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6/20/01
**Title and Subtitle:**
Genetic Susceptibility to Estrogen-Induced Mammary Cancers

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**Abstract:**
Estrogens are important in the etiology of breast cancer. We have developed the ACI rat as a novel, physiologically relevant, and genetically defined model for the study of breast cancer etiology, treatment and prevention. In this model, estrogens induce mammary cancers that are estrogen dependent and aneuploid. Susceptibility to estrogen-induced mammary cancers behaves as an incompletely dominant trait. Three loci have been identified that modify susceptibility in different genetic crosses of the ACI female with either the Copenhagen (COP) or Brown Norway (BN) male rat. We evaluated allelic imbalances (AI) among 21 chromosomes in estrogen-induced mammary cancers from female (ACI x COP) F1 progeny. AI is common and widespread in these cancers. Chromosomes 1, 5, 7, 17, 18 and to a lesser extent chromosome 11, contained significant nonrandom AI. In contrast, no AI was observed on chromosome 9. AI was common on chromosomes 5, 18 and 2, which harbor the Emca1, Emca2 and Emca3 modifiers. Preliminary evaluation of AI on chromosomes 1, 5, 9, 18 in tumors from the (ACI x BN)F1 also reveals widespread instability. These data suggest that non-random AI resulting from LOH and/or gene amplification may play a role in the etiology of estrogen induced mammary cancers.
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INTRODUCTION

Estrogens are inextricably implicated in the etiology of breast cancers in humans. Our laboratory has played a leading role in establishing the female ACI rat as a physiologically relevant animal model for the study of breast cancer etiology. We were the first to demonstrate that the naturally occurring estrogen, 17β-estradiol (E2), induces rapid development of mammary cancers in the female ACI rat (1). In contrast, the genetically related Copenhagen (COP) rat strain is relatively resistant to E2-induced mammary cancers (2). Recent data from our laboratory indicate that the unique susceptibility of the ACI rat to E2-induced mammary cancers is inherited in reciprocal ACI x COP intercrosses as an incompletely dominant trait (3-5) that is conferred in part by a locus, *Emcal*, on rat chromosome 5 (3-5). In this USAMRMC funded research we tested the hypothesis that genomic instability is an important contributing factor in the etiology of E2-induced mammary cancers. The primary experimental approach was to utilize a battery of polymorphic genetic loci to assess loss of heterozygosity (LOH) in mammary tumors induced by E2 in female F1 progeny generated by mating ACI female and COP male rats. We have demonstrated that E2-induced mammary cancers exhibit widespread genomic instability and that 6 of 21 chromosomes in this cross exhibited significant nonrandom allelic imbalance. Approximately two-thirds of the tumors examined exhibit aneuploidy (6), and all of the tumors examined exhibit allelic imbalances on one or more chromosomes. Chromosomes 1, 5, and 18, which were among those chromosomes containing nonrandom allelic imbalance in (ACI x COP) F1, were also found to contain high percentages of Al in tumors induced by E2 in female F1 progeny from an ACI x BN cross.

Using F2 progeny from the ACI x COP and ACI x BN crosses, we have mapped to rat chromosomes 5, 18 and 2, three genetic modifiers of susceptibility to E2-induced mammary cancers: *Emca1*, *Emca2* and *Emca3*, respectively. ACI alleles of *Emca1*, *Emca2* and *Emca3* act in an additive manner to increase susceptibility. *Emca1* was penetrant in all four crosses. In contrast, *Emca2* was penetrant only when the ACI allele was maternally derived, suggesting an epigenetic mechanism. *Emca3* was observed only in the ACI x BN cross.

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BODY

This progress report summarizes work completed between October 1, 1998 and October 1, 2001.

Task 1. Each mammary tumor was evaluated histologically to define its type as comedo or papillary carcinoma and to assess its extent of invasiveness.

All E2-induced mammary tumors were adenocarcinoma of the comedo, cribriform or papillary types, with the comedo form being the predominant cancer type. Invasive features were observed in a significant fraction of these mammary tumors.

Task 2. Cells were isolated from each mammary tumor and analyzed by flow cytometry to quantify cellular DNA content and assess ploidy. DNA content different from 2N and 4N were considered to be an indicator of aneuploidy.

Methods: Frozen mammary tumors and spleens from the tumor bearing animals were thawed on ice, cut into small pieces using a razor blade and mechanically dissociated in PBS using a Stomacher laboratory blender (Tekmar, Cincinnati, OH). The dissociated cells were pelleted by centrifugation, resuspended in RPMI culture media (Life Technologies, Gaithersburg, MD), filtered through Spectra/Mesh polymer macrofiltration filters (Spectrum, Laguna Hills, CA) and counted. Following dilution to approximately 1 million cells per ml in Vendelov’s reagent the cells were analyzed using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). Cells prepared from normal spleen were used as an internal ploidy standard. The resulting data were analyzed using ModFit DNA analysis software (Becton Dickinson).

