14-DAY INHALATION STUDY IN RATS, USING SIDESTREAM SMOKE.

EXPERIMENTAL PROTOCOL

(Identifier: TOX-34).

OBJECTIVE

To evaluate potential effects in animals exposed nose-only to sidestream smoke (SSS), used as a surrogate for Environmental Tobacco Smoke (ETS).

OVERVIEW

This inhalation study will examine the responses produced in rats by exposure to SSS, when exposed nose-only within 2.3 cubic meter (m^3) whole-body exposure chambers.

The study will use 5 groups, each containing 48 Sprague-Dawley rats per sex. Three target concentrations of SSS will be used, and these concentrations (expressed in milligrams [mg] of wet total particulate matter per m^3) are 0.1, 1 and 10. There will be a sham-exposure group and a chamber control group, the latter containing sentinel animals to be used for monitoring animal health. Animals will be exposed six hours per day, for 14 consecutive days. End-points will include histopathology, macrophage studies on broncho-alveolar lavage fluid (if technically feasible), CO-oximetry, plasma nicotine and cotinine, clinical pathology, organ and body weights, and adducted DNA in lung tissue. Sub-groups of animals will be kept for a further 14 days without treatment, as a reversibility study.

The total number of animals to be used in the experiment is 480. Of the 48 per sex per group, 18 will be used for dosimetry (and if deemed necessary, lavage and adducted DNA) during the exposure phase, 20 for adducted DNA and histopathology after the 90-day exposure, and 10 for the reversibility study.

INTRODUCTION

It has been shown that many carcinogens or metabolites covalently bind to DNA *in vivo* to form DNA adducts. DNA adducts of a variety of chemical carcinogens have been identified, and their ability to alter the genetic information has been observed in various test systems. Hence it is widely accepted that any chemical which forms covalent bonds with DNA of somatic cells *in vivo* or *in vitro* should be viewed as a potential mutagen or carcinogen.

Studies of covalent carcinogen-DNA interactions have typically involved isotopically labeled compounds to detect and quantitate the formation of DNA adducts (Baird, 1979). In order to extend laboratory studies of genotoxic damage to chemicals that are not readily available in the radioactive form, the biochemical method of ^{32}P-postlabeling of chemical-DNA adducts was developed (Randerath et al., 1981, Gupta et al., 1982). In this approach, radioactive ^{32}P is incorporated into DNA constituents after exposure of the DNA to a non-radioactive binding chemical. The evidence for the presence of chemically altered nucleotides is provided by the appearance of extra spots on thin layer chromatography (TLC) of digests of the chemically modified DNA, as detected by autoradiography. This method is applicable to adducts of known or unknown origin and structure.

Numerous statements have been made in the scientific literature on the biological activity of ETS, and in particular, the potential carcinogenicity of ETS. This study was designed to obtain information on the effects of the exposure of animals to SSS, at concentrations equivalent or

higher than those reported for ETS in the field. The chosen end-points are the DNA adduct technique described above, cytogenetic end-points from broncho-alveolar lavage, and respiratory tract histopathology.

FACILITIES & ADMINISTRATION

Facilities

R.J. Reynolds Tobacco Co.
Building 630/2
Winston-Salem NC 27102

Contractors

Veritas Labs : Animal Health, Necropsy, Hematology, Histopathology
Hazleton Labs America : Clinical chemistry
P.R.I. : Quality Assurance, Animal Care
Microbiological Associates : Serology

Study Administration

Study Director: C.R.E. Coggins, Ph.D., DABT
Co-Directors: A.T. Mosberg, Ph.D., DABT, C.K. Lee, Ph.D, D.J. Doolittle, Ph.D., DABT
Inhalation Toxicologist: P.H. Ayres, Ph.D., DABT
Study Pathologist: J.W. Sagartz, DVM, ACVP
Reviewing Pathologist: D.L. Dungworth, DVM, ACVP
Analytical Chemist: M.W. Ogden, Ph.D
Quality Assurance Officer: S.J. Graham, BS

RECORDS TO BE MAINTAINED

The following records will be maintained, to allow a reconstruction of the experiment : animal health, serology, randomization, identification and housing, test article, calibration of instruments, daily inhalation exposures, clinical observations, body weights, CO-oximetry, plasma nicotine and cotinine, necropsy (including clinical pathology), histopathology, statistical analyses.

