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TITLE

IN VIVO MOUSE BONE MARROW MICRONUCLEUS ASSAY OF REACTIVE SKIN DECONTAMINATION LOTION

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In Vivo Mouse Bone Marrow Micronucleus Assay of Reactive Skin Decontamination Lotion

BY:

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Prairie Biological Research Ltd.

SUBMITTED TO:
SCIENTIFIC AUTHORITY

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June 1998

CRAD

PBR PRAIRIE BIOLOGICAL RESEARCH LTD.

FINAL REPORT

(DRES SOLICITATION NO. W7702-6-R599/A)

STUDY TITLE:

IN VIVO MOUSE BONE MARROW MICRONUCLEUS ASSAY OF REACTIVE SKIN DECONTAMINATION LOTION

PREPARED BY: **Ram D. Mehta, Ph.D. P.Biol.**

PREPARED FOR: DEFENCE RESEARCH ESTABLISHMENT LTD.
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Attn: Dr. Thomas Sawyer

COMPLETION DATE: June 8, 1998

STUDY NUMBER: 970805/3

DATE: June 30, 1998

Ram D. Mehta, Ph.D. P.Biol.
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IN VIVO MOUSE BONE MARROW MICRONUCLEUS ASSAY OF REACTIVE SKIN DECONTAMINATION LOTION

Authorized Representative:	**Dr. Thomas Sawyer**
Contract Laboratory:	**Prairie Biological Research Ltd.** **4290-91A Street, Block C** **Edmonton, Alberta** **Canada T6E 5V2**
Test Substance:	**Reactive Skin Decontamination Lotion**
Test Article Lot No.:	**Lot 5D11449**
PBR Study No.:	**970805/3**
Test Substance Description:	**yellow colored lotion**
Storage Condition:	**under refrigeration (3-6°C)**
Test Article Receipt:	**August 05, 1997**
Study Initiation:	**April 15, 1998**
Study Completion:	**June 08, 1998**
Study Director:	**Ram D. Mehta, Ph.D. P.Biol.**
Technologist:	**Ebenezer Sowa**

STATEMENTS OF COMPLIANCE

Prairie Biological Research Ltd.'s Study N0. 970805/3 was conducted in compliance with the OECD Principles of Good Laboratory Practice (GLP), in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not determined by the testing facility.

Ram D. Mehta, Ph.D. P.Biol.
Study Director

June 30/1998
Date

QUALITY ASSURANCE STATEMENT

Procedures, documentation, equipment records, etc., of the study (No. 970805/3) are examined in order to assure that the study is performed in accordance with the regulations specified as above and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures. This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Frank Kozar, Ph.D. P.Biol.
Quality Assurance

98-06-30
Date

TABLE OF CONTENTS

SUMMARY

The test article, Reactive Skin Decontamination Lotion, was tested in the *in vivo* mouse (Balb/cCr//Alt BM virus, antibody free) bone marrow micronucleus assay. The assay was conducted by exposing a test group of 10 mice (5 male and 5 female) to each of 5 doses of the test article; 2000, 1000, 500, 250, and 125 mg/kg. Sterile isotonic saline was used as the solvent and negative control. The test article was readily soluble at the highest dose. Mice were injected once intraperitoneally and bone marrow samples were extracted 24 hours post-injection and smeared onto slides for analysis. The frequency of micronucleated polychromatic erythrocytes (PCE's) in the polychromatic erythrocyte population was used as the criterion for evaluating genotoxicity.

At the dose levels tested, no apparent toxicity was observed. All the mice in treatment, control, and environmental groups were healthy during the course of the study. Cytotoxic effect of the treatment on the bone marrow cells was negligible as the percentage of PCE's among total erythrocytes at all dose levels tested was comparable with the negative control values.

There was no statistically significant increase in the frequency of micronucleated polychromatic erythrocytes (PCE's) relative to the solvent control ($p > 0.05$) at the dose range tested.

The frequency of micronucleated PCE's in the negative control group was within the range of historical solvent control. The positive control, (7,12-dimethylbenz(a)anthracene) produced a statistically significant positive response for micronucleus induction ($p > 0.05$). Thus, the positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report and based on the evaluation criteria applied, Reactive Skin Decontamination Lotion elicited a negative (non-genotoxic) response in the *in vivo* mouse bone marrow micronucleus assay.