Results and Discussion: Flow cytometric analysis of DNA content within cells isolated from mammary cancers induced in female ACI rats by E2 indicated that the majority of these tumors exhibit genomic instability. Within a group of fifteen E2-induced mammary cancers, five (33%) of the tumors exhibited a normal DNA profile, where the majority of cells displayed a diploid DNA content. Seven (47%) tumors contained a significant fraction of cells which exhibited a hyperdiploid DNA content, two (13%) tumors contained a large fraction of cells that were tetraploid in their DNA content, and one (7%) of the 15 mammary tumors contained a large fraction of cells that were hypertetraploid in their DNA content. These data clearly illustrate that different E2-induced mammary carcinomas are heterogeneous with respect to ploidy, with approximately 66% of the examined tumors exhibiting clear features of aneuploidy. This observation is noteworthy as the mammary tumors that develop in rats and mice treated with dimethylbenz[a]anthracene have been reported to exhibit a diploid DNA profile. These data were included in a recent manuscript from our laboratory published in the Proceedings of the National Academy of Sciences (97:2779-2784). Support from the USAMRMC was acknowledged in that publication. A reprint of the manuscript is appended to this progress report.

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Task 3. A panel of appropriately located simple sequence repeat (SSR) loci were identified that are polymorphic between the ACI and COP and the ACI and BN strains from among those mapped in the rat genome.

Results and Discussion: This task has been accomplished on a chromosome-by-chromosome basis. Analyses of tumors in (ACIxCOP) F1 females are problematic for certain regions of the genome due to a low density of SSR markers that are polymorphic between these two rat strains. The density of polymorphic markers was greater for the analysis of (ACIxBN) F1 females.

Task 4. DNA was isolated from each mammary tumor as well as from the spleen of each mammary tumor bearing F1 animal. Each of the polymorphic SSR loci identified in #3 was amplified using polymerase chain reaction (PCR) and commercially available primers. The amplified DNAs were subjected to electrophoresis on denaturing acrylamide gels and the amount of DNA product generated from the ACI and COP or ACI and BN alleles at each locus was quantified using a phosphorimager. LOH was revealed as a strain specific loss of an SSR locus in the mammary tumor relative to the spleen from the same animal. Suspected LOH was confirmed by repeating the analyses at flanking loci.

and,

Task 5. The resulting data was analyzed to determine: 1) the extent of LOH occurring across the rat genome; 2) whether the observed LOH occurred selectively in specific regions of the genome; and 3) whether LOH within a specific region of the genome correlated with tumor type or invasiveness.

Methods: The rat tissues used in this project were from experiments supported by NIH grant CA77876. In the NIH funded experiments, mammary tumors were induced in (ACIxCOP) F1 and (ACIxBN) F1 female rats by treatment with E2, administered from subcutaneous Silastic implants as described by us previously (1). No funds from the USAMRMC were used for purchase or housing of live animals.

Methods for evaluating LOH were established and optimized. Genomic DNA was isolated from 10 mg of rat spleen and 25 mg of rat mammary tumor using QIAamp tissue kits (QIAGEN). Each DNA (30 ng) was amplified by PCR using Platinum Taq DNA Polymerase (Gibco) and standard methods as described in our original application; [32P]dCTP (Amersham) was included in each PCR reaction, resulting in generation of labeled PCR products. Oligodeoxynucleotide primers for amplifying the different polymorphic SSR loci on each of the rat chromosomes were obtained from Research Genetics, Inc. The PCR products were separated on 5% or 8% denaturing polyacrylamide sequencing gels. Each PCR reaction was assayed in duplicate. Following drying, the gels were visualized by autoradiography on a Phosphorimager (Molecular Dynamics), and the intensities of the DNA bands generated from the ACI and COP alleles were quantified using ImageQuant software (Molecular Dynamics). The average (± standard deviation, n = 2) pixel intensity generated from the ACI and COP alleles from spleen and

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mammary tumor DNA was determined for each of several SSR loci. An LOH index for each polymorphic locus was calculated by: 1) dividing the intensity of the ACI product by the intensity of the COP product generated from each mammary tumor and spleen DNA; 2) comparing the ACI/COP ratio from the mammary tumor with the ACI/COP ratio from the spleen using Student’s t test; and, 3) subsequently dividing the ACI/COP ratio from each mammary tumor by the ACI/COP ratio from each spleen. Despite individual variation in the spleen PCR products obtained from each animal, the standard deviation among all ACI/COP ratios was only 13.8% among all 21 rats evaluated by 75 different markers. An LOH index that was equal to or greater than 1.25 was interpreted to suggest loss of the COP allele within the genome of the mammary tumor at the locus assayed. An LOH index that was equal to or less than 0.75 was interpreted to suggest loss of the ACI allele within the genome of the mammary tumor at the locus assayed. Additional statistical interpretation was applied using methods developed by Newton, et al (10). Nonrandom variation of the rate of allelic imbalance was evaluated across all chromosomes with a Monte Carlo interpretation of Fisher’s exact test. In those chromosomes exhibiting nonrandom variation, log-likelihood ratios (LOD scores) were calculated for each marker to determine whether the gain or loss of alleles was significantly different from what may be expected as background rates of gain or loss.

Results and Discussion: Analyses of allelic imbalance (AI), resulting from deletion (LOH) or gene amplification, in mammary tumors induced by E2 in female (ACIxCOP) F1 progeny was completed on all rat chromosomes 1-20 and X. Data indicated that AI in these tumors is common and nonrandom. Allelic imbalance (AI) was present in 19 of 21 chromosomes with 59 of 75 markers (78.7%) showing instability in at least one of the 21 tumors. A total of 206 allelic imbalance events were recorded among 1469 possible tumor-marker pairs. Among the 21 F1 mammary tumors evaluated by 75 total markers 53.8% events were attributable to COP loss of alleles compared to 45.6% ACI loss. Loss of ACI alleles was most evident in chromosomes 1, 4, 10, 12, 13, 14 and 17. Loss of COP influence was most notable in chromosomes 2, 3, 5, 7, 8, 15, 18 and 20. The AI observed in chromosome 11 was characterized by an equal loss of COP and ACI alleles. No AI events were noted in chromosomes 9 and 19 due to apparent stability of the genome or paucity of available SSR polymorphic markers, respectively. Chromosomes 1, 5, 7, 17, 18, in addition to chromosome 11, were also found to contain statistically significant nonrandom variation in the (ACIxCOP) F1 rat. Microsatellite instability was an inconsistent feature of the observed AI.