This study will not be listed as a regulated study and the results are not intended to be submitted to any regulatory agency.

Data will be entered into the Xybion Path/Tox software (Xybion Medical Systems, Cedar Knolls, NJ) running under the VMS operating system on a VAX 8820 computer. This software is designed for the acquisition and management of toxicology and pathology data. System control is maintained by a computer resident protocol in order to ensure data integrity and compliance with the Good Laboratory Practice guidelines of the FDA and EPA.

EXPERIMENTAL DESIGN

The experimental design is based on the OECD guideline, *Repeated-dose inhalation toxicity: 14/28-day study*, # 412, adopted 12 May 1981.

Three groups of animals will be exposed to SSS in whole-body inhalation chambers (Moss et al., 1982). During exposure, animals will be restrained in nose-only tubes in the chambers to minimize deposition of smoke on fur and subsequent oral ingestion during preening. There will be a sham-exposed group, placed in restraint tubes but exposed only to dilution air. A further group of animals will be kept as chamber controls (without tube restraint) and as sentinels for the detection of disease. Animals will be exposed six hours per day, for fourteen consecutive days. The groups will be "stagger-started" at intervals of one day, to prevent saturation of limited resources at necropsy. Animals in satellite groups (10 per sex) will be kept for a further 14 days without treatment to assess reversibility.

EXPERIMENTAL ANIMALS

A minimum of 560 (280 male and 280 female) animals, weighing 125-150 grams and aged 38-41 days will be ordered, to provide the required number of animals for testing. The vendor will be Charles River Raleigh (Raleigh, NC). Animals will be housed individually in transparent polycarbonate cages and acclimated to laboratory conditions in room # 47 for 2-3 weeks prior to the first exposure.

Animal Selection and Justification for Test System

The Sprague-Dawley rat (Crl:CD/BR, VAF/Plus) was chosen as the experimental animal, because they have frequently been used in inhalation studies and there is a large amount of background data available in the scientific literature. The test system is similar to that used in earlier studies performed in house and at contract facilities.

Animal Health, Serology

Two days after delivery, 5 animals per sex will be randomly chosen and killed for collection of sera, which will be tested for the following antibodies to disease : Reovirus Type 3, cilia associated respiratory bacillus, Kilham's rat virus, Toolan's H-1 virus, pneumonia virus of mice, Sendai, rat coronavirus / sialodacryoadenitis virus, lymphocytic choriomeningitis virus, *Mycoplasma pulmonis*. Antibody testing will again be made on sera obtained from 5 animals per sex in group 2 at the beginning and end of the inhalation part of the experiment, and at reversibility (total of 20 animals per sex). The lungs from the sentinel animals will be taken and examined histopathologically, to ascertain health status.

The start of the inhalation part of the study is dependent upon negative serology data being obtained on the pre-study samples, and upon a histopathology statement on the animals killed at delivery, releasing the animals from quarantine.

Group Allocations

Any animals with questionable health (as assessed by a veterinarian or experienced assistant) will be excluded from the following procedure. One week after delivery the animals will be allocated by sex into groups, such that the body weights in the groups are as homogeneous as possible. The allocation will be made using the "A" module of the Xybion software. A record will be kept of quarantine number, weight at group allocation and permanent identification number. Surplus animals from the above procedure will be removed from the study.

Animal Identification

During the week after allocation into groups, animals will be tail-tattooed (Animal Identification & Marking Systems, Piscataway, NJ) with their permanent identification number. The animals will be returned to cages with cards attached, recording the study number, animal number, sex, pre-study number, and study director. Additional cards will be attached on the outside of the inhalation chambers. The following animal identification numbers will be used :

Sham	Male	101-148	Female	151-198
Room		201-248		251-298
SSS Low		301-348		351-398
SSS Medium		401-448		451-498
SSS High		501-548		551-598

Of the 48 animals per sex, 20 will be randomly allocated for the 14-day necropsy and subsequent histopathology. Ten animals per sex per group will be randomly allocated for the reversibility study. The remaining 18 animals will be used for dosimetry and special studies throughout the study.