PURPOSE

The purpose of this study is to evaluate the *in vivo* genotoxic potential of the test article, Reactive Skin Decontamination Lotion (RSDL), based on the frequency of micronuclei induction in mouse polychromatic erythrocytes in the bone marrow.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test article, Reactive Skin Decontamination Lotion (RSDL), was received by Prairie Biological Research Ltd. on August 05, 1997 and assigned the code number 97-BZ-1.

Upon receipt, the test substance was described as bright yellow colored free flowing liquid with no distinct odor and was stored at 3 to 6°C in a tightly sealed opaque plastic container protected from moisture.

Isotonic saline (0.9% sodium chloride solution in distilled water) was used as a vehicle to dissolve and deliver the test article, RSDL. The positive control compound used in this assay was 7,12-dimethylbenz(a)anthracene (DMBA), a known clastogen, supplied by Sigma Chemical Co. (CAS 57-97-6). Dimethylsulfoxide (CAS 67-68-5) was used to solubilize and deliver DMBA.

MATERIALS AND METHODS

Test System

The mouse bone marrow micronucleus test is a mammalian *in vivo* test which detects damage to the chromosomes or the mitotic apparatus (resulting in micronucleus formation) in the bone marrow cells of the animals exposed to a test article (Cihak, 1979; Schmid, 1975; Matter and Schmid, 1971). Micronuclei are formed as a consequence of acentric fragments of chromosomes or entire chromosomes lagging behind at the anaphase stage of cell division due to chromosome breakage or mitotic spindle apparatus damage (Schmid, 1975). When an erythroblast develops into an erythrocyte, the main nucleus is extruded and micronuclei remain behind in the cytoplasm.

Mouse polychromatic erythrocytes (PCEs), extracted from femur bone marrow were analyzed in this assay. Low levels of micronucleated PCEs in the bone marrow do occur naturally during erythrocyte maturation. The positive response of the assay is based on an increase in the frequency of micronucleated PCEs in the bone marrow of treated animals.

Experimental Animals

Healthy, male and female BALB/cCr//Alt BM, inbred, virus antibody free mice (supplied by Health Science Laboratory Animal Services, University of Alberta, Edmonton, Canada), approximately 8-9 weeks old weighing 21 – 29 g (mean weight 22 g), were selected and randomized before assignment to treatment and control groups. The animals were quarantined for 5 – 6 days and were certified by clinical veterinarian to be disease free prior to the study. The animal treatment and bone marrow sample collection phase of the study was conducted at the Health Sciences Laboratory Animal Services, University of Alberta, Edmonton Alberta, under a contractual agreement with Prairie Biological Research Ltd.

Number and Sex

In the assay, 6 female and 6 male mice were employed per treatment dose level and control group. A group of 3 female and 1 male was kept as environmental sentinel. For genotoxicity evaluation only five female and five male mice were scored for each treatment and control group.

Dose Levels

The dose levels were determined on the basis of average body weight (22 g) of the test animals. The treatment groups were administered with 5 dose levels of the test article, the maximum dose being the maximum allowable (limit or biologically relevant) dose of 2000 mg/kg for this assay (Hayashi et.al., 1994). Four additional doses used were: 1000, 500, 250, and 125 mg/kg. The delivery volume of the test article for injections was 1ml per animal.

The test article stock solution was prepared at a concentration of 44 mg/ml to deliver the highest dose level, with succeeding lower doses prepared from the highest dosing solution by serial (2-fold) dilution in the isotonic saline. The test article was found to be readily soluble in isotonic saline and at the highest dosing solution appeared as a light yellow colored transparent fluid. All solutions were filter sterilized using 0.22 μm membrane filter into pre-cleaned and sterilized 20 ml injection vials.

The positive control compound (DMBA), was dissolved in Dimethylsulfoxide (DMSO) and used at a concentration of 120 mg/kg. Two negative controls were used, DMSO and isotonic saline. The delivery volume for injection per animal was 0.1 ml for DMBA and DMSO and 1.0 ml for the isoltonic saline.