An abstract describing these findings was presented at the 2001 meeting of the American Association for Cancer Research (7). A copy of this abstract is appended to this progress report. We anticipate that a manuscript describing these findings will be submitted early in 2002. (8)

Using techniques developed in the analysis of AI in the (ACIxCOP) F1 tumors, a similar analysis was conducted in 14 E2-induced tumors from the (ACIxBN)F1 progeny. These observations have so far been limited to chromosomes 1, 5, 9, 18. These chromosomes were chosen for the preliminary investigation in this intercross because: 1) they were notable in the (ACIxCOP) F1 intercross for exhibiting high rates of significant nonrandom allelic imbalance (chromosomes

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1, 5, 18); 2) they exhibit nonrandom allelic imbalance and contain loci that modify susceptibility to E2-induced mammary cancers (chromosomes 5, 18 and text below); and 3) the absence of allelic imbalance (chromosome 9) is markedly different from the widespread AI seen in the other chromosomes and may represent an important region of genomic stability or conservation.

In these four chromosomes 1, 5, 9, 18 from the (ACI x BN)F1, AI is common. The percentage of AI was 22%, 31%, 15%, and 22%, respectively. AI was observed in at least 28% and as many as 72% of tumors in each chromosome. Loss of BN alleles (or gain of ACI alleles) is predominate in each chromosome. These observations contrast to those from the (ACI x COP) F1 in the following manner: 1) chromosome 9 does not appear to exhibit genomic stability and 2) the majority of the AI in chromosome 1 is due to under-representation of alleles from the tumor-resistant paternal BN strain instead of alleles from the tumor- susceptible ACI maternal strain.

Emca1, a modifier of susceptibility to E2-induced mammary cancer, has been mapped by us to rat chromosome 5 in the (ACIxCOP) intercross (unpublished data). For each of 17 polymorphic markers evaluated on rat chromosome 5, AI was observed in at least 1 and as many as 7 of the 21 tumors. On average, an individual tumor exhibited AI at 20% (range 0-82%) of the markers. Approximately 65% of the observed AIs resulted from loss of COP alleles (or gain of ACI alleles). Only 1 tumor did not exhibit any AI, 2 tumors exhibited AI at approximately 50% of the markers and 2 tumors exhibited AI at a majority of the chromosome 5 markers. Within the 4 tumors exhibiting AI at 50% or more of the markers, 3 tumors exhibited consistent loss of COP alleles and 1 exhibited consistent loss of ACI alleles.

AI was evaluated at four polymorphic markers on rat chromosome 18, onto which the Emca2 modifier of susceptibility to E2-induced mammary cancers has been mapped by us in the (ACI x COP) intercross. AI was observed at each of these markers in a minimum of 3 and a maximum of 5 of the 21 mammary tumors. A total of 16 AI events were observed within the 83 informative marker/tumor pairs evaluated, an overall frequency of 19%. Of the 16 observed events, 19% resulted from loss of ACI alleles, whereas 81% resulted from loss of COP alleles.

We have mapped to rat chromosome 2 a locus, Emca3, that modifies susceptibility to E2-induced mammary cancers in an ACI x BN intercross (unpublished data). We have evaluated AI at 6 polymorphic loci on chromosome 2. AI was observed at 14% of the informative marker/tumor pairs. Each of the markers exhibited AI in at least 1 and as many of 4 tumors. Eleven of the 21 tumors did not exhibit AI at any marker. AIs resulting from loss of ACI alleles were observed at the same frequency as AIs resulting from loss of COP alleles.

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Task 6. The data was communicated to the scientific community through presentation at appropriate meetings and publication in peer reviewed journals.

The data indicating that mammary tumors induced in ACI rats by E2 exhibit aneuploidy were communicated by Dr. Shull in an invited presentation at the Gordon Conference on Hormonal Carcinogenesis held in August of 1999 and were included in a recent manuscript published in the Proceedings of the National Academy of Sciences 97:2779-2784, 2000. Support from the USAMRMC was acknowledged in that publication. A reprint of the manuscript is appended to this progress report. Data summarizing the patterns of Al in the ACI, COP and BN intercrosses and the presence of loci modifying tumor susceptibility will be presented by Dr. Shull at an upcoming meeting at the Cold Spring Harbor Laboratory, and manuscripts describing these data are in preparation for publication.

An abstract describing the genome wide screen of Al was submitted for presentation at the 2001 meeting of the American Association for Cancer Research. A copy of this abstract is appended to this progress report. We anticipate that a manuscript describing these findings will be submitted early in 2002.

Task 7. All required progress reports and communications were prepared and submitted to the Army Breast Cancer Research Program.

All required annual and midterm reports have been submitted to meet prior deadlines. This final report was submitted November 1, 2001.

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KEY RESEARCH ACCOMPLISHMENTS

1. Flow cytometric analyses of E2-induced mammary tumors have been performed indicating that a large fraction of these tumors exhibit aneuploidy.

