ESTROUS INFLUENCE ON SURGICAL CURE OF
A MOUSE BREAST CANCER

By H. V. RATAJCZAK,* R. B. SOTHERN,‡ and W. J. M. HRUSHESKY‡

*From the *Department of Pathology, Stritch School of Medicine, Loyola University, Maywood, Illinois 60153; and the ‡Division of Oncology, Department of Medicine, University Hospitals and the Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota 55455*

It is difficult to understand why some premenopausal women with breast cancer succumb quickly to metastatic cancer after local treatment, whereas others with apparently comparable stages of disease are cured by resection or other local treatment. Breast cancers arising in human beings and mice are often highly responsive to changes in sex hormone concentrations or drugs that change the ability of these hormones to bind to their receptors or transduce the resultant message to the genome of the breast cancer cell. These responses frequently include total disappearance of all evidence of breast cancer for many years. The menstrual cycle in women, or estrous cycle in the female mouse, is responsible for precisely timed high amplitude and well-coordinated fluxes of a series of powerful hormones that have many important and predictable physiologic, biochemical, and immunologic consequences (1). With this in mind, we have studied the relationship between the estrous cycle in the mouse and the metastatic potential of a transplantable estrogen receptor–bearing mammary adenocarcinoma.

The C₃HeB/FeJ mouse is an inbred animal that allows growth of a transplanted mammary tumor that originated spontaneously in a C₃H inbred mouse. The C₃HeB/FeJ mouse does not harbor the virus that causes mammary adenocarcinoma in the C₃H mouse and therefore will not transmit the virus to its young by suckling. The mammary adenocarcinoma used in this study has repeatedly been shown to be relatively rich in estrogen receptors (2). As is the case in the human disease, when the C₃HeB/FeJ mouse is implanted with this mammary adenocarcinoma and the tumor is surgically removed after several weeks of growth, not every mouse dies of subsequent metastases (3).

In an attempt to better understand the basis for this biological heterogeneity, we hypothesized that the stage of the estrous cycle of an individual animal at the time of tumor implant and/or primary tumor resection might influence metastatic potential. In addition, this study defined the estrous periodicity of cell types in vaginal smears of the C₃HeB/FeJ mouse, determined if this periodicity changed upon introduction of an estrogen receptor–bearing mammary adenocarcinoma, and deter-

mined whether the estrous periodicity changed again upon removal of the tumor-bearing leg or amputation of a normal leg in control mice.

Our results demonstrate that the presence of a transplanted syngeneic estrogen receptor–bearing tumor predictably affects the estrous cycle and, more importantly, that the timing within the estrous cycle of surgical removal of this tumor, regardless of its size, profoundly influences the expression of subsequent tumor cell metastasis.

Materials and Methods

Experimental Approach.   176 female C_3HeB/FeJ mice (The Jackson Laboratory, Bar Harbor, ME) at least 10 wk old were housed five per cage with lights on for 12 h of each 24-h day (lights on 06^{00}-18^{00}, lights off 18^{00}-06^{00}, local time) with food (laboratory chow) and water available ad libitum. At daily intervals, a total of 9,761 vaginal smears were obtained between 2 and 6 h after light onset (49–66 smears/mouse) (Table I). A drop from a Pasteur pipette of sterile PBS (pH 7.2) was added to the vagina and the fluid was aspirated immediately and transferred to a glass slide. Slides were stained with hematoxylin and eosin.

The tumor used in these studies was a spontaneous mammary adenocarcinoma that arose in a female C_3H mouse and that was repeatedly carried by subcutaneous transplantation in female C_3HeB/FeJ mice. Malignant cells were isolated from a tumor ~10 mm in diameter, minced with scissors, and suspended in Medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with penicillin (P, 50 U/ml) and streptomycin (S, 50 μg/ml). Cell viability was assessed by trypan blue dye exclusion. Transplantation was accomplished by subcutaneous injection of 2×10^5 viable cells suspended in 0.1 ml medium into the left hind leg at the base of the tibia. 14–17 d after inoculation, the primary tumor was first measured (length × width in millimeters) and then removed by amputation of the leg at the proximal end of the femur while the animal was under light Metofane® anesthesia. 30 normal animals had their left hind legs similarly amputated and they served as a control for assessing the effect of operation alone.

Mice were singly housed after amputation and daily vaginal smears were obtained at the same time of day for the ensuing 28 d. At this point all 132 surviving original tumor bearers were killed by cervical dislocation and lung metastases were noted and measured with a caliper (length × width in millimeters).