Test article and control solutions were prepared about 2 hours prior to injecting the animals.

Frequency and Route of Administration

Treatment group of mice were exposed to the test article and the control compounds by intraperitoneal injection once. All injections were given under light metofane anaesthesia.

Housing and Feeding Conditions

Mice were caged by sex group prior to the experiment for acclimatization. During the course of the experiment, treatment and control groups were caged separately. All animals were observed daily for the duration of the study. Any abnormal behavior was recorded. Husbandry, temperature, humidity and light cycles were controlled and monitored as dictated by good animal husbandry practices. Diet and drinking water (certified Diet #5002 and PETWA water) were supplied *ad libitum.*

Micronucleus Assay

The micronucleus assay was conducted by exposing mice treatment groups to five dose levels of the test article, 2000, 1000, 500, 250, and 125 mg/kg. In addition, both positive and negative (solvent) control groups were employed. The test article was administered once at each dose level. After 24-hour exposure to the test substance, the mice were sacrificed and the bone marrow was obtained from femurs, smeared onto randomly coded slides and stained. A minimum of 2000 PCEs per animal were scored for the presence of micronuclei. Only the PCEs will have micronuclei induced by a recent treatment due to the duration of erythropoiesis in mouse bone marrow (Mavournin *et al.*, 1990). The genotoxic potential of the chemical was assessed by comparing the frequency of micronucleated PCEs in the exposed animals with that of the solvent control group.

Bone Marrow Extraction and Microscope Slide Preparation

At approximate 24 hours after final dose administration, mice were euthanized with carbon dioxide (CO_2) and mortality confirmed with the pinch-toe reflex technique. Both femurs were removed and cleaned free of muscle. A pair of scissors was then used to make a cut at the proximal end to expose the bone marrow canal. A 3cc syringe with a 26-gauge needle containing 1 ml of fetal bovine serum (FBS) was inserted into the proximal end. The marrow was exuded from the distal end into a 1.5 ml Eppendorf tube by slowly flushing the syringe. Using the same FBS-marrow mixture and syringe, the above was repeated for the other femur as well. Eppendorf tubes containing samples were then centrifuged at 1000 rpm for ten minutes. The majority of the supernatant was discarded and the cells in the pellet were resuspended carefully by repeated aspiration with a Pasteur pipette. A small drop of the suspension was placed at one end of the slide and spread by pulling the material behind a clean slide held at an angle of about 45°. Four slides were prepared per animal and air-dried for at least 48 hours.

Staining of Prepared Slides for Analysis

Staining method described by Schmid (1975) was used for slide preparation. Slides were stained in May-Grünwald's Stain (BDH, supplied by VWR, Ontario, Canada) for 3 minutes followed by further staining with 1:1 May-Grünwald stain : distilled water solution for one minute and then in Giemsa (1 part Giemsa to 6 parts phosphate buffer, pH 6.8) for about 10 minutes. Slides were rinsed thoroughly in distilled water, air-dried, cleared with xylene, and mounted in Cytosol™ (Stephens Scientific, Riverdeale, NJ, USA). Giemsa was obtained from Sigma Chemical CO., Missouri, USA. Phosphate buffer was prepared from 0.71 g Na_2HPO_4 and 0.68 g KH_2PO_4, dissolved in 1 litre distilled water with pH adjusted to 6.8.

Scoring for Micronucleated Polychromatic Erythrocytes (PCE's)

The number of micronucleated PCEs was scored out of at least 2000 PCEs counted per animal. Ten exposed animals (5 male and 5 female) were scored for each treatment and control group.

Bone marrow cytotoxicity was determined by calculating percentage of PCEs among total erythrocytes.

Slides were randomly numbered by an individual not involved in the scoring process. Slides were observed under 40X objective and/or oil immersion objectives (60X and 100X). Due to the staining, PCEs appear purple while normochromatic erythrocytes (NCE's) appear orange in color. This difference in staining characteristics is due to the ribosomal RNA present in PCEs, and lacking in NCEs (Mavournin *et al.*, 1990). Micronucleated cells usually appear quite similar to normal cells and mostly contain only one micronucleus. Micronuclei appear as round, darkly stained regions within erythrocytes (Schmid, 1975).

