935
(R)

712359

FEB 19 1982

Dr. Edmund S. Copeland
Chemical Pathology Study Section
Division of Research Grants
National Institutes of Health
Westwood Building, Room 353
Bethesda, Maryland 20205

Dear Dr. Copeland:

ORNL RESEARCH GRANT PROPOSAL, "CARCINOGENESIS OF XENOTRANSPLANTED HUMAN EPITHELIA" (INTERAGENCY AGREEMENT DOE NO. 40-1175-81, NIH NO. 1 R01 CA 31952-01)

Enclosed for your consideraton are two copies of revised budget pages for the subject research grant proposal, prepared by the DOE's Oak Ridge National Laboratory (ORNL). These revisions reflect reductions in animal and histology costs, based on suggestions during the recent site visit that the experiments described in specific aims No. 3 and No. 4 be eliminated. These revisions also reflect changes in indirect costs, resulting from our current negotiations with the DHHS Atlanta Regional Office. Please note also that the NIEHS Interagency Agreement listed on original grant application page 11 will not provide support for Dr. Klein-Szanto after June 30, 1982.

Technical questions concerning the enclosure should be directed to A. J. P. Klein-Szanto, ORNL Principal Investigator, telephone (FTS) 624-3971. Administrative matters should be addressed to J. E. Carr, ORNL Work for Others Coordinator, telephone (FTS) 624-6062.

Sincerely,

Original Signed by
William R. Bibb

BEST COPY AVAILABLE

William R. Bibb, Director
Research Division

ER-13:WRB

Enclosure:
Revised Budget Pages (2)

cc w/encl:
W. P. Snyder, CC-10, ORO

cc w/encl:
H. Postma, ORNL
J. E. Carr, ORNL
A. J. P. Klein-Szanto, ORNL

REPOSITORY Oak Ridge Operations Records Holding area
COLLECTION Documents 1944-94
BOX No. B-87-7 Bldg. 2714-H
FOLDER 40-1175-81 NIH
40-1175-81

CONCURRENCE
RTG. SYMBOL ER-13
INITIALS/SIG. Bibb
DATE WRB
RTG. SYMBOL
2-19-82
INITIALS/SIG.
DATE
RTG. SYMBOL
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ER-13:WRBibb:bci:6-0742:2-18-82

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DOE F 1325.10 (7-79)

OFFICIAL FILE COPY

FORM APPROVED
O.M.B. NO. 68-R0249

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
Carcinogenesis of Xenotransplanted Human Epithelia

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT [X] NO [ ] YES (If "YES," state RFA number and/or announcement title)

3. PRINCIPAL INVESTIGATOR PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
Klein-Szanto, Andres J. P.

3b. SOCIAL SECURITY NUMBER
[redacted]

3c. MAILING ADDRESS (Street, city, state, zip code)
Oak Ridge National Laboratory
Biology Division
P. O. Box Y
Oak Ridge, Tennessee 37830

3d. POSITION TITLE
Medical Staff Scientist

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Biology Division

3f. TELEPHONE (Area code, number and extension)

3g. MAJOR SUBDIVISION
Cancer and Toxicology Section

4. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA INVOLVED
[ ] NO [ ] YES (If "YES," form HHS 596 required)

5. RECOMBINANT DNA RESEARCH SUBJECT TO NIH GUIDELINES
[X] NO [ ] YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
From: April 1, 1982 Through: March 31, 1985

7. TOTAL DIRECT COSTS REQUESTED FOR PROJECT PERIOD (from page 5)
$ 238,845

8. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$ 73,560

9. PERFORMANCE SITES (Organizations and addresses)
Biology Division
Oak Ridge National Laboratory
P. O. Box Y
Oak Ridge, TN 37830

10. INVENTIONS (Competing continuation application only)
Were any inventions conceived or reduced to practice during the course of the project?
[ ] NO [ ] YES - Previously reported
[ ] YES - Not previously reported

11. APPLICANT ORGANIZATION (Name, address and congressional district)
Oak Ridge National Laboratory
P. O. Box Y
Oak Ridge, TN 37830

Third Congressional District

12. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT (See instructions)
Code 6 0 Description

13. ENTITY IDENTIFICATION NUMBER
60 - Department of Energy

14. TYPE OF ORGANIZATION (See instructions)
[ ] Private Nonprofit
[X] Public (Specify Federal, State, Local): Federal

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number.)
William R. Bibb
Director, Research Division
Department of Energy
Oak Ridge, TN 37830
FTS 626-0727, (615) 576-0727

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
William R. Bibb
Director, Research Division
Department of Energy
Oak Ridge, TN 37830
FTS 626-0727, (615) 576-0727

17. PRINCIPAL INVESTIGATOR PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable)
[signature]

DATE
2-16-82

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)
William R. Bibb

DATE
2-19-82

10189003

PHS-398
Rev. 5/80

PRINCIPAL INVESTIGATOR/PROGRAM DIRECT: Klein-Szanto, Andres J.P.

DETAILED BUDGET FOR FIRST 12 MONTH BUDGET PERIOD DIRECT COSTS ONLY

FROM: 4-1-82 THROUGH: 3-31-83

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) (See instructions)		TIME/EFFORT				
NAME	TITLE OF POSITION	%	Hours per Week	SALARY	FRINGE BENEFITS	TOTALS
A.J.P. Klein-Szanto	Principal Investigator	35	14	12,950	3,890	16,840
M. Terzaghi	Co-Investigator	10	4	3,700	1,110	4,810
D. Martin	Technician	30	12	6,900	2,070	8,970
To be named	Postdoctoral Fellow	100	40	15,000	1,500	16,500
	SUBTOTALS →			38,550	8,570	47,120

CONSULTANT COSTS (See instructions)

EQUIPMENT (Itemize)

SUPPLIES (Itemize by category)

Animals and Animal Care = 14,040
EM and Histology Supplies = 2,000
Chemicals (reagents, carcinogens, tissue culture media) = 3,000
ARG + Photographic material = 1,500
Histology Service = 2,000

22,540

TRAVEL	DOMESTIC	AACR Meeting - Principal Investigator	800
	FOREIGN		
PATIENT CARE COSTS	INPATIENT		
	OUTPATIENT		

ALTERATIONS AND RENOVATIONS (Itemize by category)

CONTRACTUAL OR THIRD PARTY COSTS (See instructions)

OTHER EXPENSES (Itemize by category)

Publication costs = 600
Maintenance and Service of Equipment = 2,000
Human Tissues Shipping Expenses = 500

3,100

TOTAL DIRECT COSTS (Also enter on page 1, item 8) → $73,560

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		47,120	50,890	54,960		
CONSULTANT COSTS		-				
EQUIPMENT		-				
SUPPLIES		22,540	24,344	26,291		
TRAVEL	DOMESTIC	800	880	950		
	FOREIGN	-				
PATIENT CARE COSTS	INPATIENT	-				
	OUTPATIENT	-				
ALTERATIONS AND RENOVATIONS						
CONTRACTUAL OR THIRD PARTY COSTS						
OTHER EXPENSES		3,100	3,350	3,620		
TOTAL DIRECT COSTS		73,560	79,464	85,821		

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Also enter on page 1, item 7) → $ 238,845

JUSTIFICATION (Use continuation pages if necessary): Briefly describe the specific functions of the personnel and consultants. For all years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and contractual or third party costs. For future years, justify any significant increases in any category. In addition, for COMPETING CONTINUATION applications, justify any significant increases over current level of support. If a recurring annual increase in personnel costs is anticipated, give percentage.

The three-year budget is calculated on the basis of an annual increase of 8% in expenses.

Animals - The animals are bred and housed in a specific-pathogen-free facility, a requirement for precision research in respiratory carcinogenesis. Animal costs are calculated on a cage basis no matter how many animals per cage; the standard number in experimental cages is 5 rats/cage or 10 mice/cage. The cost unit in the Biology Division is MCE, i.e. "mouse cage equivalent"; 1 rat cage = 2 MCE. The SPF animal facilities are an integral part of the Respiratory Carcinogenesis Unit; therefore, cost for animals used for experiments must include the cost of maintaining the animal line, breeding cages, and animals held in stock until the proper age (8-12 weeks old). The stock-holding number is determined by the anticipated needs of the investigators. It is important to note therefore, that costs must represent some steady-state level with numbers of animals being used constantly and equivalent numbers entering from the breeding colony. The animal costs incurred also include those used fairly rapidly for making tracheal implants and finally the experimental cages that are long-term holdings. For breeding purposes, 4.34 MCE/rat/week and 2.85 MCE/nude mouse/week are calculated. The minimum number of animals which can be effectively delivered to each investigator in order to be able to carry out this experiment with animals of a uniform age is 4 MCE for rats and 1 MCE for nude mice.

Justification (continued)

The projected annual cost per average MCE is $234.00. This cost is determined from utilities, supplies, disease testing, manpower expenses (animal management, veterinarian, caretakers). It should be noted that fringe benefits and Biology Division overhead are included in these costs. The average estimated needs and cost of the experiments per year are:

SPF Rats	
Breeding (12 mo) 24 MCE	$ 5,616.00
Use (4 mo) 48 MCE	3,744.00
Nude Mice	
Breeding (12 mo) 8 MCE	$ 1,872.00
Use (12 mo) 12 MCE	2,808.00
Total	$14,040.00

Histology Service - Most of our material will be prepared as continuous sections of tracheal explants to identify lesions. Slides are charged on cost per "slide equivalent" basis. We anticipate an average of 1.2 slide equivalents/slide at a cost of $6.25 per slide equivalent, 320 slides = $2,000.

Human Tissues Shipping Expenses - Priority mail only - from Indianapolis to Oak Ridge = $500.00.

107890b

CHECKLIST

This is the required last page of the application.

Check the appropriate boxes and provide the information requested.

TYPE OF APPLICATION:

☐ NEW application *(This application is being submitted to the PHS for the first time.)*

☐ COMPETING CONTINUATION of grant number: _____.
(This application is to extend a grant beyond its original project period.)

☐ SUPPLEMENT to grant number: _____.
(This application is for additional funds during a funded project period.)

☐ REVISION of application number: _____.
(This application replaces a prior version of a new, competing continuation or supplemental application.)

☐ Change of Principal Investigator/Program Director.
Name of former Principal Investigator/Program Director: _____.

ASSURANCES IN CONNECTION WITH:

Civil Rights	Handicapped Individuals	Sex Discrimination	Human Subjects General Assurance *(If applicable)*	Laboratory Animals *(If applicable)*
☒ Filed	☒ Filed	☒ Filed	☐ Filed	☐ Filed
☐ Not filed	☐ Not filed	☐ Not filed	☐ Not filed	☐ Not filed

INDIRECT COSTS:

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submit it to the appropriate DHHS Regional Office. Indirect costs will not be paid on foreign grants, construction grants, and grants to individuals, and usually not on grants in support of conferences.

☐ DHHS Agreement Dated: _____.
_____ % Salary and Wages or _____ % Total Direct Costs.

Is this an off-site or other special rate, or is more than one rate involved? ☐ YES ☐ NO
Explanation: _____

☒ DHHS Agreement being negotiated with Atlanta Regional Office.

☐ No DHHS Agreement, but rate established with _____ Date _____

☐ No Indirect Costs Requested.

Indirect costs are projected at 95% of the annual total direct cost for this proposal.

1078907

JUN 19 1981

Division of Research Grants
National Institutes of Health
Bethesda, Maryland 20014

Gentlemen:

ORNL GRANT PROPOSAL, "CARCINOGENESIS OF XENOTRANSPLANTED HUMAN EPITHELIA" (INTERAGENCY AGREEMENT DOE NO. 40-1175-81)

Enclosed for your consideration are six copies of the subject research proposal, prepared by the DOE's Oak Ridge National Laboratory (ORNL). Also enclosed are an executed Grant Certification and an executed Cost Sharing Agreement in the format of HEW Form 490.

The objective of the proposed study is to further develop an in vivo experimental model using previously unexposed xenotransplanted human epithelia cells. The system will then be employed to analyze the early and late effects of chemical carcinogens. The proposal requests a three-year period of performance beginning April 1, 1982. The initial twelve-month funding will require $99,637 for direct costs.

Your favorable consideration of the proposal should be reflected in a funding authorization to this office. Upon its receipt, we will authorize ORNL to proceed with the research project. Monthly billings will be accomplished by Standard Form 1081 to reimburse the DOE appropriation for actual cost incurred.

Technical questions concerning the proposal should be directed to A. J. P. Klein-Szanto, ORNL Principal Investigator, telephone FTS 624-3971. Administrative questions should be addressed to J. E. Carr, ORNL Work for Others Coordinator, telephone FTS 624-6062.