2. Methods for evaluating LOH have been established and optimized.

3. Appropriate polymorphic loci have been identified for evaluating LOH in mammary tumors induced in ACI/COP F1 rats by chronic treatment with E2.

4. AI has been evaluated on chromosomes 1-20 and X in the (ACIxCOP)F1 rat, revealing that AI is common and nonrandom in E2-induced mammary cancers. Microsatellite instability is not a significant feature of the AI in these tumors. Preliminary observations on four of 20 autosomes from the (ACIxBN)F1 rat suggest a similar pattern of widespread AI.

5. AI suggestive of loss of COP alleles were highly prevalent at markers linked to Emcal and Emca2, suggesting that somatic events at these loci may contribute to E2-induced mammary carcinogenesis.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: One manuscript has been published. Several oral presentations have included references to the data generated with support of this award. A minimum of one additional manuscript will be submitted in early 2001.

Patents: None.

Degrees: None.

Development of cell lines: None.

Informatics such as databases and animal models: None.

Funding applied for: None at present. Additional applications anticipated.

Employment or research opportunities: None.

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CONCLUSIONS

Data generated to date suggest that genomic instability and AI are common events during E2-induced mammary tumorigenesis. It is anticipated that this information will enhance our understanding of the molecular events leading to mammary cancer development. Our research on estrogen-induced mammary tumorigenesis has illustrated for the first time the physiological relevance of the ACI rat as an animal model for the study of breast cancer etiology.

Mapping of the (ACIxBN)F2 genome is being completed. Experiments to evaluate cell autonomous vs. host genetic and host hormonal mechanisms of tumorigenesis are planned. Additional studies to determine the extent of AI in the (ACIxBN)F1 genome may be pursued. These data together with greater characterization of the three loci modifying tumor susceptibility may reveal novel mechanisms regarding the heritability of tumor susceptibility and the molecular processes which contribute to mammary tumorigenesis.

REFERENCES


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8. Flood et al., manuscript in preparation.


Rat strain-specific actions of 17β-estradiol in the mammary gland: Correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers


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The genetically related ACI and Copenhagen (COP) rat strains display diametrically opposed susceptibilities to mammary cancer development when treated chronically with 17β-estradiol (E2). Here, we compare the actions of E2 on cell proliferation and lobuloalveolar development in the mammary glands of female ACI and COP rats. After 12 wk of E2 treatment, the mammary glands of ACI rats exhibited a significantly greater proliferative response to E2, compared with COP rats, as evidenced by quantification of S phase fraction and development of lobuloalveolar hyperplasia. Focal regions of atypical epithelial hyperplasia were observed in ACI, but not COP, rats. These strain differences were not because of differences in circulating E2, progesterone or, prolactin. Two-thirds of the induced mammary cancers in ACI rats exhibited aneuploidy. The E2-induced mammary cancers regressed when hormone treatment was discontinued, indicating that they were estrogen-dependent. Progesterone receptor was expressed by the great majority of epithelial cells within the E2-induced atypical hyperplastic foci and the mammary carcinomas, suggesting a link between these lesions. These data demonstrate a correlation between E2 action in the induction of mammary cell proliferation and atypical epithelial hyperplasia and genetically conferred susceptibility to E2-induced mammary cancers.

Epidemiologic studies suggest that estrogens are critical factors in breast cancer etiology (1–3). Supporting this link are the observations that early menarche, late menopause, late first full-term pregnancy, and nulliparity are each associated with increased risk of the disease, whereas oophorectomy prior to menopause significantly decreases breast cancer risk. A recent clinical trial demonstrated that the antiestrogen tamoxifen significantly reduces the incidence of breast cancer in women at high risk of developing the disease (4). One theory consistent with these data is that breast cancer risk is proportional to the cumulative number of ovulatory cycles and the repetitive stimulation of mammary cell proliferation by ovarian estrogens. At present, the molecular mechanisms through which estrogens contribute to the development of breast cancers are unknown.

The ACI rat appears unique among rat strains in that it is highly susceptible to estrogen-induced mammary cancers but rarely develops mammary cancers spontaneously (5–15). We have demonstrated that chronic treatment with 17β-estradiol (E2) induces mammary cancers in virtually 100% of ovari-intact ACI rats with a median latency of approximately 20 wk (15). In contrast, the Copenhagen (COP) rat is resistant to the development of estrogen-induced mammary cancers (5–8, 16). This difference between the ACI and COP rat strains is particularly striking because these strains are closely related genetically; the ACI strain was derived from a cross between the August and COP strains (17). Because the ACI and COP rat strains exhibit diametrically opposed susceptibilities to E2-induced mammary cancers (15, 16), they provide valuable models for studying the molecular mechanisms through which estrogens contribute to mammary cancer development.

In the present study, we have compared the ability of E2 to stimulate mammary cell proliferation and lobuloalveolar development in the mammary glands of female ACI and COP rats. Data presented herein reveal quantitative and qualitative differences in the manner in which the mammary epithelia of ACI and COP rats respond to E2, suggesting that genetic control of estrogen action in the mammary epithelium may contribute to the differing susceptibilities of these rat strains to E2-induced mammary cancers.