Evaluation of Test Results

The data presented for each treatment and control group include micronucleated PCEs/1000 PCEs. Statistical analysis of the frequency of micronucleated PCEs was performed using the Analysis of Variance (ANOVA) test. In the event of a positive ANOVA test, the Cochran-Armitage trend test was applied to measure dose responsiveness. Furthermore, the response of male and female mice in each dose group was compared using Wilcoxon Signed Rank statistic for any sex associated difference in induction of micronuclei (Urlando and Heddle, 1990).

A positive genotoxic effect of the test article was based upon a statistically significant dose-related increase ($p \leq 0.05$) in the number of micronucleated PCEs relative to the

solvent control. A significant increase at any one dose level only with no dose response was considered equivocal.

If the test article showed no statistically significant increase in the number of micronucleated PCEs compared with the solvent control, the test article was considered non- genotoxic in this system.

Positive results in the micronucleus assay indicate that the test article induces micronuclei, which could have resulted from chromosomal or mitotic apparatus damage.

Criteria for a Valid Test

Solvent controls

The frequency of micronucleated PCEs per 1000 PCEs in the solvent controls should fall within the range of historical negative control data of PBR and/or within the range reported in the literature (Shelby *et al.*, 1989; Mavournin *et al.*, 1990).

Positive Control

The frequency of micronucleated PCEs in the positive control group must be statistically increased ($p \leq 0.05$) relative to the solvent control.

Archives

Upon completion of the final report, specimens, all raw data and quality assurance/quality control report are maintained in Prairie Biological Research Ltd.'s archives located at 4290-91A Street, Block C, Edmonton, Alberta, T6E 5V2, Canada.

RESULTS AND DISCUSSION

Solubility test

Isotonic saline was determined to be the solvent of choice based on solubility of the test article and compatibility with the test animals. The test article was soluble in the isotonic saline at the maximum concentration of 44 mg/ml. The osmolality of the solvent control was 262 mOsm/kg and the osmolality of the top dose, 44 mg/ml, was 457 mOsm/kg as determined by Microosmo Meter (Precision Systems). The pH of the test article stock solution was 10.5 that was adjusted to pH 7.2 with 1M HCl.

Test article Toxicity

All mice in treatment, control, and environmental groups were healthy during the course of the study (Table 1). The percentage of PCE's among total erythrocytes at all dose levels tested was comparable to the negative control values, showing that there was negligible cytotoxic effect of the treatment (Table 2). Since micronuclei can only form during cell division, bone marrow cytotoxicity may inhibit cell division and thus suppress the percentage of PCEs among total erythrocytes resulting in unreliable counts for micronucleated PCE's (Mavournin *et al.*, 1990). The percentage of PCEs among total erythrocytes should not be less than 20% of the control (Hayashi *et al.*, 1994).

Micronucleus Assay

The results for the rodent bone marrow micronucleus assay are presented in Table 3. A minimum of 2000 PCEs per animal were scored for the presence of micronuclei for the negative controls (isotonic saline and DMSO), the RSDL test doses (2000, 1000, 500, 250 and 125 mg/kg), and DMBA (the positive control).

The positive control (DMBA at 120 mg/kg) showed a significant increase in frequency of micronuclei ($p<0.05$, ANOVA). The frequency of micronucleated PCE's in the negative controls (isotonic saline and DMSO) was within the acceptable range of control values (<5/1000 PCE's).

Compared to the respective negative (solvent) control values, there was no significant increase in the frequency of micronucleated PCE's induced by the test article at the dose range tested ($p\geq0.25$, ANOVA) . Based on the Wilcoxon Signed Rank Statistic analysis, there was no significant difference between the observed response for micronucleus induction in the male and female test animals ($p>0.05$).

CONCLUSION

The positive controls fulfilled the requirements for a valid test. Under the conditions of the assay described in this report and based on the evaluation criteria applied, Reactive Skin Decontamination Lotion was found to exhibit a negative (non-genotoxic) response in the *in vivo* mouse bone marrow micronucleus assay.

