AD_____

Award Number DAMD17-94-J-4201

TITLE: Effects of Hormone Replacement Therapies and Dietary Phytoestrogens on the Mammary Gland of Macaques

PRINCIPAL INVESTIGATOR: J. Mark Cline, D.V.M., Ph.D.

CONTRACTING ORGANIZATION: Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157-1040

REPORT DATE: July 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Final (1 Jun 94 - 30 Jun 99)

4. TITLE AND SUBTITLE
Effects of Hormone Replacement Therapies and Dietary Phytoestrogens on the Mammary Gland of Macaques

5. FUNDING NUMBERS
DAMD17-94-J-4201

6. AUTHOR(S)
J. Mark Cline, D.V.M., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Bowman Gray School of Medicine
Winston-Salem, North Carolina 27157-1040

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The purpose of this study has been to use histomorphometric and immunohistochemical techniques to study the incidence and characteristics of mammary gland hyperplasia, dysplasia and other possible indicators of breast cancer risk, in cynomolgus macaques given long-term hormonal treatments. Treatments evaluated to date include conjugated estrogens (CEE), medroxyprogesterone acetate (MPA), the combination of CEE and MPA, tamoxifen, estradiol (E2), 17α-dihydroequilenin (DHEN), soybean phytoestrogens (SBE), and SBE + E2. Pathologic evaluation, histomorphometric evaluations, and staining for estrogen receptor, progesterone receptor, and the proliferation marker Ki-67 MIB were done. Results are as follows: The addition of MPA to CEE therapy increases, rather than decreases, mammary gland proliferation. Tamoxifen treatment does not induce mammary gland proliferation beyond that seen in controls; this is in contrast to a marked uterotrophic effect. DHEN does not induce mammary gland or endometrial proliferation. Soybean estrogens do not induce mammary or endometrial proliferation when given alone, and exert an antagonistic effect on E2-induced proliferation. New work includes dietary modulation of hormonal effects on mammary gland, interactions of tamoxifen and estradiol, identification of p53expression in CEE-treated macaque mammary gland, assessment of expression of estrogen receptor beta, and further agonist/antagonist effects of soy phytoestrogens.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, estrogen, hormone replacement therapy, macaques, phytoestrogens, progesterone, tamoxifen	49
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JMC Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JMC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

J Mark Cline 7/29/99

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

• Background

Postmenopausal estrogen replacement has been shown to have major beneficial effects in the prevention of coronary heart disease (Avila, 1990; Bush 1987; Henderson 1988; Hunt 1987; Petitti 1986; Stampfer 1985) and osteoporosis (Ettinger 1985; Kiel 1987; Weiss 1980; Ravnikar 1992; Colditz 1990). Unfortunately, the public health impact of these benefits to postmenopausal health has been small because of poor patient compliance. In the United States, 15% of naturally postmenopausal women 45 to 54 years of age use hormone replacement therapy (HRT), while the number decreases to 6% at ages 55 and older. A recent review of this problem indicates that **concern over the risk of breast cancer is the greatest single disincentive for the use of HRT** (Ravnikar 1992). Identification and quantification of this risk is compromised by a lack of basic knowledge of the hormonal regulation of the breast.

The concerns of women regarding breast cancer risk associated with hormonal therapies have some basis in the results of recent epidemiologic studies (Colditz 1993; Colditz 1995; Coope 1992; Pike 1993). Colditz et al found a significant association between current estrogen replacement therapy and increased breast cancer risk (Colditz 1992, 1993). The mechanistic basis for this increased risk is unknown. The mitogenic effects of estrogens on both breast and endometrial tissue are well recognized, as are the beneficial effects of progestins on endometrial cell proliferation and cancer risk. The action of progestins on breast tissue is more controversial; the literature offers a number of conflicting results both *in vitro* (Mauvais-Jarvis 1986; Haslam 1988; Papa 1990; Moore 1991), and in vivo (Anderson 1989; de Lignieres 1992; Gompel 1993; Bergkvist 1989; Ohi 1992). The assumption that breast and uterus are regulated similarly leads to the conclusion that the combined hormone replacement therapy designed to decrease the risk of endometrial cancer (i.e. estrogen + a progestin) is also appropriate for breast. However, a recent meta-analysis of studies including women treated with estrogen plus a progestin did not show a protective effect of the use of progestin (Colditz 1993). Recently, data from the Nurse's Health Study has shown a similar result (Colditz 1995). A particularly disturbing paper published recently indicates that the overall survival advantage provided by hormone replacement therapy (HRT) is lost after 10 years of treatment, largely as a result of increased breast cancer incidence (Grodstein et al., 1997).

Oral contraceptive use is generally considered to have minimal effect on breast cancer risk (WHO, 1992), although there is some evidence for increased risk in long-term current users (La Vecchia, 1995).

Studies of breast regulation, particularly in the postmenopause, are limited. Most studies have involved one of the following:
1. in vitro models which do not adequately mimic the hormonal milieu of the breast
2.in vivo studies in rodent models which are different in many ways from women
3. observational and epidemiologic studies of women which are confounded by lack of experimental rigidity.
4. studies in women using minimally invasive techniques such as fine-needle aspiration, which do not allow study of spatial/paracrine relationships within the breast.

Experimental studies using human tissues are subject to confounding errors: The apparently normal breast tissue in breasts operated for benign or malignant lesions may be subject to paracrine influence from the tumor, and the breasts of reduction mammoplasty operated women contain considerably more adipose tissue than the breasts from normal average women. Fine needle aspiration biopsies from healthy women also have limitations: Different epithelial areas of the mammary gland can not be safely distinguished in such samples. The ideal model would be based

on surgical samples from healthy women, which is a practical impossibility.

Thus the problem lies in the lack of an adequate experimental system in which to test hypotheses relating to breast cancer risk. Our work is designed to use the cynomolgus macaque model to answer questions relating to breast regulation and breast cancer risk.

The associations between proliferative breast lesions and breast cancer risk are quite strong in women. Within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation result in increased risk of transformation to the neoplastic phenotype (Cohen 1991, Butterworth 1992). Among women with benign breast lesions, ductal hyperplasia with atypia is a strong risk factor for the development of overt breast carcinoma (London 1992, Page 1992). Within populations of cells *in vitro* and *in vivo*, high rates of cellular proliferation increase the risk of transformation to the neoplastic phenotype. It is likely that this general phenomenon applies to the breast as well (Moolgavkar 1980).

- **Scope of the present work**

The effects of various hormone therapies on atherosclerosis and osteoporosis in female monkeys have been studied for some time at our institution (Adams 1991, Clarkson 1989). **The work described herein further uses this model system for study of the breast.** We have evaluated several studies which provide a unique opportunity for evaluation of the effects of estrogens, progestins, and androgens on mammary gland and endometrium *in vivo*, using animals given doses equivalent to those used in women. Therapies used include postmenopausal estrogen (Premarin) with and without a progestin (MPA); postmenopausal treatment with Premarin, MPA, the combination, or tamoxifen; premenopausal administration of Triphasil; premenopausal Triphasil followed by postmenopausal HRT; and postmenopausal nandrolone, an androgenic steroid. Our studies have also assessed the effects of dietary (soy) phytoestrogens. This collection of material represents a unique resource for the study of proliferative lesions induced in target tissues by hormonal replacement therapy.

Macaques are similar to women in many aspects of reproductive physiology and anatomy. Macaques have a distinct menarche and menopause, at about 3 and 20 years of age, respectively. They have a 28-day menstrual cycle, with a hormonal profile similar to that of women (Mahoney 1970). Their endometrial responses to endogenous and exogenous hormones parallel those of women (Kaiserman-Abramof 1989). Mammary glands in these animals differ from the human breast grossly, but microscopically the mammary tissues of women and female macaques are quite similar (Schultz 1937, MacPherson 1974, Benirschke 1978). Primate mammary gland has unique cytokeratin phenotype which is identical in macaques and women, but is not shared by more distant species (Tsubura 1991). In this and othe aspects of breast biology relevant to cancer risk (such as proto-oncogene and tumor suppressor gene expression), the mammary gland of macaques is quite similar to that of women (Cline, unpublished data). Mammary neoplasms are uncommon in macaques (Benirschke 1978, Warner 1979). However, there are occasional reports of mammary gland tumors in macaques (Beniashvilli 1989), and a recent paper by Uno indicates that long-term evaluation of macaques may reveal relatively high incidences of mammary and colonic neoplasms (Uno, 1997). We believe that the female macaque model provides a unique alternative for measurement of responses of the breast to exogenous and endogenous hormones. This model also allows for extensive studies of treatment effects in normal breast which could not be done in women, for example serial biopsy studies or determinations of regional variations in breast regulatory processes.

Very few studies have dealt with the responses of macaque mammary glands to exogenous hormones (Speert 1948, Tavassoli 1988) Findings to date are that estrogens, progestins, and growth hormone can induce mammary gland proliferation, and that high doses of estrogens may

induce neoplasms (Tavassoli 1988).

- **Purpose of the present work**

The specific aim of this work is to assess the effects of oral contraceptives and hormonal therapies on the incidence and severity of hyperplastic and dysplastic lesions in the mammary gland of macaques, and to assess regulatory alterations such as changes in sex steroid receptor expression. The following hormone therapies are being evaluated (specific doses are listed on page18).

Treatments given to premenopausal animals
Triphasil
Ethinyl estradiol (the more estrogenic component of Triphasil)
Levonorgestrel (the more progestinic component of Triphasil)
Soybean Phytoestrogens

Treatments given to postmenopausal animals
Estradiol
Premarin (conjugated equine estrogens)
Premarin + medroxyprogesterone acetate
Medroxyprogesterone acetate
Nandrolone
Tamoxifen
Tamoxifen+Estradiol
17α- dihydroequilenin
Soybean Phytoestrogens
Soybean phytoestrogens + estradiol

METHODS

Our basic approach is the use of intermediate markers of breast dysregulation in macaques (hyperplasia, dysplasia, epithelial proliferation measured by Ki-67 expression, changes in estrogen and progesterone receptor expression, and expression of the p53 tumor suppressor gene product) in order to identify which hormonal treatments might induce a greater risk of breast cancer in women.

Study Design

Animals subjected to a variety of hormonal manipulations are included in this work, as well as control monkeys from these studies, which has allowed concurrent study of the normal menstrual cycle. Studies from which tissues are being collected include the following:

Three studies dealing with postmenopausal hormone replacement therapy:
88-14 Estrogen replacement/secondary intervention trial
91-20 Estrogen replacement primary intervention trial
93-16 17α-dihydroequilenin study

One study using premenopausal (contraceptive) steroids and postmenopausal comparison of estrogen replacement versus dietary soy supplementation:
91-12 Oral contraceptive atherosclerosis primary prevention trial/ Soy as an estrogen alternative trial

One study of contraceptive steroids alone:
91-24 Oral contraceptive study (Triphasil and its components)

One study of androgenic steroids used to prevent osteoporosis:
92-04 Anabolic steroid study

The above-listed studies were described in the original grant application. Additional opportunities arising during the past 3 years also include three very exciting studies of potential estrogen antagonists, namely:

Two studies of soybean phytoestrogens, given alone and in combination with estradiol:
93-18 Effects of Soybean Estrogens in peripubertal macaques
94-33 Interactions of Mammalian and Plant Estrogens

One study of concurrent administration of estradiol and tamoxifen:
95-13 Interactions of Estrogen and Tamoxifen

A brief description of each study is given on the following pages.

Experiment 88-14

Estrogen replacement/atherosclerosis secondary intervention trial (Conjugated estrogens +/- medroxyprogesterone acetate)

Study design

Progression phase - surgically postmenopausal, adult female monkeys were fed an atherogenic diet, to allow progression of atherosclerosis and osteoporosis for 22 months.

↓

Animals were then randomized into 3 groups:
Ovariectomized control (n = 26)
Premarin (n = 22)
Premarin + Cycrin (n = 21)

↓

Treatment phase (24 months)
Diet changed to low fat and low cholesterol

↓

Euthanasia and necropsy
Termination date: July, 1993

↓

Assessment of:

Benefits: Atherosclerosis regression, arrest of osteoporosis progression.

Risks: Hyperplastic, dysplastic or neoplastic lesions in breast and endometrium. In particular, the relative effect of Premarin and Premarin + MPA has been assessed.

Experiment 91-20: Primary atherosclerosis intervention trial (Conjugated estrogens +/- medroxyprogesterone acetate; tamoxifen)

Termination date: April 1995

Study design

Surgically postmenopausal, female cynomolgus monkeys

↓

Randomization to 5 groups:
- Ovariectomized control (n = 15)
- Premarin (conjugated equine estrogens, CEE)(n = 15)
- Cycrin (medroxyprogesterone acetate, MPA) (n = 15)
- Premarin + Cycrin (n = 15)
- Tamoxifen (n = 15)

↓

Treatment - fed moderately atherogenic diet, 35 months

↓

Euthanasia and necropsy

↓

Assessment of:

Benefits: Cardioprotective effect of Premarin alone and with Cycrin. Direct comparison of cardioprotective effect of Premarin and Tamoxifen.

Risks: Hyperplastic, dysplastic, and neoplastic lesions in breast and endometrium. This study is of particular interest because a) it allows comparison of the effects of Premarin and Premarin + MPA with MPA alone, and b) it provides an opportunity to examine the effect of Tamoxifen on normal mammary gland.

Experiment 93-16: 17α-dihydroequilenin study

Termination date: December 1993

Study design

Young, female rhesus monkeys

↓

Randomization to 3 groups:

Cycling control (n = 16)
Ovariectomized control (n = 17)
17α-dihydroequilenin (n = 17)

↓

Treatments were given for 21 weeks. Animals received a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Assessment of:

Benefits: Cardioprotective effect of 17α-dihydroequilenin.

Risks: Mammary hyperplasia, dysplasia, and neoplasia relative to either control group.