Materials and Methods

Care and Treatment of Animals. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Female ACI and COP rats were obtained from Harlan-Sprague–Dawley and National Cancer Institute Breeding Program (Frederick, MD), respectively. The animals were housed in an accredited barrier facility under controlled temperature, humidity, and lighting conditions, and were fed a diet that was formulated in accordance with guidelines established by the American Society of Nutritional Science (18) and prepared as described previously (19, 20). Treatment with E2 was initiated at approximately 9 wk of age. Silastic tubing implants, empty or containing 27.5 mg of E2, were surgically inserted into the interscapular region as described previously (15, 16). Thereafter, each rat was examined twice weekly for palpable mammary cancers. Four hours preceding killing, each rat received an i.p. injection of BrdUrd (Sigma; 50 mg/kg body weight) solubilized in sterile PBS.

Collection, Processing, and Immunohistochemical Evaluation of Mammary Tissues. Following treatment with E2 for the indicated period of time, each animal was killed by decapitation, trunk blood was collected, and serum was stored at −80°C. The anterior pituitary gland was removed and weighed. Serum E2 (15), prolactin (PRL) (15), and progesterone (21) were measured by radioimmunoassay as described previously. The location and size of each mammary tumor were recorded at necroscopy.

Abbreviations: COP, Copenhagen; DES, diethylstilbestrol; E2, 17β-estradiol; PR, progesterone receptor; PRL, prolactin.

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tumors were frozen in liquid nitrogen and stored at −80°C; spleens from these animals were also frozen and stored at −80°C. Sections from each mammary tissue specimen were stained with hematoxylin and eosin for histologic characterization. The density of the mammary parenchyma within each gland was estimated by computer-assisted (Optimas, Seattle, WA) quantification of the percentage of total cross-sectional area comprised of mammary parenchyma. Mammary glands from each animal were also prepared as whole mounts stained with alcian blue (Sigma) (22). Mammary cells incorporating BrdUrd during the 4-hr period preceding killing were quantified immunohistochemically as described previously (16, 19). A minimum of 2,000 mammary cells per animal were scored for incorporation of BrdUrd. To identify cells expressing progesterone receptor (PR), mammary sections were deparaffinized, rehydrated in water, and heated in citrate buffer (pH 6.0) for 20 min. Tissues were then incubated as follows: (i) 20 min at room temperature in 3% hydrogen peroxide; (ii) 20 min at room temperature in goat serum diluted 1:100 in PBS; (iii) 60 min at 37°C in rabbit anti-PR serum (C-19; Santa Cruz Biotechnology) diluted 1:100 in PBS. Bound primary antibody was detected by using biotinylated goat anti-rabbit IgG followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories) and visualized by using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Sections were counterstained with Mayer's hematoxylin.

Analysis of Cellular DNA Content by Flow Cytometry. Frozen mammary tumors and spleens from tumor-bearing animals were thawed on ice, minced, and mechanically dissociated in PBS using a Stomacher laboratory blender (Tekmar, Cincinnati). The dissociated cells were pelleted by centrifugation, resuspended in RPMI culture media (Life Technologies, Gaithersburg, MD), filtered through Spectra/Mesh polymer macrofiltration filters (Spectrum, Laguna Hills, CA), and counted. Following dilution to approximately 1 million cells per ml in Vendaev's reagent (23), the cells were analyzed by using a FACScalibur flow cytometer (Becton Dickinson). Cells prepared from normal spleen were used in parallel samples as an internal ploidy standard. The resulting data were analyzed by using MODFIT DNA analysis software (Becton Dickinson).

Statistical Analysis of Data. Data are presented as the mean ± SEM. Differences between means were assessed by using two-way ANOVA and Newman–Keul's post hoc test. P values < 0.05 were considered significant.

Results

Responsiveness of Mammary Epithelium to E2 Is Rat Strain-Specific. The mammary epithelium of the female ACI rat exhibited a more robust proliferative response to E2 than did that of the female COP rat. After 12 wk of treatment with E2 (a time point that precedes appearance of palpable mammary cancers), cell proliferation in the mammary glands of female ACI rats was increased approximately 8-fold relative to that observed in untreated controls (Fig. 1A). Whereas approximately 0.5% of mammary cells of control ACI rats stained positive for BrdUrd, 3.9% of mammary cells of E2-treated ACI rats were BrdUrd-positive (P < 0.01). By contrast, E2 treatment increased cell proliferation within the mammary glands of female COP rats 3.5-fold (P < 0.01). Approximately 0.8% and 3.0% of mammary cells of untreated and E2-treated COP rats, respectively, were BrdUrd-positive (Fig. 1A). The 30% difference in the S phase fraction between the E2-treated ACI and COP rats was significant (P < 0.01).

The difference in the proliferative response of the ACI and COP mammary epithelia to E2 was reflected histologically (Fig. 1B, and see Fig. 2). The mammary glands of untreated virgin ACI (Fig. 2A and C) and COP (Fig. 2E and G) females were similar in appearance and consisted of branched ducts extending throughout the mammary fat pad and terminating in alveolar buds. Treatment with E2 for 12 wk induced ductal branching and extensive lobuloalveolar hyperplasia in both strains. However, the induction of lobuloalveolar hyperplasia was clearly more pronounced in the ACI rats (Fig. 2B and D) than in the COP rats (Fig. 2F and H). Quantitative analysis of stained sections indicated that the mammary parenchyma comprised 70% of cross-sectional area in E2-treated ACI rats compared with 53% in the treated COP rats (Fig. 1B). This difference, reflecting the greater density of lobules in the ACI glands, was statistically significant (P < 0.01) and was also evident upon examination of mammary gland whole mounts (Fig. 2D and H). Secretory activity was apparent in the mammary glands of treated ACI and COP rats.
COP rats and, although variable, appeared more pronounced in the COP strain compared with the ACI strain (Fig. 2 B and F).