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Table 1: Clinical Signs Following Administration of RSDL

[1]Treatment	Clinical Observation	Number of Animals with Clinical Signs/Total Number of Animals Dosed		Number of Animals Died/Total Number of Animals Dosed	
		Males	Females	Males	Females
Solvent control ([2]Isotonic saline) 45ml/kg	Normal	0/5	0/5	0/5	0/5
[3]RSDL Doses					
125mg/kg	Normal	0/5	0/5	0/5	0/5
250mg/kg	Normal	0/5	0/5	0/5	0/5
500mg/kg	Normal	0/5	0/5	0/5	0/5
1000mg/kg	Normal	0/5	0/5	0/5	0/5
2000mg/kg	Normal	0/5	0/5	0/5	0/5
Solvent control [4]DMSO 4.5ml/kg	Normal	0/5	0/5	0/5	0/5
Positive control [5]DMBA 120mg/kg	Normal	0/5	0/5	0/5	0/5

[1]Treatment: administered doses of test article and negative and positive control

[2]Normal saline containing 0.9g NaCl/100ml distilled water

[3]RSDL = Reactive skin decontamination lotion (Treatment article)

[4]DMSO = Dimethyl sulfoxide (solvent for positive control)

[5] DMBA = 7, 12-dimethylbenz[a]anthracene (positive control)

Table 2 **Percent PCEs of Total Erythrocytes**

	[1]% PCE							
	Treatment Article: Reactive Skin Decontaminant Lotion						Positive Control: [2]DMBA	
[3]Group	1	2	3	4	5	6	9	10
Dose mg/kg	2000	1000	500	250	125	0	120	0
Mouse								
1	46	46	31	44	51	49	61	50
2	49	46	39	52	59	50	50	44
3	48	47	64	43	55	47	45	46
4	45	49	74	42	46	53	59	38
5	40	52	54	47	48	52	63	49
Mean % PCEs	46	48	52	46	52	50	56	45
% of Negative control	92	96	104	92	104	100	124	100

[1] %PCE = (Polychromatic erythrocytes scored/Total erythrocytes scored) x 100.

[2]DMBA = 7, 12-dimethylbenz[a]anthracene;

[3]Group = Set of mice treated with test article, positive and negative controls.
Group 6 is Saline control and Group 10 is DMSO control.

Table 3: Micronucleus Frequency in Bone Marrow of Mice Treated with RSDL

[1]Treatment	Sex	[2]Time (hr)	Number of Mice	Total [3]MPCE	Total [4]PCE	Micronucleated Polychromatic Number per 1000 PCEs	p-value
Solvent control **Isotonic Saline** 45ml/kg	M	24	5	29	10856	2.7 ± 0.17	-
	F	24	5	28	10587	2.6 ± 0.79	-
RSDL Doses							
125mg/kg	M	24	5	29	10272	2.8 ± 0.09	0.68
	F	24	5	26	10346	2.5 ±1.64	0.89
250mg/kg	M	24	5	25	10577	2.4 ± 0.09	1.00
	F	24	5	27	10888	2.5 ± 0.15	0.89
500mg/kg	M	24	5	28	10610	2.6 ± 0.27	0.79
	F	24	5	30	10716	2.8 ± 0.28	0.89
1000mg/kg	M	24	5	32	10907	2.9 ± 0.21	0.27
	F	24	5	38	10679	3.7 ± 0.08	0.27
2000mg/kg	M	24	5	38	10759	3.5 ± 0.40	0.25
	F	24	5	37	10849	3.4 ± 0.04	0.32
Solvent control ***DMSO** 4.5ml/kg	M	24	5	25	10766	2.3 ± 0.18	-
	F	24	5	31	11354	2.7 ± 0.16	-
Positive control ****DMBA** 120mg/kg***	M	24	5	87	10924	8.0 ± 0.88	0.00
	F	24	5	75	10455	7.2 ± 0.42	0.00

[1]Treatment: administered doses of test article and negative and positive control

[2]Time (hr) denotes the exposure time

[3]MPCE indicates the micronucleated polychromatic erythrocytes scored

[4]PCE is the polychromatic erythrocytes scored from each sex group

*DMSO = Dimethylsulfoxide

**DMBA = 7, 12-dimethylbenz[a]anthracene

*** Positive control dose with p-value <0.05 (Analysis of variance test)

APPENDIX 1 : STUDY PROTOCOL

PBR PRAIRIE BIOLOGICAL RESEARCH LTD.