Bio-assay and Vaginal Cell Quantification.   Each mouse without visible metastases was further studied using a bio-assay for metastases. The lungs of each apparently tumor-free animal were minced in RPMI-1640 (Gibco Laboratories) supplemented with P and S and half of the suspension was injected subcutaneously into each of the shaved flanks of a normal syngeneic mouse (one donor per recipient). These recipient mice were subsequently observed for tumor growth for 4 mo. The frequencies of grossly visible metastases and bio-assay positivity for metastatic disease were then compared with regard to the size of tumor at resection and also with regard to the cellular pattern in the vaginal smear (estrous stage) at the time of initial tumor implant and the time of surgical removal of the primary transplanted tumor by amputation.

It is well established that cell types of vaginal smears indicate stage of the estrous cycle. In this investigation, no studies were done to correlate cell types in vaginal smears with hormonal status or reproductive changes physiologically. Therefore, in this investigation vaginal cell types were scored on a plus basis: leukocytes (L) were either absent, 1^+, 2^+, or 3^+. Likewise, cornified epithelial cells (C) were either absent or 1^+, 2^+, or 3^+. Combined cellularity of the vaginal smears with regard to these two cell types was then scored between 0 and 6. Nucleated epithelial cells were relatively constant in number and were not included in this analysis. An arbitrary division separating the cellularity equally was made at 0–3 and 4–6, roughly approximating proestrus/estrus and metestrus/diestrus, respectively. The incidence of surgical cure was then compared as a function of whether few or many leukocytes and/or cornified epithelial cells were present in the vaginal smear on the day of tumor transplant and tumor resection or amputation. Seven mice with recurring local tumor growth at the site of resection and six mice that died early in the study from surgical complications had

TABLE I

Study Design and Details

Group	Number of mice	Age at start	Tumor bearing?	First smear	Tumor inoculation	Leg/tumor amputation	Sacrifice	Smears/ mouse	Died	Recurs	Evalu- able
		wk									
1A	20	12	Yes	Nov 16 (←4 d)	Nov 12 (17 d→)	Nov 29 (28 d→)	Dec 27	49	0	1	19
1B	39	20	Yes	Nov 16 (←4 d)	Nov 12 (17 d→)	Nov 29 (28 d→)	Dec 27	49	3	3	33
2	57	18	Yes	Nov 30 (←10 d)	Nov 19 (14 d→)	Dec 03 (28 d→)	Dec 31	52	1§	2	55§
3A	9	27	Yes	Dec 29 (22 d→)	Jan 19 (14 d→)	Feb 02 (28 d→)	Mar 02	64	3	0	6
3B	6	11	Yes	Dec 29 (22 d→)	Jan 19 (14 d→)	Feb 02 (28 d→)	Mar 02	64	0	0	6
4	15	11	Yes	Dec 29 (24 d→)	Jan 21 (14 d→)	Feb 04 (28 d→)	Mar 04	66	0	1	14
Totals	146								7	7	133§
5	10	27	No	Dec 29 (22 d→)	—	Jan 19 (42 d→)	Mar 02	64	1	—	9
6	10	11	No	Dec 29 (36 d→)	—	Feb 02 (28 d→)	Mar 02	64	0	—	10
7	10	11	No	Dec 29 (38 d→)	—	Feb 04 (28 d→)	Mar 04	66	1	—	9
Totals	30								2	0	28
Totals	176								9	7	161

One mouse died before amputation, six mice died shortly after amputation, and three died before date of sacrifice (two with a recurring primary tumor). One mouse (§) died 4 d before it would have been killed and had metastases; it was included in final calculations. The primary tumor recurred at the amputation site in another six mice. All of these mice were eliminated from metastatic calculations. Values given in parentheses between study dates indicate interval (in days) and direction (before [←] or after [→]) of following column.

to be excluded from analysis for metastatic potential. One mouse died with metastases present 24 d after leg amputation and was included in final calculations.