**Strain Differences in Lobuloalveolar Hyperplasia Are Independent of Circulating E2, Progesterone, and Pituitary Tumor-Associated Hyperprolactinemia.** The levels of E2, progesterone, and PRL in trunk blood serum were measured to ascertain their relationships to the observed strain difference in E2-induced lobuloalveolar hyperplasia. Serum E2 levels in treated animals did not differ between the two strains, averaging $165 \pm 24$ pg/ml and $306 \pm 119$ pg/ml (mean $\pm$ SEM) in ACI and COP rats, respectively (data not shown). Progesterone levels were within the physiologic range and did not differ significantly as a consequence of E2 treatment or rat strain (data not shown). Treatment with E2 for 12 wk induced pituitary growth and hyperprolactinemia in both female ACI and COP rats. Pituitary weight was increased 3.4-fold ($P < 0.01$), from $12.0 \pm 0.4$ to $40.9 \pm 2.9$ mg, in female ACI rats, and 2.4-fold ($P < 0.01$), from $14.2 \pm 0.4$ to $33.5 \pm 3.6$ mg, in female COP rats (Fig. 3A). Similarly, treatment with E2 increased serum PRL from $4 \pm 1$ to $640 \pm 114$ ng/ml ($P < 0.01$) in female ACI rats, and from $9 \pm 5$ to $524 \pm 118$ ng/ml ($P < 0.01$) in female COP rats (Fig. 3B). The differences in pituitary weight (Fig. 3A) and serum PRL (Fig. 3B) between E2-treated ACI and COP rats were not significant. Therefore, the observed strain differences in E2-induced lobuloalveolar hyperplasia do not appear to result from differences in the levels of circulating E2, progesterone, or PRL in the E2-treated ACI and COP rats.

**Mammary Cancers Induced in ACI Rats by E2 Exhibit Genomic Instability.** Flow cytometric analysis of cellular DNA content within mammary cancers induced in ACI rats by prolonged treatment with E2 indicated that the majority (67%) of these tumors exhibited aneuploidy (Fig. 4). Of 15 mammary cancers analyzed, 5 (33%) exhibited a normal DNA profile where the great majority of cells displayed a diploid DNA content (Fig. 4A); 7 (47%) cancers contained a significant fraction of cells that exhibited a hyperdiploid DNA content (Fig. 4B); 2 (13%) contained a large fraction of cells with tetraploid DNA content (Fig. 4C); and 1 (7%) contained a large fraction of cells that were hypertetraploid in their DNA content (Fig. 4D).
standards for diploid and tetraploid DNA content. (A) Mammary cancer from cells within tumor-bearing animal were included in parallel samples to provide internal vertical axis indicates relative cell number. Cells from the spleen of each fluorescence intensity, which is directly proportional to DNA content. The tumor ment with E2. The horizontal axis represents an arbitrary scale of relative regressed completely within 38 days of implant removal, whereas the largest Fig. 4. Mammary cancers induced in ACI rats by E2 exhibited aneuploidy. palpable mammary tumors. All of the mammary cancers re- increase in the mammary glands of female ACI rats as a function exogenous E2 is required for maintenance of established maam- treatment may be precursors of the E2-induced carcinomas. The Estrogen-Induced Mammary Cancers in ACI Rats Are Dependent on Exogenous E2. To determine whether continuous exposure to exogenous E2 is required for maintenance of established mammary cancers, the E2-containing implants were removed from a group of 4 tumor-bearing ACI rats harboring a total of 14 palpable mammary tumors. All of the mammary cancers re-gressed following implant removal. The regression of four tumors in one animal is illustrated in Fig. 5. In that animal, tumors 1, 2, 3, and 4 were detected following 98, 133, 147, and 154 days of E2 treatment, respectively. The E2-containing implant was removed on day 158 relative to initiation of estrogen treatment, when the tumors ranged from 0.4 to 1.1 cm in diameter. Within 38 days of implant removal, the largest of the four tumors had regressed to 0.2 cm in diameter and the three smaller tumors were no longer detectable by palpation. Regression of the largest tumor was complete within 59 days after removal of the E2-containing implant. These data, which are representative of those from three additional tumor-bearing animals, indicate that maintenance of elevated levels of circulat- ing E2 are required for continued growth of E2-induced mammary cancers.