Sincerely,

Original Signed by
William R. Bibb

William R. Bibb, Director
Research Division

ER-13:WRB

Enclosures:
1. Proposal (6)
2. Grant Certification
3. Cost Sharing Agreement

cc w/encls:
W. P. Snyder, CC-10, CRO

cc w/o encls:
H. Postma, ORNL
J. E. Carr, ORNL
A. J. P. Klein-Szanto, ORNL

B 2224 ER-13:WRBibb:mac:6-0742:5-21-81

DOE F 1325.10
(7-79)

OFFICIAL FILE COPY

1018908

CONCURRENCE
RTG. SYMBOL ER-13
INITIALS/SIG WRB
DATE 6-19-81

COST SHARING AGREEMENT

Awarding Office:
National Institutes of Health

Application or Award Number:

Applicant:
U. S. Department of Energy
Oak Ridge Operations Office
Research Division
Post Office Box E
Oak Ridge, Tennessee 37830

Proposed Project Period:
From April 1, 1982 to March 31, 1985

ORNL RESEARCH GRANT PROPOSAL, "CARCINOGENESIS OF XENOTRANSPLANTED HUMAN EPITHELIA" (INTERAGENCY AGREEMENT DOE NO. 40-1175-81)

In order to satisfy HHS cost sharing requirements, the U. S. Department of Energy agrees to bear 17.18 percent of the total cost of the above project.

Total project cost is the sum of the total allowable direct and indirect cost incurred by the recipient and sub-recipients of cost-type contractors, plus the value of any third party in-kind contributions which benefit the project. These amounts will be calculated according to the rules in the HHS grant administration regulations at 45 CFR, Part 74.

If the project period consists of more than one budget period, the agreed-upon rate will apply to the project period as a whole, including any non-competitive extensions but not necessarily to each individual budget period.

If HHS stops funding this project prior to the close of the originally agreed-upon project period, the rate will apply to the actual period of support.

If the project period is extended competitively, another cost sharing agreement will be negotiated.

William R. Bibb

William R. Bibb, Director
Research Division

6-19-81
Date

1078909

Certification Required by PHS: 1-515-40B

for

CARCINOGENESIS OF XENOTRANSPLANTED HUMAN EPITHELIA

To Be Performed By

The Department of Energy's Oak Ridge National Laboratory

I certify that:

1. The Department of Energy has the statutory authority to carry out the research study set forth in the proposal for the research project with the above title.

2. The Department of Energy will be legally, financially and administratively responsible for the conduct of the research project with the above title if a research grant is awarded to Oak Ridge Operations of the Department of Energy for this purpose.

3. Dr. William R. Bibb, Director, Research Division, Oak Ridge Operations, is delegated by me to administer the grant for the Department of Energy.

R. J. Hart
Manager
Oak Ridge Operations
Department of Energy
JUN 19 1981

1078910

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION

POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

OFFICE OF THE DIRECTOR

May 27, 1981

Approved by Maddox by phone 6-18-81

Department of Energy
Oak Ridge Operations
Attention: Mr. J. A. Lenhard, Assistant Manager
for Energy Research and Development
Post Office Box E
Oak Ridge, Tennessee 37830

Gentlemen:

Grant Proposal to the National Institutes of Health, "Carcinogenesis of Xenotransplanted Human Epithlia"
(Interagency Agreement No. 40-1175-81)

Enclosed for your review and approval is a research grant proposal being submitted to the National Institutes of Health. The proposal requests a three-year period of performance beginning April 1, 1982, and a total direct funding of $323,165. The initial twelve-month funding will require $99,637 plus indirect costs of approximately $30,890.

The objective of the proposed study is to further develop an *in vivo* experimental model using previously unexposed xenotransplanted human epithelial cells, and to employ this system to analyze the early and late effects of chemical carcinogens.

The following information is provided to assist in your review and approval of this proposal:

a. This research effort is described in the approved ORNL Institutional Plan on page 52, "Work for Others (Excluding NRC)." The personnel required for this research are included in the direct FTE's in the "Summary of Resources" on page 53.

b. A. J. P. Klein-Szanto will serve as the principal investigator devoting 35% of his effort to this research; the remaining 65% will be devoted to NIEHS (40-662-77).

c. Existing space and resources are available to conduct this research without interfering with existing DOE efforts.

40-1175-21

1078911

d. This research will serve the DOE related objectives in Activity HA 02 02 01 0, "Health Effects Research in Biomedical Systems - Carcinogenesis."

After your approval and approval of the Office of Health and Environmental Research, please forward six (6) copies to the Division of Research Grants, National Institutes of Health, Bethesda, Maryland 20014.

If you have any questions concerning this proposal, please contact A. J. P. Klein-Szanto, ext. 4-3971.

Sincerely yours,

Herman Postma

Herman Postma
Director

HP:TV:ep

Enclosure

cc: J. E. Carr
R. J. M. Fry
R. A. Griesemer
R. F. Hibbs
A. J. P. Klein-Szanto
J. N. Maddox, DOE-GTN
W. R. Ragland
C. R. Richmond
File - RC

1078912

DETACH AND CLIP TO THE SIGNED FACE PAGE OF THE APPLICATION

PERSONAL DATA ON

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

The Public Health Service has a continuing commitment to monitoring the operation of its review and award processes to detect—and deal appropriately with—any instances of real or apparent inequities with respect to age, sex, race, or ethnicity of the proposed principal investigator/program director.

To provide the PHS with the information it needs for this important task, the principal investigator/program director is requested to complete the form below and attach a single copy to the signed face page of the application.

Upon receipt and assignment of the application by the PHS, this form will be detached from the application. It will NOT be duplicated and will NOT be a part of the review process. Data will be confidential, and will be maintained in Privacy Act record system 09-25-0036, "Grants: IMPAC (Grant Contract Information)." All analyses conducted on the data will report aggregate statistical findings only and will not identify individuals.

If you decline to provide this information, it will in no way affect consideration of your application.

Your cooperation will be appreciated.

Date of Birth: [redacted] (Month/Day/Year)

Sex: ☐ Female ☒ Male

Race and/or Ethnic Origin:

Check one:

- ☐ American Indian or Alaskan Native
- ☐ Asian or Pacific Islander
- ☐ Black, not of Hispanic origin
- ☐ Hispanic
- ☒ White, not of Hispanic origin

NOTE: The category that most closely reflects the individual's recognition in the community should be used for purposes of reporting mixed racial and/or ethnic origins. Definitions are on the back of form.

1078913

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

ABSTRACT OF RESEARCH PLAN

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION (Same as Item 11, page 1)

Oak Ridge National Laboratory, P.O. Box Y, Oak Ridge, Tennessee 37830

TITLE OF APPLICATION (Same as Item 1, page 1)

Carcinogenesis of Xenotransplanted Human Epithelia

Name, Title and Department of all professional personnel engaged on project, beginning with Principal Investigator/Program Director

A. J. P. Klein-Szanto	Medical Staff Scientist	Biology Division, Cancer and Toxicology Section
M. Terzaghi	Staff Scientist	Biology Division, Cancer and Toxicology Section

ABSTRACT OF RESEARCH PLAN: Concisely describe the application's specific aims, methodology and long-term objectives, making reference to the scientific disciplines involved and the health-relatedness of the project. The abstract should be self-contained so that it can serve as a succinct and accurate description of the application when separated from it. DO NOT EXCEED THE SPACE PROVIDED.

The overall objective of the proposed research is to develop an in vivo experimental model for toxicological and carcinogenesis studies using xenotransplanted-reconstituted human epithelia. Epithelial cells obtained from immediate autopsies of full term fetuses will be isolated and inoculated into de-epithelialized rat tracheas, which will be subsequently sealed, and transplanted subcutaneously into athymic nude mice. The main part of this project comprises the study of normal and carcinogen-treated xenotransplanted-reconstituted human respiratory epithelium. Tumor development as well as other late and early changes induced by 7,12-dimethylbenz(a)anthracene will be studied sequentially during 18 months after initiation of exposure. A series of putative tumor markers of great clinical interest will be studied at different timepoints using smears of tracheal secretions and tissue sections. The following markers will be monitored by histoenzymological and histoimmunochemical techniques: gamma-glutamyl transferase, carcinoembryonic antigen, ectopic hormones, loss of blood group antigens, and hematoporphyrin binding. In addition, the in vitro evaluation of altered growth potential of in vivo carcinogen exposed cells will be carried out. Sequential morphological changes will also be evaluated by light microscopy, histometry, and transmission electron microscopy. The effects of nickel subsulfide and arsenic trioxide both poorly soluble particulate materials implicated in human carcinogenesis, will be similarly studied. In addition, human epithelial cells from organs other than lung (such as esophagus, colon, bladder, skin, and cervix uteri) will be isolated and transplanted into devitalized organs to study the feasibility of using these cells for future toxicological and carcinogenesis studies.

LABORATORY ANIMALS INVOLVED. Identify by common names. If none, state "none"

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

SECTION 1.	PAGE NUMBERS
Face Page, Abstract, Table of Contents	1-3
Detailed Budget for First 12 Month Budget Period	4
Budget Estimates for All Years of Support	5
Biographical Sketch-Principal Investigator/Program Director (*Not to exceed two pages*)	7-8
Other Biographical Sketches (*Not to exceed two pages for each*)	9-10
Other Support	11
Resources and Environment	12

SECTION 2.

Introduction (*Excess pages; revised and supplemental applications*)	
Research Plan	
A. Specific Aims (*Not to exceed one page*)	14
B. Significance (*Not to exceed three pages*)	14-16
C. Progress Report/Preliminary Studies (*Not to exceed eight pages*)	16-18
D. Methods	19-25
E. Human Subjects, Derived Materials or Data	13
F. Laboratory Animals	36
G. Consultants	
H. Consortium Arrangements or Formalized Collaborative Agreements	
I. Literature Cited	26-31
Checklist	37

SECTION 3. Appendix (*Six sets*) (*No page numbering necessary for Appendix*)

Number of publications: 0 Number of manuscripts: 0

Other items (list):

Application Receipt Record, form PHS 3830
Form HHS 596 if Item 4, page 1, is checked

PRINCIPAL ESTIGATOR/PROGRAM DIRECTOR Klein anto, Andres J. P.

DETAILED BUDGET FOR FIRST 12 MONTH BUDGET PERIOD DIRECT COSTS ONLY

FROM 4-1-82 THROUGH 3-31-83

DOLLAR AMOUNT REQUESTED (Omit cents)

PERSONNEL (Applicant organization only) (See instructions)		TIME EFFORT				
NAME	TITLE OF POSITION	%	Hours per Week	SALARY	FRINGE BENEFITS	TOTALS
A.J.P. Klein-Szanto	Principal Investigator	35	14	12,950	3,630	16,580
M. Terzaghi	Co-Investigator	10	4	3,700	1,040	4,740
D. Martin	Technician	30	12	6,900	1,930	8,830
To be named	Postdoctoral Fellow	100	40	15,000	1,500	16,500
	SUBTOTALS →			38,500	8,100	46,650

CONSULTANT COSTS (See instructions)

EQUIPMENT (Itemize)

SUPPLIES (Itemize by category)

Animals and Animal Care = 29,084
EM and Histology Supplies= 2,000
Chemicals (reagents, carcinogens, tissue culture media) = 3,000
ARG + Photographic material = 1,500
Histology Service[d] = 3,043

38,627

TRAVEL	DOMESTIC AACR meeting - Principal investigator	700
	FOREIGN	
PATIENT CARE COSTS	INPATIENT	
	OUTPATIENT	

ALTERATIONS AND RENOVATIONS (Itemize by category)

CONTRACTUAL OR THIRD PARTY COSTS (See instructions)

OTHER EXPENSES (Itemize by category)

Publication costs = 600
Utilities (300 sq. ft.) = 4,000
Maintenance and Service of Equipment = 2,000
Division administration = 7,060

13,660

TOTAL DIRECT COSTS (Also enter on page 1, Item 8) → $ 99,637

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT REQUESTED
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		1st BUDGET PERIOD (from page 4)	ADDITIONAL YEARS SUPPORT REQUESTED			
			2nd	3rd	4th	5th
PERSONNEL (Salary and fringe benefits.) (Applicant organization only)		46,650	50,380	54,410		
CONSULTANT COSTS		-				
EQUIPMENT		-				
SUPPLIES		38,627	41,721	44,757		
TRAVEL	DOMESTIC	700	760	820		
	FOREIGN	-				
PATIENT CARE COSTS	INPATIENT	-				
	OUTPATIENT	-				
ALTERATIONS AND RENOVATIONS						
CONTRACTUAL OR THIRD PARTY COSTS						
OTHER EXPENSES		13,660	14,750	15,930		
TOTAL DIRECT COSTS		99,637	107,611	115,917		

TOTAL FOR ENTIRE PROPOSED PROJECT PERIOD (Also enter on page 1, item 7) → $ 323,165

JUSTIFICATION (Use continuation pages if necessary): Briefly describe the specific functions of the personnel and consultants. For all years, justify any costs for which the need may not be obvious, such as equipment, foreign travel, alterations and renovations, and contractual or third party costs. For future years, justify any significant increases in any category. In addition, for COMPETING CONTINUATION applications, justify any significant increases over current level of support. If a recurring annual increase in personnel costs is anticipated, give percentage.