STUDY PLAN

In Vivo MOUSE (BONE MARROW) MICRONUCLEUS ASSAY SINGLE ADMINSTRATION

1.0 PURPOSE

The purpose of this study is to evaluate the genotoxic potential of a test article identified as Reactive Skin Decontamination Lotion (RSDL) based upon its ability to induce micronuclei in rodent polychromatic erythrocytes (PCEs).

2.0 SPONSOR

2.1 Name: Defence Research Establishment Ltd.

2.2 Address: P.O. Box 4000
Medicine Hat, Alberta
T1A 8K6

2.3 Representative: Dr. Thomas Sawyer

2.4 Sponsor Project No.: W7702-6-R599/B

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: Reactive Skin Decontamination Lotion (RSDL)
NSN4230-21, Lot 5D11449

3.2 Controls:

Negative: Test article solvent

Positive: 7,12-dimethylbenz(a)anthracene (DMBA)

3.3 Determination of Strength, Purity, etc.

The sponsor will be directly responsible for: (a) determination and documentation of the analytical purity and composition of the test article, and (b) the stability and strength of the dosing solutions.

3.4 Test article Sample Retention

The Sponsor will be responsible to retain a reserve sample of the test article.

4.0 TESTING LABORATORY & KEY PERSONNEL

4.1 Name: Prairie Biological Research Ltd.

4.2 Address: 4290-91A Street
Edmonton, Alberta
Canada, T6E 5V2

4.3 Study Director: Ram D. Mehta, Ph.D. P.Biol.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Starting Date: April 15, 1998

5.2 Proposed Experimental Completion Date: May 30, 1998

5.3 Proposed Report Date: June 15 , 1998

6.0 TEST SYSTEM

The micronucleus test is a mammalian *in vivo* test, which detects damage to the chromosomes, or the mitotic apparatus induced by chemicals (Cihak, 1979; Schmid, 1975; Matter and Schmid, 1971). Micronuclei are chromatin bodies considerably smaller than the principal nucleus, consisting of acentric fragments of chromosomes or entire chromosomes which lag behind at the anaphase stage of cell division due to chromosome breakage or mitotic spindle apparatus damage (Schmid, 1975). When an erythroblast develops into an erythrocyte, the main nucleus is extruded and may leave micronuclei in the cytoplasm. Rodent polychromatic erythrocytes (PCEs) extracted from femur bone marrow are used in this assay. Low levels of micronucleated PCEs do occur naturally. The assay is based on an increase in the frequency of micronucleated PCEs in the bone marrow of treated animals. BALB/cCr//Alt BM mice (virus antibody free; 8-9 weeks old) are used for this assay.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The micronucleus assay is conducted by exposing mice treatment groups to a minimum of three dose levels of the test article including the maximum tolerated dose. In addition, both positive and solvent (negative) control groups are employed. The test article is administered once at each dose level. After 24-hour exposure to the test substance, the mice are sacrificed and the bone marrow is obtained from femurs, smeared onto randomly coded slides and stained. At least 2000 PCEs per animal are scored for the presence of micronuclei. The percentage of polychromatic erythrocytes to the total population of erythrocytes is used to assess cytotoxicity of the treatment. The genotoxic potential of the chemical is assessed by comparing the frequency of micronucleated PCEs in the exposed animals with that of the solvent control group.

7.1 Selection of Solvent

Unless the Sponsor has specified the test article solvent, a solubility determination is conducted to determine the maximum soluble concentration (up to a maximum of 5000 mg/ml) in a variety of solvents compatible with the test system. Solvents compatible with this test system, in order of preference include, but are not limited to, isotonic saline, vegetable oil, and dimethylsulfoxide (DMSO). The solvent of choice for the test article is the one selected in order of preference that permits preparation of the highest stock concentration, up to a maximum of 44 mg/ml, (based on the maximum 2000 mg/kg dose for a 22 g test subject and a maximum of 1 ml injection volume). Fresh solutions of the test article are prepared within two hours of injection.

