AD_____

GRANT NUMBER DAMD17-97-1-7040

TITLE: Preventive Role of Specific Dietary Factors and Natural Compounds Against DNA Damage and Oxidative Stress

PRINCIPAL INVESTIGATOR: Shunji Morita, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5400

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE
Preventive Role of Specific Dietary Factors and Natural Compounds Against DNA Damage and Oxidative Stress

5. FUNDING NUMBERS
DAMD17-97-1-7040

6. AUTHOR(S)
Shunji Morita, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

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 21702-5400

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)*

This project is examining the hypothesis that certain antioxidant vitamins (C and E) and natural compounds (caffeic acid phenethyl ester [CAPE] and epigallocatechin gallate [EGCG] may be protective against mammary carcinogenesis, at lest in part, by decreasing the extent of DNA damage in the target tissue. This DNA damage may be caused by chemical carcinogens in the environment (e.g., benzo(a)pyrene) and/or dietary factors that increase endogenous production of reactive oxygen species. We are developing sensitive monoclonal antibodies and assays for measuring the oxidative DNA damage, 5-hydroxymethyldeoxyuridine (5HMU). Several hybridomas have been developed. A pilot study has investigated the effects of CAPE treatment on benzo(a)pyrene-DNA adducts in MCF-7 cells. A dose-dependent decrease in DNA damage with increasing CAPE concentration was observed indicating that the immunologic method is appropriate for these types of studies and supporting the original hypothesis.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
8

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.

__✓__ 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.

__✓__ 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.

Shinji Morita 8/1/98

PI - Signature Date

Table of contents

Introduction	5
Results	5
Conclusions	7
References	8

Introduction

It is well established that covalent binding of chemical carcinogens to DNA is the initiating event in chemical carcinogenesis. While exposure to environmental contaminants in the air, food, and cigarette smoke may result in significant levels of DNA damage, it was more recently recognized that endogenous sources of damage also play an important role in initiating carcinogenesis. The work in this project concentrates on one model environmental contaminant, benzo(a)pyrene (BP), and one type of endogenous oxidative DNA damage, 5-hydroxymethyldeoxyuridine (5HMU). Prior studies have demonstrated that both types of DNA damage result in mutations (1,2). There is also extensive epidemiologic and laboratory data indicating that various dietary antioxidants protect cells against DNA damage. In this project we are specifically investigating the role of four antioxidants, vitamins C and E, caffeic acid phenethyl ester (CAPE), and epigallocatechin gallate (EGCG) in inhibiting DNA damage. These antioxidants are found in natural products such as fruits and vegetables (vitamins C and E), the popular medicine honeybee propolis (CAPE), or green tea (EGCG). Studies carried out to date suggest that these compounds may have a role in chemoprevention of cancer.

The goal of this project is to test the hypothesis that certain antioxidant vitamins (C and E) and natural compounds (CAPE and EGCG) may be protective against mammary carcinogenesis, at least in part, by decreasing the extent of DNA damage in the target tissue. This DNA damage may be caused by chemical carcinogens in the diet (e.g., BP) and/or dietary factors that increase the endogenous production of reactive oxygen species. To facilitate these studies we are developing sensitive methods that can be used to assess the extent and types of DNA damage in mammary epithelial cells that can result from specific chemicals in the diet, or from factors in the diet that affect oxidative damage to cellular DNA. A monoclonal antibody and highly sensitive ELISA will be developed to accurately measure 5HMU in mammary epithelial cells and other types of cells and tissues. A quantitative immunohistochemical method will also be developed.

To test the hypothesis that vitamins C and E, CAPE, and EGCG are protective against DNA damage, their effects on formation of BP-DNA and 5HMU in nontumorigenic MCF-10A and tumorigenic MCF-7 human mammary epithelial cells treated with or without BP or H_2O_2 will be determined. Adducts will be monitored by immunoassay using a previously developed antiserum generated against BP diol epoxide modified DNA (BPDE-DNA) and the antibodies to be developed in this proposal against 5HMU.

Results

Laboratory work was begun in October 1997 by Dr. Yasmina Ait Amara-Mokrane. Unfortunately, in April of 1998, for personal reasons, she decided to return to France. Permission was obtained to replace Dr. Amara by Dr. Shunji Morita. On June 18, 1998, Dr. Morita began work on this project.

For development of monoclonal antibodies to 5HMU, the ribose form of this oxidized base was coupled to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) by the periodate oxidation procedure (3). Levels of coupling were determined by the absorbance at 280 nm for the protein and at 260 nm for the base. Levels of 5HMU on KLH and BSA were 17 and 22-660, respectively. The value for KLH is expressed as a range given the variability in molecular weight reported for this protein. These antigens were both used for the immunization of BALB/c mice after emulsification in Freund's adjuvant. After three immunizations, animals were tail bled and sera screened for the presence of antibodies by noncompetitive enzyme-linked immunosorbent assay (ELISA). For screening the 5HMU-BSA immunized animals plates were coated with KLH or 5HMU-KLH. For the 5HMU-KLH immunized animals, BSA and 5HMU-BSA were used. Serial dilution of the mouse sera indicated several animals immunized with each antigen had responded with significant titer against the conjugate.