Results

Expression of Metastases vs. Time of Primary Tumor Removal.   While the eventual incidence of pulmonary metastases was not influenced by vaginal smear content (estrus stage) on the day of tumor implantation (x^2 = 0.40, p = 0.53), the time of resection of the tumor-bearing limb markedly influenced the incidence of pulmonary metastases 28 d later. 36 of 133 mice (27%) were apparently disease free by gross inspection of the lungs 28 d after amputation of the primary tumor (Fig. 1, *left*). 40% (24 of 60) of the mice with small amounts (0 to 3) of L+C cells on day of tumor resection were grossly disease free, while only 16.4% (12 of 73) with large amounts (4–6) of L+C cells on day of resection were disease free at this point of the study (x^2 = 9.26, p = 0.002). Bioassay using the lungs of animals that were grossly free of metastases resulted in some tumor induction in recipient mice, reducing the final bio-negative, disease-free totals to 16 of 60 (27%) for L+C = 0–3, and 9 of 73 (12.3%) for L+C = 4–6 (x^2 = 4.44, p = 0.035) (Fig. 1, *right*; Table II).

Expression of Metastases vs. Primary Tumor Size.   A statistically significant difference in mean tumor sizes at resection among the five groups of tumor-bearing animals was found by analysis of variance (ANOVA: F = 132.6, p < 0.0001). In addition, two distinct but separate normal distributions of tumor sizes were found at resection
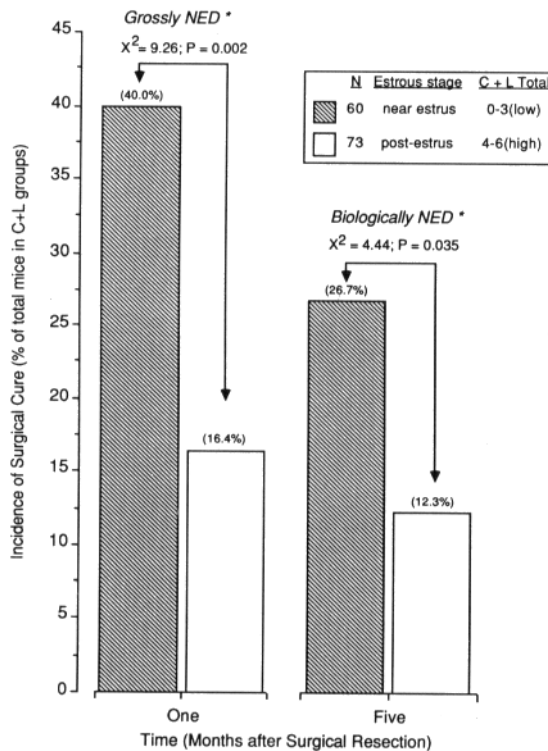

FIGURE 1.   Cell types in vaginal smear at time of surgical removal of transplanted estrogen receptor-bearing tumor significantly affect whether or not metastases ultimately appear in female mice. Grouping consisted of mice with low total cell counts (0–3), indicating near or in estrus (highest fertility) and mice with high total cell counts (4–6), indicating a post-estrus stage (lowest fertility). 132 mice were killed 28 d after amputation and metastases in the lungs were counted (one mouse with metastases died 4 d before it would have been killed, and was included for a total of 133 mice). At this time, 40% of mice with low cell counts were disease free as compared with only 16.4% of the mice with high total cell counts (x^2 = 9.26, p = 0.002). 5 mo after leg amputation, 26.7% of mice with low cell counts were classified as bio⁻ (disease free), as compared with only 12.3% of mice with high total cell counts (x^2 = 4.44, p = 0.035).
* No evidence of disease.

TABLE II

Metastasis depends upon time of tumor removal

Sub- grouping	Metastases	Score of L + C/number of mice (percent of total):		χ^2	p value
		0–3	4–6		
1	Yes (gross and bio⁺)	44 (73.3%)	64 (87.7%)	4.44	0.035
	No (bio⁻)	16 (26.7%)	9 (12.3%)		
2	Yes (gross only)	36 (60.6%)	61 (83.6%)	9.26	0.002
	No* (bio⁺ and bio⁻)	24 (40.0%)	12 (16.4%)		
3	Yes (gross only)	36 (60.0%)	61 (83.6%)		
	No* (bio⁺)	8 (13.3%)	3 (4.1%)	9.50	0.009
	No (bio⁻)	16 (26.7%)	9 (12.3%)		

Metastatic incidence grouping mice by numbers of leukocytes (L) and cornified (C) cells observed in vaginal smear on day of primary tumor removal. Mice with grossly apparent metastases on day 28 were coded as "yes (gross)." Lung tissue, disease free at this time, was minced and implanted into naive mice (bioassay), who were observed for 4 mo. If, after this span, tumors appeared, the animal from whom lungs were taken was coded as "bio⁺" and if no tumor appeared the donor animal was coded as "bio⁻."