ORNL uses the Uniform Cost Accounting System recommended by the Federal Cost Accounting Standards Board. This system makes use of Cost Collect Centers in which salaries of scientific and technical personnel performing similar tasks are averaged; associated costs (fringe benefits and Biology Division Administration) are calculated as percentages (28% and 30%, respectively) of that average cost.

Indirect costs have been established at a rate of 35% through an agreement negotiated between the NIH and the DOE. Because this percentage covers only part of costs that are often included by other institutions as indirect charges and because the DOE requires recovery of full costs, certain charges (utilities and Division Administration) are listed as Direct Costs on this form (shown under "Other Expenses").

The three-year budget is calculated on the basis of an annual increase of 8% in expenses.

Animals - The animals are bred and housed in a specific-pathogen-free facility, a requirement for precision research in respiratory carcinogenesis. Animal costs are calculated on a cage basis no matter how many animals per cage; the standard number in experimental cages is 5 rats/cage or 10 mice/cage. The cost unit in the Biology Division is MCE, i.e. "mouse cage equivalent"; 1 rat cage = 2 MCE. The SPF animal facilities are an integral part of the Respiratory Carcinogenesis Unit; therefore, cost for animals used

Justification (continued)

for experiments must include the cost of maintaining the animal line, breeding cages, and animals held in stock until the proper age (8-12 weeks old). The stock-holding number is determined by the anticipated needs of the investigators. It is important to note therefore, that costs must represent some steady-state level with numbers of animals being used constantly and equivalent numbers entering from the breeding colony. The animal costs incurred also include those used fairly rapidly for making tracheal implants and finally the experimental cages that are long-term holdings. A proper breakdown of charges determined from long-term management of these facilities is to charge an additional 70% of the cost of the experimental animals and those used for breeding and stock rats and to charge 200% for breeding and stock nude mice. The nude mice are more costly since the litters are a mixture of "nudes" and "hairys."

The projected annual cost per average MCE for March 1981 is $175.20. This cost is determined from utilities, supplies, disease testing, manpower expenses (animal management, veterinarian, caretakers). It should be noted that fringe benefits and Biology Division overhead are included in these costs. The average estimated needs and cost of the experiments per year are:

SPF Rats

	Rats	MCE
Use	200	80
Breeding		56
	Total Rats	136 x 175.20 = 23,828

Nude Mice

	Mice	MCE
Use	100	10
Breeding	100	20
	Total Mice	30 x 175.20 = 5,256

Histology Service: Most of our material will be prepared as continuous sections of tracheal explants to identify lesions. Slides are charged on cost per "slide equivalent" basis. We anticipate an average of 1.2 slide equivalents/slide at a cost of $6.34 per slide equivalent, 400 slides=$3,043.

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Andres J. P. Klein-Szanto	Medical Staff Scientist	[redacted]

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
[redacted]			

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications, especially those most pertinent to this application. Do not exceed 2 pages.

1966-1968: Instructor of Pathology, Department of Pathology, University of Buenos Aires, School of Medicine
1968-1974: Chief Instructor, Department of Pathology, University of Buenos Aires, Schools of Medicine and Dentistry
1970-1974: Staff Scientist, Pathology Section AEC, Buenos Aires
1974-1976: Oberassistant, Department of Structural Biology, University of Zurich
1976-1978: Head, Pathology Section, Argentine AEC, Buenos Aires
1978-present: Medical Staff Scientist, Oak Ridge National Laboratory, Biology Division

Selected Publications (from a total of 63 articles in refereed journals and books):

Unidirectional scanning for the microspectrophotometric investigation of enzyme reactions in squamous epithelium. R. L. Cabrini, A. J. P. Klein-Szanto, and M. E. Itoiz, Acta Histochem. 36:399-403, 1970.

Microspectrophotometric study of oxidative enzymes in irradiated epidermis. A. J. P. Klein-Szanto and R. L. Cabrini. Internatl. J. Radiat. Biol. 18(3):235-241, 1970.

Microspectrophotometric study of histoenzymic reactions in rat epidermis subjected to 0.5 Mrads of deuteron radiation. R. L. Cabrini, M. E. Itoiz, J. Mayo, E. E. Smolko, and A. J. P. Klein-Szanto. Internatl. J. Radiat. Biol. 18(5):414-421, 1970.

An oncogenic virus carried by hamster kidney cells. J. Mayo, J. H. Lombardo, A. J. P. Klein-Szanto, C. Conti, and J. L. Moriera. Cancer Res. 33:2273-2277, 1973.

Ultramorphological features of an experimental hamster sarcoma. A. J. P. Klein-Szanto, C. Conti, and J. Mayo. Z. Krebsforsch. 80:277-284, 1973.

Ultrastructure of irradiated nuclei: A. J. P. Klein-Szanto, B. M. de Rey, C. Conti, and R. L. Cabrini. Strahlentherap. 147:263-270, 1974.

Enzyme variations produced in epidermis by soft X-ray collimated beams. A. J. P. Klein-Szanto, R. L. Cabrini, M. E. Itoiz, and H. E. Volco. Int. J. Radiat. Biol. 26:285-288, 1974.

Microspectrophotometric study of acid phosphatase activity in irradiated squamous epithelium. M. E. Itoiz, C. Frasch, H. E. Volco, and A. J. P. Klein-Szanto. Strahlentherap. 147:643-648, 1974.

Metaplastic conversion of the differentiation pattern in oral epithelium affected by leukoplakia simplex. A stereologic study. A. J. P. Klein-Szanto, J. Banoczy, and H. E. Schroeder. Pathol. Eur. 11:189-210, 1976.

Graded alterations of the epithelial differentiation pattern in buccal mucosa affected by lichen planus. A. J. P. Klein-Szanto, L. Andersen, and H. E. Schroeder. Virchows Arch. Cell Pathol. 22:245-261, 1976.
Stereologic baseline data of normal human epidermis. A. J. P. Klein-Szanto. J. Investig. Derm. 68:73-78, 1977.
Architecture and density of connective tissue papillae of the human oral mucosa. A. J. P. Klein-Szanto and H. E. Schroeder. J. Anat. (Lond.) 123:93-109, 1977.
Clear and dark basal epithelial cells in human epidermis. A stereologic study. A. J. P. Klein-Szanto. J. Cutan. Pathol. 4:275-281, 1977.
Ultrastructural stereology of irradiated epidermis. B. M. de Rey, A. J. P. Klein-Szanto, and R. L. Cabrini. Radiation Res. 77:103-116, 1979.
Ultrastructural characteristics of carcinogen induced non-dysplastic changes in tracheal epithelium. A. J. P. Klein-Szanto, D. C. Topping, C. A. Heckman, and P. Nettesheim. Am. J. Pathol. 98:61-82, 1980.
Ultrastructural characteristics of carcinogen-induced dysplastic changes in tracheal epithelium. A. J. P. Klein-Szanto, D. C. Topping, D. A. Heckman, and P. Nettesheim. Am. J. Pathol. 98:83-100, 1980.
Studies on the mechanism of action of antitumor promoting agents: Their specificity in two-stage promotion. T. J. Slaga, A. J. P. Klein-Szanto, S. M. Fischer, C. E. Weeks, K. Nelson, and S. Major. Proc. Natl. Acad. Sci. USA 77:2251-2254, 1980.
Sexual differences in the distribution of epithelial alterations in vitamin A deficient rats. A. J. P. Klein-Szanto, J. N. Clark, and D. H. Martin. Int. J. Vitam. Nutr. Res. 50:61-69, 1980.
Cutaneous manifestations in rats with advanced vitamin-A deficiency. A. Klein-Szanto, D. H. Martin, and A. H. Pine. J. Cutan. Pathol. 7:260-270, 1980.
Studies of neoplastic development in respiratory tract epithelium. P. Nettesheim, A. J. P. Klein-Szanto, A. C. Marchok, V. E. Steele, M. Terzaghi, and D. C. Topping. Archiv. Pathol. 105:1-10, 1981.
Reestablishment of a mucociliary epithelium in tracheal organ cultures exposed to retinyl acetate - A biochemical and morphometric study. J. N. Clark, A. J. P. Klein-Szanto, A. H. Pine, K. B. Stephenson, and A. C. Marchok. Europ. J. Cell Biol. 21:261-268, 1980.
In vivo differentiation of normal and cultured preneoplastic tracheal epithelial cells. The importance of epithelial mesenchymal interactions. M. Terzaghi and A. J. P. Klein-Szanto. J. Natl. Cancer Inst. 65:1039-1048, 1980.
Induction of dark keratinocytes by 12-O-tetradecanoyl-13-phorbol-acetate and mezerein as an indicator of tumor-promoting efficiency. A. J. P. Klein-Szanto, S. K. Major, and T. J. Slaga. Carcinogenesis 1:399-406, 1980.
Quantitative analysis of disturbed cell maturation in dysplastic lesions of the respiratory epithelium. A. J. P. Klein-Szanto, P. Nettesheim, D. C. Topping, and A. Olson. Carcinogenesis 1:1007-1016, 1980.
Distribution and ultrastructural characteristics of dark cells in squamous metaplasias of the respiratory tract epithelium. A. J. P. Klein-Szanto, P. Nettesheim, A. Pine, and D. Martin. Am. J. Pathol. (in press).
Modulation of growth, differentiation and mucous glycoprotein synthesis by retinyl acetate in cloned carcinoma cell lines. A. C. Marchok, J. N. Clark, and A. J. P. Klein-Szanto. J. Natl. Cancer Inst. (in press).
Dark epithelial cells in preneoplastic lesions of the human respiratory tract. A. J. P. Klein-Szanto, P. Nettesheim, and G. Saccomanno (submitted).
Skin tumor promoting activity of benzoyl peroxide, a widely used free radical generating compound. T. J. Slaga, A. J. P. Klein-Szanto, L. Triplett, L. Yotti, and J. E. Trosko. Science (in press).
Experimental models for the study of lung cancer morphogenesis. In: Morphogenesis of Lung Cancer. Eds., Y. Shimosato, M. Melamed, and P. Nettesheim. P. Nettesheim and A. J. P. Klein-Szanto, CRC Press, Boca Raton (in press).
Heterotopic tracheal transplants: Techniques and applications. In: Pulmonary Toxicology. Ed., G. Hook. A. J. P. Klein-Szanto, B. Pal, M. Terzaghi, and A. Marchok, Raven Press, NY, (in press).

1018920

BIOGRAPHICAL SKETCH

Give the following information for key professional personnel listed on page 2, beginning with the Principal Investigator/Program Director. Photocopy this page for each person.

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Terzaghi, Margaret	Staff Scientist	[redacted]

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
[redacted]	[redacted]	[redacted]	[redacted]

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to recent representative publications, especially those most pertinent to this application. Do not exceed 2 pages.

Experience

Undergraduate NSF Fellowship, Institute of Molecular Biology, University of Oregon, 1963. Supervisor, Prof. Franklin Stahl. Project in phage genetics.

Teaching Assistant, Boston University, 1969.

Research Assistant, Peter Bent Brigham Hospital, 1968.

Research Assistant, Cancer and Toxicology Program, Biology Division, Oak Ridge National Laboratory, 1975-present.

Honors

[redacted]

Publications

Terzaghi, M. Master's Thesis, Boston University. Abscopal effects of radiation in hamster cheek pouch tumors. (Data presented at Radiation Research Meeting) 1971.

Terzaghi, M. and J. B. Little. Interactions between radiation and benzo(a)pyrene in an in vitro model for malignant transformation. In: Experimental Lung Cancer. Carcinogenesis and Bioassays. (E. Karbe and J. Park, eds.) Springer-Verlag, 1974.

McGandy, R. B., A. R. Kennedy, M. Terzaghi, and J. B. Little. Experimental respiratory carcinogenesis: Interaction between alpha-radiation and benzo(a)pyrene in the hamster. In: Experimental Lung Cancer. Carcinogenesis and Bioassays. (E. Karbe and J. Park, eds.) Springer-Verlag, 1974.

Terzaghi, M. Doctoral Thesis, Harvard University. Radiation-induced oncogenic transformation in mammalian cells. 1974.

Terzaghi, M. and J. B. Little. Repair of potentially lethal radiation damage in mammalian cells is associated with enhancement of malignant transformation. Nature 253: 548-549, 1975.

Terzaghi, M. and J. B. Little. Characterization of a hamster lung adenocarcinoma and its tissue culture adapted lines. J. Nat. Cancer Inst. 55, 1975.