Experiment 91-24
Atherosclerosis/contraceptive steroids primary prevention trial

Termination dates: June 1993 (interim sacrifice) and fall 1995

Study design

Premenopausal, female cynomolgus monkeys

↓

Randomization to 4 groups:

Control (intact, normally cycling)
Triphasil (n = 24)
Cyclic ethinyl estradiol (n = 24)
Cyclic levonorgestrel (n = 24)

↓

Treatment was given for 35 months. Animals received a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Assessment of:

Benefits: Cardioprotective effect of premenopausal estrogen use.

Risks: Mammary hyperplasia, dysplasia and neoplasia, and whether such effects relates to the ethinyl estradiol or levonorgestrel component of Triphasil.

Experiment no. 92-04
Osteoporosis primary prevention trial

Termination date: June 1994

Study design

Pre- and postmenopausal female cynomolgus monkeys

↓

Randomization:
Cycling control (n = 15)
Ovariectomized control (n = 15)
Nandrolone in year 1 after ovariectomy (n = 15)
Nandrolone in year 2 after ovariectomy (n = 15)

↓

Treatment was given for 24 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Benefits: Prevention/treatment of osteoporosis

Risks: Coronary artery atherosclerosis exacerbation. Adverse effects of androgenic/anabolic steroids on mammary gland and endometrium.

Experiment No. 93-18
Effects of Soybean Estrogens

Termination date: January 1995

Study design

Peripubertal female cynomolgus monkeys

↓

Randomization:
Untreated controls (n = 13)
Soybean estrogens (n = 14)

↓

Treatment was given for 12 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Benefits: Prevention/treatment of atherosclerosis.

Risks: Potential adverse estrogenic effects of soybean estrogens, such as induction of mammary or endometrial proliferation.

Experiment No. 94-33
Interactions of Mammalian and Plant Estrogens

Termination date: October 1995

Study design

Postmenopausal female cynomolgus monkeys

↓

Randomization:
- Ovariectomized control (n = 15)
- Estradiol (n = 15)
- Soybean estrogens (n = 15)
- Estradiol + Soybean estrogens (n = 15)

↓

Treatment was given for 6 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Benefits: Prevention/treatment of atherosclerosis; potential additive effect of soy and estradiol in the prevention of arterial and bone disease. Potential antagonistic effect of soy and estradiol, which might prevent breast and endometrial proliferation caused by estradiol.

Risks: Potential antagonistic effect of soy and estradiol, which might reduce the effectiveness of coronary artery protection. Potential additive effect of soy and estradiol on mammary gland and endometrium, resulting in increased proliferation and cancer risk.

Study No. 95-13
Interactions of Estrogen and Tamoxifen

Termination date: June 1996

Study design

Postmenopausal female cynomolgus monkeys

↓

Randomization:
Ovariectomized control (n = 6)
Estradiol (n = 6)
Estradiol + tamoxifen (n = 6)

↓

Treatment was given for 2 months. Animals were fed a moderately atherogenic diet.

↓

Euthanasia and necropsy

↓

Benefits: Protective effect of tamoxifen on breast, which will presumably be reflected in lower proliferation in breast .

Risks: Endometrial proliferation induced by tamoxifen and estradiol, leading to hyperplasia and increased risk of neoplasia.

Experiment 91-12
Oral contraceptives/Soy as an Estrogen Alternative
Termination date October 1993 (interim) and December 1996

Study design (phase I)
Premenopausal, female cynomolgus monkeys

↓

Randomization to 2 groups:
Control (n = 100)
Triphasil (n = 100)

↓

Treatment phase - Animals were fed a moderately atherogenic diet and treated for 24 months.

↓

Assessment of:
Benefits - cardioprotective effect of premenopausal estrogen use, particularly for stressed females.
Increase in peak bone mass from premenopausal estrogen use.

↓

Study design (phase II)

Surgically postmenopausal monkeys

↓

Randomization of the two groups from part I (estrogen use or not) into three groups:
1) Control (n = 63)
2) Premarin (conjugated equine estrogens, CEE) (n = 63)
3) Soy phytoestrogens (n = 63)

for a treatment period of 36 months.

↓

Final necropsy

↓

Assessment of:

Benefits: Does premenopausal estrogen use add to the postmenopausal hormone replacement therapy effects on atherosclerosis and osteoporosis? Possible protective effect of premenopausal contraceptive use on endometrium; possible protective effects of soy phytoestrogens on breast and endometrium.

Risks: Indicators of breast or endometrial cancer risk associated with CEE or oral contraceptives. Possible uterotrophic and mammotrophic effects of phytoestrogens.

Diets/Drug Dosing
For all studies, the hormones were administered twice daily in the diet, with the exception of nandrolone. Most animals consume a moderately atherogenic diet (40% of calories from fat, 0.2 mg of cholesterol per Calorie). Monkeys were fed approximately 120 Calories per kg of body weight per day. Doses were as follows:

Drug	Abbreviation	Dose equivalent per woman per day
Conjugated equine estrogens	CEE	0.625 mg
Medroxyprogesterone acetate	MPA	2.5 mg
17β-Estradiol	E2	2 mg (experiment 94-33) 0.25 mg (experiment 95-13)
Tamoxifen	TAM	20 mg
Ethinyl estradiol	EE	Days 1-6: 0.03 mg Days 7-11: 0.04 mg Days 12-21: 0.03 mg Days 22-28 : no drug
Levonorgestrel	LN	Days 1-6: 0.05 mg Days 7-11: 0.075 mg Days 12-21: 0.125 mg Days 22-28: no drug
17α-Dihydroequilenin	DHEN	0.312 mg/kg
Soybean estrogens	SBE	99.7 mg (experiment 91-12) 148 mg (experiments 94-33)
Nandrolone	-	No daily equivalent; animals were given injections of 25 mg nandrolone decanoate by intramuscular injection every 3 weeks.

Drug doses were computed as:
human dose divided by 1800 Calories/woman/day = dose per Calorie of diet
Doses arrived at by this means were therefore consistently scaled, and adjusted for metabolic rate. They are similar to the dose calculated by scaling on the basis of body surface area (Mordenti 1986).

Tissue collection
Mammary glands were collected at the end of each study, when all monkeys are euthanized and necropsied. Tissues were fixed in 4% buffered paraformaldehyde at 4°C. The tissue was removed from paraformaldehyde after 24 hours, stored in 70% ethanol at 4°C, and later trimmed to 3 mm in thickness, embedded in paraffin using standard histologic procedures, and sectioned at 5 μm for immunostaining. Endometrial and ovarian tissues were also collected, in parallel with breast samples.

Histopathology
Mammary gland slides were subjectively classified as atrophic, hyperplastic, or neither. The treatment group of each animal was obscured during the procedure to prevent observer bias. Hyperplasia, atypia, cystic lesions, and secretory activity were noted. Lesions were independently graded as none, mild, moderate or severe. The criteria of the World Health Organization were used for tumor classification (WHO, 1982)

Morphometry
Mammary gland thickness, mammary lobular size, and area fraction of the mammary tissue occupied by glands are measured from histologic sections using video microscopy and a

Macintosh-based video imaging system and public domain software (NIH Image, available via the Internet by anonymous FTP [file transfer protocol] from zippy.nimh.nih.gov).

Stereology
In early studies prior to acquisition of the image analysis system, estimates of the relative proportions of tissue components in the mammary gland were made by point counting, after the method of Chalkley (Chalkely, 1945). These included percentage of gland occupied by epithelium, connective tissue and fat. Numbers of points intercepting each lobule were also recorded, as a relative indicator of lobular size.

Sex steroid receptors and proliferation marker staining methods.
Staining procedures were done on fixed, paraffin-embedded tissues. The basic staining procedure uses an avidin-biotin-peroxidase method (Wordinger 1987) modified for antigen retrieval from paraffin-embedded tissue. The estrogen receptor and progesterone receptor analyses were performed with either antibodies from Dako laboratories (Dako Corporation, Carpinteria, CA, USA), and Immunotech laboratories (Immunotech, Marseille, France), or the mouse monoclonal antibodies NCL-ER-LH2 and NCL-PGR antibodies for detection of estrogen and progesterone receptor expression respectively(Novocastra, Newcastle–upon–Tyne, U.K.), depending on the study.

Assessment of proliferation (Ki67-MIB)
We first used the KI-67 MIB-1 (MIB) monoclonal antibody (Immunotech, Marseille, France) that gives an immunostaining identical to Ki-67 antibody and which can be used on paraffin embedded tissue sections (Cattoretti 1992). We have also used the NCL-Ki-67-MM1 mouse monoclonal antibody (Novocastra, Newcastle–upon–Tyne, U.K.), which has provided us with identical results. As for the receptor analysis, the MIB basic staining procedure is done by an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin embedded tissue. The murine monoclonal antibody Ki-67 reacts with a human DNA-binding protein that is present in proliferating cells but absent in quiescent cells. A detailed cell cycle analysis showed that the Ki-67 antigen is expressed in G1, S, G2 and mitosis (with maximum levels during G2 and M phases) but not in G0 and using this antibody an exact determination of the growth fraction of a given human cell population, regardless of whether it is normal or malignant, has been possible (Gerdes 1991).

Immunostaining for p53 expression
The antibody that was used for p53 was DO-7 (Dako A/S Glostrup Denmark) and the staining procedure was an avidin-biotin-peroxidase method modified for antigen retrieval from paraffin-embedded tissues, similar to that used for Ki-67 staining.

Quantification of immunohistochemical staining
Immunostained cells were quantified by cell counting in sections, by an observer blinded to treatments. Epithelial cells lining the alveoli, the terminal and major ducts were considered separately in order to assess regional differences. Labeled cell nuclei were identified as unlabeled (0), weakly (+), moderately (++), or intensely (+++) labeled. At least 100 cells per slide were counted at 3 different sites for each combination of animal, tissue site and stain type. Major ducts and alveoli were easily identifiable, but clearly defined terminal ducts could not be identified in some cases.

Statistical methods
Statistical analysis is performed using the Mann-Whitney U-test with Bonferroni corrections for multiple comparisons, Kruskal Wallis test, Chi-square test, and Spearman's rank correlation test.

Ancillary Projects
Several ancillary projects have been carried out which enhance the understanding of mammary data collected. These include:

1) development of methods for detection of the newly-described estrogen receptor beta (ERβ) in macaque tissues.
2) development of vaginal cytology methods for prospective screening of live macaques for estrogenic effects on the reproductive tract;
3) development of a method to retrospectively approximate the reproductive history of monkeys; and
4) development of a morphometric protocol to assess treatment-related changes in ovaries of oral contraceptive-treated macaques, in order to document treatment effects.

RESULTS

A brief outline of accomplishments to date is followed by a presentation of specific experimental findings. Progress to date includes:

1. Collection of over 600 paired, frozen and fixed breast and endometrial samples, from macaques treated with conjugated estrogens with and without medroxyprogesterone acetate, tamoxifen, triphasic oral contraceptives, nandrolone, estradiol, dietary phytoestrogens, and controls.

2. Development of morphometric and cell counting methods for evaluation of breast and endometrium, including acquisition of a computerized video microscopy/image analysis system and development of standard measurement procedures.

3. Refinement and application of immunohistochemical methods for detection of the proliferation marker Ki-67, estrogen receptors and progesterone receptor; tissues from approximately 500 animals have been stained and evaluated to date.

4. Publication of a manuscript to the American Journal of Obstetrics and Gynecology detailing morphologic and immunohistochemical changes in the breast of surgically postmenopausal macaques given conjugated estrogens with or without the addition of MPA (from study 88-14). \

5. Results in mammary gland have been compared to endometrial morphology and Ki-67, ER, and PR staining in endometrium, in the same animals used for the above manuscript (via a separate grant, received from the Office of Research on Women's Health). This parallel study was the basis of a Young Investigator Award to Dr. Cline for presentation of the findings at the North American Menopause Society meeting.

6. Study of the regional variation in breast regulation, by quadrant and distance from the nipple. Identification of regional variations provides us with an assessment of the degree of random or predictable intrinsic variation in within breast tissue, a parameter of considerable interest should we plan biopsy-based studies in the future. Results of this study are published in the journal Gynecologic andObstetric Investigation (Cline, 1997)

7. Development of histopathologic criteria for retrospectively distinguishing the uteri of parous and nulliparous macaques. Since some animals in our studies were acquired as adults with an unknown reproductive history, and parity affects long-term breast regulation, this is an important source of variation in the breast which we needed to identify. This work was presented at the 1995 meeting of the American College of Veterinary Pathologists (Cline, 1995), and a manuscript is in preparation for submission to the journal Veterinary Pathology.

8. Vaginal cytologic studies demonstrated classical estrogenic effects of estradiol and conjugated estrogens in macaques, a weak estrogenic effect of tamoxifen on the vagina, and no estrogenicity of soybean estrogens in the vagina. These results were published in the journal Fertility and Sterility (Cline, 1996); the manuscript was included in the 1996 report.

9. Ovarian histomorphometric studies identified distinct atresia-inducing effects of oral contraceptives, including progestin-only contraceptives, in follicles of treated animals.

10. Whole-mount methods for assessing mammary gland development have been developed in our laboratory; based on preliminary examination of a few samples, we believe that this method will provide an important adjunct to our existing procedures for evaluating mammary gland proliferation.

11. Our demonstrated interest in dietary chemoprevention of breast cancer has led to the generation

of an invited review of the potential chemopreventive properties of phytochemicals (Cline and Hughes, 1998).

12. We have demonstrated that the tumor suppressor gene p53 is up-regulated in the mammary glands of macaques given hormone replacement therapies (Isaksson et al., in press)

13. International collaborations in conjunctionwith this work have continued; scientists in training have been sent to WFU School of Medicine from the Karolinska Institute in Stockholm, and Ernst-Moritz-Arndt University in Griefswald, Germany, to study the macaque model of breast and endometrial regulation.

14. We have recently developed a collaborative effort with Dr. Thomas Register of our department, in order to evaluate expression of the newly-described estrogen receptor beta.