The initial two fusions were carried out with the 5HMU-KLH immunized mice using standard protocols with polyethylene glycol (4). Fused cells were distributed into ten 96-well plates and hybrids selected in HAT (hypoxanthine, aminopterine, thymidine) media. For these KLH fusions multiple clones were present in almost every well. After two weeks wells were screened by noncompetitive ELISA on plates coated with BSA or 5HMU-BSA. About 20 clones for each fusion which gave high readings with 5HMU-BSA and at least 10-fold lower readings with BSA were selected for further screening by competitive ELISA. Plates were coated with 5HMU-BSA and clones first titered by serial dilution to select a concentration that would result in appropriate color development in the ELISA. These concentrations were then used to determine 50% inhibition by free 5HMU. All clones isolated from the 5HMU-KLH fusions did not recognize free 5HMU. Because of this unexpected finding, the competitive ELISA was repeated using 5HMU-BSA coated wells and 5HMU-BSA as the competitor. As expected, binding of antibodies to 5HMU-BSA coated plates could be competed off by 5HMU-BSA in solution. It is not clear why the antibodies do not recognize free 5HMU. It is possible that the coupling procedure with periodate further oxidizes the 5HMU and that the antibodies recognize this oxidized product. Alternatively, there may be recognition of the linking region between the hapten (5HMU) and the carrier protein (BSA or KLH).

We then switched to the 5HMU-BSA immunized animals. While the number of clones were lower than in the fusions with the KLH conjugates, we were successful in obtaining antibodies which could be competed off by free 5HMU. Two clones, 6D6 and 7A11, have been partially characterized. In the competitive ELISA, 50% inhibition of binding occurred with approximately 100 pmol 5HMU for both antibodies. While it is possible that sensitivity can be further increased by using a fluorescence based assay, we will also carry out additional fusions with the BSA-immunized mice in an attempt to obtain a higher affinity antibody. In prior studies, we have produced antibodies giving 50% inhibition in competitive ELISA in the low femto mole to low pico mole range for other antigens. The best clones will then be further characterized by testing for cross-reactivity with the normal nucleotides, calf thymus DNA, other oxidized DNA bases, and other carcinogen modified DNAs. This

procedure will allow us to select the most sensitive and specific clones for use in the remaining cell culture studies.

We have also carried out one pilot study on the effect of caffeic acid phenethyl ester (CAPE) on benzo(a)pyrene (BP) treated MCF-7 cells. Doses of CAPE and BP were selected by initial treatment of MCF-7 cells for 24 hr followed by determination of cytotoxicity using the MTT assay kit (Promega). All treatments resulted in >90% viability. Cells were treated with CAPE for 24 hrs before and during the 16 hr treatment with BP. DNA was isolated from the cells by standard phenol/chloroform/isoamylalcohol and RNase treatments and then assayed by competitive ELISA using a previously developed polyclonal antiserum (#29) generated in rabbits immunized with BPDE-DNA. As shown in Table 1, CAPE treatment inhibits BP-DNA adduct levels in the treated cells in a dose-dependent manner.

Table 1 Effect of CAPE treatment on BP-DNA adduct levels in MCF-7 cells

Treatment		Adducts/10^8 nucleotides
BP μM	CAPE μM	
0	0	nondetectable
2.5	0	14.6
2.5	3.5	9.1
2.5	8.9	8.5
2.5	17.5	4.3

Conclusions

Antigen has been synthesized for the development of monoclonal antibodies to the oxidized base 5HMU and several initial clones isolated. These clones will be further characterized but additional clones will also be prepared over the next few months to isolate higher affinity antibodies. Initial studies on the use of immunologic methods for monitoring the effects of antioxidants on DNA damage levels demonstrated the sensitivity of this method. Future studies will expand the number of compounds tested for both the bulky BP adducts and the oxidative damage, 5HMU.

References

1. Jeffrey, A.M. (1985) DNA modification by chemical carcinogens. Pharmac. Ther., 28: 237-72.
2. Marnett, L.J., and Burcham, P.C. (1993) Endogenous DNA adducts: potential and paradox. Chem. Res. Toxicol., 6: 771-85.
3. Erlanger, B.R., and Beiser, S.M. (1964) Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. Proc. Natl. Acad. Sci. USA, 52: 68-74.
4. Santella, R.M., Lin, C.D., Cleveland, W.L., and Weinstein, I.B. (1984) Monoclonal antibodies to DNA modified by a benzo[a]pyrene diol epoxide. Carcinogenesis, 5: 373-7.