* Not visible.

with mice in Study 2 displaying smaller tumors than those in the other four groups. Although mice in Study 2 (late November, 18 wk old at start of study) had mean tumor sizes (in mm²) nearly a third of that of the other mice (45 ± 2 [SE], n = 54), a statistically significant difference was also observed among the remaining groups of mice (F = 7.6, p = 0.0002 from ANOVA). The mean sizes of larger tumors ranged from 105 mm² ± 4 for group 4 (studied late in December) to 128 mm² ± 6 SE for group 1A (studied in mid-November). As would be expected, there was a significant, but relatively unimpressive, correlation of tumor size at resection and the number of pulmonary metastases present 28 d later (r = 0.33), as well as the absolute volume of those pulmonary metastases (r = 0.26). However, this was brought about by the two separate tumor size populations, since if small and large tumor populations (Study 2 vs. other studies) were handled separately, there was no correlation of primary tumor size and number or size of metastases. In fact, actual incidence (as opposed to number or size) of pulmonary metastases between Study 2 (with smaller tumors) and the other studies (with larger ones) was not significantly different (χ^2 = 1.81, p < 0.20) for 36 of 54 mice in Study 2 vs. 61 of 79 in the remaining studies.

To normalize tumor sizes among study groups for subsequent analyses, each individual tumor size was reexpressed as a percentage of mean tumor size of all mice in the respective study group. This resulted in a normal distribution of tumor sizes for all 133 mice, with individual sizes ranging from 26 to 204% of mean size (mean = 100% ± 2.4). After normalization of primary tumor sizes to percent of group mean sizes, there was no correlation of tumor size with number nor size of pulmonary metastases for the entire group of 133 mice (r = −0.004 for both correlations). A t test comparing primary tumor sizes of mice at resection that did not display metastases (99.97% ± 4.40) vs. those that did (100.01% ± 2.84) was not significant at 28 d (t = 0.01, p = 0.993), nor at 4 mo (100.81% ± 2.62 vs. 96.33 ± 5.84, respectively; t = 0.72, p = 0.473). A comparison of primary tumor sizes vs. vaginal cellular counts at time of resection was not significant when the 133 mice were divided
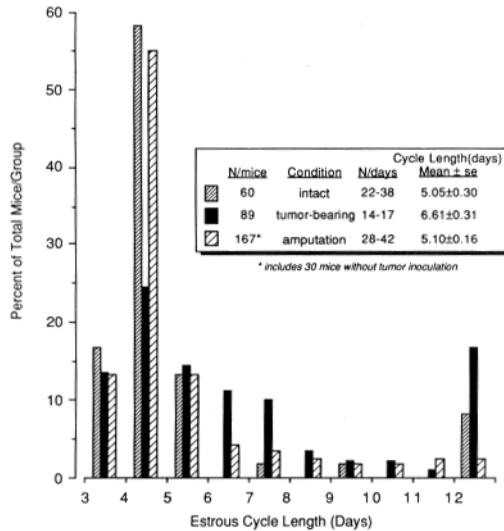
FIGURE 2. Distribution of estrous cycle lengths in C_3HeB/FeJ mice determined by using the sum of cornified cells plus leukocytes (both noted on a scale of 0–3) observed in daily vaginal smears. To estimate the average periodicity of cell type occurrence for each mouse, each of the series was divided into three parts representing the intact span (covering 22–24 d for 30 mice before tumor inoculation and 22–38 d for 30 control mice before leg amputation), the tumor-bearing span (14–17 d for 89 mice) and post amputation (28–42 days after removal of the leg for all mice, including 30 mice without tumor inoculation) (see Table I). Each time series of L or C combined scores was analyzed by the least-squares fit of cosine curves with trial periods between 3 and 12 d and with 6-h increments bet.·een trial periods (4). The length of the best-fitting curve, indicating the average time between maximal or minimal occurrence of L, C, or L+C, during the spans of interest was then compared statistically using Students t test in order to compare the data from each mouse and the behavior of the group mean period lengths.

into low counts (0–3) and high counts (4–6) (97.1% ± 3.5 for $n = 60$ vs. 102.3% ± 3.2 for $n = 73$; $t = 1.08$, $p = 0.28$). This was also true for the 26 mice with vaginal counts at time of tumor inoculation ($t = 1.24$, $p = 0.226$).