15. The work accomplished through this grant has led to an invitation from the Comprehensive Cancer Center of Wake Forest Unversity to establish a Primate Resource Core laboratory so that the type of collaborative work done in these studies can reach a broader group of cancer researchers in our institution.

The following are the specific technical objectives proposed from July, 1994 to July, 1999, accompanied by a report of what has been accomplished.

Technical Objective	**Work Accomplished**
Year 1 (1994/1995)	
Processing, staining, and measurement from tissues collected in 1993; studies 88-14 (final sacrifice), 91-24 (interim sacrifice), 91-12 (interim sacrifice).	Completed.
Collection of tissues from studies 92-04 and 93-16.	Completed.
Year 2 (1995/1996)	
Collection of tissues from studies 91-20, 91-24, and 91-12 (final sacrifices).	Completed.
Processing, staining, and measurement of tissues collected.	Completed.
Presentation and publication of interim results from studies 91-24 and 91-12, final results from study 88-14.	Final results from studies 88-14 (CEE+/-MPA) and 93-16 have been published. Preliminary data from experiments 94-33, 91-20, 93-18, and 91-24 have been presented. Interim endpoints from experiment 91-12 have been published.
Year 3 (1996/1997)	
Presentation and publication of results from studies 91-20, 91-24, and 91-12	Final results of study 91-20 have been published. Manuscripts from studies 91-24 and 91-12 were begun.
Year 4 (1997/1998)	
Measurement of tissues collected, and data analysis	Analysis of tissues and data from X91-24 and X91-12 continues. Additional manuscripts from X91-20 and ancillary projects were begun.
Year 5 (1998/1999)	Analysis of tissues and data from X91-12 completed. Publication of 6 original papers from X91-12, X91-20, X94-33, and X92-04 and ancillary studies. Publication of a book chapter and two reviews. Additional manuscripts in preparation. Invited speaker to the American Association for the Advancement of Science.
No-cost extension for completion of analyses from X91-12	

Specific Results Listed By Study

I. Conjugated Estrogens with or without MPA (Study 88-14)
This is a long-term comparison of the effects of CEE and CEE+MPA in surgically postmenopausal macaques (the study design is shown on page 9).

The most relevant finding in this study was that the addition of the progestin MPA to estrogen (CEE) treatment had differing effects on the mammary gland and endometrium of macaques: That is, MPA antagonized the proliferative effect of CEE in the endometrium, but not the mammary gland. In fact, the addition of MPA to CEE treatment *increased* prolifertion in the mammary gland (Figure 1).

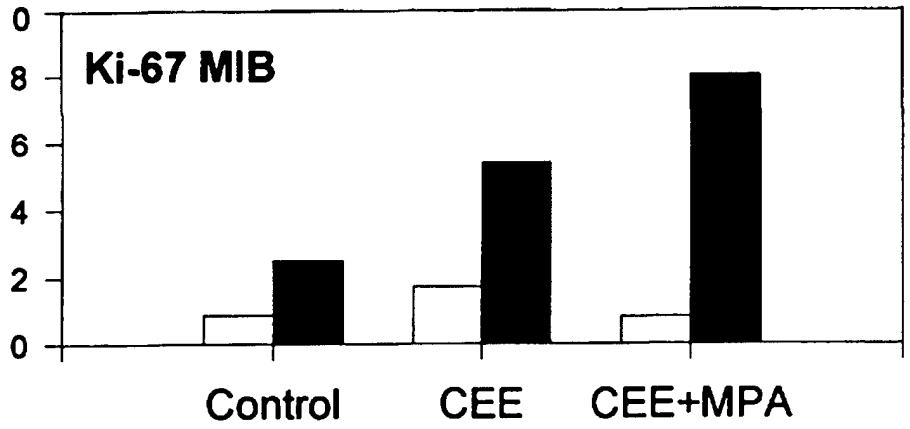

Figure 1. Divergent effects of combined estrogen/progestin treatment on the endometrium (white bars) and mammary gland (black bars) of macaques. Bars indicate the percentages of cells labeled with the proliferation marker Ki-67.

This study is essentially complete, and the results have been published (Cline et al., 1996).

II. Conjugated estrogens, MPA and Tamoxifen (Study 91-20)
This is a study of the comparative effects of CEE, MPA, CEE+MPA, and tamoxifen in surgically postmenopausal macaques. Preliminary morphometric and proliferation data were presented in previous reports and are briefly outlined below. This work has been published (Cline et al., 1996).

In this second study, results in the groups given CEE, and CEE+MPA, are similar to those in the preceding experiment. Proliferation data on these groups, and the additional groups given MPA alone and tamoxifen, are shown in Figure 2. Again, it is apparent that CEE+MPA exerts a greater mammotrophic effect than CEE alone (Figures 2 and 3), in contrast to the findings in the uterus (Figure 4). As might be expected, tamoxifen does not cause an increase in mammary gland proliferation.

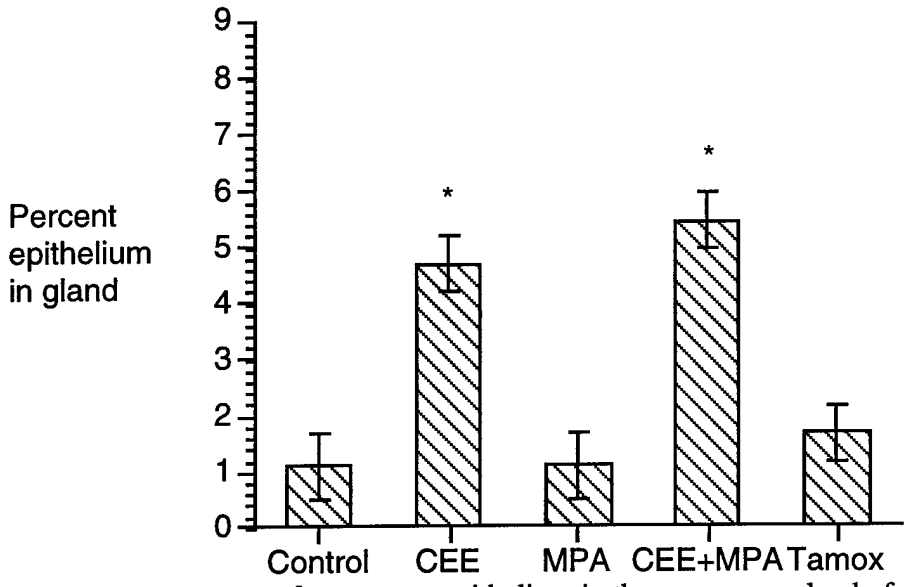

Figure 2. Percentage of mammary epithelium in the mammary gland of macaques given CEE, MPA, the combination, or tamoxifen. Combined CEE+MPA produced maximal glandular proliferation. Stars indicate groups differing from controls at $p < 0.05$.

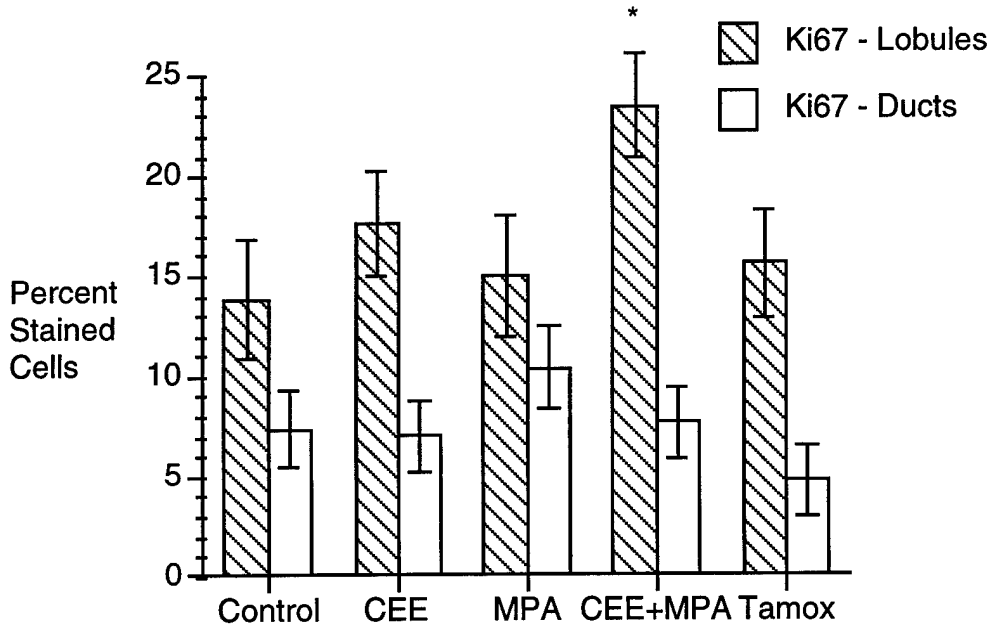

Figure 3. Ki-67 staining in mammary epithelial cells, expressed as percentage positively-stained cells. Stars indicate groups differing from controls at $p < 0.05$.

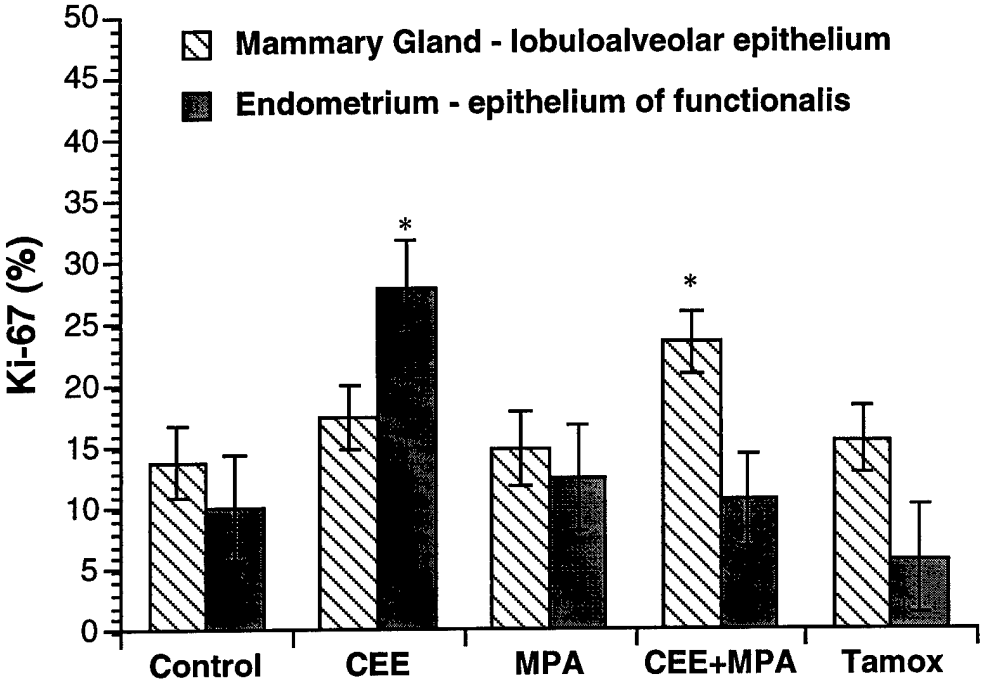

Figure 4. Ki-67 staining in mammary gland and endometrium, expressed as percentage positively-stained cells. Maximal proliferation was induced by CEE+MPA in the mammary gland, in contrast to maximal proliferation induced by CEE alone in the endometrium. Stars indicate groups differing from controls at $p < 0.05$.

Estrogen and progesterone receptor staining has been completed for the mammary glands. No significant differences in estrogen receptor expression were induced inthe mammary glands by any of the treatments. However, progesterone receptor expression was increased by both CEE and tamoxifen treatment. Adding MPA to CEE treatment diminished PR expression (Figure 5).

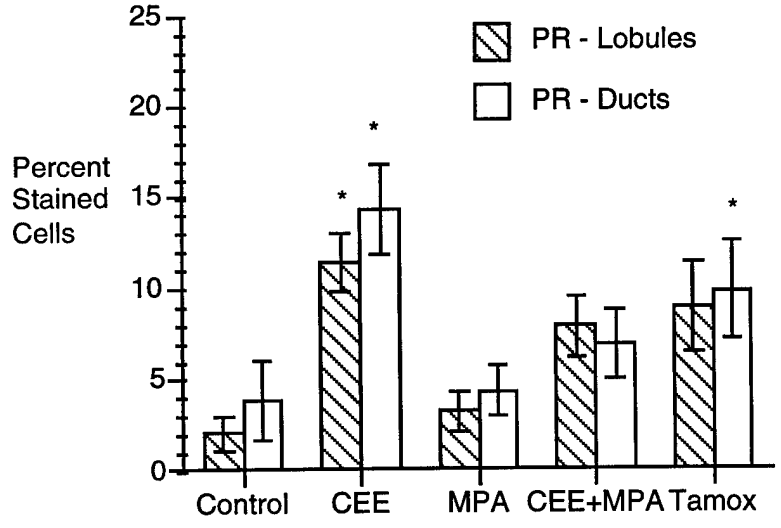

Figure 5. - Progesterone receptor (PR) staining in mammary epithelial cells, expressed as percentage positively-stained cells. Stars indicate groups differing from controls at $p < 0.05$. CEE and MPA also differ at $p < 0.05$ for both sites.

Measurement of p53:
A subset of animals in this study were used to evaluate expression of the apoptosis-inducing regulatory protein p53 in mammary gland and endometrium. Work done in collaboration with our colleagues at the Karolinska Institute in Stockholm has shown that wild-type p53 is up-regulated in the mammary glands of estrogen-treated macaques and can be detected using commercially available antibodies. This work has also shown that there are differences in the relative expression of the proliferation marker Ki67 and p53 in animals given estrogen (CEE) as opposed to tamoxifen. We found in this study that mammary and and endometrial p53 is elevated by CEE but not tamoxifen treatment (Figures 6 and 7), thus potentially pointing out a new manifestation of the mixed agonist-antagonist activity of tamoxifen. This particularly exciting finding has been the subject of another publication recently accepted by Breast Cancer Research and Treatment (Isaksson et.al., in press, 1998).

