25)</td>
<td>0.30 ± 0.13</td>
</tr>
<tr>
<td>WN (15)</td>
<td>0.40 ± 0.16</td>
</tr>
<tr>
<td>Cop (30)</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SE.

Table 2: Mammary cancer development following a single gastric intubation with 20 mg of DMBA to female F1 hybrids produced by breeding inbred COP to other inbred strains of rats

<table>
<thead>
<tr>
<th>F1 hybrid produced by breeding $^b$</th>
<th>Mammary cancers/rat $^a$</th>
<th>Resistance ratio $^c$</th>
</tr>
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<tbody>
<tr>
<td>OM × Cop</td>
<td>0.40 ± 0.15</td>
<td>12.5</td>
</tr>
<tr>
<td>NSD × Cop</td>
<td>0.50 ± 0.15</td>
<td>8.3</td>
</tr>
<tr>
<td>WF × Cop</td>
<td>0.50 ± 0.15</td>
<td>5.5</td>
</tr>
<tr>
<td>BUFF × Cop</td>
<td>0.35 ± 0.20</td>
<td>6.3</td>
</tr>
<tr>
<td>Fischer × Cop</td>
<td>0.20 ± 0.15</td>
<td>6.0</td>
</tr>
<tr>
<td>AUG × Cop</td>
<td>0.10 ± 0.16</td>
<td>4.0</td>
</tr>
<tr>
<td>ACI × Cop</td>
<td>0.05 ± 0.02</td>
<td>6.0</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SE.

$^b$ Resistance ratio = number of mammary cancers per rat in the non-COP parental strain divided by number of mammary cancers per rat in the F1 hybrid.

$^c$ Ratio of number of mammary cancers per rat in the non-COP parental strain divided by number of mammary cancers per rat in the F1 hybrid.

$^d$ 20 Female F1 hybrids per cross-breeding.

Effects of Cross-breeding on DMBA-induced Mammary Adenocarcinogenesis. Previous studies had demonstrated that cross-breeding COP rats with the highly susceptible NSD and WF inbred strains resulted in female F1 hybrids which were resistant to DMBA-induced mammary adenocarcinogenesis (1). Genetic analysis demonstrated that this resistance is due to the inheritance of a single copy of a dominant, autosomal genetic allele from the COP genome (1). To test the generality of the ability of this COP allele to suppress DMBA-induced mammary adenocarcinogenesis, two additional highly susceptible inbred strains, the OM and BUFF, were cross-bred to COP rats and the resultant female F1 hybrids exposed to 20 mg of DMBA p.o. at 50 days of age. These experiments (Table 2) demonstrated that these female F1 hybrids were between 6- and 12-fold more resistant to DMBA-induced mammary adenocarcinogenesis than their highly susceptible BUFF and OM parental female rats, respectively. Included in Table 2, for comparison, are the data for the female NSD × COP and WF × COP hybrids (1). These data demonstrate that the inheritance of a single COP allele by F1 hybrids, produced by cross-breeding highly susceptible inbred strains to COP, consistently results in a greater than 5-fold suppression of DMBA-induced mammary adenocarcinogenesis.

In order to test if inheritance of the COP allele suppressed the development of mammary cancer in female F1 hybrids regardless of the absolute starting susceptibility of the non-COP parental strain, additional female F1 hybrids produced by breeding various intermediate (i.e., Fischer) and low susceptibility (i.e., AUG and ACI) inbred strains to the COP strain were exposed to DMBA (Table 2). These results demonstrate that regardless of the starting susceptibility, all of the female F1 hybrids are more resistant to DMBA than their respective non-COP parental strains.