At 28 d after resection, the mean tumor size for mice not displaying pulmonary metastases was 101.9% ± 5.9 for 29 mice with C+L = 0 and 3, and 94.1% ± 5.4 for 12 mice with C+L = 4–6. The mean tumor sizes for mice with metastases at 28 d were 94.0% ± 4.4 for 36 mice with C+L = 0–3 and 104.0% ± 3.7 for 61 mice with C+L = 4–6. A two-way ANOVA comparing the interaction of the resected tumor size with vaginal cellular counts at resection and expression of pulmonary metastases showed no effect of primary tumor size for 133 mice at 28 d (F = 2.50, $p = 0.117$), nor at 4 mo (F = 0.49, $p = 0.486$). These results were similar when comparing resected tumor size and metastatic incidence with vaginal cell counts available for 26 mice at inoculation.

It was thus clear that resected tumor size was not solely responsible for the development of subsequent pulmonary metastases and that at least one other important factor was influencing and overriding the anticipated correlation of tumor size itself with metastatic potential. This factor was the timing of resection within the mouse's estrous cycle.

Estrous Cycle Length. In defining the periodicity of L and C cells in vaginal smears of the C_3HeB/FeJ mouse, cosines with trial periods between 3 and 12 d were tested with 6-h increments between periods (4). The best-fitting estrous periodicity before tumor transplant was defined as being, on average, 5.05 ± 0.30 d (Fig. 2 and Table III). Presence of a very small and growing tumor increased the average period length to 6.61 ± 0.31 d ($t = 3.42$, $p = 0.001$). Lengthening of periodicity of cells in vaginal smears by presence of a tumor is not a novel observation and was reported as early as 1922 (5). Tumor removal by amputation of the tumor-bearing leg resulted in the

TABLE III

Average Period Length of Estrous Cycle for Mice Before, During, and After Bearing an Implanted Mammary Adenocarcinoma and for Control Mice Before and After Removal of a Non-Tumor-bearing Hind Leg

| Group | Treatment | N | Average length of estrous period (days ± SE) observed for cell types: | | |
			Leukocytes (L)	Cornified cells (C)	L + C
Normal	None	30	5.09 ± 0.44	7.50 ± 0.58	4.88 ± 0.38
	Surgery	28	4.15 ± 0.07	5.60 ± 0.58	4.59 ± 0.35
Tumor	None	60	4.83 ± 0.25	7.67 ± 0.40	5.05 ± 0.30
	Tumor	60	6.33 ± 0.28	7.42 ± 0.36	6.61 ± 0.31
	Surgery	167	4.57 ± 0.10	5.86 ± 0.21	5.10 ± 0.16

| | | Significance (p value from t test): | | | | | |
	Spans compared	t	p	t	p	t	p
Normal	None vs. surgery	2.08	0.042	2.31	0.024	0.55	0.586
Tumor	None vs. tumor	3.85	<.001	0.46	0.460	3.42	0.001
	Tumor vs. surgery	7.47	<.001	3.76	<.001	4.73	<.001
	None vs. surgery	1.20	0.230	4.05	<.001	0.15	0.877

Periodicity of cell types in vaginal smears determined by best-fitting cosine curves (with trial windows from 3 to 12 d).

very precise return to a normal period length of 5.10 ± 0.16 d (t = 4.73, p < 0.001). It is interesting to note there were five mice (8%) that had 12-d periods in the intact group, and 15 mice (17%) with 12 d periods in the tumor-bearing group. The reason for these long cycles is not known with certainty but probably relates to individual mice infrequently skipping estrus. Fig. 2 clearly demonstrates the behavior of estrus in these outliers as well as the overall lengthening and shortening of estrous cycling in the remainder of the animals. Amputation of a tumor-bearing leg caused most animals to revert to shorter cycles. The length of the period of 30 normal animals (4.6 ± 1.4 d) was unaffected by surgery (t = 0.55, p = −0.59) and was not significantly different from mice whose tumors had been resected (t = 1.57, p = 0.12). These analyses are a refinement of results reported earlier on reversion of the estrous cycle to normal duration after surgical removal of the tumor (6).

If L alone or C alone were used in the described periodicity analysis, very similar results occurred (Table III). L alone followed the same pattern as the combination of L+C with significant differences between the estrous cycle length of tumor bearers and those before or after tumor. The estrous period defined by C alone showed no significant change when animals were given tumor but did shorten significantly when the tumor was removed.