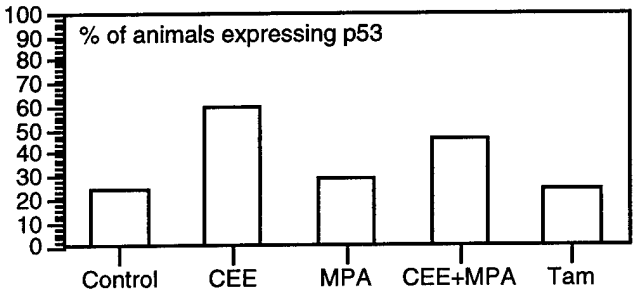

Figure 6. Immunohistochemical detection of of the p53 tumor suppressor gene product in animals givenconjugated equine estrogens (CEE). MPA - medroxyprogesterone acetate; Tam - tamoxifen

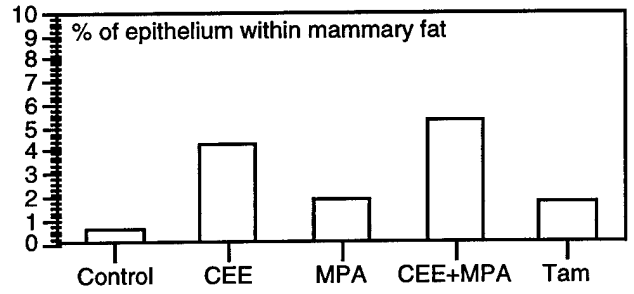

Figure 7. Morphometric assessment of mammary gland proliferation in the same animals shown in the preceding figure.

III. DHEN versus Conjugated Estrogens (Study 93-16)

This is a study of the relative effects of 17α-dihydroequilenin (DHEN) in pre- and post-menopausal macaques (the study design is shown on page 11). DHEN has considerable potential for use as a "selective" estrogen, exerting beneficial effects on the cardiovascular system and bone without producing increased breast and endometrial proliferation. In this study, DHEN did not exert mammotrophic or uterotrophic effects. The data was given in the 1996 report, and are summarized in Figure 8. Endometrial data from this study have been published (Washburn et al., 1996).

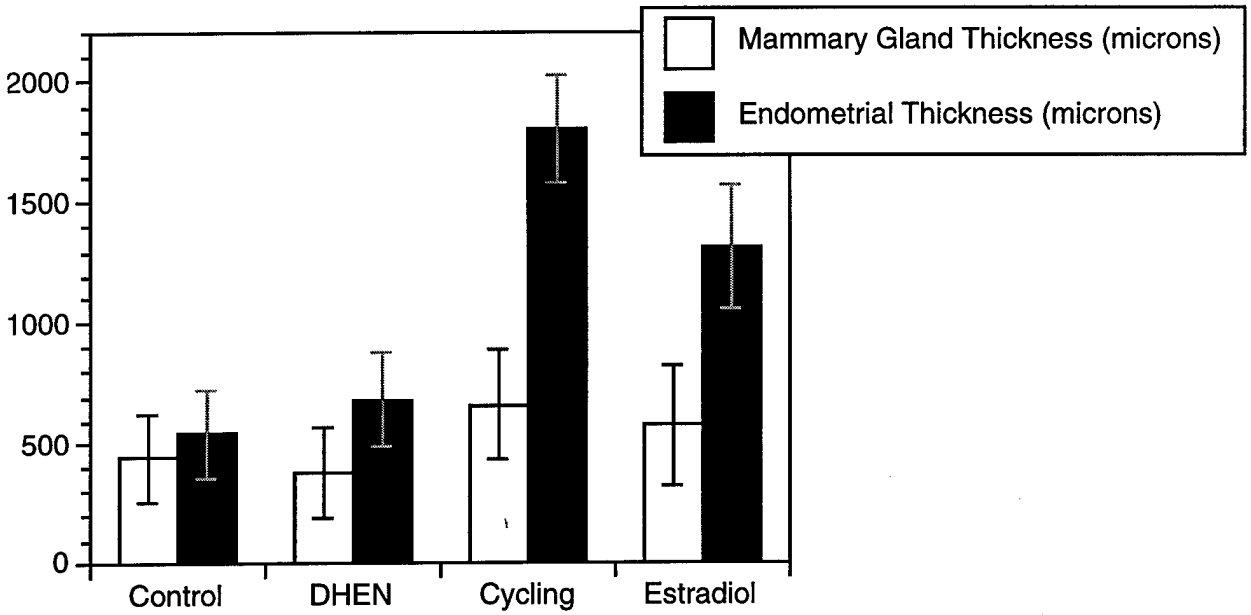

Figure 8. Dihydroequilenin does not induce proliferation in mammary gland or endometrium of macaques (not statistically different from controls) .

IV. Triphasic Oral Contraceptives (Experiment 91-24)

This study seeks to explore the possible role of triphasic oral contraceptive use on chronic disease risk in the monkey model (see experimental design, page12). A particularly interesting aspect of this study is the concurrent evaluation of typical triphasic oral contraceptives (modeled after the widely used Triphasil ™), and the individual components of the contraceptive, namely ethinyl estradiol and levonorgestrel. To date, no statistically significant treatment-related differences have been identified in mammary glands of animals treated with the whole preparation or its components (Figuers 9 and 10).

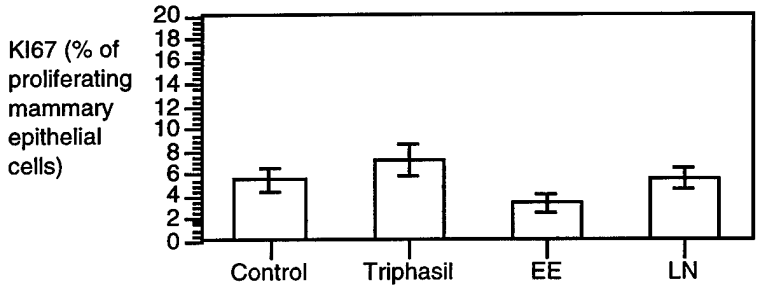

Figure 9. Effects of Triphasil (TRI), ethinyl estradiol (EE) and levonorgestrel (LN) on proliferation in lobuloalveolar tissue of the macaque mammary gland. There are no statistically significant differences.

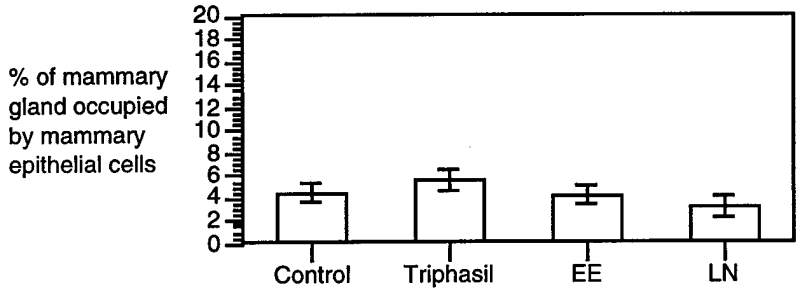

Figure 10. Effects of Triphasil (TRI), ethinyl estradiol (EE) and levonorgestrel (LN) on a morphologic indicator of glandular hyperplasia.There are no statistically significant differences.

It is of interest to contrast this study with experiment 88-14, and in particular to note that the combination of an estrogen and a progestin does not increase epithelial proliferation in these premenopausal animals. This reflects the biology of breast cancer risk in women, in which oral

contraceptive use does not produce profound changes in breast cancer risk. Possible sources of this difference include 1) differences between breast regulation in pre- and post-menopausal animals; 2) differences due to the specific estrogen and progestins used; or 3) differences resulting from cyclic, as opposed to continuous, administration of hormones.

V. Effects of Nandrolone (Experiment 92-04)

The design of this study is givenon page 13; it is a trial of the osteoporosis preventive anabolic androgenic steroid nandrolone. Mean estradiol concentrations in the two nandrolone groups after treatment began ranged from 76.8 to 171.0 pg/ml and 60 to 130.2 pg/ml for the OVX+ND and OVX+NDdelay groups, respectively whereas concentrations in the ovariectomized control group ranged between 0 and 9.08 pg/ml. These estradiol concentrations correlated with increased uterine weight (Obasanjo, 1998), and were interpreted as resulting from conversion of nandrolone to estradiol (Obasanjo 1996). Mammary gland effects of nandrolone were minimal, consisting of an insignificant trend for greater glandular epithelial area (Figure 11). Endometrial changes were more pronounced, and consisted of increases in uterine weight, mucometra, and adenomyosis ; we have published these results (Obasanjo,1998).

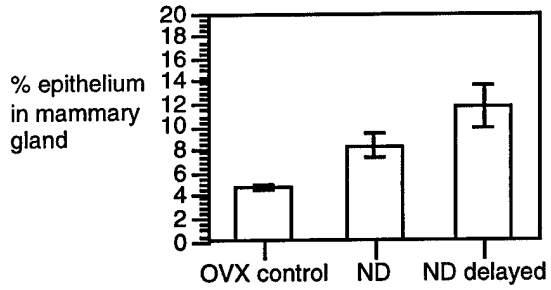

Figure 11. Percentage of mammary tissue occupied by glandular epithelium in ovariectomized macaques given nothing (OVX control), nandrolone immediately after ovariectomy (ND) or nandrolone beginning 1 year after ovariectomy (ND delay). No groups are statistically different.

VI. Effects of Soy Phytoestrogens on Peripubertal Macaques (Experiment 93-18)

Our recent work with dietary modulation of intermediate markers of cancer risk has produced some intriguing results. This first of our soy studies (see page 14 for study design) was done as a pilot project in a small cohort of female monkeys fed soy phytoestrogens, and demonstrates (as do the vaginal cytology data) that soy phytoestrogens do not induce increases in mammary or endometrial proliferation, as estimated by morphometric measurement of the percentage of each tissue made up of epithelial cells.

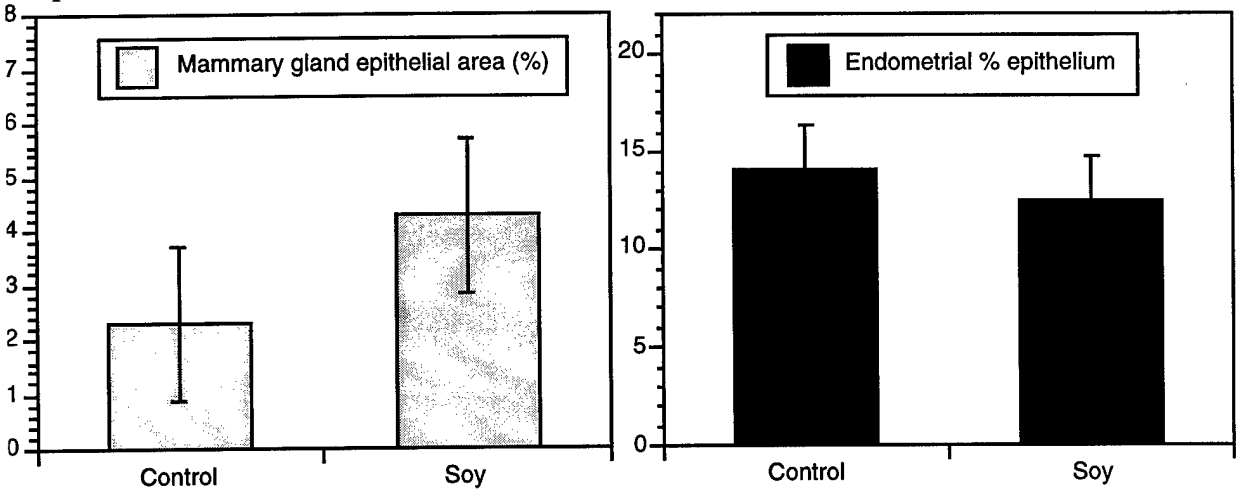

Figure 12. Soy phytoestrogens do not induce mammary gland or endometrial hyperplasia.

VII. Interactions of Mammalian and Plant Estrogens (Experiment 94-33)
Further explorations of soy effects on mammary gland led us to examine the effects of estradiol in concert with dietary soy supplementation. The study design is outlined on page 15 Findings to date indicate that soy phytoestrogens are not themselves classically "estrogenic" (i.e. proliferation - inducing) in mammary gland or endometrium (Figures 13-15). Other details of this study are given in the attached manuscript, in press in the American Journal of Clinical Nutrition (Foth and Cline, 1998).

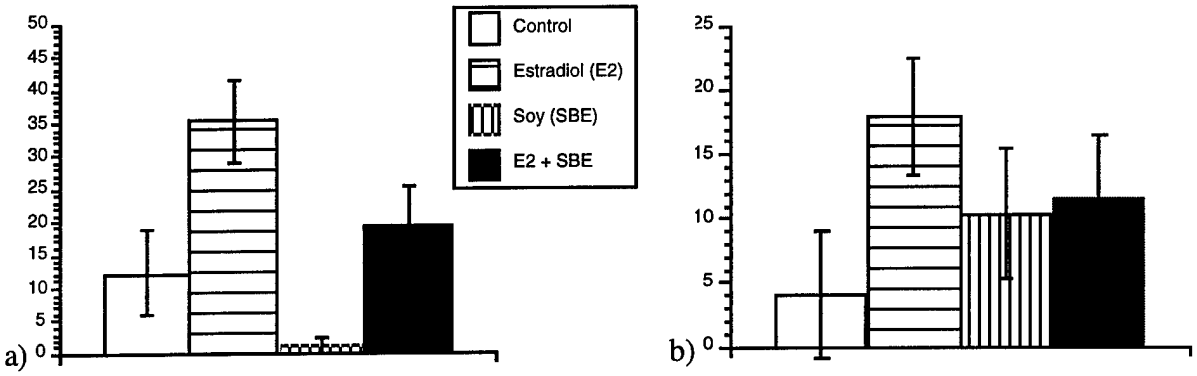

Figure 13. - Immunohistochemical staining for the proliferation marker Ki-67 in a) endometrium and b) mammary gland of cynomolgus macaques. Estrogen-induced proliferation is antagonized by the addition of soybean estrogens (SBE). Soy phytoestrogens do not induce mammary gland proliferation alone, and exert an antagonistic effect on estrogen-induced proliferation. Only the estrogen-treated group differs from controls at $p < 0.05$.