Animal Housing & Care

The animals will be housed and cared for in accordance with the Animal Welfare Act of 1970 and amendments (Public Law 91-579), as set forth in CFR Title 9, Part 3 Sub-part E, *Specifications for the humane handling, care, treatment, and transportation of warm-blooded animals other than dogs, cats, rabbits, hamsters, guinea pigs and non-human primates*. Reference will also be made to the DHHS document *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23).

After quarantine, the animals will be housed and exposed in animal room # 46 in the 630/2 building, with limited access. The rooms will have controlled lighting (12 hours of darkness, from 6 p.m.), temperature (20-24°C), and humidity (40-60% relative humidity, RH). Seven-day continuous recordings will be kept of RH and temperature. Animals will be housed individually in the inhalation chambers, with the doors closed (except when wheing animals and transferring them between chambers).

Feed & Water

Animals will have unrestricted access to certified feed (Purina Rodent Chow # 5002, presented as pellets) and distilled water. No feed will be available during inhalation exposures. Feed will be withheld overnight prior to necropsy. Chemical analyses of feed, water or bedding will not be performed, because it is unlikely that contaminants would adversely affect the experiment.

EXPOSURE REGIMEN

Test Material

The test material is SSS from the 1R4F reference cigarette (Tobacco & Health Research Institute, Lexington, KY). The SSS will be generated through use of the AMESA 1300C smoke generator (CH Technologies, Westwood, NJ).

Aerosol Concentrations

The target WTPM concentrations are 0, 0.1, 1, and 10 mg/m^3. In the event that WTPM sampling causes artifacts, equivalent concentrations will be assayed by CO and/or solanesol.

Animal Exposures

Each treatment group will be exposed to aerosol for 6 hours per day, for 14 consectutive days. Animals that die during the experiment will not be replaced. The reversibility animals will be kept for a further 14 days in the same room, without any treatment.

INHALATION EXPOSURE SYSTEM

Calibration of Air Sampling Instruments

The measurement of flow rates is of critical importance in inhalation toxicology. The inhalation exposure system will use a variety of different instruments to measure flow rates on each of the exposure days.

Two primary standards are used : bubble flow meters (Series 823, Mast Development Co., Davenport, IL) for flow rates up to 5 liters per minute (LPM), and certified dry gas meters (Singer AL-1400, American Meter Company, Nebraska City, NE) for flow rates above 5 LPM. A variety of secondary measuring instruments, including pressure differential devices such as the CME 60-5-40 and 40-5-3A (CME Corp., Manassas, VA) will be used for routine measurements; these instruments will be calibrated against the primary standards.

Aerosol generation apparatus

An AMESA smoke generator will be fitted with a stainless steel SSS collection cone. The SSS will be drawn into a common plenum (3" diameter plastic tubing throughout), from which SSS and dilution air be will drawn through the chambers. Dilution air will be drawn from the animal room, through combination HEPA/charcoal filters (IES-8810; Kunz, Winston-Salem). Temperature and RH of the aerosol will be measured with a condensation dew point hygrometer (Model 1100 DP, General Eastern Instrument Co., Watertown, MA). A single generator will provide test material for each of the three chambers (see Figure 1).

Animal Exposure Apparatus

The whole-body inhalation chamber has been described previously (Moss et al., 1982); female rat nose-only restraint tubes will be used to minimize contamination of the pelt with deposited SSS which could then be ingested during preening (Langård and Nordhagen, 1980). A total of 5 chambers will be used, with a sixth chamber being sanitized and rotated daily. Each chamber has 6 trays each containing 16 individual cages: a system for rotating the animals within the chamber over the duration of the experiment will be devised, to minimize any effects of cage position within the chamber.