Discussion

Presently, premenopausal women with larger primary tumor (>1-inch diameter) or axillary lymph node involvement with tumor (clinical stage II breast cancer) are routinely subjected to high-dose intensity multi-agent chemotherapy given every 3 to 4 wk for 6–12 mo after total resection of the primary tumor. This approach has

been proven to prevent breast cancer relapse and improve long-term survival of these high risk patients. A consensus meeting of breast cancer experts has agreed that the benefit conferred by aggressive adjuvant chemotherapy in these women raises their probability of long-term disease-free survival by between 9 and 30% (7). Our data clearly show that, according to how the fertility cycle is partitioned and referenced, tumor resection is curative at least two or three times as frequently depending upon when in the mouse's cycle the operation is performed. Surgical cure is reproducibly most frequent when the operation is performed just before and during the span commonly associated with ovulation. This phase is a time associated with high amplitude rhythmic surges of estrogen. Fatal metastatic disease occurs most reproducibly when the primary tumor is resected at the time in the cycle associated with the lowest fertility, metestrus in mice, which is comparable to the time around menstruation in women, a time of rhythmic estrogen withdrawal.

Patients, who are identical with regard to the size of the primary cancer, stage of disease at diagnosis, hormonal receptor status, and the number of axillary lymph nodes involved by cancer, frequently have vastly different outcomes. These observations have led to studies concerned with the factors that influence the "metastatic potential" of tumor cell clones. A great deal of this work has focused upon the cellular, biochemical, and genetic basis by which tumor cells differ in their metastatic potential (8–10). Another large body of work highlights the host, its defenses, its neurohumoral influence over the cancer cells, and how these factors might help to determine the ultimate clinical outcome. This report focuses on the biological consequences of rhythmic hormonal changes in the host and how those hormonal changes affect the interaction of tumor cells and host tissues.

It has already been shown, for example, that the balance between host and tumor changes predictably as a function of time of day and from season to season, as well as over the course of disease progression (11–19). Our present data indicate that a complex "crosstalk" between this transplanted malignancy and its murine host results in a predictable effect of the tumor upon the mouse's estrous cycling and a predictably different metastatic behavior depending upon when in the animal's estrous cycle the primary tumor is resected. While the time of estrous cycle of tumor implant played no role in eventual metastasis of the tumor, ultimate outcome is apparently the result of the interaction of stress hormones and sex hormones at the time of surgery.

Surgically induced changes in the balance between the host and cancer are therefore associated with predictable stress/sex hormone interactions of probable importance to: the biology of host tissues destined to receive circulating metastatic cells (in this case, the lung); the biology of the circulating tumor cells themselves (their ability to implant, traverse boundaries, produce autocrine or paracrine growth factors, induce new vessel formation and enter and remain in active cell division phase); and/or upon the ability of the host immune system to effectively counter the metastatic process (20). The experiments summarized here cannot sort this out, but point the way toward pertinent research in each of these separate areas. Because estrogenic effects upon NK activity have been suggested (21), experiments investigating cellular immunity, as well as host and tumor tissue at different estrous stages, are necessary to fully elucidate the complex interactions responsible for the estrous stage dependence of metastatic potential.

In addition, the presence of this transplanted mammary tumor also reversibly affects

the periodicity of cell types found in vaginal smears of mice. The more precise nature of the substance or substances that these tumor cells produce, or cause other cells to produce, which lengthens the mouse's estrous cycle, remains mysterious. The identification of such a substance could open the door to the discovery of a new class of tumor markers for certain endocrine-dependent breast cancers. The isolation, purification, identification, and production of a "natural" substance that modulates estrous cycling, if novel, also has potential implications for fertility control.

The differences in lengths of estral periods defined by L alone or C alone found in this study conflict somewhat with the findings of Voss (22) in which quantities of L and C were regularly expressed opposite to each other in magnitude. In the present study, peak quantities of these cell types were not perfectly out of phase, but overlapped considerably. This difference may be explained by the differing methodologies and by the use of different strains of mice.

Testing for Hormone Receptors. Useful tests upon tumor tissue that predict the ultimate clinical outcome of human breast cancer include tumor cell estrogen and progesterone receptor concentration determinations (23). A complex, incompletely understood, yet important relationship between mammary adenocarcinoma, hormones, and their receptors has been established (24, 25). The predictable high-amplitude hormonal fluxes occurring during each fertility cycle undoubtedly affect hormone receptor biology within tumor cells and this raises the practical questions of when in the fertility cycle to optimally perform diagnostic or prognostic biopsies, the results of which are routinely obtained to plan breast cancer therapy. There may well be a span within the menstrual cycle during which the receptor concentrations most accurately and reproducibly predict prognosis or likely response to hormonal manipulation. While 65% of patients classified as being "receptor-positive" respond to hormone therapy, more than a third do not. Obtaining biopsies at specific phases of the fertility cycle might improve the predictive accuracy of this invasive and expensive test. A data base correlating hormone receptor concentration and menstrual phase of sampling with frequency of response to hormonal treatment and ultimate outcome is required to adequately investigate this hypothesis.