As a control to test the specificity of the ability of the COP strain to confer resistance to DMBA-induced mammary adenocarcinogenesis in its F1 hybrid produced by cross-breeding to highly susceptible strains, similar F1 hybrids were produced by cross-breeding the Fischer inbred strain to the same four highly susceptible strains of rats. Female F1 hybrids produced by breeding Fischer animals to either OM, NSD, WF, or BUFF rats were exposed to 20 mg of DMBA at 50 days of age (Table 3). Each of four different F1 hybrids was just as highly inducible...
as the respective high-susceptibility non-Fischer parental strain (i.e., no resistance was induced). These results demonstrate that dominantly acting genetic alleles are responsible for the high susceptibility to DMBA-induced mammary adenocarcinogenesis in the OM, NSD, WF, and BUFF inbred strains. This is important since if high susceptibility to DMBA-induced mammary adenocarcinogenesis had been due to recessive alleles, then cross-breeding high-susceptibility strains with intermediate or low-susceptibility inbred strains would produce less susceptible female F₁ hybrids. Since the results in Table 3 demonstrate that dominantly acting genetic alleles are responsible for the high susceptibility of the OM, NSD, WF, and BUFF female rat, this means that the resistance of the female F₁ hybrids produced by breeding either OM, NSD, WF, or BUFF to COP strain animals (Table 2) is not due to a recessive lack of susceptibility but to an active process of suppression of the high susceptibility to DMBA-induced mammary adenocarcinogenesis in these female F₁ hybrids.

Site of Action of the COP Suppression Gene. Dao and Sinha have demonstrated that direct exposure to DMBA of mammary glands, growing in vitro in organ culture, can lead to the development of mammary adenocarcinomas (16). This demonstrates that the rat mammary glands themselves can be directly induced to develop into a cancer without the effects of systemic factors present in vivo in the host rat. In vivo systemic factors (e.g., ovarian and pituitary hormones), however, can effect the ability of DMBA to induce mammary adenocarcinogenesis (17–19). It is thus crucial to determine if the site of action of the COP gene responsible for suppression of DMBA-induced mammary adenocarcinogenesis is in the mammary gland itself or at the systemic level.

Initially, to do this, a series of factors was compared between 50-day-old female rats of the highly susceptible NSD versus the resistant COP strain. The female NSD rat is a larger animal at 50 days of age than the corresponding COP rat, and therefore it has more mammary tissue than the COP female rat at 50 days of age (Fig. 1). Since the NSD × COP F₁ hybrids are intermediate in both body weight and mammary gland volume as compared to their parental strains (Fig. 1) but are still resistant to DMBA-induced mammary carcinogenesis, this suggests that a larger body weight or larger mammary volume alone is not able to increase the susceptibility to mammary cancer development in these female F₁ hybrids.

It is known in a variety of other organ systems that cell proliferation at the time of exposure is a critical element in determining whether chemical carcinogens can induce cancer development (20–22). With regard to the mammary gland specifically, it is known that one of the reasons why 50-day-old female rats of certain strains are so susceptible to DMBA-induced mammary carcinogenesis is that at this time of exposure, the mammary ductal epithelial cells are proliferating rapidly (23–26). Therefore, as an index of the proliferation rate of these ductal cells, the volume doubling time of the ductal network of the right abdominal-inguinal mammary glands of the female NSD, COP, and their F₁ hybrids were compared. These volume doubling times were estimated by plotting the log of volume of right inguinal-abdominal mammary gland ductal network versus host age in days for the three groups of animals (Fig. 2). Such plots reveal that between 20 and 50 days of host age, there is a linear relationship between the increase in log of ductal volume and host age (i.e., exponential growth) for each group. Therefore, by dividing the slope of this line determined between 20 and 50 days of life by the log 2, the volume doubling time during that time period can be estimated for each of the three groups of animals (Fig. 2). Such analysis reveals that at 50 days of age (i.e., time of DMBA exposure), the mammary ductal network of the female NSD, COP, and their F₁ hybrid progeny are growing essentially equally and at a rapid rate (i.e., 10- to 13-day doubling time). In addition, similar analysis during the period after 50 days of life reveals that the mammary ductal network volume doubling time increases substantially (i.e., growth rate decreases) as the host rat ages. This increase in mammary gland volume doubling time, however, is seen equally in the NSD, COP, and F₁ hybrids (Fig. 2). These results suggest that there is no major difference in the