Terzaghi, M. and J. B. Little. Oncogenic transformation in vitro: Influence of repair processes. In: The Biology of Radiation Carcinogenesis. Raven Press, 1975.

Terzaghi Publications (cont.)

Terzaghi, M. and J. B. Little. Radiation-induced transformation in a C3H mouse embryo derived cell line. Cancer Res. 36: 1367-1374, 1976.

Terzaghi, M. and J. B. Little. Oncogenic transformation in vitro following split-dose X-irradiation. Int. J. Rad. Biol. 29: 583, 1976.

Kennedy, A. R., Desrosiers, A., Terzaghi, M., Little, J. B. Morphometric and histological analysis of the lungs of Syrian Golden hamsters. J. Anatomy, 125: 527-553, 1978.

Nettesheim, P., Marchok, A. C., and Terzaghi, M. In vivo-in vitro studies of epithelial neoplasia. In: Polycyclic Hydrocarbons and Cancer: Chemistry, Molecular Biology and Environment, Vol. 2, pp. 307-329 (P. Ts'o and H. V. Gelboin, eds.), Academic Press, 1978.

Terzaghi, M. In vivo-in vitro studies with respiratory tract epithelium exposed to carcinogen. II. Methods for defining altered behavior of epithelial cell populations. In: In vitro Carcinogenesis. Guide to the Literature, Recent Advances and Laboratory Procedures, (U. Saffiotti, H. Autrup, eds.). NCI Technical Report Series No. 44, 1978.

Terzaghi, M., Nettesheim, P., and Williams, M. L. Repopulation of denuded tracheal grafts with normal, preneoplastic and neoplastic epithelial cell populations. Cancer Res. 38: 4546-4553, 1978.

Terzaghi, M. and Nettesheim, P. The dynamics of neoplastic development in carcinogen exposed tracheal mucosa. Cancer Res. 39: 4003-4010, 1979.

Terzaghi, M. and Klein-Szanto, A. J. P. Differentiation of normal and cultured preneoplastic tracheal epithelial cells in rats. Importance of epithelial mesenchymal interactions. J. Natl. Cancer Inst. 65: 1039-1048, 1980.

Terzaghi, M., Nettesheim, P., Yarita, T., and Williams, M. L. Epithelial focus assay for early detection of carcinogen altered cells in various organs of rats exposed in situ to N-nitrosoheptamethyleneimine. J. Natl. Cancer Inst. (submitted).

Klein-Szanto, A. J. P., Pal, B., Terzaghi, M., and Marchok, A. Heterotopic tracheal transplants: Techniques and applications. In: Pulmonary Toxicology, Ed., G. Hook. Raven Press, NY, (in press).

OTHER SUPPORT

(USE CONTINUATION PAGES IF NECESSARY)

For each of the professionals named on page 2, list, in three separate groups: (1) active support; (2) applications pending review and/or funding; (3) applications planned or being prepared for submission. Include all Federal, non-Federal, and institutional grant and contract support. If none, state "NONE." For each item give the source of support, identifying number, project title, name of principal investigator/program director, time or percent of effort on the project by professional named, annual direct costs, and entire period of support. (If part of a larger project, provide the titles of both the parent grant and the subproject and give the annual direct costs for each.) Briefly describe the contents of each item listed. If any of these overlap, duplicate, or are being replaced or supplemented by the present application, justify and delineate the nature and extent of the scientific and budgetary overlaps or boundaries.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: A. Klein-Szanto

(1) ACTIVE SUPPORT:

A. NIEHS Interagency Agreement (40-662-77). Environmental Carcinogenesis Studies.
PI: A. Klein-Szanto Effort: 80% Entire Period of Support: 6/80 - 6/84.
Direct Annual Cost (6/81-6/82): 132,700.

B. DOE Seed Money No. 3203-0214. In vivo Model for Chemical Carcinogenesis Studies Using Xenotransplanted Normal Human Tissues.
PI: A. Klein-Szanto and M. Terzaghi Effort: 10%
Entire Period of Support: 9/80 - 9/81
Annual Direct Costs: 49,070

C. NCI No. Y01-CP-90211. Dynamics of neoplastic development in the respiratory system.
PI: M. Terzaghi. Effort: 10%
Entire Period of Support: 9/79 - 8/81.
Annual Direct Costs: 269,500.

(2) PENDING SUPPORT:

NIH CA-29556-01. Importance of Dark Cells in Skin Carcinogenesis.
PI: A. Klein-Szanto Effort: 25%
Annual direct costs: 61,250

A reduction in the effort on the NIEHS Interagency agreement is foreseen in order to invest that effort in the pending and the presently submitted grant proposals.

(3) SUBMITTING: None

M. Terzaghi

(1) ACTIVE SUPPORT:

A. NCI No. Y01-CP-90211. Effort: 85% (See above)

B. DOE Seed Money No. 3203-0214. Effort 15% (See above)

(2) APPLICATION PENDING: NIH CA30271-01. Sensitivity of Initiated Cells to Carcinogenic Insults. Effort: 50%. Direct annual costs: 145,375.

(3) Application planned: Neoplasia in human respiratory tumors. In vitro model.

RESOURCES AND ENVIRONMENT

FACILITIES: Mark the facilities to be used and briefly indicate their capacities, pertinent capabilities, relative proximity and extent of availability to the project. Use "other" to describe facilities at other performance sites listed in Item 9, page 1, and at sites for field studies. Using continuation pages if necessary, include a description of the nature of any collaboration with other organizations and provide further information in the RESEARCH PLAN.

[X] Laboratory: A histopathology laboratory (equipped with microtomes, cryotomes, ultramicrotomes, ovens) and a tissue culture laboratory will be used in this project.

[ ] Clinical:

[X] Animal: Included in the Respiratory Carcinogenesis Unit is a modern barrier facility designed and operated to maintain rodents free of pathogens. It contains 12 holding rooms and a service area. The rodent production and holding rooms are operated on a strict barrier basis.

[ ] Computer:

[X] Office: Office space adjacent to the laboratories.

[ ] Other (_____):

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location, and pertinent capabilities of each.

Histopathology: Hitachi HU11B electron microscope, cryotomes, LKB Ultratome III-fluorescence microscope

Tissue culture: 72" open faced hood, 72" sterigard hood, three double door CO_2 incubators, inverted microscopes

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultants, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

Secretarial assistance is paid from indirect costs.

Maintenance and service are available upon request and are paid by means of direct costs.

DEPARTMENT OF HEALTH AND HUMAN SERVICES

PROTECTION OF HUMAN SUBJECTS ASSURANCE/CERTIFICATION/DECLARATION

[X] ORIGINAL [ ] FOLLOWUP [ ] REVISION

[X] GRANT [ ] CONTRACT [ ] FELLOW [ ] OTHER

[X] NEW [ ] RENEWAL [ ] CONTINUATION

APPLICATION IDENTIFICATION NUMBER *(If known)*

STATEMENT OF POLICY: Safeguarding the rights and welfare of subjects at risk in activities supported under grants and contracts from DHHS is primarily the responsibility of the institution which receives or is accountable to DHHS for the funds awarded for the support of the activity. In order to provide for the adequate discharge of this institutional responsibility, it is the policy of DHHS that no activity involving human subjects to be supported by DHHS grants or contracts shall be undertaken unless the Institutional Review Board has reviewed and approved such activity, and the institution has submitted to DHHS a certification of such review and approval, in accordance with the requirements of Public Law 93-348, as implemented by Part 46 of Title 45 of the Code of Federal Regulations, as amended, (45 CFR 46). Administration of the DHHS policy and regulation is the responsibility of the Office for Protection from Research Risks, National Institutes of Health, Bethesda, MD 20205.

1. TITLE OF PROPOSAL OR ACTIVITY

Carcinogenesis of Xenotransplanted Human Epithelia

2. PRINCIPAL INVESTIGATOR/ACTIVITY DIRECTOR/FELLOW

Klein-Szanto, Andres J. P.

3. DECLARATION THAT HUMAN SUBJECTS EITHER WOULD OR WOULD NOT BE INVOLVED

[ ] A. NO INDIVIDUALS WHO MIGHT BE CONSIDERED HUMAN SUBJECTS, INCLUDING THOSE FROM WHOM ORGANS, TISSUES, FLUIDS, OR OTHER MATERIALS WOULD BE DERIVED, OR WHO COULD BE IDENTIFIED BY PERSONAL DATA, WOULD BE INVOLVED IN THE PROPOSED ACTIVITY. (IF NO HUMAN SUBJECTS WOULD BE INVOLVED, CHECK THIS BOX AND PROCEED TO ITEM 7. PROPOSALS DETERMINED BY THE AGENCY TO INVOLVE HUMAN SUBJECTS WILL BE RETURNED.)

[X] B. HUMAN SUBJECTS WOULD BE INVOLVED IN THE PROPOSED ACTIVITY AS EITHER: [X] NONE OF THE FOLLOWING, OR INCLUDING: [ ] MINORS, [ ] FETUSES, [ ] ABORTUSES, [ ] PREGNANT WOMEN, [ ] PRISONERS, [ ] MENTALLY RETARDED, [ ] MENTALLY DISABLED. UNDER SECTION 6. COOPERATING INSTITUTIONS, ON REVERSE OF THIS FORM, GIVE NAME OF INSTITUTION AND NAME AND ADDRESS OF OFFICIAL(S) AUTHORIZING ACCESS TO ANY SUBJECTS IN FACILITIES NOT UNDER DIRECT CONTROL OF THE APPLICANT OR OFFERING INSTITUTION.

[x] We will use autopsy material from the designated pathology service.

4. DECLARATION OF ASSURANCE STATUS/CERTIFICATION OF REVIEW

[ ] A. THIS INSTITUTION HAS NOT PREVIOUSLY FILED AN ASSURANCE AND ASSURANCE IMPLEMENTING PROCEDURES FOR THE PROTECTION OF HUMAN SUBJECTS WITH THE DHHS THAT APPLIES TO THIS APPLICATION OR ACTIVITY. ASSURANCE IS HEREBY GIVEN THAT THIS INSTITUTION WILL COMPLY WITH REQUIREMENTS OF *DHHS Regulation 45 CFR 46*, THAT IT HAS ESTABLISHED AN INSTITUTIONAL REVIEW BOARD FOR THE PROTECTION OF HUMAN SUBJECTS AND, WHEN REQUESTED, WILL SUBMIT TO DHHS DOCUMENTATION AND CERTIFICATION OF SUCH REVIEWS AND PROCEDURES AS MAY BE REQUIRED FOR IMPLEMENTATION OF THIS ASSURANCE FOR THE PROPOSED PROJECT OR ACTIVITY.

[X] B. THIS INSTITUTION HAS AN APPROVED GENERAL ASSURANCE (DHHS ASSURANCE NUMBER G1716) OR AN ACTIVE SPECIAL ASSURANCE FOR THIS ONGOING ACTIVITY, ON FILE WITH DHHS. THE SIGNER CERTIFIES THAT ALL ACTIVITIES IN THIS APPLICATION PROPOSING TO INVOLVE HUMAN SUBJECTS HAVE BEEN REVIEWED AND APPROVED BY THIS INSTITUTION'S INSTITUTIONAL REVIEW BOARD IN A CONVENED MEETING ON THE DATE OF 4-18-80 IN ACCORDANCE WITH THE REQUIREMENTS OF THE *Code of Federal Regulations on Protection of Human Subjects (45 CFR 46)*. THIS CERTIFICATION INCLUDES, WHEN APPLICABLE, REQUIREMENTS FOR CERTIFYING FDA STATUS FOR EACH INVESTIGATIONAL NEW DRUG TO BE USED (SEE REVERSE SIDE OF THIS FORM).

THE INSTITUTIONAL REVIEW BOARD HAS DETERMINED, AND THE INSTITUTIONAL OFFICIAL SIGNING BELOW CONCURS THAT:

EITHER [X] HUMAN SUBJECTS WILL NOT BE AT RISK; OR [ ] HUMAN SUBJECTS WILL BE AT RISK.

5. AND 6. SEE REVERSE SIDE

7. NAME AND ADDRESS OF INSTITUTION

Oak Ridge National Laboratory
P.O. Box Y
Oak Ridge, TN 37830

8. TITLE OF INSTITUTIONAL OFFICIAL	TELEPHONE NUMBER
SIGNATURE OF INSTITUTIONAL OFFICIAL: Charles P. Richmond	DATE: 15 MAY 81

HS-596 (Rev. 5-80)

ENCLOSE THIS FORM WITH THE PROPOSAL OR RETURN IT TO REQUESTING AGENCY.

1078925

(A) Specific Aims

The objective of the proposed study is to further develop an in vivo experimental model using previously unexposed xenotransplanted human epithelial cells, and to employ this system to analyze the early and late effects of chemical carcinogens.

