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TOX R 003868

Glyphosate / Tox

RELEASABLE

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JUL 6 1984

OFFICE OF PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Mutagenicity studies with glyhosate
Caawell #: 661A

To: Robert Taylor
Product Manager #25
Registration Division (TS-767)

THRU: Christine E. Chaisson
Head, Review Section IV
Toxicology Branch
Hazard Evaluation Division (TS-769)

FROM: William Dykstra, Ph.D. William Dykstra 6/12/84
Toxicology Branch
Hazard Evaluation Division (TS-769) 7/6/84

The DER's submitted by Dynamac on the mutagenicity studies with glyphosate have been reviewed by me and I agree with Dynamac evaluations of these studies. The reviewed studies are listed below:

1. gene mutation (CHO/HGPRT)
2. DNA repair (rat hepatocyte)
3. in vivo bone marrow cytogenetics
4. Range finding study for the effects of glyphosate on rat bone marrow cells
5. Pharmacokinetics

Caswell #: 661A

EPA: 68-01-6561
TASK: 61
May 29, 1984

DATA EVALUATION RECORD

GLYPHOSATE

Pharmacokinetics

CITATION: Ridley, W.P. A Study of the plasma and bone marrow levels of glyphosate following intraperitoneal administration in the rat. An unpublished report (Study No. 830109) prepared by the Environmental Health Laboratory, Monsanto Company for Monsanto Company, dated 10-24-83.

REVIEWED BY:

Nicolas P. Hajjar, Ph.D.
Senior Scientist
Dynamac Corporation

Signature: Nicolas P. Hajjar
Date: May 29, 1984

I. Cecil Felkner, Ph.D.
Mgr. Genetic Toxicology Dept.
Dynamac Corporation

Signature: I. Cecil Felkner
Date: 5-29-84

Cipriano Cueto, Ph.D.
Department Director
Dynamac Corporation

Signature: Cipriano Cueto
Date: 5-29-84

APPROVED BY:

William Dykstra, Ph.D.
EPA Scientist

Signature: William Dykstra
Date: 5/31/84

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DATA EVALUATION RECORD

STUDY TYPE: Pharmacokinetics study in rats.

CITATION: Ridley, W.P. A Study of the plasma and bone marrow levels of glyphosate following intraperitoneal administration in the rat. An unpublished report (Study No. 830109) prepared by the Environmental Health Laboratory, Monsanto Company for Monsanto Company, dated 10-24-83.

ACCESSION NUMBER: 251737.

LABORATORY: Environmental Health Laboratory, Monsanto Company, St., Louis MO.

QUALITY ASSURANCE STATEMENT: Present, signed and dated October 20, 1983.

TEST MATERIAL: The test material used was a mixture of [^{14}C-methyl] N-(phosphonomethyl) glycine sodium salt, and the protonated acid of the unlabeled test material. Radiolabeled glyphosate had a specific activity of 5 mCi/mmole and a radiochemical purity of 98 percent, whereas the purity of unlabeled glyphosate was 98.7 percent.

PROTOCOL:

1. Nine male and 9 female Crl:CD BR rats were obtained from Charles River Laboratories, Wilmington, MA. The animals were acclimatized to laboratory conditions for a period of 7 days, then placed in stainless steel metabolism cages for four days prior to dosing, and for the duration of the study. Purina Rat Chow and water were available ad libitum. The rats were fasted for a period of 22-24 hours prior to dosing. The animals were 8-9 weeks old and the average weight of the male was 264 gm and of females 186.0 gm at dosing.

2. A dosing solution containing 12.25 gm and 5.487 mg of the unlabeled and [^{14}C] labeled glyphosate, respectively, in Hank's Balanced Salt Solution was prepared. The final pH of this dosing solution was adjusted to 7.18 in a final volume of about 70 ml. The specific activity was determined to be 29.8 dpm/µg glyphosate based upon the protonated acid weight.