### Table 3

<table>
<thead>
<tr>
<th>Cross-Breed</th>
<th>Mammary Cancers/ rat†</th>
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<tr>
<td>OM × Fischer ‡</td>
<td>4.70 ± 0.50</td>
</tr>
<tr>
<td>NSD × Fischer</td>
<td>2.40 ± 0.15</td>
</tr>
<tr>
<td>WF × Fischer</td>
<td>2.45 ± 0.80</td>
</tr>
<tr>
<td>BUFF × Fischer</td>
<td>2.35 ± 0.25</td>
</tr>
</tbody>
</table>

* Mean ± SE.
‡ 20 Female F₁ hybrids per cross-breed.
growth rate of the target mammary ductal cells at the time of DMBA exposure (i.e., day 50) or subsequently between the highly susceptible NSD or the resistant COP and F₁ hybrids.

Since the growth of mammary gland is under hormonal control, the serum levels of 17β-estradiol, progesterone, and prolactin were compared between 50-day-old NSD and COP female rats. Since at 50 days of age, both NSD and COP female rats are continuously proceeding through the estrus cycle, the serum levels for each of the hormones were determined at 7 a.m. and 7 p.m. each day of the estrus cycle for both the NSD and COP female rats (Fig. 3). These data demonstrate that there is no substantial difference in any of the serum hormone levels at any point in the estrus cycle. These hormonal findings are thus consistent with the equal growth rate of the mammary glands of the NSD and COP female rats at 50 days of age (Fig. 2). In addition, it was determined that following exposure to 20 mg of DMBA to 50-day-old NSD or COP female rats, both strains maintained their normal 4- to 5-day estrus cycle.

In order to evaluate if the difference between the high susceptibility of the NSD versus the resistance of the COP female 50-day-old rat was due to differences in the metabolism of DMBA by the two strains, the temporal pattern of serum concentrations of DMBA and its polar metabolites was determined for both strains during the 24-h period following oral feeding of 10 mg of DMBA (Fig. 4). The level of DMBA in the serum reaches a peak by 1 h after oral feeding and remains detectable for 24 h. The serum level of polar DMBA metabolites rose rapidly, however, the highest levels were observed between 16 and 24 h after feeding. Again, no major differences were observed between female rats of the NSD and COP strains.

The temporal pattern of total amount of DMBA-related compounds, the amount of unbound ether-soluble DMBA-related compounds, the amount of unbound water-soluble DMBA-related compounds (i.e., polar DMBA metabolites), and the amount of macromolecular-bound DMBA-related compounds were also determined for the mammary gland and liver of female NSD versus COP strains between 6 and 24 h following oral feeding of 10 mg of DMBA (Fig. 5). These results demonstrate that between 6 and 24 h following feeding, the amount of macromolecular-bound DMBA-related compounds is rather constant and represents only a small portion of the total amount of DMBA-related compounds present in the mammary glands of either the NSD or COP female rat. Again, there is no major difference between the mammary glands of the NSD versus COP female rat with regard to any of the parameters measured. Interestingly, the level of total DMBA-related compounds in the liver is lower, regardless of host strain, than those found in the mammary glands. The level of macromolecular-bound DMBA-related compounds is, however, very similar between the liver and mammary glands of each strain. The major difference between these two organs is the large amount of ether-
The amount of binding to protein, RNA, or DNA was not until 1 yr had elapsed following DMBA direct exposure. Mammary tumor development was not seen in the transplanted glands until either (a) a tumor developed in the transplanted glands during the first 24 h following gastric intubation of 10 mg of DMBA to 50-day-old female rats of the NSD and COP strains.

The macromolecular-bound DMBA-related compounds determined in Fig. 5 were further subdivided into those binding to protein, RNA, and DNA (Fig. 6). These results demonstrate that between 6 and 24 h following 10 mg p.o. of DMBA, most of the DMBA-related compounds are bound to protein with less being bound to RNA and even less being bound to DNA. The amount of binding to protein, RNA, or DNA was not different between the two strains.