Implications of Timing of Breast Cancer Surgery. The implications of this interactive communication between tumor and host biology are of substantial basic and clinical interest. If the dynamic neurohumoral milieu associated with the lowest incidence of metastatic disease can be precisely defined, our understanding of some of the fundamental processes governing the spread of cancer will be improved. Pharmacologic mimicking of the neurohumoral state associated with the greatest likelihood of surgical cure before removal of the primary cancer also becomes a real possibility. At the very least, this kind of knowledge makes it possible to plan an excisional biopsy or mastectomy to coincide with the occurrence of the menstrual stage associated with the optimal state of natural resistance to metastatic spread of breast cancer. Krzanowski (26) observed a statistically significant cycling in immunity as expressed by T4/T8 lymphocyte ratios throughout the menstrual cycle in healthy women and concluded that the state of sex cycle at time of surgery may influence the outcome. Preliminary results showing that NK activity in the spleens of C_3HeB/FeJ mice changes predictably during the estrous cycle also argue for the likely relevance of the effects of these hormonal cycles upon cellular immunity (27).

If our work is confirmed by simple retrospective and prospective epidemiologic

studies in human beings that determine the timing of the woman's last menstrual period relative to the time of primary resection, careful consideration of when in the fertility cycle to biopsy or resect the breast cancers of cycling women has the potential to save many thousands of lives worldwide.

Summary

We have studied the effect of estrous stage, as reflected by vaginal cellularity, at the time of surgical resection of an estrogen receptor-bearing mammary adenocarcinoma upon the metastatic potential of that tumor in the C_3HeB/FeJ mouse. Presence of the tumor prolonged the length of the estrous cycle by $\sim$25% and removal of the tumor returned the cycle to its usual duration. Neither estrous stage at tumor implant nor size of tumor at resection (within a small range) had significant independent effects upon differences observed in the incidence of subsequent pulmonary metastases. However, estrous stage at time of surgical removal of the tumor, as reflected by cell types in vaginal smear, markedly affected whether or not metastases ultimately appeared. Because the estrous cycle in mice, comparable to the human menstrual cycle, reflects high-amplitude, rhythmic changes in hormone concentrations, it may be that the hormonal status of a woman at the time of tumor resection is an important determinant of whether or not that breast cancer ultimately metastasizes.

References

1. Nicol, T., and B. Vernon-Robert. 1965. The influence of estrous cycle, pregnancy and ovariectomy on RES activity. *J. Reticuloendothel. Soc.* 2:15.
2. Fisher, B., N. Gunduz, S. Zheng, and E. A. Saffer. 1982. Fluoresceinated estrone binding by human and mouse breast cancer cells. *Cancer Res.* 42:540.
3. Ratajczak, H. V., M. G. Lewis, and K. Duggal. 1986. Increased chemotaxis of leukocytes from mice bearing tumors. *Cancer Res.* 46:2225.
4. Nelson, W., Y. L. Tong, J. K. Lee, and F. Halberg. 1979. Methods for cosinorrhythmometry. *Chronobiologia.* 6:305.
5. Allen, E. 1922. The oestrous cycle in the mouse. *Am. J. Anat.* 3:297.
6. Ratajczak, H. V., R. B. Sothern, and W. J. M. Hrushesky. 1986. Single cosinor analysis of vaginal smear cell types quantifies mouse estrous cycle and its alteration by mammary adenocarcinoma. *Annu. Rev. Chronopharmacol.* 3:223.
7. Wittes, R. E. 1986. Proceedings of the NIH Consensus Development Conference on Adjuvant Chemotherapy and Endocrine Therapy for Breast Cancer. *Natl. Cancer Inst.* 1:165.
8. Fidler, I. J., and M. L. Kripke. 1977. Metastasis results from preexisting variant cells within a malignant tumor. *Science (Wash. DC).* 197:893.
9. Poste, G., J. Doil, and I. J. Fidler. 1981. Interactions among clonal subpopulations affect stability of the metastatic phenotype in polyclonal populations of B16 melanoma cells. *Proc. Natl. Acad. Sci. USA.* 78:6226.