Estrogen-Induced Atypical Epithelial Hyperplasia and Mammary Cancers in ACI Rats Express PR. The mammary glands of ACI rats treated with E2 for as few as 12 wk contained focal regions of atypical epithelial hyperplasia in which the cells exhibited enlarged nuclear and cellular volumes and dense eosinophilic cytoplasmic staining (Fig. 6A). In contrast, no such foci were observed in the mammary glands of COP rats that were treated with E2 for 12 wk (data not shown). The majority of epithelial cells within the atypical foci observed in E2-treated ACI rats displayed strong immunoreactivity to an antibody to the PR (Fig. 6B). Similarly, the majority of epithelial cells within the mammary cancers that developed in response to prolonged E2 treatment also stained intensely for the presence of PR, regardless of whether they were of the comedo (Fig. 6C), papillary (Fig. 6D), or cribriform (data not shown) types. By contrast, only 20–30% of the epithelial cells within the hyperplastic lobules of ACI rats treated for 12 wk (Fig. 6E) or longer (Fig. 6D) with E2, or normal ductal epithelial cells of age-matched untreated controls (Fig. 6F) stained positive for PR. These data suggest that the atypical foci observed within 12 wk of initiation of E2 treatment may be precursors of the E2-induced carcinomas. The number of focal regions of atypical epithelial hyperplasia and the degree of cellular atypia within the foci were observed to increase in the mammary glands of female ACI rats as a function of the length of E2 treatment beyond the 12-wk time point. Consequently, atypical hyperplastic foci were numerous in ACI rats bearing E2-induced mammary cancers (data not shown). Discussion The genetically related ACI and COP rat strains exhibit diametrically opposed phenotypes with respect to susceptibility to E2-induced mammary cancers (15, 16). In the present study, we have demonstrated that the mammary gland of the highly susceptible ACI rat exhibits a greater proliferative response to administered E2 compared with the resistant COP rat. This difference was evidenced by quantification of the number of mammary cells incorporating BrdUrd as well as by histologic indicators of lobuloalveolar hyperplasia. The number of BrdUrd-positive cells was 30% greater in E2-treated ACI rats than in E2-treated COP rats; the difference (fold induction) between basal and E2-stimulated mammary cell proliferation in ACI rats was more than twice that observed in the COP strain; and the percentage of the mammary gland cross-sectional area comprised of parenchyma was 30% greater in E2-treated ACI rats than in treated COP rats. Moreover, focal regions of atypical epithelial hyperplasia were observed in the mammary glands of ACI, but not COP, rats that were treated with E2 for 12 wk. The associations between estrogens, cell proliferation, and breast cancer risk in human populations and mammary cancer devel-
Proliferation, lobuloalveolar hyperplasia, and atypical epithelial mammary carcinomas were also uniform in expressing PR, from an untreated, 21-wk-old, ovary-intact ACI rat; age-matched control for the mammary tissues of the ACI and COP rats. Estrogenic involvement in the etiology of mammary hyperplasia has been reported previously (16) and illustrated that PR induces proliferation and associated hyperplasia in female COP rats but does not induce a high incidence of mammary cancers. Moreover, ovariectomy (15) and dietary energy restriction (38) each inhibit development of E2-induced mammary cancers in female ACI rats without inhibiting development of pituitary tumors and associated hyperplactinemia. Therefore, hyperprolactinemia is insufficient to induce a high incidence of mammary cancers in estrogen-treated rats. It is interesting to note that the genetic loci that confer and/or modulate susceptibility to E2-induced mammary cancers in F2 progeny from crosses between the highly susceptible ACI strain and two resistant rat strains, COP and Brown Norway, appear to be distinct from those that influence development of estrogen-induced pituitary tumors, indicating that these two estrogen-induced tumorigenic processes may be genetically separable (unpublished data).

In the present study, female ACI and COP rats exhibited similar increases in pituitary weight and circulating PRL in response to 12 wk of E2 treatment. By contrast, male ACI rats exhibit a significantly greater pituitary growth response to diethylstilbestrol (DES) than do male COP rats (39). The ACI rat appears not to display a significant sexual dimorphism in relation to the pituitary growth response to administered estrogen (unpublished data). Consequently, the amount of E2-induced pituitary growth exhibited by female COP rats closely approached that exhibited by female ACI rats.

The majority of the E2-induced mammary cancers examined in this study exhibited aneuploidy. To our knowledge, this is the first report of genetic instability in mammary cancers induced in any rat strain by administered E2. Preliminary data suggest that loss of heterozygosity (LOH) is a common feature of E2-induced mammary cancers in ACI rats (unpublished data). The roles of aneuploidy and LOH in the development of E2-induced mammary cancers are not understood. Aneuploidy and LOH are commonly observed in human breast cancers (40).

Mammary cancers induced by E2 in ACI rats regressed upon removal of the E2-containing implants, indicating that these cancers are dependent upon exogenous estrogen for their maintenance. Shellabarger et al. (41) noted a similar observation for mammary cancers induced in ACI rats by DES, although data were not presented to indicate how rapidly regression occurred. Not established in these studies is whether and to what extent production of estrogens by the ovaries resumed after cessation of prolonged estrogen treatment.

The mammary glands of ACI rats treated with E2 for as few as 12 wk contained focal regions of atypical epithelial hyperplasia. The majority of cells comprising these foci were quite uniform in their expression of PR, relative to cells in the adjacent hyperplastic lobules. The epithelial cells within E2-induced mammary carcinomas were also uniform in expressing PR, suggesting a possible link between the atypical hyperplastic foci and the carcinomas. Atypical epithelial hyperplasia of this type has not been described previously in the mammary glands of estrogen-treated ACI rats. The functional significance of PR and its hormone ligands within the atypical hyperplastic foci and mammary cancers induced in ACI rats is unknown. It is interesting to note that cell proliferation in the human breast is greatest during the luteal phase of the menstrual cycle, when circulating progesterone is highest (42–43). Moreover, ovariectomy markedly inhibits development of mammary cancers in