Taken together, these results did not demonstrate that there were differences between the systemic environment of highly susceptible NSD female rats compared to the resistant COP animal. In order to determine if the site of action of the COP suppression allele, the expression of which determines the susceptibility versus resistance to DMBA-induced mammary adenocarcinogenesis, is within the mammary glands themselves, a mammary gland transplantation method was used. The right inguinal-abdominal mammary gland was separately transplanted into the interscapular region of 30-day-old female rats of the NSD and COP strains.

The right inguinal-abdominal mammary gland was removed from 10 NSD and 10 COP female rats when the donor animals were 30 days of age. These donor glands were individually transplanted into the interscapular region of 30-day-old female NSD x COP F₁ hybrids. At 50 days of age (i.e., 20 days following transplantation), an incision was made in the skin over the transplanted mammary glands in order to expose the tissue. One mg of DMBA was then "dusted" according to the direct application method of Sinha and Dao (11). The incision was closed and the female F₁ animal hosts were allowed to live until either (a) a tumor developed in the transplanted glands and reached a size of 1–2 cc, or (b) if no tumor became palpable, until 1 yr had elapsed following DMBA direct exposure. Macroscopically detected tumors were processed for histological classification as to whether they were an adenocarcinoma, sarcoma, or fibroadenoma, as described in "Materials and Methods." When no macroscopically detectable tumor was present at 1 yr following exposure, the implanted donor mammary gland was removed, fixed in buffered formaldehyde, and embedded in paraffin. The embedded tissue was then step-sectioned at 50-μm intervals and at least 4 sections at different levels within each tissue were processed for histological examination. In this way, the incidence of any microscopic foci of cancer could be evaluated within donor glands which had no macroscopic indication of tumor.

The reason the transplanted gland was directly exposed to DMBA, as opposed to gastric intubation of the F₁ hybrid host with DMBA, was to increase the likelihood of mammary cancer induction. Following the usual gastric intubation with DMBA, 2.48 mammary cancers per inbred NSD female rat develop (Table 1), however, since there are 12 individual mammary glands per female rat, this means that using this feeding method, the probability that any one individual mammary gland will develop mammary cancer is only approximately 20% (i.e., 2.48 mammary cancers of 12 individual mammary glands at risk per rat = 0.20 mammary cancers/individual mammary gland per rat). In contrast, previous studies (1) have demonstrated that 100% of inguinal-abdominal mammary glands of NSD female rats directly exposed to 1 mg of DMBA develop mammary adenocarcinomas, as opposed to a 0% incidence in such directly exposed COP mammary glands.

Since the NSD and COP donor mammary glands were both transplanted into female F₁ hybrid recipients, if systemic factors are critical in controlling resistance to DMBA-induced mammary adenocarcinogenesis, none, or very few, mammary adenocarcinomas should develop in the donor glands since the
female NSD × COP F₁ hybrid recipients are of the resistant genotype (Table 2). In direct contrast to this prediction, 7 of 10 DMBA-treated donor NSD mammary glands developed into continuously growing, macroscopically detectable mammary adenocarcinomas within 1 yr (Table 4). A representative histology section of one of these DMBA-induced mammary adenocarcinomas developing from a donor NSD gland is presented in Fig. 7. For comparison, a histological section from one of the two DMBA-exposed donor NSD mammary glands which did not develop either a continuously growing macroscopically detectable or microscopically detectable mammary adenocarcinoma is presented in Fig. 8. Examination of Fig. 8 suggests that what is maintained following transplantation of donor mammary glands is the mammary ductal network surrounded by fat cells. There is little evidence of alveolar development, however, ductal end buds are present. This is important since previous studies have identified the terminal end buds of the mammary ducts as the target of DMBA-induced adenocarcinogenesis (26).