The specific aims of this study are:

(1) To develop and improve an in vivo experimental model using isolated human epithelial cells from the tracheo-bronchial tree. A pilot study has shown that it is feasible to repopulate previously deepithelialized rat tracheas with human epithelial cells isolated from the tracheobronchial tree, when this system is transplanted into the subcutaneous tissue of nude mice. The possibility of eventual time-dependent changes, and long-term survival of the xenotransplanted reconstituted epithelia will be investigated.

(2) To study the early and late effects (including eventual induction of neoplasia) of DMBA on the xenotransplanted human tracheobronchial cells. The progressive changes taking place after carcinogen exposure will be evaluated by light and electron microscopy, morphometry, histochemical evaluation of tumor markers of clinical importance (carcinoembryonic antigen, hormones, enzymes, etc.), and by an in vitro assay of in vivo acquired alterations of growth potential.

(3) Study the early and late effects of nickel and arsenic on the xenotransplanted human respiratory cells. This study, using similar procedures as in specific aim 2, will be attempted in order to compare the effects of DMBA (a well known chemical carcinogen) and those produced by particulate materials which have been implicated in the etiology of human cancer, i.e. arsenic trioxide and nickel subsulfide.

(4) The possibility of developing similar xenotransplanted reconstituted models with human epithelia from organs other than the lung will be attempted. Isolated epithelial cells from the bladder, skin, esophagus, cervix uteri, and colon will be employed; the evolution and changes of these epithelia will be studied as function of time after transplantation.

(B) Significance

An important objective of biomedical research is to further advance our understanding of the mechanisms of human disease in order to implement more effective preventive measures as well as develop improved therapeutic methods. Toward this end, a number of experimental models designed to approximate the intact human subject have been developed. The most widely used and sensible approach to study general mechanisms of action of noxious agents, which are basically the same for most mammals, has been the development of experimental models using laboratory rodents. However, due to metabolic peculiarities of the different mammalian species (and eventually to some other less known factors, i.e. immunological, genetic, etc.) it is often difficult to reliably extrapolate from the experimental animals to humans. This is especially a problem in the fields of chemical carcinogenesis and toxicology where some substances are apparently innocuous or less effective to some species and very toxic or carcinogenic to others (1,2). In order to adequately evaluate the susceptibility of human tissues and to compare it to that of other mammals, a number

of experimental models using human tissues have been developed.

Several investigators have employed organ cultures of human tissues, i.e. lung, colon, pancreas, esophagus to study the effects and metabolism of polycyclic hydrocarbons (3-8). Other studies using human cell cultures have demonstrated malignant transformation after in vitro treatment with chemical carcinogens (9-10). The most recent approach has been the in vivo exposure of xenotransplanted human tissues to xenobiotics. The availability of severely immunosuppressed athymic nude mice, which usually do not reject transplanted normal human cells (11), has made this approach possible. Recently, several investigators have transplanted normal and pathologic human skin into the dorsum of athymic nude mice (12-14). In one of these studies hyperplasias and neoplasias probably originated in human skin grafts were observed after systemic initiation with urethane and topical frequent promotion with 12-O-tetradecanoyl phorbol-13-acetate (TPA) (14).

Several laboratories have shown that human bronchus can be transplanted to nude mice and maintained subcutaneously for many months (15-17). By introducing 1-2 mg of 3 methylcholanthrene into the lumens of bronchial grafts, Shimosato et al. (16) induced squamous metaplasias in many bronchial transplants. Between 2 to 4 months after transplantation some dysplastic (2/25) and neoplastic lesions (2/25) were seen. Similar findings were also described by Okamoto et al. (15). These authors used bar-shaped beeswax pellets containing 3.2 mg 7,12-dimethylbenz(a)anthracene (DMBA), which were inserted into the bronchial graft's lumen. Both dysplastic (3/11) and neoplastic (3/11) lesions, which were absent in the untreated human grafts, appeared between 9 and 32 weeks, after insertion of the DMBA pellet. One of the major complications of these studies, in which adult tissues from surgical specimens were used (mostly from tuberculosis or cancer patients), is that it is impossible to ascertain that the transplanted airway segments were normal. In order to avoid this difficulty we intend to use epithelia derived from newborns or fetuses which have not died from hereditary of respiratory diseases and have in all probability not been exposed previously to any chemical carcinogens.

Another obstacle in most experimental models thus far devised for studying normal human epithelial cell populations which cannot be continuously subcultured, is the difficulty in finding a reliable and homogeneous supply of viable human tissues. This seriously limits the size and nature of experiments which can be realistically carried out. It is our propsed goal to develop an experimental model in which normal human tissues can be continuously generated from a single tissue sample by adapting an "in vivo culture system" previously developed in our laboratory for studying neoplastic development in rat respiratory tissues (18). This will enable a theoretically unlimited supply of human epithelial cells (see D, methods) from the same donors, thus allowing repetitions and variations of in vivo experiments using human cells of the same origin.

The development of these experimental in vivo models using epithelial cells from human organs will provide a reliable and ethically acceptable method to study the basic mechanisms of action of chemicals and other noxious agents, for interspecies comparisons as well as for assay purposes. The first attempts in these directions will be the evaluation of the early and late effects of DMBA, arsenic trioxide and nickel subsulfide. DMBA was selected because of its effects on the rat tracheal epithelium have been extensively studied in our laboratory. A detailed comparison of effects would then be possible. Arsenic trioxide was selected because to date no adequate animal model has been developed to study the effects of arsenic compounds which are known toxic and carcinogenic for humans (19). Nickel occupies an intermediate position, i.e. although numerous epidemiological and pathological studies have implicated nickel compounds in respiratory cancer pathogenesis (20-21), there

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are only a few experimental reports on the weak carcinogenicity of this metal (22-24).

The endpoints to be evaluated in the course of these studies are a) tumor induction, b) early and late morphological changes induced by chemicals, c) appearance and distribution of biological markers of neoplasia, and d) alterations of the *in vitro* growth potential.

Evaluation of tumor incidence is obviously a very important component of this study, but due to the short life span of the nude mice it is not the most reliable endpoint to study in this experimental model. Although tumors are expected to occur after exposure to carcinogens, the final incidence after 12 months of experimentation will not be comparable to similar experiments carried-out in rat or hamster tracheal transplants in which the induction of tumors was studied throughout a period of time equivalent to the entire life span of the donor animals (25-26). Although this rationale also is valid for the other endpoints to be studied, the morphological (27-28), biological, and especially the in vitro detectable markers of neoplastic changes (29) appear relatively early after carcinogen exposure and will be detected during the first months after carcinogen exposure.

The study of these markers will be performed periodically during the entire course of the experiments, and will provide a better understanding of human neoplastic development induced by chemicals. Although there are numerous clinico-pathological and cytological follow-up studies of human lung cancer and its preliminary stages (30-33), the present approach allows a wider and more controlled screening of newly developed markers which although of great practical importance in clinical pathology, to date have not been analyzed in detail during the process of neoplastic development in human respiratory tract epithelium.

(C) Preliminary Studies

(C1) Establishment of xenotransplanted normal human epithelia.

Trachea and bronchi from three human fetuses (6 to 9 months of pregnancy) were used in this pilot study. The tissues were obtained from immediate autopsies and sent to our laboratory. The human epithelial cells were isolated and inoculated into the lumina of deepithelialized rat tracheas (time between death and inoculation into the tracheas was less than 48 hours). The tracheal ends were closed and the organs were transplanted sc. in nude mice of Balb background (see methods). Approximately 10 to 20 denuded rat tracheas were repopulated with cells from a single human donor. Two to three weeks after transplantation, normal mucociliary epithelium covered most of the luminal surface. Some areas of simple columnar, cuboidal or squamous epithelium were also seen (Fig. 1A). Three to four weeks after transplantation the histologic picture was the same. At this timepoint, normal epithelial cells from 10 transplants were again isolated and inoculated into 30 new denuded rat tracheas and transplanted sc. into nude mice. This process permits the establisment of an "*in vivo*" culture system which can be passaged without any apparent change in cellular characteristics for at least 5 consecutive passages. Mucociliary differentiation is maintained throughout these passages, and at least until passage 3, the last passage in which chromosome studies were carried out, normal human karyotypes were found (Fig. 1B).

(C2) Induction of metaplastic-dysplastic changes in xenotransplanted human epithelia.

Human epithelial cells from the respiratory tract of three donors were exposed

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to DMBA. Cells from the in vivo passages 2 to 4 were employed. The chemical carcinogen was delivered using beeswax pellets as a vehicle. Pellets containing two different doses of DMBA were used, i.e. 200 and 500 μg. The epithelial lining of the tracheas was studied 2, 4, and 8 weeks (2 tracheal grafts per timepoint) after initiation of carcinogen exposure. Both doses induced an intense toxic reaction including partial ulceration of the epithelium and an acute inflammatory response. This was observed at 2 and 4 weeks with 500 μg DMBA and to a lesser extent at 4 weeks with 200 μg DMBA. The non-ulcerated epithelium was either hyperplastic or metaplastic, with orthokeratinized epidermoid metaplasia predominating (fig. 2). Eight weeks after insertion of the carcinogen pellet, the inflammatory and toxic reaction had subsided, but not disappeared, and a large percentage of the luminal surface of the grafts was covered with epidermoid metaplasias with mild or moderate atypia (fig. 3). Since the use of beeswax as a vehicle allows a very rapid release of DMBA (33), eliciting an extremely intense toxic reaction, in another pilot experiment we used a matrix composed fo stearyl alcohol-beeswax (ratio 9:1) which markedly retards the release of DMBA (34) (Fig. 4). The use of this matrix in pellets containing 200 μg DMBA markedly reduced the toxic reaction and induced uniform epithelial lesions (hyperplasias and metaplasias) largely devoid of an inflammatory component.

(C3) Detection of morphological and biological markers

Previous studies carried out in our laboratory have detected the presence of intensely stained cells (dark cells) in the carcinogen induced preneoplastic lesions of the rat tracheal epithelium. The number of these cells increased proportionally to the degree of atypia (35). Similar changes have been detected by us in human autopsy material (36). Dark cells similar to those seen in these two previous studies have also been observed in the mild and moderate epidermoid metaplasias induced by DMBA in xenotransplanted human epithelia. Table I shows the comparative percentages of dark cells in rats and humans. This preliminary morphological study of dark cells in the xenotransplanted epithelia indicates that these cells constitute a heterogeneous population of both involutional and active cells. The active dark cells are characterized by few cytoplasmic organelles, abundant free ribosomes, a large nucleus and a prominent nucleolus. The nature of these cells, which have been postulated to be dedifferentiated precursors of cancer cells, both in epidermal and respiratory preneoplastic lesions (35-38), is still an open question. Further study of dark cells in the human xenotransplanted model will help elucidate their nature and role in carcinogenesis, as well as give a broader base for the eventual use of this morphological marker of atypia in human diagnostic pathology. Another area in which this model could yield additional data is exfoliative cytology. In the course of the pilot experiments described in C2 smears of the tracheal graft content were prepared and stained with the Papanicolau technique, thus permitting a good comparison between the histopathological findings and the cytological picture. In the few cases studied thus far, a tight correlation was found between the cytological and histopathological observations. Although this type of study has been previously done in patients (30-33) the present model permits a closer and better controlled correlation and could eventually be used to detect biological markers in smears (see D4). Previous studies on rat tracheal epithelium have shown that the ^{3}H Tdr labeling index (LI) of the normal mucociliary epithelium (approximately 0.004) increases markedly after DMBA exposure (up to approximately 0.30 in the basal layer and 0.10 in the suprabasal layer of the dysplastic metaplastic lesions) (35). In this same study the LI of the suprabasal layers increased gradually from approximately 0.03 in the epidermoid metaplasias without atypia to approximately 0.11 in the carcinoma in situ. The LI from the normal human xenotransplanted reconstituted epithelium described in C1 (0.005) was very similar to that of the normal rat tracheal epithelium and the LI of the epidermoid metaplasias obtained after treating this

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epithelium with 200 μg DMBA was markedly higher and in the same order of magnitude as the rat lesions.