**Fig. 6.** Atypical epithelial hyperplasias and mammary cancers induced in ACI rats by E2 express PR. Cells expressing PR were identified immunohistochemically as described in Materials and Methods. (A) Mammary gland, sectioned and stained with hematoxylin and eosin, from a 21-wk-old female ACI rat treated with E2 for 12 wk. Illustrated is a focal region of atypical epithelial hyperplasia and, to the right, adjacent acinar structures. The epithelial cells within the atypical hyperplasia exhibited enlarged nuclei and dense eosinophilic cytoplasmic staining. The illustrated atypical hyperplasia was minimally deviated from the surrounding lobules. The number of atypical hyperplastic foci and the degree of cellular atypia were observed to increase as a function of the duration of E2 treatment beyond 12 wk (data not illustrated). (B) A serial section to that in A that has been immunostained for PR. The majority of cells within the atypical epithelial hyperplasia exhibited immunoreactivity to PR, whereas fewer cells in the adjacent acinar structures stained positive for PR (arrow). (C) Mammary comedo carcinoma from an ACI rat treated with E2 for 193 days. The majority of the cancer cells exhibited immunoreactivity to PR. The arrow indicates necrotic debris characteristic of these cancers. (D) Mammary papillary carcinoma from an ACI rat treated with E2 for 216 days. The majority of the epithelial cells within the cancer exhibited immunoreactivity to PR. The arrow indicates adjacent acinar structures where a subset of the epithelial cells were immunoreactive to PR. (E) Mammary gland from an ACI rat treated with E2 for 12 wk exhibited lobuloalveolar hyperplasia. A subset of the epithelial cells stained positive for PR. (F) Mammary gland from an untreated, 21-wk-old, ovary-intact ACI rat; age-matched control for the E2-treated animals illustrated in A, B, and E. A subset of epithelial cells within the normal ductal structures exhibited immunoreactivity to PR.
ACI rats treated with E2 (15) or DES (9). These data suggest that estrogens and progestins may interact in regulating mammary cell proliferation in the human and rat. However, Segaloff (9) demonstrated that treatment of ovariectomized ACI rats with DES and progesterone did not induce mammary cancer development to the extent observed in DES-treated, ovary-intact ACI rats. Because circulating progesterone was not measured by Segaloff (9), it should not be assumed that progesterone levels in the treated ovariectomized rats were restored to within the physiologic range of ovary-intact rats.

In summary, the mammary epithelia of ACI and COP rats exhibited quantitative and qualitative differences in their responsiveness to E2. These differences correlated with the differing susceptibilities of these two rat strains to E2-induced mammary cancers. Because the ACI rat appears unique in its high degree of susceptibility to E2-induced mammary cancers, whereas the genetically related COP rat is resistant to these cancers, these two rat strains provide useful models for studying the role of E2 in mammary cancer development. Ongoing genetic studies are addressing the mechanism underlying these strain differences in the belief that this information will prove relevant to our understanding of the etiology of human breast cancers.

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ALLELIC IMBALANCE IN MAMMARY TUMORS INDUCED IN ACI/COPENHAGEN F1 RATS BY 17β-ESTRADIOL

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The ACI and Copenhagen (COP) rat strains exhibit opposing susceptibilities to mammary cancers induced by continuous treatment with 17β-estradiol (E2). We have recently demonstrated that the unique susceptibility of the ACI rat to E2-induced mammary cancers behaves as an incompletely dominant trait in reciprocal crosses to the COP strain. Although virtually all of the female ACI/COP F1 progeny develop mammary cancers in response to E2, latency is delayed and tumor burden is reduced in F1 females relative to that observed in ACI females. Linkage analysis reveals on rat chromosome 5 a locus, Emcal (Estrogen-induced mammary cancer), that modifies susceptibility to E2-induced mammary cancers in ACI x COP intercrosses. The mammary tumors that develop in E2 treated rats are estrogen-dependent and frequently exhibit aneuploidy. We hypothesize that deletion of COP alleles within Emcal and/or elsewhere in the genome may contribute to E2-induced mammary carcinogenesis in ACI/COP F1 females. A panel of 21 mammary tumors induced in F1 females by E2 was examined for allelic imbalance (AI) at polymorphic loci distributed across the rat genome. DNA from each tumor was amplified using PCR and primer pairs flanking mapped microsatellite repeats, the radiolabeled DNA products were separated on acrylamide gels, and the amount of products arising from the ACI and COP alleles were quantified on a phosphorimager and compared to results generated from DNA prepared from the spleen. AI, defined as an ACI/COP allele ratio in a tumor that differs from that of the spleen by ≥ 25%, was evaluated for each chromosome, and representative data are summarized here. For each of 17 markers evaluated on rat chromosome 5, AI was observed in at least 1 and as many as 7 of the 21 tumors. On average, an individual tumor exhibited AI at 20% (range 0-82%) of the markers. Approximately 65% of the observed AIs resulted from loss of COP alleles (or gain of ACI alleles). Only 1 tumor did not exhibit any AI, 2 tumors exhibited AI at approximately 50% of the markers and 2 tumors exhibited AI at a majority of the chromosome 5 markers. Within the 4 tumors exhibiting AI at 50% or more of the markers, 3 tumors exhibited consistent loss of COP alleles and 1 exhibited consistent loss of ACI alleles. AI was also observed in 14-55% of the 21 tumors at each of 6 markers on chromosome 18. Only 2 tumors did not exhibit AI at at least 1 marker, whereas 5 tumors exhibited AI at 50% or more of the chromosome 18 markers. Approximately 75% of the AI events on chromosome
18 resulted from loss of COP alleles. We conclude that widespread genomic instability is a common feature of E2-induced mammary cancers. Supported by grants DAMD17-98-1-8217, R01-CA68529 and R01-CA77876.

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