10. Fidler, I. J., and G. Poste. 1982. The heterogeneity of metastatic properties in malignant tumor cells and regulation of the metastatic phenotype. *In* Tumor Cell Heterogeneity, A. H. Owens, D. S. Coffey, and S. B. Baylin, editors. Academic Press, New York. 127–145.

11. Eilber, F. R., and D. L. Morton. 1970. Impaired immunologic reactivity and recurrence following cancer surgery. *Cancer.* 25:362.

12. Eilber, F. R., A. Nizze, and D. L. Morton. 1975. Sequential evaluation of general immune competence in cancer patients: correlation with clinical course. *Cancer.* 35:660.

13. Hughes, A. H., H. I. Jacobson, R. K. Wagner, and P. W. Jungblut. 1976. Ovarian-independent fluctuations of estradiol receptor levels in mammalian tissues. *Mol. Cell. Endocrinol.* 5:379.

14. Lakatua, D., E. Haus, K. Labrosse, C. Veit, and L. Sackett-Lundeen. 1986. Circadian rhythm in mammary cytoplasmic estrogen receptor contact of BALB/c female mice with and without pituitary isografts. *Chronobiol. Int.* 3:213.

15. Hrushesky, W. J. M., T. Teslow, F. Halberg, D. Kiang, and B. J. Kennedy. 1979. Temporal components of predictable variability along the 1-year scale in estrogen receptor concentration of primary human breast cancer. *Proc. Am. Soc. Clin. Oncol. Annu. Meet.* 20:331.

16. Cohen, P., Y. Wax, and B. Modan. 1983. Seasonality in the occurrence of breast cancer. *Cancer Res.* 43:892.

17. Jacobson, H. I., and D. T. Janerich. 1980. Is seasonality in human reproduction related to seasonality in tissue levels of estrogen receptor? *In* Functional Correlates of Hormone Receptors in Reproduction. Elsevier Science Publishing Co., Inc., New York. 573–578.

18. Hrushesky, W. J. M., E. Haus, D. Lakatua, N. Vogelzang, and B. J. Kennedy. 1983. Seasonality in testicular cell proliferation and seminoma incidence. *Proc. Am Soc. Clin. Oncol. Annu. Meet.* 24:18.

19. Hrushesky, W., D. Lannin, and R. Olshefski. 1983. Transplantable tumor growth depends upon: circadian stage, anatomic site and size of tumor inoculum. *Am. Assoc. Anatomists.* 205:85A. (Abstr.)

20. Krzych, U., H. R. Strausser, J. P. Bressler, and A. L. Goldstein. 1978. Quantitative differences in immune responses during various stages of the estrous cycle in female BALB/c mice. *J. Immunol.* 121:1603.

21. Seaman, W. E., T. D. Gindhart, J. S. Greenspan, M. A. Blackman, and N. Talal. 1979. Natural killer cells, bone and the bone marrow: studies in estrogen treated mice and in congenitally osteopetrotic mice. *J. Immunol.* 122:2541.

22. Voss, H. E. 1930. Der postpartum-oestrus der Nagetiere. *Biol. Generalis.* 6:433.

23. Jenson, E. V., E. R. Desombre, and P. W. Jungblut. 1967. Estrogen receptors in hormone-responsive tissues and tumors. *In* Endogenous Factors Influencing Host-Tumor Balance, R. W. Wissler, T. L. Dao, and S. Wood Jr., editors. University of Chicago Press, Chicago. 15–30.

24. Wittliff, J. L. 1984. Steroid-hormone receptors in breast cancer. *Cancer.* 53:630.

25. Lakatua, D., E. Haus, H. Berg, and L. Sackett-Lundeen. 1984. Mesor elevation in circadian rhythm in estrogen receptor activity in the uterus of BDF1, female mice carrying an MXT3.2 transplantable breast cancer. *In* Chronobiology 1982–1983. E. Haus and H. Kabat, editors. S. Karger Publishers, New York. 49–54.

26. Krzanowski, M. 1985. Low human T4:T8 lymphocyte ratios around ovulation time. *Chronobiologia.* 12:254.

27. Hrushesky, W. J. M., and R. L. Simmons. 1988. Metastatic potential and splenocyte NK activity are each estral stage dependent. *Int. Natural Killer Workshop, 5th, Hilton Head Island, SC.*