(C4) In vitro detectable alterations of the growth potential

The in vivo exposure of tissues to chemical carcinogens induces the appearance of detectable cells with altered in vitro growth potential. These cells have been retrieved from carcinogen treated brain (39), kidney (40), trachea (41), and salivary glands (42). Under normal conditions, cells for these organs survive in vitro for only a limited number of cell doublings. The cells from carcinogen treated organs acquired an unlimited capacity to grow in culture, and this phenomenon can be detected many months before the first tumors develop and even before preneoplastic alterations are observed. In our laboraotry, the epithelial focus assay (EF) was developed for the purpose of analyzing the process of neoplastic development and progression as it occurs in vivo after DMBA exposure of the rat's tracheal epithelium (29). Unexposed rat tracheal epithelium yield no or only very few cells which grow in vitro. Epithelial cells derived from DMBA treated rat tracheas give rise in vitro to numerous epithelial foci capable of sustained growth (10 to 100 times more than the unexposed epithelia). Cell populations derived from these primary epithelial colonies can be further tested for the progressive loss of the constraint of growth, i.e., possibility of subculturability, anchorage independent growth, and tumorigenicity in vivo (29). The capacity of the epithelial foci to be subcultured (EFs) and the capacity of these subculturable foci to grow in soft agarose (EFs,ag+) appear to reflect the severity of carcinogen-induced changes in tracheal epithelium. The frequency of these foci (EFs and EFs,ag+) increase both with DMBA dose and with increased time after exposure, indicating that these foci expand in size and number for many months after cessation of carcinogen exposure.

Using human originated material as described in C1 and C2 we have obtained preliminary data on the primary epithelial focus production of normal and DMBA treated human epithelium. Normal human respiratory epithelium exhibited very few foci (∿1 per graft) whereas the DMBA treated epithelium (200 and 500 μg) showed 10 to 50 times more foci than the unexposed epithelium. Subculturability and anchorage independent growth are presently being tested.

(C5) Effect of carcinogens on rat tracheal transplants

Most of these studies using carcinogen exposed rat tracheas have been carried out in our laboratory during the last ten years and constitute an important background material as well as a great source of practical experience for the execution of the proposed experiments in grafts containing xenotransplanted-reconstituted human epithelia. The data on the carcinogenicity of DMBA, the dose response, as well as the morphology and cell kinetics of the induced lesions have been previously reported in the literature (27-28,35,43-44). Similarly, the effects of nickel subsulfide have been studied in our laboratory (24). Data from these experiments as well as from other investigators (22-23) showed that despite the epidemiological evidence showing that nickel exposed persons have a markedly elevated risk of developing respiratory tract cancers (21), only a low incidence of carcinomas was obtained in experimental animals. Even more dramatic is the dichotomy between the epidemiological data indicating that arsenic is an important human carcinogen (19) and the few negative experimental data available (45-47). In our laboratory we have exposed 50 rat tracheal transplants to arsenic trioxide, and after more than 15 months of exposure (1 mg in silastic pellets) neither tumors nor preneoplastic lesions (at 6-12 months) were observed although release of most of the arsenic was demonstrated by spectrophotometric analysis of the pellets (Klein-Szanto, unpublished data). Both the silastic and the stearyl alcohol beeswax pellets permit a sustained release of arsenic and nickel respectively for period of more than 8 weeks (Pal and Klein-Szanto, unpublished data).

(D) Methods

(D1) General

In all our experiments six to eight-week-old female athymic nude mice of Balb/c background will be used as recipients. The donor tracheas which will act as containers will be obtained from 12 to 16 week old female Fischer 344 from our SPF breeding colony. All the human tissues used will be obtained from immediate autopsies of full term fetuses (without evidences of genetic and/or respiratory disease) and maintained at 4°C for a maximum period of 48 hrs. until the epithelial cells can be isolated and inoculated into the denuded rat tracheas.

(D2) Specific aim No. 1:

(D2a) *In vivo* "culture technique for the establishment of normal human respiratory epithelium in nude mice.

Two alternative procedures will be tested for the establishment of denuded rat tracheas repopulated with human epithelial cells. One will involve the direct inoculation of suspensions obtained from human material into denuded tracheas, as previously described for repopulation with rat epithelial cells (29). Cells will be removed from the intact tissues by incubation with 1% pronase in physiological saline solution for 15 minutes at 37°C followed by gentle scraping of the mucosal surface with a scraping needle fashioned from a 16 gauge needle. The separated cells will then be collected by rinsing the tracheal lumen with 10 ml Hanks BSS containing 0.5 ml 10% BSA, the resulting cell suspension will then be centrifuged at 1000 rpm for 10 minutes, the supernatant removed, and fresh medium added. The centrifugation procedure will be repeated two more times in order to remove all pronase from the epithelial cells. The cells will be counted in a hemocytometer and 10^4-10^5 cells inoculated into each denuded rat trachea. The deepithelialization of rat tracheas is achieved by multiple sequential freeze thawing of fresh adult rat tracheas (48). In this manner, all viable rat tissue is eliminated, leaving only the devitalized trachea to act as a passive container for the inoculated human cells. The tracheas containing the human cells will be sealed at both ends with vascular clips and transplanted subcutaneously in congenitally athymic (nude) mice of Balb/c background. The transplants will then be left in place as long as is necessary for a normal appearing, well differentiated epithelium to cover the entire luminal surface.

Preliminary studies have shown that in tracheas inoculated with 10^4-10^5 epithelial cells, approximately 2 weeks is required to establish a normal mucociliary epithelium. In order to amplify the original number of tracheas containing xenotransplanted repopulated epithelial cells, the cells from the fully reconstituted epithelium will be again isolated and 10^4-10^5 viable cells inoculated into denuded tracheas (fig. 5). Cells from each original tracheal graft can be inoculated into 3 to 5 new denuded tracheas. This procedure can be performed every 2 to 3 weeks for at least 5 *in vivo* "passages" each of them permitting an amplification of 3 to 5. An alternative pathway (fig. 5) to further expand the population of human cells prior to inoculation of cells into the first series of tracheas, is to establish outgrowth cultures from small (1-2 mm^2) pieces of autopsy-derived tissues. The epithelial cells which grow out from each piece can be collected by trypsinization at 1-2 week intervals. The suspensions of trypsinized human cells obtained in this manner will then be inoculated into denuded tracheas as described above. Following sequential passage *in vivo* of epithelial transplantation lines initially established by each of the above alternative techniques, it will be imperative to perform cytogenetic studies in order to determine the presence of a normal human

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karyotype. A final choice between the two techniques described above for initiation of the human transplantation lines will be based on the results of the cytogenetic studies and on the relative efficiency with which the original cell pool can be expanded by each technique. Once the total tracheal transplant population has been expanded to at least 20 transplants containing cytogenically normal human cells from each original donor, 1/3 will be kept as a reservoir for further expansion of the transplantation line and 2/3 will be used for experimental purposes.

(D2b) Evolution of normal xenotransplanted reconstituted human epithelia from the respiratory tract.

In order to study the evolution and eventual changes taking place in the xenotransplanted epithelia as function of time of each in vivo culture line, 5-8 grafts containing human cells from at least four different donors will be studied every 8 weeks during the lifetime of the animals (12-18 months). The eventual morphologic changes will be evaluated by light and electron microscopy and changes in cell proliferation will be studied by ^{3}H Tdr pulse autoradiograms (see D3b for technique). Prior to sacrifice, all animals will be injected with 2 μCi/g of ^{3}H-thymidine.

(D3) Specific aim No. 2: Early and late effects of DMBA on xenotransplanted reconstituted human respiratory epithelium

(D3a) Protocol.

The effects of this well known polycyclic aromatic hydrocarbon will be studied using pellets permitting a slow release (stearyl alcohol-beeswax, 9:1) of carcinogen. The dose of 200 μg has been tentatively selected, since it is a highly carcinogenic dose in rats (26) and has not elicited an intense early toxic reaction in our model (see preliminary results). A total of 140 tracheal transplants containing xenotransplanted reconstituted epithelium (originated from 4-6 different donors, ∿25 each) will be treated with 200 μg DMBA 3 weeks after seeding of human cells. Except for those animals carrying tracheal grafts which will be used for further in vitro studies, all animals will be injected with 2 μCi/g ^{3}H Tdr one hour prior to sacrifice. Forty tracheal grafts will be maintained in the animals for up to 18 months (survival or tumor incidence study). Sixty grafts will be used for a serial sacrifice study (tentative timepoints: 1-3-6-9-12-15 months). The 12 grafts studied at each timepoint will be processed for routine light and electron microscopy for detection of putative markers of malignant transformation. The remaining 40 grafts will be used for the in vitro EF assay (tentative timepoints 3, 6-9-12 months). In addition to the unexposed controls described in section D2c, a group of 40 tracheal grafts (10 from each donor) will be exposed to stearyl-alcohol-beeswax blanks and studied by histology ARG and the in vitro EF assay at 1-3-6 and 12 months after insertion of the blank pellets. Another group of 40 grafts will be maintained for 18 months to discard the possibility of "spontaneous" or matrix induced tumor production (no exposure group).

(D3b) Morphological studies (light and electron microscopy)

Smears from the luminal contents of all tracheal grafts will be performed in order to study the exfoliative cytological picture and to compare it with the histopathology of the same transplants. For this purpose smears will be fixed with ether-ethanol and stained with the Papanicolaou technique. Other smears will be used for the detection of γ-glutamyl-transpeptidase (GGT), hematoporphyrin derivative, and carcinoembryonic antigen (CEA) (see below). The tracheal transplants (12 per time point) will be cut perpendicularly to the longitudinal axis in approximately 10, one to two mm thick rings. Each alternate ring will be fixed in a

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glutaraldehyde-formaldehyde fixative (49) and subsequently processed for light and electron microscopical observation. The remaining alternate rings will be used both for obtaining frozen sections (for detection of hematoporphyrin derivative blood group antigens, and γ glutamyl transpeptidase), and paraffin embedded blocks (for detection of ectopically produced hormones, CEA and glycogen in the DMBA induced lesions). The rings to be embedded in plastic will be further processed as follows; washing the tissues with 0.1 M sodium cacodylate solution for 30 minutes, the tissues will be post-fixed overnight in 2% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in graded ethanol solutions and embedded in Epon 812. The blocks will be oriented flat at the bottom of reversed BEEM capsules. From these blocks 1 μm thick sections will be prepared and stained with toluidine blue. Additional sections will be used to prepare autoradiograms employing a rapid method of scintillation autoradiography (50) in which the sections are exposed for approximately 14 days and will subsequently be stained with toluidine blue. For the quantitative determinations of labeling indices, only cells with five grains or more will be considered as labeled. The number of labeled and unlabeled nucleated cells will be determined in autoradiograms stained with Toluidine blue. Ultrathin sections will be cut from these blocks with LKB Ultratome III, stained with uranyl acetate and lead citrate, and observed in a Hitachi HU11B electron microscope. In addition to a general morphologic evaluation of the epithelium (which will be followed by a quantitative study, see D3c). Special attention will be focused on the number and characteristics of dark cells in the different epithelial lesions. In addition, glycogen which has been seen especially in human carcinoma in situ of the airways (51) will be investigated in periodic acid-Schiff-stained sections with and without diastase digestion. This will be performed in the same type of sections in which hormones will be investigated, since recently human-chorionic gonadotropin and glycogen have been localized in the same areas of severely atypical epithelia (52).

(D3c) Histometry.

The percentage of luminal surface occupied by normal, atrophic, hyperplastic, metaplastic, dysplastic, or neoplastic epithelium will be evaluated by histometric-planimetric technqiues using 1 μm thick Epon sections stained with toluidine blue. The sections will be evaluated by projecting them with a Zeiss projecting-microscope to the screen of a Ladd graphic digitizer interphased with a Monroe 1860 calculator. This analytical system permits a precise planimetric determination by specifically following with a cursor the contours of the total circumference of the organ's lumina and that of the fraction covered by the different types of epithelium. Means per trachea can be automatically calculated.

(D3d) Histochemical demonstration of phenotypic markers of malignancy.

(D3d1) Gamma glutamyl-transpeptidase: This enzyme, which catalyzes the transfer of gamma glutamyl groups of peptides to other peptides and amino acids has been used successfully to detect putative preneoplastic cell populations in liver (53-55), as well as neoplastic cells in several other organs (56-59). Although this enzyme can be found in several embryonic tissues, it is usually not seen in the adult normal cells (60). Recently, for example, GGT has been found in neoplastic cells of skin squamous carcinoma (56) and in preneoplastic epithelial lesions of the oral mucosa (59) but not in the normal stratified squamous epithelium (56,59). Similarly, GGT has been seen in fetal lung epithelia and in lung tumors but only a slight enzyme activity was noted in the epithelial lining of the adult respiratory tract (60). Although no observations on the presence of GGT in preneoplastic lesions of the respiratory tract have been reported in the literature, previous investigations in liver, skin, and lung suggest that this enzyme might be a useful marker for these putative precursor lesions of lung cancer. In this context, smears and fresh tissue

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sections obtained with a cyrotome will be fixed in acetone at -20°C, air dried and processed according to the histochemical technique of Rutenburg et al. (61) for the detection of GGT (incubation for 10-20 minutes in a medium containing γ-glutamyl-4-methoxy naphthalamide as a substrate, washing in 0.85% NaCl solution, dipping in 0.2 M cupric sulfate solution washed in water and mounting in glycerine-agar).