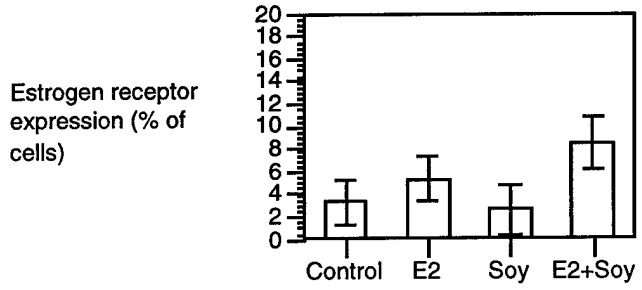

Figure 14. - Immunohistochemically detectable ER expression induced by E2 and Soy treatment.

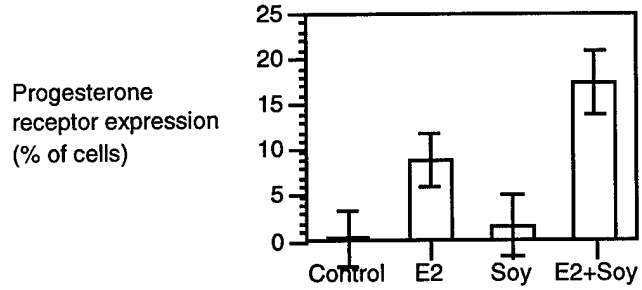

Figure 15. - Immunohistochemical staining for progesterone receptor in mammary gland of cynomolgus macaques. Both estrogen-treated groups differ from controls at $p < 0.05$.

It is of particular interest that soy phytoestrogens diminish proliferation in the breast but do not alter progesterone expression; since PR is a classical indicator of estrogenic effect, these results

indicate that the antiproliferative effect of soy phytoestrogens may be independent of any estrogen-antagonist effects.

VIII. Effects of Estradiol and Tamoxifen in Combination (Experiment 95-13)
This study (design given on page 16) was a unique opportunity to assess low-dose estradiol (0.25 mg/woman/day equivalent) and the combination of estradiol and tamoxifen.

Findings to date indicate first that different threshholds for proliferative responses are operative for mammary gland and endometrium; serum concentrations for estradiol were in the range of 50-60 pg/mL, which induced a significant proliferative response in endometrium but not breast (Figures 16 and 19). Secondly, it is apparent that a dose of tamoxifen which has no effect or even an antiproliferative effect on estrogen-induced proliferation does not antagonize the induction of progesterone receptor (Figures 18 and 19).

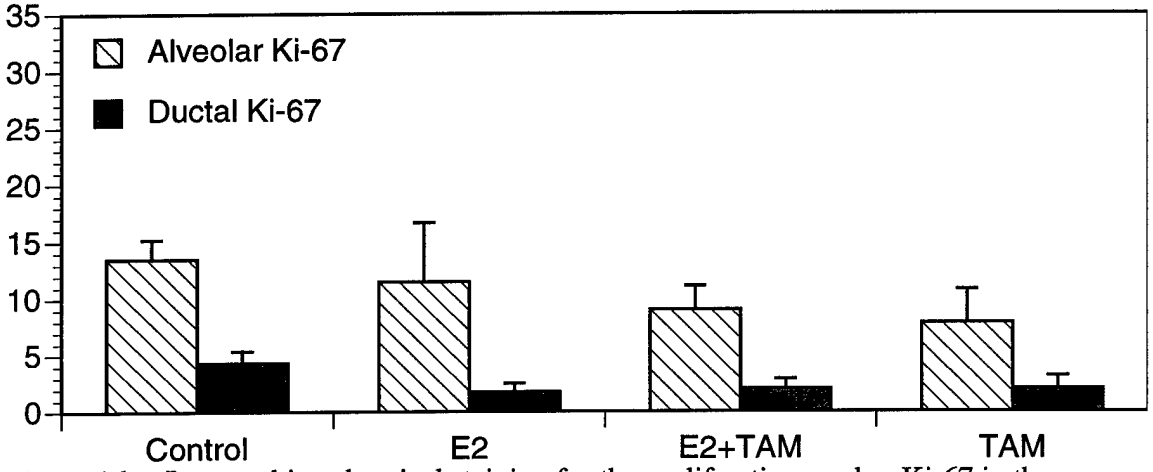

Figure 16. - Immunohistochemical staining for the proliferation marker Ki-67 in the mammary gland of cynomolgus macaques. No groups are statistically different.

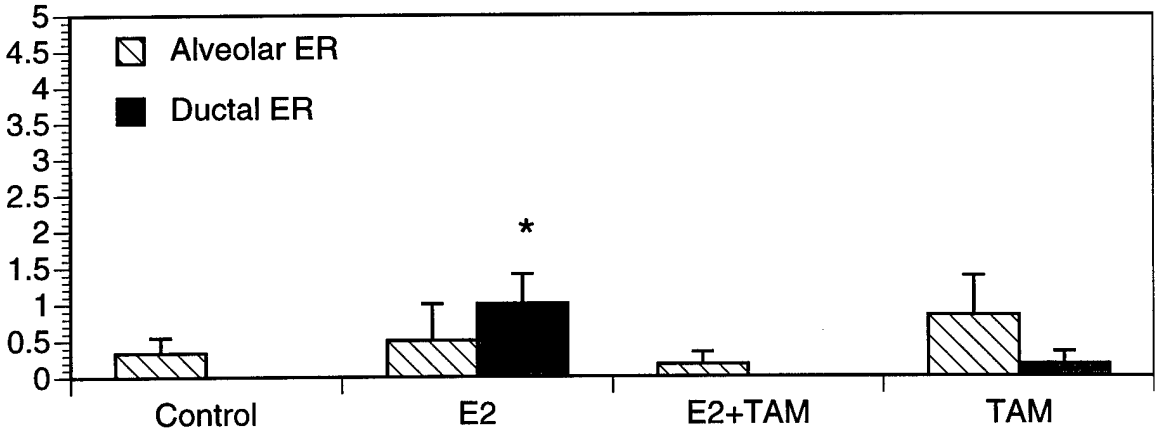

Figure 17. - Immunohistochemical staining for estrogen receptors in the mammary gland of cynomolgus macaques. Asterisks indicate statistical differences from controls at $p<0.05$.

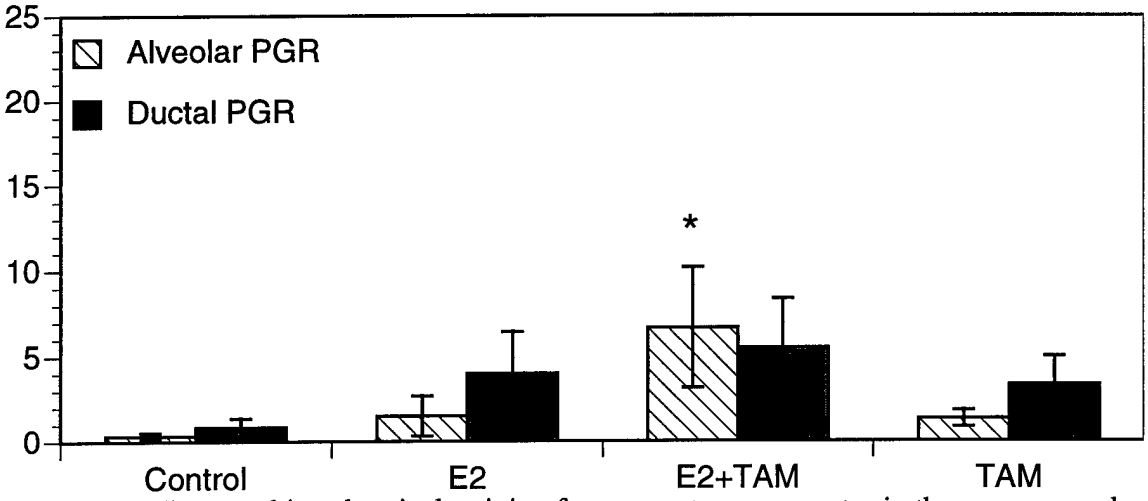

Figure 18. - Immunohistochemical staining for progesterone receptor in the mammary gland of cynomolgus macaques. Asterisks indicate statistical differences from controls at $p<0.05$.

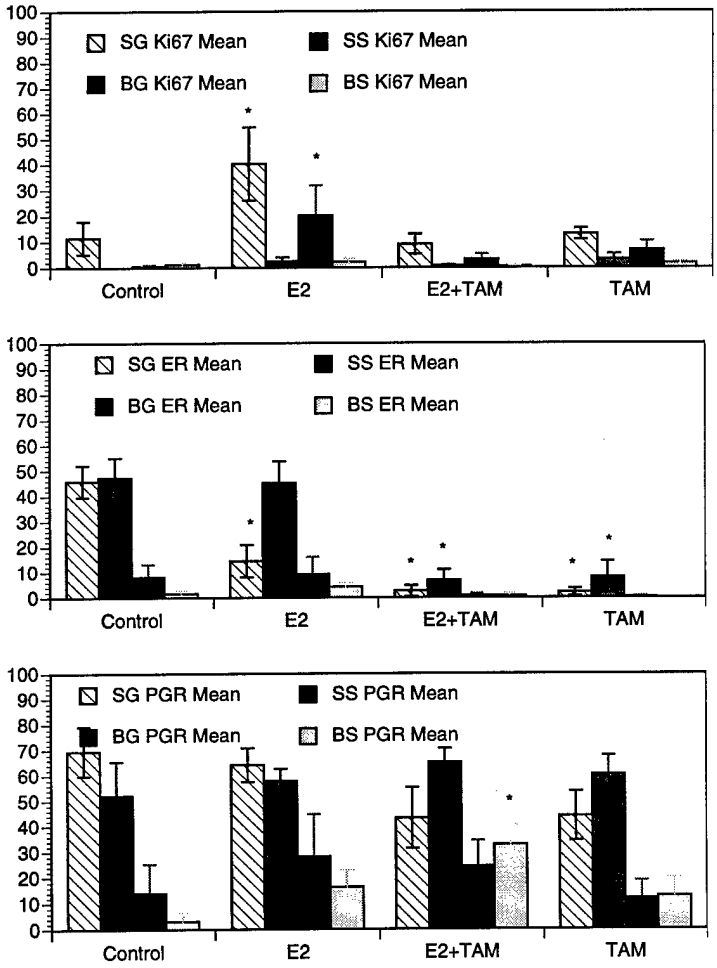

Figure 19. Endometrial findings. Ki67 (top), ER (middle), and PR (bottom) from endometrium of animals given estradiol, tamoxifen or the combination (Experiment 95-13). SG = superficial glandular tissue; BG = basal glandular tissue; SS = superficial stroma; BS = basal stroma. The marked effect of treatment on endometrial proliferation (Ki67) and ER expression stands in contrast to the minimal effect seen in the mammary gland. PR was induced in both tissues, although to a lesser degree in endometrium than mammary gland.

IX. Oral contraceptives/Soy as an Estrogen Alternative (Experiment 91-12)

Tissue collection for this study was completed approximately 1 year behind the planned schedule due to unavoidable circumstances. Final data were completed during the past year, supported by a no-cost extension to the grant period. Final results are presented in this report; manuscripts are in preparation.

Data analyzed to date indicate that dietary soybean phytoestrogens (SBE) do not induce breast or uterine proliferation in the ovariectomized macaque model, in contrast to the proliferative responses of both tissues to estrogen replacement therapy by conjugated estrogens (CEE).

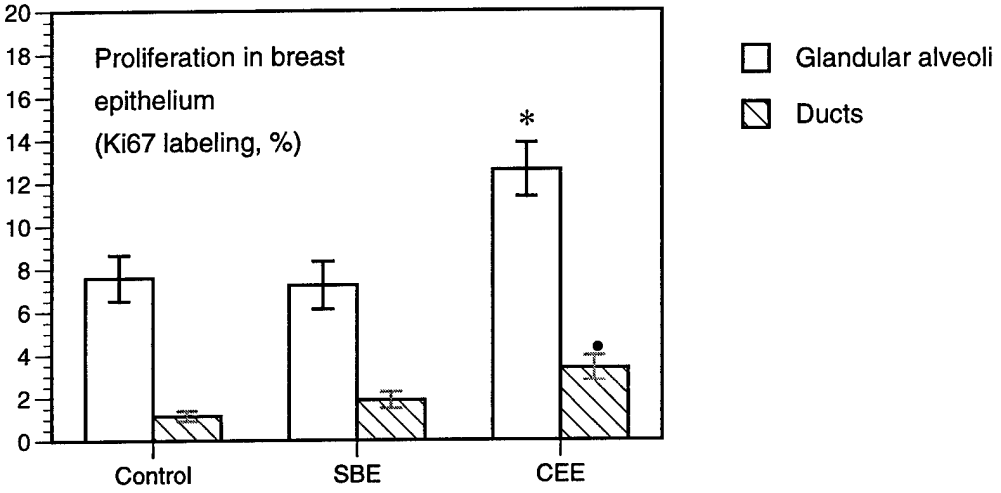

Figure20: Ki-67 labeling in breast epithelium of cynomolgus macaques given conjugated equine estrogens (CEE) or soybean estrogens (SBE). * = different from both other groups at $p<0.05$; • = different from controls only at $p<0.05$.