7.2 Dose Selection

For the initial assessment of genotoxicity, mice treatment groups are administered 5 dose levels, the maximum dose being the limit dose 2000 mg/kg, with four additional doses of 1000, 500, 250, and 125 mg/kg If the maximum tolerated dose (MTD) is <500 mg/kg, the test is repeated to include at least two dose levels below the MTD. The 2000 mg/kg dose is the maximum allowable (limit) test article dose that can be administered to the test subjects as agreed upon by a consensus group (Hayashi *et al.*, 1994). This limit dose has also been accepted by OECD. The delivery volume for injections is limited to 1ml of test solution per animal.

7.3 Frequency and Route of Administration

Mice treatment groups are exposed to the test article and control compounds by intraperitoneal injection once. All injections are given under light metofane anesthesia for maximum safety.

7.4 Experimental Animals

Healthy, young adult BALB/cCr//Alt BM mice (8-9 weeks old) are selected and randomized before assignment to treatment and control groups.

7.4.1 Number and Sex

In the assay, 5 female and 5 male subjects are employed per treatment dose level and control group.

7.4.2 Housing and Feeding Conditions

Mice are caged by sex group prior to the experiment for acclimatization. During the course of the experiment, treatment and control groups are caged separately. All animals are observed daily for the duration of the study. Any abnormal behaviour is recorded. Husbandry, temperature, humidity and light cycles are controlled and monitored as dictated by good animal husbandry practices. Diet and drinking water are supplied *ad libitum.*

7.5 Controls

7.5.1 Solvent Control

Two solvent control groups are included, one each for isotonic saline and dimethylsulfoxide (DMSO), the solvents for the test article and positive control respectively.

7.5.2 Positive Control

DMBA (7,12-dimethylbenz(a)anthracene) is used as the positive control and employed once at any of the following three dose levels; 120, 60, and 30 mg/kg. The solvent vehicle for DMBA is DMSO. The delivery volume per animal for the positive control is limited to no more than 0.1 ml solution in order to prevent toxic effects from the solvent (Schmid, 1975). The above dose levels for the positive control are determined by previous historical control data derived from earlier studies (McFee *et al.*, 1989; Shelby *et al.*, 1989).

7.6 Labelling Procedure for Identification

All treatment and control groups are labeled with a permanent-marking pen by the PBR study number and a code system to designate the treatment condition.

7.7 Treatment and Sampling of Test Animals

The test article is administered by intraperitoneal injection. For a single dosage, the treatment group is sampled once after 24 hours. Both positive and negative controls are sampled at approximately the same time as the treatment groups.

7.8 Preparation of Microscope Slides for Analysis

At the sampling time, mice are sacrificed using CO_2 euthanasia and both femurs are removed and cleaned free of muscle. A pair of scissors is then used to make a cut at the proximal end to expose the bone marrow canal. A 3 cc syringe with a 26-gauge needle containing 1 ml of fetal bovine serum (FBS) is inserted into the proximal end. The marrow is exuded from the distal end into a 1.5 ml Eppendorf tube by slowly flushing the syringe. Using the same FBS-marrow mixture and syringe, the above is repeated for the other femur as well. Eppendorf tubes containing samples are then centrifuged at 1000 rpm for ten minutes. The majority of the supernatant is discarded and the cells in the pellet are resuspended carefully by repeated aspiration with a Pasteur pipette. A small drop of the suspension is placed at one end of the slide and spread by pulling the material behind a clean slide held at an angle of about 45°. The slides (4 per animal) are air-dried for at least 48 hours and stained as follows (Schmid, 1975).

Materials

May-Grünwald stain
Phosphate buffer; 0.71 g Na_2HPO_4, 0.68 g KH_2PO_4, 1 litre distilled water, adjusted to pH 6.8
1 part Giemsa stain to 6 parts Phosphate buffer
Staining Dishes
Distilled Water
Mountant, Cytosol™ (Stephens Scientific, Riverdeale, NJ, USA)
Xylene

a) Stain slide in May-Grünwald's Stain (BDH, supplied by VWR, Ontario, Canada) solution for 3 minutes.

b) Stain slide for a further one minute in 1:1 May-Grünwald stain solution (diluted with distilled water).

c) Drain the excess stain from the slide.

d) Stain in 1 part Giemsa(Sigma Chemical CO., Missouri, USA) solution to 6 parts phosphate (pH6.8) buffer for 5 to 10 minutes.

e) Rinse thoroughly in phosphate buffer or distilled water.

f) Air-dry, clear in xylene and mount in Cytosol™.