Of the 10 DMBA-exposed donor COP mammary glands, only 1 developed into a continuously growing macroscopically detectable adenocarcinoma (Table 4). Histologically, this COP-derived cancer (Fig. 9) was very similar to those induced in the donor NSD glands (Fig. 7). These results demonstrate that it is the genetic makeup of the glands themselves, not that of the host animal, which determines the susceptibility versus resistance to DMBA-induced mammary adenocarcinogenesis.

Two of the donor COP glands developed into continuously growing macroscopically detectable sarcomas (Table 4). The seven remaining F₁ hybrids, which had no indication of any macroscopically detectable continuously growing tumors in the donor COP glands, were followed for 1 yr after DMBA exposure before being killed. Of these donor COP mammary glands, 2 were completely normal while 5 of these glands did contain microscopic foci of cancer (Fig. 10). Histologically, these foci of microscopic cancers are similar in overall appearance to those of the continuously growing macroscopic mammary adenocarcinomas induced by DMBA exposure (compare Figs. 7 and 9 with Fig. 10). The only major difference, so far detectable, between these microscopic foci and the continuously growing macroscopic cancers is the lower number of mitotic figures present in the microscopic foci as compared to the continuously growing macroscopic cancers. This fact may explain why these microscopic lesions did not produce continuously growing macroscopically detectable tumors by 1 yr following DMBA treatments.

These results suggest that 60% of the donor COP mammary glands (i.e., 50% microscopic plus 10% macroscopic cancers) and 80% of the donor NSD mammary glands (i.e., 10% microscopic plus 70% macroscopic cancers) underwent at least some of the steps involved in DMBA-induced mammary adenocarcinogenesis. The fact that the percentage of DMBA-exposed COP donor glands which have undergone at least some of the steps involved in mammary adenocarcinogenesis is not massively different from that of the NSD donor gland, as compared to the 7-fold difference in the incidence of continuously growing macroscopically detectable cancer between these two strains, suggests that the suppression of DMBA-induced malignant transformation affected by the COP resistance allele does not involve prevention of the initial step in the process, but instead, affects some malignant event or events distal to those required for morphological transformation.

**DISCUSSION**

In a previous study, it was demonstrated that at least two malignant transformation events are required to induce mammary adenocarcinogenesis in 50-day-old female outbred SD rats exposed to either DMBA or MNU (27). The outbred female SD rat, like its corresponding inbred NSD counterpart, is of the high-susceptibility genotype to DMBA-induced mammary adenocarcinogenesis (1). The OM, WF, and BUFF are also highly susceptible to DMBA-induced mammary adenocarcinogenesis (Table 1). Using genetic breeding techniques, Gould has demonstrated that high susceptibility to DMBA-induced mammary adenocarcinogenesis in inbred female WF rats is due to a group of independently segregating dominant genes, the presence of one or more of which confers high susceptibility to DMBA-induced mammary adenocarcinogenesis in F₁, hybrid females produced by breeding highly susceptible WF to intermediate susceptible Fischer rats (3).

The generality of the dominant nature of the high-susceptibility genotype has been further demonstrated in the present studies by the fact that cross-breeding three additional high-susceptibility strains of inbred rats (i.e., OM, NSD, and BUFF) to the resistant COP strain were all found to be resistant to DMBA exposure. These results confirmed the generality of the previous studies (1) demonstrating that resistance of COP-derived F₁ hybids is not due to a recessive lack of susceptibility but to an active process of suppression of the high susceptibility to DMBA-induced mammary adenocarcinogenesis induced by the inheritance of a single dominant autosomal allele from the COP genome.