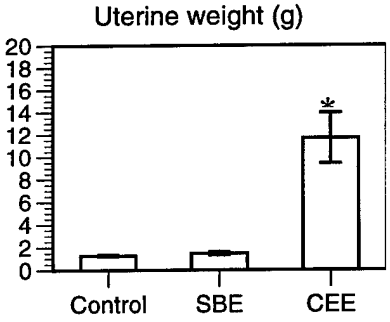

Figure 21: Uterine weight of animals given conjugated equine estrogens (CEE) or soybean estrogens (SBE). * = different from both other groups at $p<0.05$.

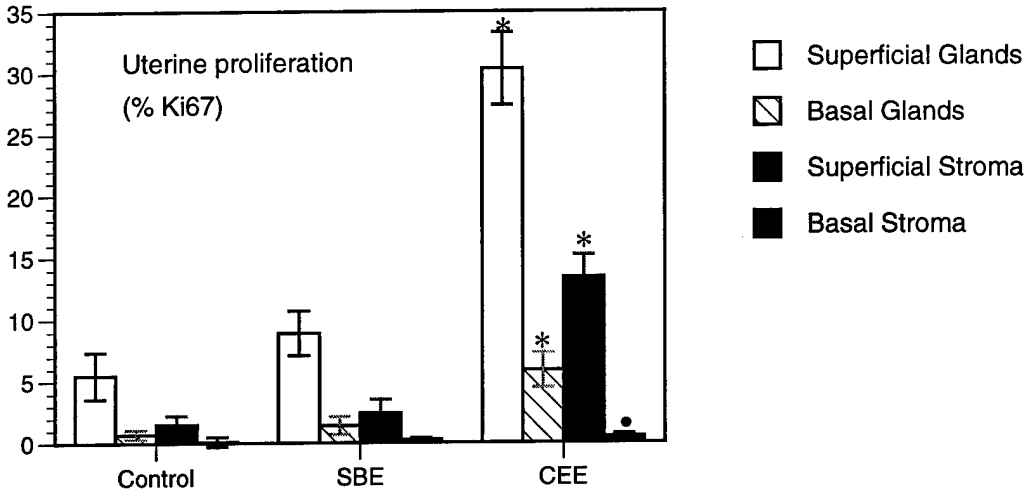

Figure 22: Ki-67 labeling in endometrial epithelium and stroma of cynomolgus macaques given conjugated equine estrogens (CEE) or soybean estrogens (SBE). * = different from both other groups at $p<0.05$; • = different from controls only at $p<0.05$.

Oral contraceptive effects

Final analyses of the data collected in this study have given little evidence for an interaction of oral contraceptive use with subsequent responses to estrogen replacement therapy; the only significant main effects of oral contraceptive use were on mammary gland thickness (a relatively weak measure of mammotrophic effect; $p = 0.0225$), and modulation of the induction of estrogen and progesterone receptors in the endometrial epithelium ($p = 0.025$ and 0.012, respectively)

Soy/Estrogen Interactions

A unique aspect of experiment 91-12 is that the experimental groups were large enough to allow within-group comparisons of the interactions between soy and endogenous estradiol and estrone concentrations, in the group of animals treated with soy alone. At the low levels of estrogens present in these ovariectomized macaques, few effects were apparent; however, it is of interest that on such effect is the lowering of serum estrone concentrations (Figure 23). Since estrone is presumably formed in these ovariectomized animals from peripheral aromatization of adrenal androgens, it is possible that dietary soy consumption in the long term may lower breast cancer risk by lowering serum estrogens. This effect has been reported in premenopausal women by Lu et al. (1996), but a similar observation has not been made in postmenopausal women. Local aromatization in the breast is also a potential factor in breast cancer promotion (Blankenstein et al., 1992). Aromatase inhibition by soy isoflavones has been observed *in vitro* (Adlercreutz, 1993), and aromatase inhibition is postulated to be a chemopreventiv emechanism (Kelloff et al., 1996).

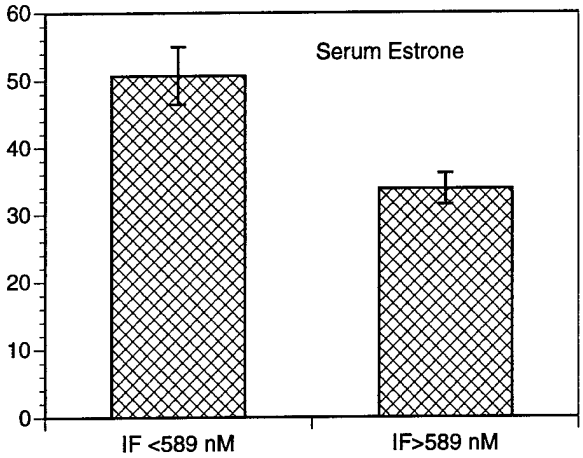

Figure 23. Serum concentrations of estrone (ng/ml) from monkeys in the soy-fed group of experiment 91-12 are shown on the y axis; animals having serum soy isoflavone concentrations (IF) above themedian value of 589 nM had lower serum estrone concentrations. This may indicate attenuation of estrone synthesis by soy isoflavone intake.

Another interesting finding from the intra-group analysis of soy-fed animals in experiment 91-12 is that mammary gland lobular area was diminshed in animals in the upper half of serum isoflavone concentrations. (Figure 24).

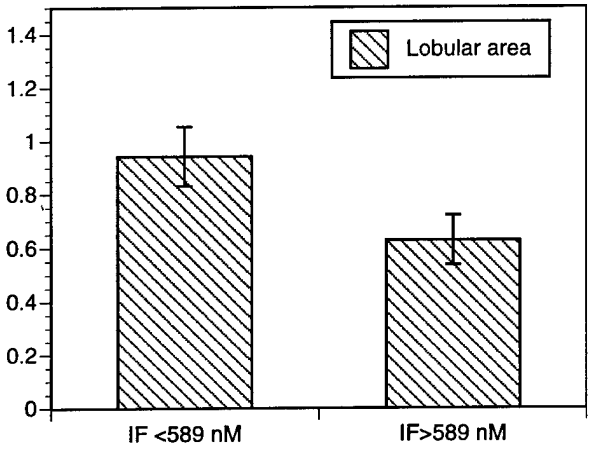

Figure 24. Mammary epithlelial (lobular) area in sections from monkeys in the soy-fed group of experiment 91-12 are shown on the y axis; animals having serum soy isoflavone concentrations (IF) above themedian value of 589 nM had a lower proportion of the mammary tissue occupied by epithelium.

Correlation analyses of serum isoflavones have yielded perplexing results. Using parametric analysis, (Pearson's correlation coefficient) serum genistein seems to be correlated with a weak increase in mammary gland epithelial proliferation in both lobuloalveolar tissue ($r = 0.3670$, $p = 0.0039$) and ductal epithelium ($r = 0.2570$, $p = 0.475$). However, with nonparametric methods this effect disappears. For total isoflavone concentrations, there is a negative association between lobular area and serum isoflavone concentration ($r = -0.2641$, $p = 0.0415$). The difference between these two findings may point to differential effects of different isoflavones, and may help to explain the difference between animal carcinogenesis studies of whole soy diets which indicate a chemopreventive effect (Barnes et al., 1995), and the studies of breast cancer cells in which

genistein is shown to promote the growth of MCF-7 cells (Makela et al., 1994).

X. Ancillary Projects

Measurement of ER beta in macaque tissues
The background of data provided by this project has led to increased interest in use of the macaque model to understand estrogen-mediated events. In 1996 we successfully competed for intramural pilot project funds to assess the possiblity that the newly described ER beta occurs in macaques. We found that the beta receptor is expressed in macaque tissues, in a pattern similar to that described in humans. Data from this study were presented at the Triangle Conference on Reproductive Biology and further work is in progress in preparation for publication.

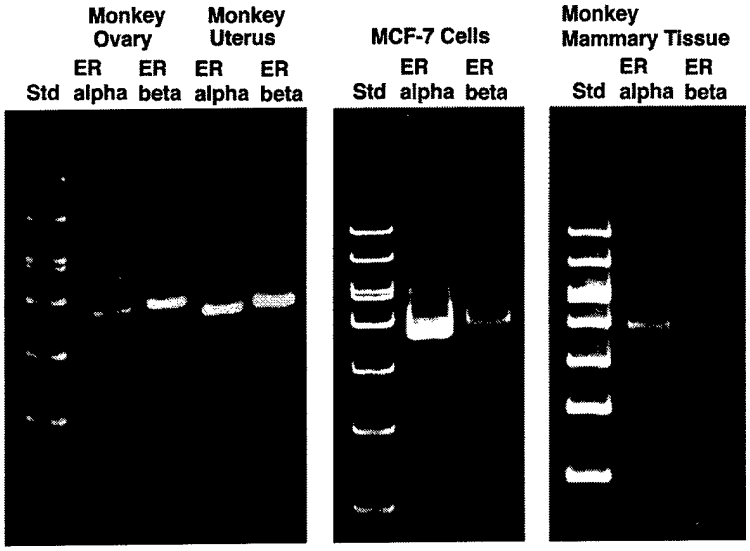

Figure 24. Expression of ER beta mRNA in macaque tissues, as determined by RT-PCR. ER beta expression is similar to that of ER alpha in ovary and uterus, but less in MCF-7 mammary carcinoma cells and mammary gland tissue. Courtesy of Dr. Thomas Register.

Vaginal cytologic evaluation of hormone-treated animals
Vaginal cytologic examinations have been made on animals in studies 91-12 during the latter half of the experiment (see page 17 for the study design), and in study 91-20 (the study design is on page10). Our published results indicate that CEE and tamoxifen exert an estrogenic effect on the vaginal epithelium, and that SBE does not (Cline et al.,1996).

Identification of Previously Pregnant animals
The presence of perivascular extracellular mucinous matrix deposits was identified as an indicator of prior pregnancy. Data summarized in Table 1 indicate that parity status of macaques can be determined from histopathologic examination of the uteri, with a reasonable degree of certainty.

Table 1. Accuracy of retrospective determination of parity status inmacaques, based on uterine histopathologic examination.

	Cynomolgus macaques		Rhesus macaques		Stumptailed macaques	All animals	
	Nulliparous	Parous	Nulliparous	Parous	Parous	Nulliparous	Parous
Number evaluated	13	24	13	38	5	67	26
Mean age	5.0	8.4	5.8	9.8	7.3	5.44	9.16
Range	3.1-10.2	3.8-15	3.3-12.3	4.7-16.7	5.4-8.9	4.75-12.3	8.5-16.7
Mean parity number	-	3.1	-	2.3	1.6	0	2.5
Range	-	1-7	-	1-6	1-3	-	1-7
Months since last delivery	-	8.9	-	24.8	15.6	-	18.45
Range	-	1-32	-	3-78	7-35	-	1-78
Number correctly classified	12	23	12	33	5	24	61
False positives	1	-	1	-	-	2	-
False negatives	-	1	-	5	0	-	6

Assessment of oral-contraceptive-related changes in monkey ovaries
This project was originally undertaken by a summer student in my laboratory, and was not funded by this grant; nonetheless, it has produced interesting data indicating that oral contraceptives do in fact alter the ovarian function of macaques at the doses given. This work has resulted in beneficial collegial interactions between investigators at Bowman Gray, Duke University, and the National Institute of Environmental Health Sciences. In the past year, this collaboration has led to the finding that progestin-containing oral contraceptives induce increased apoptosis in the ovarian surface epithelium. This observation has important implications for understanding the ovarian cancer-preventive effects of oral contraceptives, and was the subject of a publication (Rodriquez, 1998) and a plenary presentation to the Society of Gynecologic Investigation.

•Recommendations

The data presented herein clearly demonstrate that the macaque mammary gland can be used to provide a model of breast regulation in the post-menopausal period. This model is unique in that it provides an in vivo assessment of hormone effects on the primate breast, and can also be used to make comparisons of breast and endometrial effects in the same subject.

Several significant findings have been made to date. In the first year of this grant, we observed that the addition of MPA to conjugated estrogens did not result in suppression of the mammotrophic effects of CEE, but instead in a greater proliferative response than is seen with CEE alone. This finding addresses precisely the type of question the project is designed to target, providing a result which is of great relevance to public health, but which can only be explored with great difficulty in human subjects. This is in agreement with recent reports such as that of the Nurses' Health Study (Colditz 1995).

During the second year, we made the observation that dietary soy supplementation has the potential to protect the mammary gland from the tumor-promoting proliferative effects of estrogens (Foth and Cline, 1998). This finding has broad implications for the use of dietary modulation of breast cancer risk.

During the third year, we explored the effects of tamoxifen alone and in concert with estrogens on the endometrium and breast, and observed that cyclic oral contraceptives do not induce the same type of mammary hyperplasia inducedby postmenopausal hormone replacement therapy (Cline et al., 1998).

During year 4 of the grant, we collected tissues from the largest study in the project (X91-12, study design on page17), which was unfortunately delayed for logistical reasons beyond our control. Also during this year, we submitted for publication a unique observation of differential effects of estrogens, progestins, and tamoxifen on p53 expression in the normal macaque breast (Isaksson et al., in press), and continued our studies of ERβ expression in breast.

During year 5 of the grant (no-cost extension), we evaluated proliferation, histomorphometry, and sex steroid receptor expression tissues from the delayed study X91-12. This study has provided our group with vital information regarding the mixed estrogen agonist/antagonist of soy phytoestrogens, which we intend to pursue through humantrials of soy phytoestrogen effects on the breast, through collaborations with our clinical colleagues. Manuscripts are in preparation from experiment 91-12 and other studies completed in 1998-1999.