(D3d2) Carcinoembryonic antigen (CEA): This substance is present in relatively large amounts in tumors of the alimentary tract (62) as well as in some fetal tissues (63). Recently CEA has been detected in other human tumors including those of the lung (64,65) and has been seen in hyperplastic metaplastic and dysplastic lesions of the human respiratory tract (66). As this substance seems to increase with increasing degrees of atypia (66) it probably is an adequate marker of preneoplasia in smears and in tissue sections. This will be investigated in our experimental model using the triple bridge peroxidase-antiperoxidase method of Sternberger (67). Briefly, formaldehyde or Bouin fixed tissues and smears will be used. The tissues will be embedded in paraffin, and the sections after deparaffinization, as well as the smears of the tracheal secretions will be a) treated with hydrogen peroxide solution to destroy any endogeneous peroxidase activity, b) incubation with swine serum to suppress nonspecific binding of immunoglobulins, c) treatment with primary antibody (rabbit anti human CEA), d) after rinsing the specimens are treated with the link antibody (swine anti-rabbit immunoglobulin) which will bind to any primary rabbit antibody remaining in the section. This antibody has two binding sites and one of them remains free to attach to the reagent of the next step, e) after rinsing, the specimens are treated with horseradish peroxidase-rabbit-antihorseradish peroxidase complex, and f) after washing the peroxidase is histochemically demonstrated with a substrate containing H_2O_2 and amino-ethylcarbazole.

(D3d3) Ectopic hormone localization: The ectopic production of hormones by lung tumor cells has been confirmed in numerous reports and has been frequently used as a clinical-laboratory aid for diagnosis (63,68). Although oat cell carcinomas are the tumors which most frequently produce hormones, practically all histological types of human lung tumors, produce to a greater or lesser extent some type of hormonal marker (68). The following hormones have been frequently found to be elevated in patients with epidermoid bronchogenic carcinoma (the most frequent lung tumor): ACTH, parathohormone and human chronic gonadotropin. Small cell carcinomas were associated with elevated levels of ACTH, anti-diuretic hormone, and calcitonin, and adenocarcinomas patients exhibited high levels of ACTH, and human chronic gonadotropin (68). Although most patients with small cell carcinoma present high levels of ectopic hormone production, other types of tumors exhibit a lower incidence, for example high levels of calcitonin were found only in 30% of squamous carcinoma patients (69). These clinical data plus the recent observations on neuroendocrine differentiation patterns in several human and experimental lung tumors (70-71) suggests the possibility of finding particular hormone secretion patterns in some preneoplastic lesions. The localization of hormones in tissue sections can be carried out using the triple bridge peroxidase antiperoxidase technique of Sternberger et al. (67) as described in the preceding subsection (D3d2). In the present investigation primary antibodies against ACTH, calcitonin, chronic gonadotropin and parathormone will be used in tissue sections of preneoplastic lesions and tumors. If these are positive, similar histoimmunological detection techniques will be applied to smears of tracheal secretions.

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(D3d4) Loss of blood group antigens: Previous investigations have shown that normal human epithelial tissues express blood group isoantigens. Dabelsteen et al. (72) and George et al. (73) reported the presence of these blood group related substances in the parabasal layer of normal oral epithelium. Furthermore, these authors described the loss of blood group related antigens in preneoplastic lesions and tumors of the oral mucosa. In another study, Katoh et al. (74) demonstrated the presence of these antigens in surface and glandular epithelia of the normal human bronchus, and suggested that these might be lost during malignant transformation. To prove this hypothesis we will attempt to demonstrate the presence or absence of blood group related antigens in preneoplastic lesions induced by DMBA in xenotransplanted-repopulated human respiratory epithelium. Blood group reactivity will be demonstrated histochemically on fresh frozen sections using human anti-A and anti-B sera, following this treatment the section will be washed and treated with peroxidase- or fluorescein-labeled anti-human IgG (73-74). Blood group 0 antigen will be tested with fluorescein labeled lectin from Lotus tetragonobulus (direct technique) (73).

(D3d5) Hematoporphyrin derivative: Hematoporphyrin derivative (HPD) prepared from crude hematoporphyrin by glacial acetic acid and sulfuric acid treatment and subsequent neutralization with 3% sodium acetate (75), is a fluorescent compound which is preferentially taken-up by malignant epithelial cells (75-76). The possibility of detecting small lung tumors and carcinoma in situ has been demonstrated using a fiberoptic bronchoscope by Profio and Doiron (77). Similar studies using an experimental model of respiratory carcinogenesis in the airways of dogs have shown the usefulness of this approach for the detection of small tumors and its potential therapeutic value when used in combination with a krypton laser beam or an argon dye laser beam (78). In view of these findings, we propose to study in our model the localization of HPD in the early and late lesions induced by DMBA in the human xenotransplanted repopulated respiratory epithelium. According to previous publications a dose of 2 to 10 mg/kg of HPD was used in order to discriminate between malignant and normal tissues (77). A preliminary study will be performed in our experimental model to determine which dose (in the range of 1-15 mg/kg of HPD) is the best to adequately disciminate between normal and malignant cells. After intravenous inoculation of HPD, the tissue fluorescence is good for at least several hours (77). The fluorescence of this compound will be observed directly in fresh frozen sections with a fluorescence microscope. As in all other techniques to demonstrate markers of malignancy contiguous sections will be stained to assess the type of epithelium in which the marker will eventually be localized.

(D3e) In vitro evaluation of in vivo induced changes of growth potential (Epithelial focus or EF assay).

In vitro techniques offer new possibilities for the study of sequential changes presumed to occur during the in vivo development of neoplasia. It has been shown that shortly after carcinogen exposure of rat tracheal transplants, epithelial cells which have a markedly increased growth capacity can be isolated from the tracheal mucosa (41). A series of techniques collectively referred to as the EF assay has been devised to quantitate in vitro the emergence of carcinogen-altered and neoplastic cell populations in vivo in tracheas exposed to carcinogen. This technique originally devised for the study of carcinogen altered rat cell populations (29) has been easily adapted to be used with human xenotransplanted cells. In essence, the protocol is the same and can be summarized briefly as follows: At various time intervals after initiation of exposure, beginning prior to the development of frank neoplastic or even before the appearance of preneoplastic lesions,

tracheal grafts will be removed from host animals. Luminal epithelial cells will be dispersed enzymatically and seeded into tissue culture dishes in order to establish primary cultures. Four weeks after the establishment of the cultures the number of EF (proliferating epithelial cell foci) per dish will be scored. Each EF containing approximately 5 x 10^4 cells will be removed from the primary dish enzymatically and seeded into a separate secondary culture dish. The fraction of EF which can be subcultured two or more times will be noted. All subculturable EF (EFs) will be tested for the capacity to grow in soft agarose. Using this system with rat cells, the capacity to grow in soft agarose has been found to be well correlated with the capacity of a cell population to yield tumors when inoculated i.m. into immunosuppressed rats (29).

The EF-assay has made it possible to monitor the development in vivo of cell compartments endowed with different proliferative and neoplastic potentials in vitro. Epithelia from normal non-carcinogen exposed tracheas yield few EF in primary culture. Epithelia from carcinogen-exposed tracheas yield 10-100 times more EF than control cultures both using rat or human cells (see C4). The capacity of EF to be subcultured (EFs) and the capacity of EFs to grow in soft agarose (EFs,ag+) appear to reflect the severity of carcinogen-induced changes in rat tracheal epithelium. The frequency of EFs and EFs,ag+ populations increase both with carcinogen dose and increased time after exposure (29). Applying this model to the study of the effects of carcinogens on human tissues, we expect to find similar changes, i.e. progressive changes which will be more and more severe with time after in vivo exposure to carcinogen.

(D4) Specific aim No. 3: Effect of particulate material on xenotransplanted repopulated human respiratory epithelium

The effects of nickel and arsenic on human xenotransplanted epithelium will be studied by inserting into the lumina of the tracheal grafts pellets containing Ar_2O_3 (in silastic pellets) and Ni_3S_2 (in stearyl alcohol-beeswax, ratio 9:1, pellets) respectively. These pellets will be inserted in the grafts 3 weeks after seeding the human cells into the lumina.

Although in principle 1 mg Ni_3S_2 is a carcinogenic dose and produces large numbers of sarcomas in rats (24) a pilot experiment using 10 to 20 tracheas will be carried out in order to determine at the histological level the toxicity of 0.1, 1 and 10 mg of Ni_3S_2. The same type of dose response study will be carried out with Ar_2O_3. Once the nontoxic dose is determined, the following protocol using cells from at least 4 different donors will be carried out:

Arsenic Trioxide in Silastic Pellets:

40 tracheal transplants → Survival study (18 month duration)
40 tracheal transplants → Serial sacrifice (1, 3, 6, 12 month timepoints)
30 tracheal transplants → EF assay (1-3-6 months)

Nickel subsulfide in Stearyl Alcohol Pellets:

The same number of tracheal transplants (one hundred ten) will be used as for the study with Ni_3S_2 and the same type of studies will be carried out.

In both experiments a control group of 30 to 40 tracheal grafts carrying epithelial cells from 4-6 different donors will be exposed to the respective blank pellets for 18 months in order to study the unlikely possibility of a deleterous effect of either silastic or stearyl alcohol (this last control is common with

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the one described in D3a). A small number of tracheas (fifteen - thirty) will be affected to the study of effects of silastic blanks during the course of the experiment. In this context, histology and EF assays will be performed after 3, 6, and 12 months exposure to the blank pellets.

(D5) Specific aim No. 4: Development of xenotransplanted repopulated epithelia from other organs

In order to establish the possibility of using this system for the *in vivo* "culture" of human epithelial cells of other organs we will attempt to isolate cells from the esophagus, epidermis, bladder, colon, and cervix uteri from immediate autopsies of the same donors (as in D2a). Although the ideal number of cells to be inoculated per trachea, and the possibility of establishing initial primary explant cultures to expand the epithelial population will have to be explored independently for each organ, the basic procedures of cell isolation and seeding in denuded tracheas will be similar to that described for the respiratory tract tissues (see D2a).

In a recent publication, we have described the possibility of seeding rat respiratory epithelial cells in denuded trachea, esophagus, intestine, bladder, or ad hoc manufactured dacron containers (48). The possibility of using these other rat organs or even the dacron containers for seeding and reconstituting epithelia from other human tissues will be explored. Since respiratory epithelial cells grow better in denuded tracheas than in other tubular organs, it is possible that epithelial cells from other human organs will grow more efficiently in homologous denuded rat organs than in tracheas.

TABLE I

Percentage of Dark Basal Epithelial Cells

	Human		Rat
Lesion	"Spontaneous" (autopsy material)	DMBA treated (xenotransplants)	(DMBA treated)
Regular metaplasia	9 ± 2	6 ± 2	18 ± 4
Mild dysplasia	13 ± 3	--	--
Moderate dysplasia	26 ± 5	21 ± 4	43 ± 12
Severe dysplasia	27 ± 4	28 ± 5	57 ± 6
Carcinoma in situ	34 ± 11	--	71 ± 6

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[redacted]

LEGENDS TO FIGURES

Fig. 1A: Mucociliary epithelium from the trachea of a human donor covering the tracheal lumen of a deepithelialized rat trachea transplanted subcutaneously for 3 weeks after inoculation of human epithelial cells into the implant's lumen. Epon Toluidine blue x 400.

Fig. 1B: Normal human karyotype from the same specimen as shown in figure 1A. Small explants of the implanted trachea were harvested 3 weeks after transplantation. The explants were cultured for 14 days and then incubated in colchicine (1 μg/ml medium) for 15 hours. Giemsa x 1200.

Fig. 2: Epidermoid metaplasia produced in human xenotransplanted-reconstituted epithelium, after 4 weeks exposure with a pellet containing 500 μg DMBA. Note hyalinization and fibrosis of the subepithelial tissues. Paraffin HE x 400.

Fig. 3: Epidermoid metaplasia with moderate atypia after 8 weeks exposure with a pellet containing 500 μg DMBA. Note lack of stratum corneum and hyalinization of the lamina propia. Paraffin HE x 400.

Fig. 4: *In vivo* cumulative release of DMBA from beeswax and stearyl alcohol-beeswax pellets. Each point represents the mean (± standard deviation) of 4 rat tracheal transplants.

Fig. 5: Diagrammatic representation of the method of xenotransplantation and reconstitution of human epithelial cells in denuded rat tracheas.