On exposure days, individual animals will be taken from their cage in the chamber, placed inside a nose-only restraint tube, and replaced in the tube into the same cage. The orientation of

the tube will ensure that urine and feces drain from the tube, through the wire floor of the cage, and onto the paper-lined catch pans placed under each of the six trays of cages. The ventilation holes on the tubes will be covered with duct tape. The cages to be used were designed for 500 g rats; the use of the female rat restraint tube in the cage does not cause any spatial problems. The restraint tubes designed for female rats are sufficiently large for use with juvenile male rats.

Chambers will be operated at flow rates equivalent to at least 15 changes per hour.

Pre-exposure Characterization

Before the animal exposures begin, satisfactory achievement of uniformly distributed concentrations at or near the target concentrations will be documented. Temporal and spatial characterization of each chamber will be made for aerosol concentrations, along with a measurement of particle size distribution.

Daily Characterization of Inhalation Exposures

During animal exposures probes will be used to monitor the aerosol presented. This monitoring will be by collection of aerosol on glass-fiber pads and gravimetric estimates of aerosol concentrations, and on-line monitoring using the output from the RAM-1 instruments. The output from the RAM-1 will be displayed on a chart recorder. Glass-fiber pads will be weighed on Cahn C-31 balances (Cahn, Cerritos, CA).

Ports will be used for monitoring CO and oxygen concentrations. The analytical instrument used for CO will be the Horiba PIR-2000 CO-analyzer (Horiba Instruments Inc., Irvine, CA), calibrated daily with certified gas mixtures of CO in nitrogen (AIRCO Welding Supply, Greensboro, NC). The output from the Horiba will be displayed as voltage on a chart recorder and logged on the printer of the computer-control unit as voltage and calculated parts per million (ppm, based on prior calibration). Oxygen concentrations (%) will be monitored by a Horiba PMA-200 instrument, also calibrated with a certified gas mixture. Additional ports will be used to measure chamber concentrations of carbon dioxide (CO_2) and ammonia (Miran IR).

Measurement of particle size distribution will be made using a Mercer-style cascade impactor (Mercer et al., 1970; In-Tox Products, Albuquerque, NM). The impactor will have cut-off diameters in the range of 0.4-2.5 µm under the conditions of use; calculations of mass median aerodynamic diameter (MMAD) will be made by the use of probit analysis. The cover slips (uncoated) used to collect the aerosol for impactor analysis will be weighed using Cahn C31 microbalances.

Additional analyses will be made by the ETS group; these will be documented separately.

Data Handling

The chart recordings for aerosol concentration from each exposure will be archived, along with summary sheets for each exposure. Summary inhalation data will be entered into the "I" sub-module of the Xybion "G" software.

IN-LIFE MEASUREMENTS AND OBSERVATIONS

The "A" and "G" modules of the Xybion software will be used for data acquisition and analysis.

Clinical Observations

Animals will be visually inspected for signs of overt toxicity as they are being transferred from their cages to the restraint tubes, and when being transferred back to their cages. More detailed clinical observations will be made on each animal once every week, before the exposure and 2 hours after the end of the exposure. Data on clinical observations will be entered into the "A" module of the Xybion software.

Viability Checks

Viability checks will be made twice daily at intervals of at least 4 hours, 7 days per week. Moribund animals will be anesthetized with 70 % CO_2 in air and exsanguinated via the *vena cava*; they will not be necropsied and the carcasses will be discarded. Early deaths will not be replaced.

Body Weights

Individual body weights will be determined within 48 hours of receipt, at randomization, and at weekly intervals thereafter. Body weight data will be acquired using the "A" module of the Xybion software, in conjunction with Mettler PM 2000 balances (Mettler Instrument Corporation, Hightstown, NJ) linked to the VAX by "Y" cables. Animals will be weighed before their daily exposures.

Dosimetry

A total of 18 animals will be used for dosimetry during the exposure phase of the experiment. These dosimetry animals will not be necropsied and the carcasses will be discarded. Tissues will be available for adducted DNA work and for other specialist end-points (e.g. lung lavage).