3. The rats were dosed by intraperitoneal injection and the precise amount administered was calculated from the difference in weight of the syringe and needle before and after dosing. The males received 1150 ± 3.3 mg/kg containing 9.013 ± 0.09 x 10^6 dpm and the females received 1150 ± 7.5 mg/kg containing 6.394 ± 0.20 x 10^6 dpm of test material.

4. Blood samples were collected by orbital sinus puncture from six males and six females 15 minutes after dosing. Additional samples were collected from three animals of each sex at approximately 0.5, 1, 2, 4, 6, and 10 hours after dosing. No more than 3 blood samples were collected from any one rat during that period. The whole blood samples were centrifuged and 0.1 ml of plasma were radioassayed in 15 ml of Instagel.

   At approximately 0.5, 4, and 10 hours after dosing, three males and three females were sacrificed by cervical dislocation, and the bone marrow from both the right and left femur of each animal collected. The bone marrows were weighed, digested in soluene-350 at 50° C for 5-6 hours then allowed to sit at room temperature overnight. The samples were decolorized, 15 ml of Dimilune-30 added, and then were allowed to equilibrate to temperature and light in the liquid - sintillation counter prior to counting. Counting efficiencies were determined by means of an external standard and corrections were made for quenching. The results were reported both in dpm/gm tissue and µg glyphosate equivalents/gm tissue (ppm).

RESULTS:

A maximum concentration of radiolabeled material in male and female plasma was noted 30 min after ip administration. This corresponded to a level of 1867 ± 160 ppm and 2019 ± 83 ppm of glyphosate and/or its metabolites in males and females, respectively. The concentration of radiolabel in plasma decreased subsequently. Linear regression analysis of the data indicated that the decrease in radioactivity occurred with a half-life of approximately 0.99 and 1.0 hours in males and females, respectively.

The concentration of radiolabel measured in the bone marrow 30 minutes after administration was 267 ± 31 and 413 ± 39 ppm for males and females, respectively. Assuming first order kinetics, the decrease in radioactivity occurred with a half-life of 7.6 and 4.2 hours for the males and females, respectively.

DISCUSSION:

The study was conducted in order to "confirm that glyphosate" reaches the bone marrow following intraperitoneal injection. The amounts reaching the bone marrow were considered by the authors sufficient to justify cytogenetic evaluation. However, identification of the radiolabeled material in the bone marrow was not conducted, and only 0.0044 (13 µg/rat) and 0.0072

(15.4 µg/rat) percent of the dose administered intraperitoneally reached the bone marrow in males and females, respectively.

CONCLUSIONS:

Thirty minutes following intraperitoneal administration of [^{14}C]-glyphosate to male and female Charles River rats at 1150 mg/kg, the concentration of radiolabel present in bone marrow was 267 ± 31 and 413 ± 39 ppm, respectively (equivalent to 0.0044 and 0.0072 percent of the dose). Assuming first order kinetics the decrease in radioactivity occurred with a half-life of 7.6 and 4.2 hours for the males and females, respectively. Similarly, the half-lives of radiolabel in the plasma were approximately 1 hour in both sexes.

CORE CLASSIFICATION: Acceptable.

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EPA: 68-01-6561
TASK: 61
May 29, 1984

Caswell #: 661A

DATA EVALUATION RECORD

GLYPHOSATE

Mutagenicity (Gene Mutation, CHO/HGPRT)

CITATION: Li, A.P., Kier, L.D., and Folk, R.M. CHO/HGPRT gene mutation assay with glyphosate. Unpublished Study No. ML-83-155, Monsanto Project No. 830079 prepared by Monsanto Co. Environmental Health Laboratory for Monsanto Company. Dated 10-20-83.