[redacted]

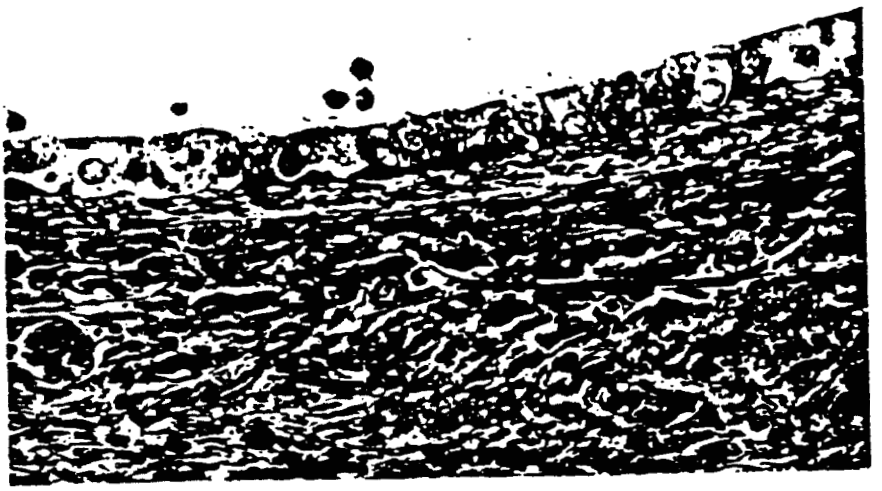

Fig. 1A

Fig. 1B

Fig. 2

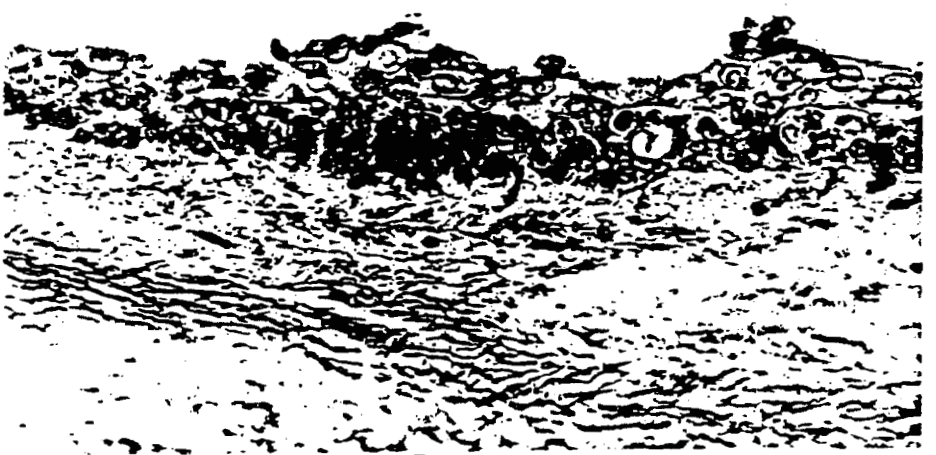

Fig. 3

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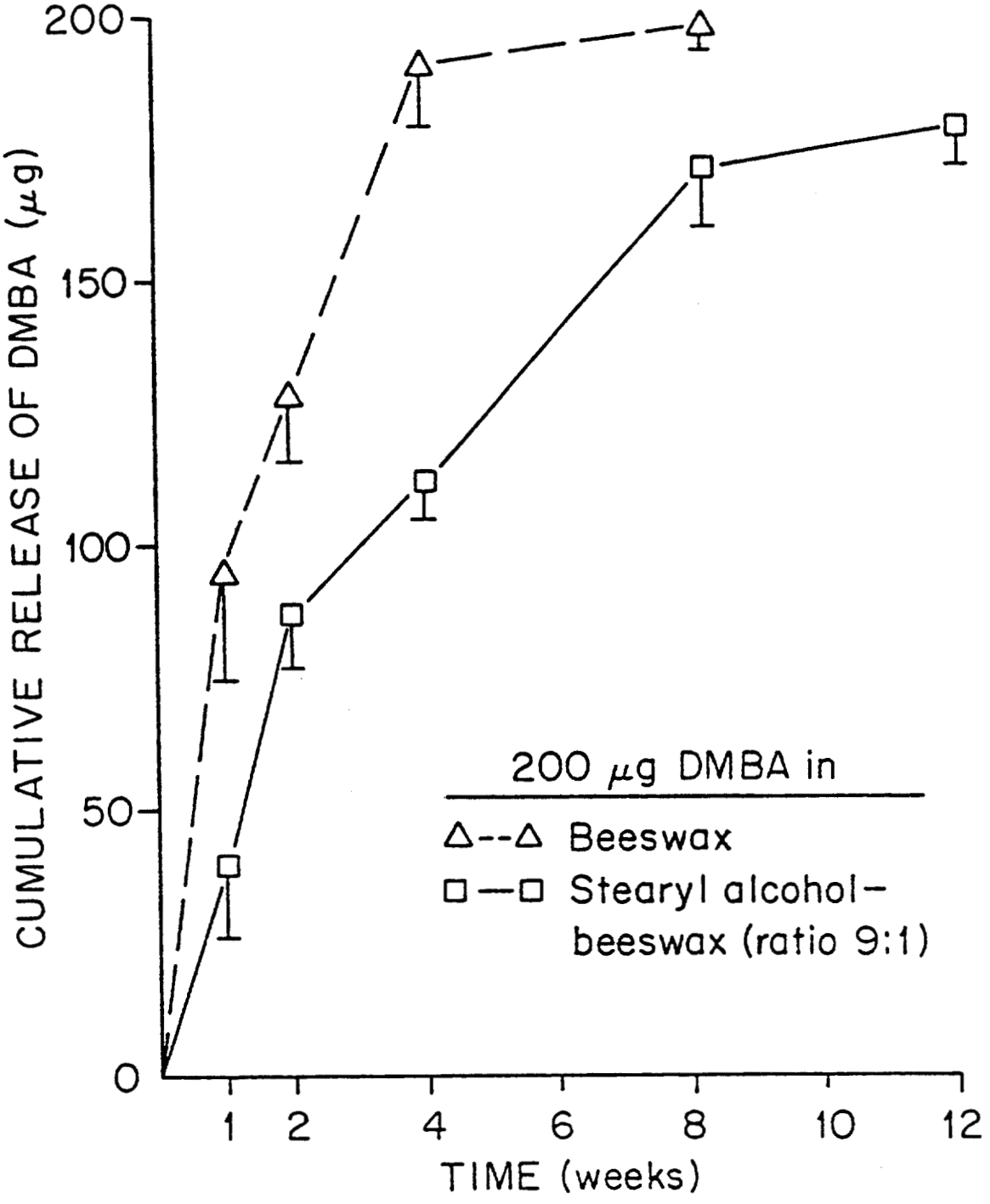
200
150
100
50
0
CUMULATIVE RELEASE OF DMBA (μg)
1
2
4
6
8
10
12
TIME (weeks)
200 μg DMBA in
Beeswax
Stearyl alcohol-
beeswax (ratio 9:1)

Fig. 4

CARCINOGENESIS STUDIES USING HUMAN CELLS

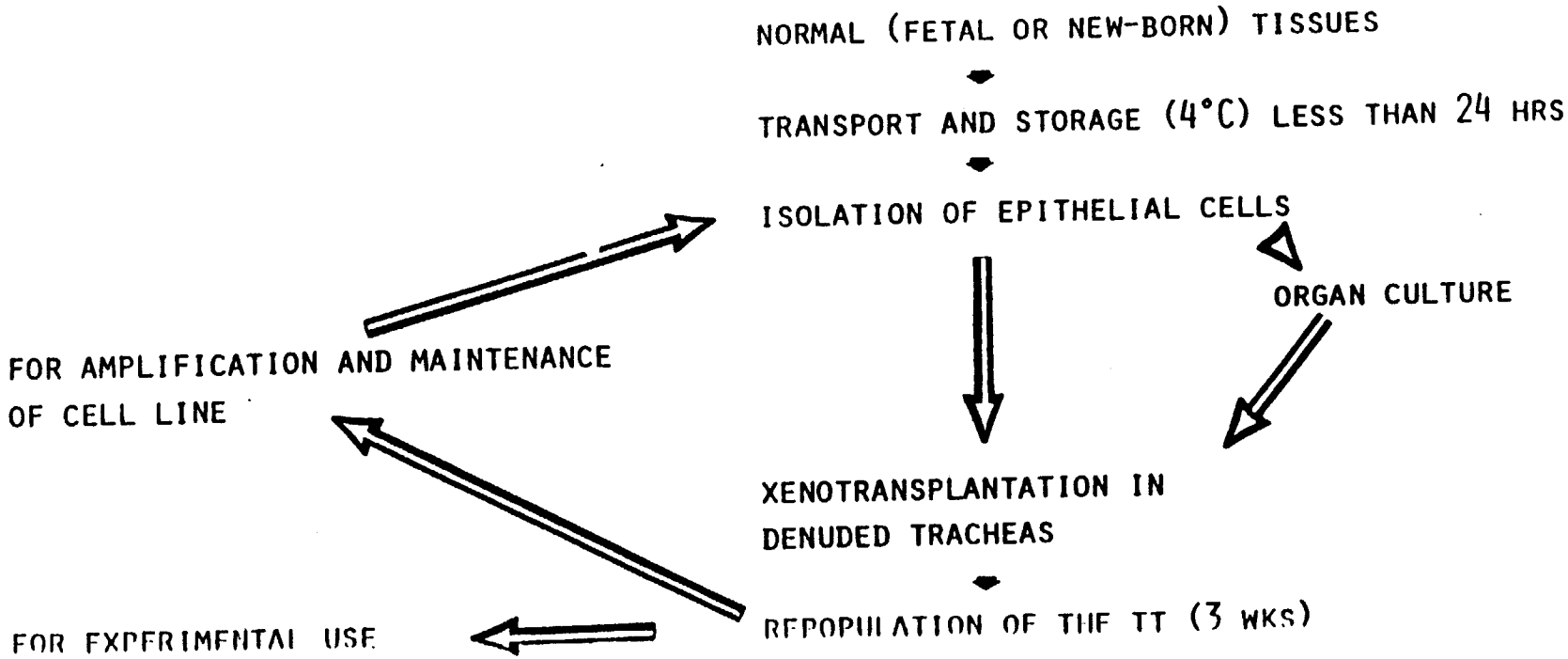

Fig. 5

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INTRA LABORATORY CORRESPONDENCE

OAK RIDGE NATIONAL LABORATORY

August 5, 1980

To: Animal Users

From: G. A. Bingham, D.V.M. GAB

Subject: NIH Animal Use Policy

This memorandum is to assure you that the Biology Division is in compliance with the U.S. Public Health Service policy concerning the care and use of laboratory animals as prescribed in their instruction sheet for grant applications (PHS 398 Rev. 10/79). We have received their acceptance of our letter of assurance of compliance with their animal policy. In keeping with this assurance we have an active committee chaired by Dr. R. A. Popp that reviews our animal policies and operations and our animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

GAB:cr

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CHECKLIST

This is the required last page of the application.

Check the appropriate boxes and provide the information requested.

TYPE OF APPLICATION:

[XX] NEW application *(This application is being submitted to the PHS for the first time.)*

[ ] COMPETING CONTINUATION of grant number: _____.
(This application is to extend a grant beyond its original project period.)

[ ] SUPPLEMENT to grant number: _____.
(This application is for additional funds during a funded project period.)

[ ] REVISION of application number: _____.
(This application replaces a prior version of a new, competing continuation or supplemental application.)

[ ] Change of Principal Investigator/Program Director.
Name of former Principal Investigator/Program Director: _____.

ASSURANCES IN CONNECTION WITH:

Civil Rights	Handicapped Individuals	Sex Discrimination	Human Subjects General Assurance *(If applicable)*	Laboratory Animals *(If applicable)*
[X] Filed	[X] Filed	[X] Filed	[X] Filed	[X] Filed
[ ] Not filed	[ ] Not filed	[ ] Not filed	[ ] Not filed	[ ] Not filed

INDIRECT COSTS:

Indicate the applicant organization's most recent indirect cost rate established with the appropriate DHHS Regional Office. If the applicant organization is in the process of initially developing or renegotiating a rate, or has established a rate with another Federal agency, it should, immediately upon notification that an award will be made, develop a tentative indirect cost rate proposal based on its most recently completed fiscal year in accordance with the principles set forth in the pertinent *DHHS Guide for Establishing Indirect Cost Rates*, and submit it to the appropriate DHHS Regional Office. Indirect costs will not be paid on foreign grants, construction grants, and grants to individuals, and usually not on grants in support of conferences.

[X] DHHS Agreement Dated: February 14, 1979
_____ % Salary and Wages or 35 % Total Direct Costs.

Is this an off-site or other special rate, or is more than one rate involved? [ ] YES [X] NO
Explanation: _____

[ ] DHHS Agreement being negotiated with _____ Regional Office.

[ ] No DHHS Agreement, but rate established with _____ Date _____

[ ] No Indirect Costs Requested.