Samples will be collected after the entire 6 hours of smoke exposure, in exposure days 2, 4, 6, 8, 10 and 12, from 3 animals per sex per group. A sufficiently large blood volume will be drawn for the analysis of both COHb and plasma nicotine. Blood will be drawn from the retro-orbital sinus, using anesthesia with 70 % CO_2 in air and heparinized micropipettes. Blood will be held on ice in plastic cuvettes containing disodium edetate during the time between sampling and analysis. Blood COHb concentrations will be determined on 0.5 ml of the total sample, using a Model 482 CO-Oximeter (Instrumentation Laboratories, Hartford, CT) set with the calibrations for rats. Data will be entered in the "C" sub-module of the Xybion "G" software.

Sub-samples of the blood collected for COHb will be taken for the analysis of plasma nicotine and cotinine (Davis, 1985).

DNA adducts

DNA isolated from benzo(a)pyrene and/or 2-acetylamino-fluorene treated liver tissue or cells in culture will be used as positive control and will be assayed concurrently with the test article treated DNA sample.

The extraction of DNA will be performed by a modification of the method of Gupta et al., (1984) as follows:

To 0.50 to 1.00g of frozen tissue (lung, larynx) in a glass homogenizer, 10 ml of TNE buffer will be added and homogenized. The homogenate will be transferred to a 50 ml polypropylene tube to which 250 µl of 20% SDS and 500 mg/ml of proteinase K will be added.

The mixture will be incubated at 68°C for 30 min and at 37°C for 1 hour. After the incubation the homogenate will be extracted successively with equal volumes of phenol, 1:1 mixture of phenol:chloroform, and chloroform. The nucleic acids will be precipitated by adding chilled ethanol. The precipitated nucleic acids will be dissolved in RNA lysis buffer and treated with RNase A and RNase T, at 37°C to digest the residual RNA. The RNase treatment will be followed by the solvent extractions as described earlier, and the DNA will be precipitated by adding chilled absolute ethanol.

The precipitated DNA will be dissolved in 0.01 x NaCl/ Citrate/ 0.1 mM EDTA buffer and quantitated spectrophotometrically at 260 nm. The DNA will then be aliquoted and stored at -80°C. The DNA from the cell pellets (approximately 20 million cells) will be extracted as described above, except that the cells will not be homogenized.

The procedure will usually consist of the steps described below. The method is a modification of the published procedure of Gupta et al. (1982) and Gupta (1985).

2.5 µg of DNA sample will be digested with 0.6 units of micrococcal nuclease, 0.01 units of spleen phosphodiesterase in 15 µl of 10 mM sodium succinate, 5 mM calcium chloride, pH 6.0. After incubation at 37°C for 3 h, the digest will be diluted to 25 µl (0.1 µg/µl DNA) with deionized water. 20 µl of the diluted digest will be mixed with 10 µl of 100 mM ammonium formate, pH 3.5, 10 µl of 10 mM tetrabutylammonium chloride, and 60 µl of water. The mixture will be extracted twice with 100 µl of water saturated 1-butanol. The extractions will be performed in 1.5 ml conical tubes by mixing for 30 sec on a vortex mixer. The phases will be separated by centrifugation at about 5000 rpm for 1 min. The butanol layers from the two extractions will be pooled and back-extracted once with 180 µl of water. This butanol extract will be neutralized by adding 2 µl of 200 mM Tris HCl at pH 9.5, and evaporated to dryness in a speed-Vac concentrator.

^{32}P-labelling of the isolated adducts

The sample residue from above will be dissolved in 3 µl of water and mixed with 7 µl of kinase reaction mix (prepared by mixing 0.1 M bicine, 0.1 $MgCl_2$, 0.1 M dithiotreitol, 10 mM spermidine, pH 9.5, 3 units of T4 polynucleotide kinase and 225 µCi of gamma-^{32}P-ATP). The mixture will be incubated at 37°C for 30 min.