REVIEWED BY:

I. Cecil Felkner, Ph.D.
Mgr. Genetic Toxicology Dept.
Dynamac Corporation

Signature: I. Cecil Felkner
Date: 5-29-84

William L. McLellan, Ph.D.
Senior Scientist
Dynamac Corporation

Signature: James R. Plautz for
Date: May 29, 1984

Cipriano Cueto, Ph.D.
Department Director
Dynamac Corporation

Signature: Cipriano Cueto
Date: 5-29-84

APPROVED BY:

W. Dykstra, Ph.D.
EPA Scientist

Signature: William Dykstra
Date: 5/31/84

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DATA EVALUATION RECORD

STUDY TYPE: Mutagenicity (Gene Mutation; Mammalian in vitro).

CITATION: Li, A.P., Kier, L.D., and Folk, R.M. CHO/HGPRT gene mutation assay with glyphosate. Unpublished Study No. ML-83-155, Monsanto Project No. 830079 prepared by Monsanto Co. Environmental Health Laboratory for Monsanto Company. Dated 10-20-83.

ACCESSION NUMBER: 251737.

LABORATORY: Monsanto Company Environmental Health Laboratory 645 S. Newstead, St. Louis, Missouri 63110.

QUALITY ASSURANCE STATEMENT: Present, signed, and dated 10/20/83.

TEST MATERIAL: The test material was identified as Glyphosate, a white powder, Lot No. XHJ-64, submitted to Environmental Health Laboratory (EHL) and indicated to be 98.7 percent pure. It was assigned Sample No. I830044 by EHL and stored at room temperature.

MATERIALS AND METHODS:

Preparation of the Test Material: Stock solutions of glyphosate were made in Ham's F12 V medium (K.C. Biological) and neutralized to pH 7.0-7.4 with 1N NaOH until a clear solution was obtained. Test solutions of different concentrations were made by diluting the stock with Ham's F12 V medium on the testing day.

Controls: The positive controls were benzo(a)pyrene (B(a)P) for S9 activation and ethyl methane sulfonate (EMS). Both were obtained from Sigma Chemical Co., St. Louis, MO.

Cell Line: The cell line was the Chinese hamster ovary line, K_1BH_4 originally obtained from A.W. Hsie[1] at Oak Ridge National Laboratory. Cultures of these cells were maintained in Ham's F12 medium supplemented with 10 percent newborn calf serum as logarithmically growing monolayers. Growth was at 37.5° C ± 2° C at a relative humdity of 95 percent under 5 percent CO_2.

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[1] Hsie, A.W., Li, A.P. and Machanoff, R. 1977. Mutant. Res. 45:333-342.

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Cytotoxicity: At 18 - 24 hr before dosing, 0.5×10^6 cells were seeded in 25 cm^2 plastic culture flasks; on the day of dosing the growth medium was replaced with 2.5 ml of Ham's F12 medium containing neither S9 or serum. An equal volume of this medium containing 2x concentrations of the test material was added; the mixture was then incubated for 3 hr at 37.5° C ± 2° C, and then the dosing medium was removed and the cells washed with 5 ml of Hank's balanced salt solution. The cells were removed by trypsinization and scored (3 samples of 200 cells plated for assessment of cloning efficiency). All plates were then reincubated for 7 to 9 days and colonies which developed were fixed with 70 percent methanol, stained by 10 percent Giemsa and hand-scored. To calculate cloning efficiency (CE) and relative survival (RS), the following expressions were used.

Mutagenesis Assays: The K_1BH_4 cells were plated the day before dosing with the test material, positive controls, or negative solvent controls. The procedure described for the cytotoxicity assay was followed, except that an additional 10^6 cells/10 ml were subcultured in hypoxanthine-free Ham's F12 medium, supplemented with 10 percent dialyzed newborn calf serum. Subculturing was carried out every 2-3 days, followed by the 7 to 9 days period allowed for phenotypic expression. After phenotypic expression, selective medium[3] (hypoxanthine-free Ham's F12 medium supplemented with 10 µM 6-thioguanine (6TG) and 5 percent dialyzed newborn calf serum) was used to select for the 6TG-resistant mutant clones. A total of 10^6 cells were assessed for mutant development, using five 100 mm plates, each containing 2×10^5 cells in 8 ml of selective medium. After incubation for 8 to 12 days, colonies were fixed, stained and scored. The cloning efficiency was determined as previously described. Using the expression which follows, a mutation frequency (M.F.)[2] was calculated.