A great deal of important information can be gained within the scope of this project as initially written. However, after making the initial observations of hormone effects of these intermediate markers of cancer risk in breast, it will be vital to proceed on to more mechanistic studies of the role of growth factors and growth factor receptor expression in the proliferative response. The continuing controversy over breast cancer risk associated with hormonal therapies, particularly with regard to the role of progestins, indicates a lack of understanding of basic regulatory processes in the breast. The recent paper by Grodstein et al. (1997) indicates that breast cancer is likely to be a limiting factor on the use of hormonal replacement therapies in at least some subset of postmenopausal women. This has led our group to seek further exploration of alternatives to traditional hormone replacement therapy, such as dietary soy supplementation. The potential cancer chemopreventive effects of soy isoflavones is well documented in rodent models and has logical mechanistic bases in terms of the antioxidant, antiproliferative, and tyrosine-kinase inhibiting effects of these compounds (Barnes, 1995). We would also like to continue our exploration of normal breast regulation, since there is evidence that the breast of human and non-human primates shares regulatory features that are not common to other species such as rodents.

We intend to continue exploring the role of soy phytoestrogens in women's health. Soy phytoestrogen pharmaceutical and "nutri-ceutical" products are widely marketed to the public, yet the question of their safety and efficacy has not yet been answered. As a part of the recent Consensus Conference on Treatment of Estrogen Deficiency Symptoms in Women Surviving Breast Cancer, it was stated that "the SERMs (selective estrogen response modifiers) and phytoestrogens represent important new agents for long-term treatment of breast cancer survivors", with the caveat that "more data are required regarding the phytoestrogens before initiation of large clinical trials". Regardless of whether their aggregate effect elevates or diminishes breast cancer risk, widespread consumption of phytochemicals in the human diet and as supplements make it imperative that we understand their effects.

CONCLUSIONS

General Summary:

Technical objectives outlined in the initial application were met on schedule, with the exception of a single study for which we received a no-cost extension of the grant; that study has now been completed.
The morphometric and immunohistologic methods proposed in the initial application have been applied successfully to a number of mammary gland samples from macaques.
Publication of results continues, and this work has been reconized intramurally and extramurally in via meeting presentations, invited presentations, invited publications, and invitations to expand the scope of cancer-related work using the monkey model.

Specific Conclusions:

In surgically post-menopausal cynomolgus macaques,

- CEE induced PR expression and focal to diffuse lobuloalveolar proliferation
- The addition of progestins to estrogen treatment increased mammary gland proliferation, in contrast to the antagonistic effects of progestins in the endometrium of the same animals.
- Medroxyprogesterone alone induced ductal but not lobulolaveolar proliferation in macaque mammary gland.
- Low-dose E2 had a threshhold effect, inducing proliferation in the uterus but not the mammary gland.
- Tamoxifen induced mammary ER and PR, but did not induce mammary gland proliferation in macaques, in contrast to cystic hyperplasia induced in the endometrium of the same animals.
- When given with E2, tamoxifen inhibited estrogen-induced proliferation, but not PR induction.
- Dietary soy phytoestrogens do not clearly induce proliferation in mammary gland endometrium; we intend to study this further.
- Dietary soy phytoestrogens antagonized the proliferation-inducing effect of estrogens in the mammary gland and endometrium.
- Dietary soy phytoestrogens did not antagonize the induction of progesterone receptors in mammary gland or uterus by estradiol; therefore the antiproliferative effect may be independent of a classical estrogen-receptor-mediated pathway.
- 17-alpha dihydroequilenin (DHEN) did not induce mammary gland or endometrial proliferation.
- Nandrolone did not alter breast histology or histomorphometry, but induced an unusual adenomyosis-like lesion in the uterus.
- p53 expression in response to estrogen treatment is novel finding in this model, and may lead to new insights regarding the role of p53 in modulation of estrogen-induced proliferative responses and the effects of mixed estrogen agonist-antagonists such as tamoxifen.
- ER beta can be detected in macaque tissues; this new aspect of the model may allow us to explore the differential expression and function of the classical and beta receptors.

REFERENCES

Adams MR, Williams JK, Clarkson TB, Jayo MJ. Effects of oestrogens and progestogens on coronary atherosclerosis and osteoporosis of monkeys. Baillieres Clinical Obstetrics & Gynaecology 1991;5:915-34.

Anderson TJ, Battersby S, King RJB, McPherson K, and Going JJ.: Oral contraceptive use influences breast cell proliferation. Human Path 1989;20:1139-1141.

Avila MH, Walker AM, Jick H. Use of replacement estrogens and the risk of myocardial infarction. Epidemiology 1990;1:128-133.

Barnes S. 1995. Effect of genistein on in vitro and in vivo models of cancer. J Nutr 125(3 Suppl):777S-783S.

Beniashvilli DS. An overview of the world literature on spontaneous tumors in nonhuman primates. J. Med. Primatol 1989, 18:423-437

Benirschke K, Garner FM, Jones TC, eds. Pathology of Laboratory Animals, New York:Springer-Verlag 1978:1204-1206.

Bergkvist L, Adami H-O, Persson I, Hoover R, Schairer C. The risk of breast cancer after estrogen and estrogen-progestin replacement. N Engl J Med 1989;321:293-297

Blankenstein MA, Maitimu-Smeele I, Donker GH, Daroszewski J, Milewicz A and Thijssen JH. On the significance of in situ production of oestrogens in human breast cancer tissue. Journal of Steroid Biochemistry & Molecular Biology. 41:891-6, 1992.

Bush TL, Barrett-Connor E, Cowan DL, Criqui MH, Wallace RB, Suchindran CM, Tyroler HA, Rifkind BM. Cardiovascular mortality and noncontraceptive use of estrogen in women: Results from the Lipid Research Clinics Program Follow-Up Study. Circulation 1987;75:1102-1109.

Butterworth BE, Goldsworthy TL. The role of cell proliferation in multistage carcinogenesis. Proc Soc Exp Biol Med 198:683-687, 1991

Cattoretti G. Becker MH. Key G. Duchrow M. Schluter C. Galle J. Gerdes J. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992;168:357-63

Chalkley HW. Method for the quantitative morphologic analysis of tissues. J Nat Cancer Inst 1943; 4:47-53.

Clarkson TB, Adams MR, Kaplan JR, Shively CA, Koritnik DR. From menarche to menopause: coronary artery atherosclerosis and protection in cynomolgus monkeys. American Journal of Obstetrics & Gynecology 1989;160:1280-5.

Cline JM, Soderqvist G, Skoog L, von Schoultz B. Divergent effects of hormone replacement on mammary and endometrial tissues of macaques. Menopause. 1995;2:278

Cline JM, Bain FT. Uterine vascular changes indicating prior pregnancy in macaques. Vet Pathol 32:585, 1995.

Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques. American Journal of Obstetrics & Gynecology. 1996;174:93-100

Cline JM, Paschold JC, Anthony MS, Obasanjo IO, Adams MR. Effects of hormonal therapies and dietary soy phytoestrogens on vaginal cytology in surgically postmenopausal macaques. Feril Steril. 1996;65:1031-1035

Cline JM, Soderqvist G, Skoog L, von Schoultz B. Regional distribution of proliferating cells and hormone receptors in the mammary gland of surgically postmenopausal macaques given hormone replacement therapy. Gynecologic and Obstetric Investigation 1997;44:41-46.

Cline JM, Hughes CL. Phytochemicals for the prevention of breast and endometrial cancer. In Biological and Hormonal Therapies of Cancer, eds. H Muss and K Foon. Kluwer Academic Publishers, 1998.

Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of conjugated estrogens, medroxyprogesterone acetate, and tamoxifen on the mammary glands of macaques. Breast Cancer Research and Treatment 1998;48:221-229.

Cohen SM, Purtilo DT, Ellwein LB. Pivotal role of increased cell proliferation in human carcinogenesis. Modern Pathol 4:371-382, 1991

Colditz GA, Hankinson SE, Hunter DJ, Willet WC, Manson JE, Stampfer MJ, Hennekens C, Rosner B, Speizer FE. The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. NEJM 1995;332:1589-1593.

Colditz GA, Egan KM, Stampfer MJ. Hormone replacement therapy and risk of breast cancer: Results from epidemiologic studies. Am J Obstet Gynecol 1993;168:1473-1480.

Colditz GA, Stampfer MJ, Willett WC, Hennekens CH, Rosner B, Speizer FE. Prospective study of estrogen replacement therapy and risk of breast cancer in postmenopausal women. JAMA 1990;264:2648-2653.

Coope J, March J. Can we improve compliance with long-term HRT? Maturitas 1992 15:151-158.

de Lignières B, Linares G, Barrat J. Effects of progesterone on epithelial cell mitotic activity in human breast. In: Progesterone in Hormone Replacement Therapy, eds RA Lobo and F Naftolin, Parthenon Press, Carnforth, U.K. 1992:47-56.

Ettinger WB, Genant HK, Cann CE. Long-term estrogen replacement therapy prevents bone loss and fractures. Ann Intern Med 1985;102:319-324.

Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad HD. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 1991;138:867-873.

Gompel A. Progestin treatments of menopause. Revue du Praticien 1993;43(20):2645-50.
Haslam SZ. Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. Endocrinology 1988;122:464-70.

Grodstein F, Stampfer MJ, Colditz GA, Willett WC, Manson JA, Joffe M, Rosner B, Fuchs C, Hankinson SE, Hunter DJ, Hennekens CH, Speizer FE: Postmenopausal hormone replacement

therapy and mortality. The New England Journal of Medicine 336 (25): 1769-1775, 1997.

Haslam SZ. Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. Endocrinology 1988;122:464-70.

Henderson BE, Pike MC, Ross RK, Mack TM, Lobo RA. Re-evaluating the role of progestogen therapy after the menopause. Fertil Steril 1988;49(Suppl)9S-12S.

Hunt K, Vessey M, McPherson K, Coleman M. Long-term surveillance of mortality and cancer incidence in women receiving hormone replacement therapy. Br J Obstet Gynecol 1987;94:620-635.

Kaiserman-Abramof IR, Padykula HA. Ultrastructural epithelial zonation of the primate endometrium (rhesus monkey). Am J Anat 1989;184:13-30.

Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet RA, Doody LA, Malone WF, Hawk ET and Sigman CC. New agents for cancer chemoprevention. Journal of Cellular Biochemistry - Supplement. 26:1-28, 1996.

Kiel DP, Felson DT, Anderson JJ, Wilson PW, Moskowitz MA. Hip fractures and the use of estrogens in postmenopausal women. N Engl J Med 1987;317:1169-1174.

La Vecchia C, Negri E, Franceschi S, Talamini R, Amadori D, Filiberti R, Conti E, Montella M, Veronesi A, Parazzini F, et al. Oral contraceptives and breast cancer: a cooperative Italian study. International Journal of Cancer. 1995;60:163-7

London SJ, Connolly JL, Schnitt SJ, Colditz GA. A prospective study of benign breast disease and the risk of breast cancer. JAMA 1992; 267(7):941-4

Lu LJ, Anderson KE, Grady JJ and Nagamani M. Effects of soya consumption for one month on steroid hormones in premenopausal women: implications for breast cancer risk reduction. Cancer Epidemiology, Biomarkers & Prevention. 5:63-70, 1996.

MacPherson EE, Montagna W. The mammary glands of rhesus monkeys. J Invest Dermatol 1974;63:17-18.

Makela S, Davis VL, Tally WC, Korkman H, Salo L, Santti R, Vihko R and Korach KS. Dietary estrogens act through estrogen receptor mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Perspectives. 102:572-8, 1994.

Mahoney CJ. A study of the menstrual cycle in *Macaca irus* with special reference to the detection of ovulation. J Reprod Fertil 1970;21:153-163.

Mauvais-Jarvis P, Kuttenn F, Gompel A. Antiestrogen action of progesterone in breast tissue. Breast Cancer Res Treatment 1986;8:179-188.

Moolgavkar SH, Day NE, Stevens RG. Two-stage model of carcinogenesis: Epidemiology of breast cancer in females. J Natl Cancer Inst 1980; 65:559-569.

Moore MR. Hathaway LD. Bircher JA. Progestin stimulation of thymidine kinase in the human breast cancer cell line T47D. Biochimica et Biophysica Acta 1991;1096(2):170-4

Mordenti J. Dosage regimen design for pharmaceutical studies conducted in animals. J Pharmaceut Sci 1986; 75:852-857.

Obasanjo IO, Clarkson TB, Weaver DS. Effects of the anabolic steroid nandrolone decanoate on plasma lipids and coronary arteries of female cynomolgus macaques. Metabolism Clin Exp 1996;45:463-8.

Ohi Y, Yoshida H. Influence of estrogen and progesterone on the induction of mammary carcinomas by 7,12-dimethylbenz(a)anthracene in ovariectomized rats. Virchows Archiv - B - Cell Pathology 1992;62:365-270.

Page DL. Dupont WD. Indicators of increased breast cancer risk in humans. Journal of Cellular Biochemistry - Supplement. 16G:175-82,1992.

Papa V. Reese CC. Brunetti A. Vigneri R. Siiteri PK. Goldfine ID. Progestins increase insulin receptor content and insulin stimulation of growth in human breast carcinoma cells. Cancer Research 1990;50(24):7858-62

Petitti DB, Perlman JA, Sidney S. Postmenopausal estrogen use and heart disease (letter). N Engl J Med 1986;315:131-132.

Pike MC. Spicer DV. Dahmoush L. Press MF. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiologic Reviews 1993;15(1):17-35.

Ravnikar VA. Compliance with hormone replacement therapy: are women receiving the full impact of hormone replacement therapy preventive health benefits?. Womens Health Issues 1992;2(2):75-80

Rodriguez GC, Walmer D, Lessey B, Cline M, Krigman H, Hughes CL. The effect of contraceptive progestins on the ovarian epithelium:cancer prevention through apoptosis.Society for Gynecologic Investigation, March 1997.

Schultz AH. Fetal growth and development of the rhesus monkey. Carnegie Contributions to Embryology 1937;26:71-97

Speert H. The normal and experimental development of the mammary gland of the rhesus monkey with some pathologic correlations. Contributions to Embryology, The Carnegie Institute of Washington 1948;32:9-65.