Mapping of ^{32}P-labeled adducts

Eight µl of the labeled sample solution will be applied to the origin close to the center of a 2Ox2O cm PEI-cellulose sheet to which a 8 cm Whatman No. 1 wick is attached. The sheet will be developed in direction 1 (D1) for 18 h or overnight in 1.1 M lithium chloride solution to obtain the transfer of normal DNA nucleotides and free radioactivity onto the wick. After development in Dl, the wet sheet will be cut at 12 cm from the bottom, and the upper portion of the PEI sheet and the wick will be discarded. The 12 cm sheet will be washed twice in water for about 5 min each and will be dried in a current of warm air. The sheet will then be turned 90° to the Dl direction and developed to the top in 2.5 M ammonium formate, pH 3.5 (D2). The wet chromatogram will be cut at 12 cm above the bottom of D2, and smaller portion (8 cm) will be discarded. The remaining sheet will be washed twice in water for 5 min each and dried.

To resolve the chemical-DNA adducts retained at the origin, the chromatogram will be developed in direction 3 (D3) to the top in 3.0 M lithium formate, 7.0 or 8.5 M urea, pH 3.5. The urea concentration in D3 and D4 chromatography will depend on the type of adducts to be resolved. The D3 chromatography will be performed in the direction opposite to Dl. The developed sheet will be washed twice in water and dried. The D4 chromatography will be performed perpendicular to D3 in 0.8 M lithium chloride, 0.5 M Tris, 7 or 8.5 M urea, pH 8.0.

After development, the chromatogram will be washed twice in water, and dried. The direction 5 (D5) chromatogram will be developed in the same direction as D4 in 0.35 M $MgCl_2$ overnight, then washed in water and dried.

Autoradiography and measurement of ^{32}P-labeled adducts

The washed and dried chromatogram obtained from D5 will be placed in contact with XAR-5 film in a cassette equipped with intensifying screens. The exposure will be done at -80 °C for up to several hours to overnight. After the exposure time, the films will be processed in a standard X-ray film development machine. The chromatogram and the exposed film will aligned and individual spots marked. The spots will then be excised from the chromatogram and placed in a glass scintillation vial for counting the radioactivity by the Cerenkov technique. Blank areas of the chromatogram will also be evaluated, and the count rate will be subtracted from the corresponding adduct rate.

^{32}P-labeling of the total DNA nucleotides

The remaining 5 µl of the diluted enzymatic digest (0.1 µg/µl DNA) will be further diluted with water to obtain a DNA concentration of 0.02 ng/µl, and 5 µl of this solution will be evaporated to dryness in a speed-vac centrifuge. The residue will be dissolved in 1µl of water and incubated at 37°C for 30 min with 2 µl of kinase reaction mix, described previously. This reaction will be performed in parallel with adduct labeling. After the 30 min incubation, 1µl of 1:1 mixture of 40 µg/µl of potato apyrase and 4 µg/µl each of dpNp mix (deoxynucleoside 3' 5'-bisphosphates), will be added and further incubated at 37°C for 30 min. The mixture will be diluted with 10 mM Tris-HCl, 5 mM EDTA, at pH 9.5, and a 5µl aliquot (0.01 ng DNA) will be applied 1.5 cm from the bottom edge of a 10 cm long PEI-cellulose sheet. The sheet will be developed in 40 mM ammonium sulfate to about 8 cm and dried. In this system, the free ^{32}P will migrate away from the origin (OR), while total DNA nucleotides will be retained at OR. The radioactivity of the total nucleotides remaining at the OR will be determined by scintillation counting.

Quantitative estimation of DNA adduct levels

The data are expressed as relative adduct labeling (RAL) values:

RAL = radioactivity (cpm) in an adduct spot divided by the radioactivity (cpm) in the total nucleotides

The dilution of total nucleotides prior to chromatographic analysis will be taken into consideration while calculating the RAL. The RAL values will then be converted into femtomoles of adducts per µg of DNA, by multiplying the RAL by 3 million (the conversion equivalency of nucleotides per µg of DNA).

NECROPSY AND HISTOPATHOLOGY

The "N" and "P" modules of the Xybion software will be used respectively for data acquisition in necropsy (Feldman, 1988) and histopathology; acquisition and statistical analyses of other data will use the "G" module.