In the present paper, comparison of systemic hormonal and DMBA levels following gastric intubation of DMBA suggests that systemic factors are not the major reason for the resistance of COP female rats. The transplantation data demonstrate that the site of action of this COP suppression gene is within the mammary gland itself and not at the host systemic level. Since Gould demonstrated that the site of action of the high-susceptibility genes of the WF strain is likewise in the mammary gland and not at the systemic level (3), this suggests that it is the genotype of mammary epithelium which predominantly determines the susceptibility versus resistance to DMBA-induced mammary adenocarcinogenesis. These results further

<table>
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<th>Table 4 Tumor formation following direct application of 1 mg of DMBA to NSD or Cop mammary glands transplanted into F₁ hybrid female rats</th>
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<td>Mammary gland donor*</td>
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*Inguinal-abdominal mammary gland removed from female donor rat at 30 days of age and transplanted into 30-day-old Cop × NSD F₁, hybrid female rats. Twenty days later (i.e., day 50), DMBA applied to transplanted gland.

*10 Rats/group.
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demonstrate that this dominant COP suppression allele is epistatic, being capable of either preventing the expression of or nullifying the effects of the several codominant high-susceptibility alleles inherited by the F₁ hybrids produced by crossbreeding highly susceptible strain rats to COP animals. The fact that the total incidence of microscopic plus macroscopic mammary adenocarcinomas is very similar for the donor NSD and COP mammary glands after direct exposure to DMBA suggests that at least one or a few of the malignant steps involved in mammary adenocarcinogenesis can occur in the COP donor mammary glands. This demonstrates that the expression of the COP suppression allele affects some step or steps in the malignant process distal to those required for morphological transformation. These observations suggest that the expression of this COP suppression allele does not function simply to prevent the initial interaction of DMBA with the mammary epithelial cells but to suppress the progression of these initiated cells to full malignancy. Such suppression could involve hormonal effects even though peripheral hormonal levels are similar between the highly resistant COP and NSD female rats. There could still be differences between the two strains with regard to tissue metabolism and thus the effectiveness of various hormones (i.e., estrogens, progestins, and prolactins) within the mammary cells themselves. In addition, the analytic methods used to study the metabolism of DMBA within the mammary tissue of the two strains may not have been sensitive enough to detect strain differences. The fact that COP female rats are likewise resistant to MNU, a direct-acting mammary carcinogen not requiring metabolic activation (1), suggests, however, that this is probably not likely.

The demonstration that there are genes the expression of which can lead to the suppression of tumorigenicity is not a new finding (28–30). Many of the previous studies have utilized somatic cell hybrids to demonstrate that when tumorigenic cells are fused with normal cells, initially the tumorigenicity of these hybrids is suppressed but with continuous passage of the hybrids, eventually chromosomal loss occurs resulting in the reexpression of tumorigenicity. Using such cell fusion analysis, Koi and Barrett demonstrated that chemically induced neoplastic progression of Syrian hamster embryo cells involves at least three steps: (a) induction of immortality, (b) activation of transforming oncogenes, and (c) loss of a tumor-suppressor factor (30). These authors further demonstrated that the loss of tumor suppression is the last step required before the hybrids are able to produce continuously growing macroscopically detectable tumors in vivo. Cell fusion studies between highly tumorigenic DMBA-induced mammary adenocarcinomas derived from NSD mammary glands and normal mammary epi-

Fig. 7. Histology of a macroscopically detectable mammary adenocarcinoma induced by direct DMBA exposure of a 50-day-old donor NSD mammary gland transplanted at 30 days of age into a female NSD x COP F₁ hybrid. A, × 10; B, × 160.

Fig. 8. Histology of a donor NSD mammary gland which was transplanted at 30 days of age into a female NSD x COP F₁ hybrid and directly exposed to DMBA at 50 days of age, but which did not develop a macroscopically detectable mammary cancer. A, × 10; B, × 160.
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Fig. 9. Histology of a macroscopically detectable mammary adenocarcinoma induced by direct DMBA exposure of a 50-day-old donor COP mammary gland transplanted at 30 days of age into a female NSD × COP F₁ hybrid. A, × 18; B, × 160.

Epithelial cells from 50-day-old COP female rats are presently underway to test if the introduction of such a suppression function can in fact block tumorigenicity in mammary adenocarcinomas.

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