7.9 Scoring for Micronucleated Polychromatic Erythrocytes

The number of micronucleated PCEs are scored out of a total 2000 PCEs counted for each test subject, and the percentage of PCE's to total number of erythrocytes is used to assess the cytotoxicity to bone marrow cells. As a safeguard against counting artifacts, the number of micronucleated NCEs is recorded per 1000 erythrocytes. The number of micronucleated NCEs should be less than 5. If micronuclei are distributed in equal proportion over both types of erythrocytes (PCE and NCE), artifacts must be suspected (Schmid, 1975).

For analyzing slides, the percentage of PCEs among total erythrocytes should not be less than 20% of the control (Hayashi *et al.*, 1994). Slides are randomly numbered by an individual not involved in the scoring process. Slides are observed under 40X objective and/or under oil immersion with high-powered objective (60X or 100X). Due to the staining, PCEs appear blue

while NCEs appear orange in color. Micronucleated cells usually appear quite similar and mostly contain only one micronucleus. Micronuclei will appear as round, darkly stained regions within erythrocytes (Schmid, 1975).

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Solvent control

The frequency of micronucleated PCEs in the solvent control should contain nearly the same frequency of naturally occurring micronucleated PCEs (approximately 1-2 micronucleated PCEs per 1000 PCEs scored in the negative control group, Shelby *et al.*, 1989). Alternatively, historical negative control data is used to determine if the concurrent control response is acceptable.

8.2 Positive Control

The frequency of micronucleated PCEs in the positive control group must be statistically increased ($p \leq 0.05$, ANOVA) relative to the solvent control.

9.0 EVALUATION OF TEST RESULTS

The data presented for each treatment and control group include micronucleated PCEs/1000 PCEs and percent PCEs of total erythrocytes. Statistical analysis of the frequency of micronucleated PCEs is performed using the Analysis of Variance (ANOVA) test. In the event of a positive response by this test, the Cochran-Armitage trend test is used to measure dose responsiveness.

A positive genotoxic effect of a tested article is based upon a statistically significant dose-related increase ($p \leq 0.05$, ANOVA) in the number of micronucleated PCEs relative to the solvent control. A significant increase at any one dose level only with no dose response is considered equivocal.

A test article showing no statistically significant increase in micronucleated PCEs is considered to be non-mutagenic in this system.

Positive results in the micronucleus assay indicate that a test article induces micronuclei, which could have resulted from chromosomal or mitotic apparatus damage.

10.0 REPORT

A report of the results of the study is prepared by Prairie Biological Research Ltd., describing accurately all methods used for generation and analysis of data.

Results presented include, but not be limited to:

- species and strain of mice used;
- age and weight of the mice used, and the number of mice for each sex in experimental and control groups;
- test conditions: test article toxicity data, description of the treatment and sampling schedule, dose level(s) employed, route of administration; positive, and negative controls;
- criteria for identification of micronucleated polychromatic erythrocytes;
- dose/response relationship, where possible;
- discussions of results;
- conclusions;
- summary, and;
- quality control report, if requested.

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be kept in the archives maintained by Prairie Biological Research Ltd. 4290-91A Street, Edmonton, Alberta, Canada in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens will be archived in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written in accordance with OECD Guideline 474 (The OECD Guidelines for Testing of Chemicals, May 1983).

This study will be performed in compliance with the provisions of the OECD Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? YES

If so, to which agency or agencies? HEALTH CANADA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13. 0 REFERENCES

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14.0 APPROVAL

Thomas W. Sawyer June 6/98

Sponsor Representative (Signature) Date

THOMAS W. SAWYER

Name (Print or Type)

Aug 05/97

PBR Study Director (Signature) Date

Dr. Ram D. Mehta

Name (Print or Type)

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