Necropsy

Animals selected at random will be killed on the day following their last exposure, and the time interval recorded. Feed will be removed from the animals during this time interval.

At necropsy, animals will be weighed and then killed by first anesthetizing with 70 % CO_2 in air and then exsanguination via the *vena cava* prior to cessation of heartbeat. Blood samples for the various assays to be performed will be collected from either the vena cava or from the retro-orbital sinus; the time of blood collection will be recorded.

Animals will be subjected to a complete gross examination in the presence of a board-certified veterinary pathologist, with special attention paid to the respiratory tract.

Hematology

The following assays will be performed on whole blood obtained at each necropsy : red blood cell count, hemoglobin, hematocrit, mean red cell volume, mean red cell hemoglobin, mean red cell hemoglobin concentration, white cell count, differential white cell counts, reticulocyte counts, platelet count. The anticoagulant Na_2-EDTA will be used; standard hematological methods will be used by the contractor. Data will be transferred on computer tape and entered into the "H" sub-module of the Xybion "G" software.

Clinical Chemistry

The following assays will be performed on serum obtained from animals at necropsy : calcium, phosphorus, chloride, sodium, potassium, glucose, alanine amino-transferase, aspartate aminotransferase, gamma glutamyl transpeptidase, urea nitrogen, albumin, creatinine, total bilirubin, total cholesterol, triglycerides, and total protein. The time of blood sampling will be recorded, using the Xybion software. Sure-Sep II serum separators (Organon-Teknika, Durham, NC) will be used to minimize hemolysis; the time between blood collection and serum collection will be kept as short as possible. Serum samples will be transported using refrigeration with dry ice. Data will be entered into the "S" sub-module of the Xybion "G" software.

Organ Weights

The lungs (complete with trachea but excluding the larynx), brain, liver, kidneys (pair) and heart (excluding major vessels) will be weighed at each necropsy, using Mettler PM 460 balances connected to the VAX with "Y" cables. Organ weights and the (fasted) body weight recorded immediately before death will be used to calculate organ : body weight ratios. The time from removal of the organ until weighing will be minimized and tissues will be kept in saline until weighing.

Tissue Collection

Tissues will be removed from each animal and fixed in 10 % neutral buffered formalin, at a volume dilution of 1 part tissue to at least 15 parts formalin. The fixative will contain 20 ml of 1 % eosin per 20 liters of 37 % formalin, as a precaution to identify the fluid as fixative. Lungs will be perfused with fixative using gravity filling with visual assessment of total lung capacity. The trachea will be ligated after inflation.

The following tissues will be collected :

Adrenals, Aorta, Bone (sternum, femur), Brain, Cecum, Colon, Cranium, Duodenum, Epididymides, Esophagus, Eyes/optic nerve, Heart, Ileum, Jejunum, Kidneys, Larynx, Liver, Lungs, Lymph Nodes, Mammary glands, Nasopharynx, Nose/turbinates, Ovaries, Pancreas, Parathyroid, Pituitary, Prostate, Rectum, Salivary gland, Seminal vesicle, Skeletal muscle (thigh), Skin (abdominal), Spinal Cord (lumbar), Spleen, Stomach, Tail, Testes, Thymus, Thyroid, Tongue, Trachea, Urinary bladder, Uterus, Zymbal's gland.

Any organs showing gross lesions will be collected; the carcasses will be discarded.

Histopathology

Respiratory tract tissues (nasal passages, larynx, trachea, conducting airways, deep lung), heart and related lymph nodes (thymic and peribronchial) and gross changes will be examined in each of the animals allocated to histopathology.

The nasal tissues will be cut at three different locations to obtain representative sections of the different epithelia, as described previously (Young, 1981). The lungs will be sectioned so as to provide a section along the main stem bronchus of each lung lobe. A precise anatomical site for cutting the larynges is required. Serial-step sections will be taken to reach this site (Burger et al., 1989).

Tissues will be stained with hematoxylin and eosin (H&E); duplicate slides of a representative section of the anterior nasal tissues, larynx, lung and trachea will be stained with Periodic-Acid-Schiff / Alcian Blue (PAS-AB) to facilitate evaluations of mucus-secreting cells.