Stampfer MJ, Willett WC, Colditz GA, Rosner B, Speizer FE, Hennekens CH. A prospective study of postmenopausal estrogen therapy and coronary heart disease. N Engl J Med 1985;313:1044-1049.

Tavassoli FA, Casey HW, Norris HJ. The morphologic effects of synthetic reproductive steroids on the mammary gland of rhesus monkeys: Mestranol, ethynerone, mestranol/ethynerone, chloroethynyl norgestrel-mestranol, and anagestone acetate-mestranol combinations. Am J Pathol 1988 131:213-234.

Tsubura A, Hatano T, Hayama S, Morii S. Immunophenotypic difference of keratin expression in normal mammary glandular cells from five different species. Acta Anatomica 140:287-293, 1991

Uno, H. Age-related pathology and biosenescent markers in captive rhesus macques. Age. 20:1-13, 1997

Warner MR. Mammary pathology. In Aging in Nonhuman Primates, DM Bowden, ed. Van Nostrand Reinhold, New York, 1979

Washburn SA, Honoré EK, Cline JM, Helman M, Wagner JD, Adelman SJ, Clarkson TB. Effects of 17α-dihydroequilenin sulfate on atherosclerotic male and female rhesus monkeys. Am J Obstet Gynecol 1996;175:341-351.

Weiss NS, Ure CL, Ballard JH, Williams AR, Daling JR. Decreased risk of fractures of the hip and lower forearm with postmenopausal use of estrogen. N Engl J Med 1980;303:1195-1198.

Wordinger RJ, Miller GW, Nicodemus DS. Manual of Immunoperoxidase Techniques. American College of Clinical Pathologists, 1987

World Health Organization. Histological typing of breast tumors. Tumori 1982; 68:181-192.

World Health Organization Technical Report Series No. 817. Oral contraceptives and neoplasia. WHO, Geneva, 1992.

APPENDICES

Appendix A: Publications

(copies of items in **bold** are appended)

Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques. American Journal of Obstetrics & Gynecology. 1996;174:93-100

Cline JM, Paschold JC, Anthony MS, Obasanjo IO, Adams MR. Effects of hormonal therapies and dietary soy phytoestrogens on vaginal cytology in surgically postmenopausal macaques. Fertil Steril. 1996;65:1031-1035

Cline JM, Soderqvist G, Skoog L, von Schoultz B. Regional distribution of proliferating cells and hormone receptors in the mammary gland of surgically postmenopausal macaques given hormone replacement therapy. Gynecologic and Obstetric Investigation 1997;44:41-46.

Cline JM, Hughes CL. Phytochemicals for the prevention of breast and endometrial cancer. In Biological and Hormonal Therapies of Cancer, eds. H Muss and K Foon. Kluwer Academic Publishers, 1998.

Von Schoultz B, Söderqvist G, Cline M, von Schoultz E, Skoog L. Hormonal regulation of the normal breast. Maturitas 1996, 23:S23-S25

Washburn SA, Honoré EK, Cline JM, Helman M, Wagner JD, Adelman SJ, Clarkson TB. Effects of 17α-dihydroequilenin sulfate on atherosclerotic male and female rhesus monkeys. Am J Obstet Gynecol 1996;175:341-351.

Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of conjugated estrogens, medroxyprogesterone acetate, and tamoxifen on the mammary glands of macaques. Breast Cancer Research and Treatment 1998;48:221-229.

Isaksson E, Cline JM, Skoog L, Soderqvist G, Wilking N, von Schoultz E, von Schoultz B. p53 expression in breast and endometrium during estrogen and tamoxifen treatment of surgically postmenopausal cynomolgus macques. Breast Cancer Research and Treatment, in press.

Clarkson TB, Anthony MS, Williams JK, Honoré EK, Cline JM. The potential for soybean phytoestrogens for postmenopausal hormone replacement therapy. PSEBM 1998;217:365-368.

Cline JM, Soderqvist G, Skoog L, von Schoultz B. Effects of conjugated estrogens, medroxyprogesterone acetate, and tamoxifen on the mammary glands of macaques. Breast Cancer Res Treat , 1998;48:221-229.

Tansey G, Hughes CL Jr, Cline JM, Krümmer A, Walmer DK, Schmoltzer S. Effects of dietary soybean estrogens on the reproductive tract in female rats. Proc Soc Exp Biol Med 1998;217:340-344.

Obasanjo I, Cline JM, Schmotzer S, Weaver DS. Nandrolone decanoate causes pathologic changes in the uterus of surgically postmenopausal female cynomolgus macaques. Menopause 1998 5:163-168.

Foth D, Cline JM. Effects of mammalian and plant estrogens on mammary glands and uteri of macaques. Am J Clin Nutr 1998 68(suppl):1413S-7S.

G.C. Rodriguez, D. Walmer, B. Lessey, M. Cline, H. Krigman and C.L. Hughes. The effect of contraceptive progestins on the ovarian epithelium:cancer prevention through apoptosis. Journal of the Society for Gynecologic Investigation 1998 5:271-276.

M Cline, CL Hughes. Phytochemicals for the prevention of breast and endometrial cancer. In *Biological and Hormonal Therapies of Cancer*. H Muss and K Foon, editors. 1998 Kluwer; Boston.

TB Clarkson, MS Anthony, JM Cline. Clinical Efficacy of Phytoestrogens - Pharmacology/Biology. In Treatment of the Postmenopausal Woman, 2nd ed. R Lobo, editor. Lippincott-Raven, Philadelphia. In press.

JM Cline, D Foth, E Isaksson, TC Register, VL Davis, B von Schoultz. Modulation der Expression von Sexualsteroidrezeptoren durch hormonale Behandlungen. [Modulation of sex steroid receptor expression by hormonal treatments]. In: Endometrium und Hormonsubstitution. T. Römer and A.O. Mueck, editors. 1998. Thieme Verlag, Stuttgart.

C Hughes, D Walmer, JM Cline, VL Davis, D Foth. Effekte von Sojaisoflavonen (Phytoestrogenen) auf das Endometrium [Effects of soy isoflavones (phytoestrogens) on the endometrium]. In: Endometrium und Hormonsubstitution. T. Römer and A.O. Mueck, editors. 1998. Thieme Verlag, Stuttgart.

B von Schoultz, G. Söderqvist, JM Cline. Möglichkeiten der Hormonsubstitution: zyklische Therapie.[Hormone replacement therapy: cyclic therapy]. In: Endometrium und Hormonsubstitution. T. Römer and A.O. Mueck, editors. 1998. Thieme Verlag, Stuttgart.

Appendix B: Abstracts and Presentations

Cline JM, von Schoultz B, Skoog L, Clarkson TB. Can the cynomolgus macaque be used as a model for studying the effect of exogenous hormones on women? The Seventh International Congress on the Menopause, Stockholm, Sweden, June 21-25, 1993

Cline, JM; Soderqvist, G; von Schoultz, E; Skoog, L; von Schoultz, B Addition of medroxyprogesterone acetate to conjugated equine estrogens in surgically postmenopausal macaques: Divergent effects on mammary and endometrial tissue. Triangle Conference on Reproductive Biology, Research Triangle Park, NC, January 14, 1995

Divergent effects of hormone replacement on mammary and endometrial tissues of macaques. Cline, JM; Soderqvist, G; Skoog, L; von Schoultz, B Sixth Annual Meeting of the North American Menopause Society, San Francisco, CA, September 1995.

Cline JM, Bain FT. Uterine vascular changes indicating prior pregnancy in macaques. Vet Pathol 32:585, 1995.

Cline JM, Soderqvist G, Skoog L, von Schoultz B. Divergent effects of hormone replacement on mammary and endometrial tissues of macaques. Menopause. 1995;2:278

Cline JM, Foth D. Effect of mammalian and plant estrogens, tamoxifen, and medroxyprogesterone acetate on epithelial proliferation in the mammary glands and uteri of macaques. Proceedings, American Association for Cancer Research. 1996;37:284.

Cline JM, Foth D. Effects of mammalian and plant estrogens, tamoxifen, and medroxyprogesterone acetate on epithelial proliferation in the mammary glands and uteri of macaques. Triangle Conference on Reproductive Biology, Research Triangle Park, NC, January 16, 1996

Cline M. Endometrial changes induced by estrogens, tamoxifen, progestins, androgens, and soy in macaques. Vet Pathol. 1996;33:602

Cline JM, Söderqvist G, Foth D, Römer T, von Schoutz B. Effects of estrogens, tamoxifen, progestins, androgens and dietary soy on the mammary gland and endometrium of macaques The Eighth International Congress on the Menopause, Sydney, Australia, November 3-7, 1996

Cline JM, Söderqvist G, Foth D, Römer T, von Schoutz B. Effects of estrogens, tamoxifen, progestins, androgens and dietary soy on the mammary gland and endometrium of macaques The Eighth International Congress on the Menopause, Sydney, Australia, November 3-7, 1996

Davis VL, Cline JM, Tansey G, McCullough B, Hughes CL Jr. The effects of dietary isoflavones and conjugated equine estrogens on mammary proliferation in ovariectomized rats. Breast Cancer Symposium of the Comprehensive Cancer Center of Wake Forest University, Winston-Salem, NC, August 29, 1996.

Foth D, Cline JM. Effekte einer estrogenmonotherapie auf das mammagewebe von cynomolgus macaques. [The effect of an estrogen monotherapy on the mammary glands of cynomolgus macaques]. Bremen, German Society for Obstetrics and Gynecology, June 7-9, 1996.

Foth D, Cline JM. Die Wirkung von estradiol und phytoestrogenen (isoflavone) auf das mamma- und uterusgeweben von macaquen. [The effect of estradiol and phytoestrogens (isoflavones) on the mammary glands and uterus of macaques]. Congress of Obstetrics and Gynecology, Dresden, October 1-5, 1996.

Hughes CL, Davis VL, and Cline, JM. Dietary soy isoflavones antagonize conjugated equine estrogen-induced proliferation of mammary gland epithelium in ovariectomized female rats. Society for Gynecologic Investigation, March, 1997.

Cline JM, Soderqvist G, von Schoultz E, Skoog L, von Schoultz B. Conjugated estrogens, medroxyprogesterone acetate, and tamoxifen alter breast regulation in macaques. Breast Cancer Res Treat 1997;46:110

Isaksson E, Cline JM, Soderqvist G, von Schoultz E, Wilking N, von Schoultz B, Skoog L. Immunohistochemical detection of p53 in breast and endometrial tissue of surgically postmenopausal cynomolgus macaques after HRT and tamoxifen treatment. 19th Annual San Antonio Breast Cancer Symposium, December 11-14, 1996.

McCollough BA, Cline JM. Characterization of proliferation markers in cynomolgus macaques. Triangle Conference on Reproductive Biology, "Ligands and Receptors in Reproduction", Research Triangle Park, NC, January 25, 1997

Ray S, Cline JM. Combined treatment with tamoxifen and low dose estradiol in surgically postmenopausal macaques: Effects on mammary and endometrial tissue. Triangle Conference on Reproductive Biology, "Ligands and Receptors in Reproduction", Research Triangle Park, NC, January 25, 1997

Register TC, Bora T, Cline JM, Hughes C, Adams M. The Classical Estrogen Receptor (ER alpha) and the newly described estrogen receptor beta are expressed in reproductive and non-reproductive tissues of cynomolgus monkeys. Triangle Conference on Reproductive Biology, "Ligands and Receptors in Reproduction", Research Triangle Park, NC, January 25, 1997

Rodriguez GC, Walmer D, Lessey B, Cline M, Krigman H, Hughes CL. The effect of contraceptive progestins on the ovarian epithelium:cancer prevention through apoptosis. Society for Gynecologic Investigation, March 1997.

Cline, JM; Davis, VL; Hughes CL. Dietary soy has dose-dependent agonist/antagonist effects on the mammary glands and uteri of estrogen-treated rats. Proceedings, ILSI Conference on Human Diet and Endocrine Modulation, Fairfax, VA, November 19-22, 1997. ILSI Press, 1998.

Cline, JM; Foth, D. Dietary soy antagonizes the effects of estradiol on the mammary glands and uteri of macaques. Proceedings, ILSI Conference on Human Diet and Endocrine Modulation, Fairfax, VA, November 19-22, 1997. ILSI Press, 1998.

Cline, JM. Soy and Cancer Prevention. Department of Nutrition, University of North Carolina at Chapel Hill, March 3, 1998

Cline, JM. Soy and Cancer Prevention. Department of Nutrition, Arkansas Children's Hospital Research Institute, Little Rock, AR, April 9, 1998.

Cline, JM. Reproductive Toxicology of hormonal agents in primates. CL Davis Symposium, Raleigh, NC, June 15, 1998.

Cline, JM. Hormonal Effects on the Reproductive Organs of Macaques. SmithKline-Beecham, King of Prussia, PA, August 26, 1998

Register TC, Cline JM. Estrogen receptors alpha and beta are expressed in non-human primate mammary tissue. Proceedings of the 80th Annual Meeting of the Endocrine Society, P1-4, June 1998.

Register TC, J M Cline, C A Shively, M G O'Sullivan, M R Adams, C S Carlson, L Zhang, J D Wagner, C E Lewis, and L M Wilkinson. Variability in the Relative Expression of Estrogen Receptor Alpha and Beta mRNA in Reproductive and Non-reproductive Non-human Primate Tissues. Proceedings of the 81st Annual Meeting of the Endocrine Society, P3-395, June 1999.

Cline, JM. Phytochemicals and Breast Cancer Risk. American Association for the Advancement of Science, Anaheim, CA, January 15, 1999

Cline, JM; Anthony, M; Wagner, J; Williams, K; Clarkson, T. Dietary soy is cardioprotective in macaques without inducing uterine or breast proliferation. International Conference on Diet and the Prevention of Cancer, Tampere, Finland, May 28-June 2, 1999.