Tissues will be read by an ACVP board-certified veterinary pathologist, initially with knowledge of the treatment groups. Sub-sets of slides will be sent to the reviewing pathologist for confirmation of any changes described.

STATISTICAL ANALYSES

In-life functions, clinical pathology, organ weights.

Statistical evaluations will be made using the tests built into the Xybion software : Bartlett's test of homogeneity of variance, followed by analysis of variance (ANOVA) techniques.

Histopathology

The statistical evaluation of incidence data will be made by the Kolmogorov-Smirnov test (Siegel, 1954).

Significance

Statistical tests will be carried out to 5 %, two-sided criteria.

DNA Adducts

An assay normally will be considered acceptable for evaluation of test results only if all of the following criteria are satisfied. Both positive and negative control DNA samples are to be assayed concurrently with the test article-treated DNA sample.

The DNA sample from the positive control should exhibit the major adduct characteristic for benzo(a)pyrene and/or for 2-acetylaminofluorene. The radioactivity of each reported adduct spot should be significantly higher than the blank area of the chromatography sheet.

Adduct spots must be observed on the test material chromatogram which is distinctly different from spots that may be observed on the negative control chromatogram. If no spots are observed after the initial development (2-3 h photographic development) of the test article chromatogram, the chromatogram may be further developed for longer periods (up to 24 h) to visualize weak adducted spots. Concurrently, the negative control chromatogram will also be developed. Under normal assay conditions, the positive control chromatogram will develop in 2-3 h and the major adducts can be visualized.

A test article will be classified as DNA-reactive if the chromatogram shows at least one adducted spot that is different from the negative control and the radioactivity of the spot is significantly higher than the adjacent blank area.

QUALITY ASSURANCE

Experienced Quality Assurance (QA) personnel will make periodic inspections of the study, and reports on the results of these inspections will be submitted to management.

A large part of the QA techniques used in this study are related to the use of the Xybion software (e.g. the use of computer passwords to identify data entry sessions).

The QA staff have access (read only) to data stored on the Xybion system. Using the "Z" module, where data can be obtained on different aspects of the study on an individual animal basis, QA staff will make a a review of around 15 % of all of the raw data generated in the study.

REPORTING

Draft Interim Report

A summary report will be prepared as soon as possible after the final necropsy. The report will be a summary of in-life observations, body weight changes, absolute and relative organ weights, hematology and gross pathology. A section on inhalation parameters will also be included. Completed data on clinical pathology will also be included.

Final Report

A final report will be prepared in draft form as soon as possible after the final necropsy.

The QA unit will prepare and sign a statement which shall specify the dates inspections were made and findings reported to management and to the study director.

The final report will include :

- objectives and procedures, as stated in the protocol
- description of the inhalation chambers and their operating conditions and performance
- tabulation of response data

- a separate pathology report, including tabulated gross and microscopic pathology
- results of serology assays
- deviations from the approved protocol or from SOPs

DNA Adducts

The data reported will include the following:

- Number of adduct spots observed on the chromatograms from the positive, negative and test article treated DNA's.
- Radioactivity counts of the adduct and blank areas from the chromatogram
- Calculated relative adduct labeling (RAL) values for each observed spot from positive and test article treated DNA.
- Calculation of number of adducts per unit of DNA (fmol adducts/μg DNA).

PROJECTED TIMING

Attempts will be made to achieve the following target dates:

Delivery of Animals: 18 September 1990
Randomization: 2 October 1990

Exposure Starting Dates
Sham Control : 9 October 1990
Low exposure : 10 October 1990
Medium exposure : 11 October 1990
High exposure : 9 October 1990

Exposure End Dates
Sham Control : 22 October 1990
Low exposure : 23 October 1990
Medium exposure : 24 October 1990
High exposure : 22 October 1990

Necropsies
Sham Control : 23 October 1990
Low exposure : 24 October 1990
Medium exposure : 25 October 1990
High exposure : 23 October 1990
Reversibility (all): 7 November 1990

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