$$C.E. = \frac{\text{no. colonies developed}}{\text{no. cells plated}}$$

$$R.S.^2 = \frac{C.E.\ (\text{dosed})}{C.E.\ (\text{negative control})}$$

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[2] R.S. was used to express cytotoxicity to the cell line;

$$M.F. = \frac{\text{No. mutant colonies}}{\text{No. cells plated}} \times \frac{1}{CE}$$

[3] Li, A.P., Dahl, A.R., and Hill, J.O. 1982. Toxicol. Appl. Pharmacol. 64:482-485.

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Experimental Design: Two experiments were used to determine the mutagenicity of glyphosate. In Experiment A, 3 doses of test material (5, 17.5 and 22.5 mg/ml) estimated to yield 100, 50, and 10 percent survival were used in conjunction with S9 concentrations of 0, 1, 2, 5, and 10 percent. This test was to determine an initital estimate of mutagenic potential at an optimum S9 concentration. In Experiment B, 5 doses of test material (2, 5, 10, 10, and 20 mg/ml) estimated to yield 100, 70, 50, 20, and 10 percent survival were used. Since no mutagenicity were observed in Experiment A, no optimum S9 concentration was determined, therefore a 5 percent S9 concentration was chosen as representative.

Metabolic Activation: The Aroclor 1254-induced rat liver S9 fraction was purchased from Litton Bionetics and was applied to cultures in varying amounts relative to the S9-cofactors. The S9-cofactor mix contained, in addition to different amounts of S9 protein, 50 mM sodium phosphate (pH 7.5), 4 mM NADP, 5 mM glucose-6-phosphate, 30 mM KCl, 10 mg $MgCl_2$ and 10 mM $CaCl_2$. One ml of the S9-cofactor mix was added to 4 ml of medium for the cytotoxicity or mutagenicity assays.

Statistics: The method of Snee and Irr[4] was used to analyze the mutagenicity data; mutant frequency values were transformed using $Y = (X + 1)^{0.15}$ where Y is the transformed mutant frequency and X is the observed mutant frequency. Treatment data were compared to the solvent control data by the Student's t-test. Determination of dose-response relationships as linear, quadratic or higher-order was possible by Snee/Irr[4] analysis, and a program developed by Irr (DuPont) was incorporated into Monsanto's computer system.

RESULTS:

Cytotoxicity Assay: Approximately 90 percent lethality occurred at glyphosate doses between 20 and 25 mg/ml. Hence, 22.5 mg/ml and 25 mg/ml were the high doses in Exp. A and Exp. B, respectively.

In the presence of varying S9 concentrations, mutant frequencies x 10^{-6} in the negative (medium) controls were: 7.4 (0 percent), 5.9 (1 percent), 7.1 (2 percent), 4.4 (5 percent) and 9.1 (10 percent). At glyphosate doses of 5, 17.5 and 22.5 mg/ml, none of the mutant frequencies were significantly different from the control values. However, with 1 percent S9, the mutant frequencies (f) x 10^{-6} and p-values[5] at varying glyphosate doses were 5 mg/ml ($f = 4.3$, $p = 0.6695$), 17.5 mg/ml ($f = 11.6$, $p = 0.3470$) and 22.5 mg/ml ($f = 19.1$, $p = 0.1796$).

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[4] Snee, R.D. and Irr, J.D. 1981. Mutat. Res. 85:77-93.

[5] Probability to be the same as control by the method of Snee and Irr (1981).

In the absence of S9 at various glyphosate doses, the mutant frequencies x 10^{-6} were: 2 mg/ml (f = 3.5, p = 0.1789), 5 mg/ml (f = 11.3, p = 0.9994), 10 mg/ml (f = 10.8, p = 0.6314), 15 mg/ml (f = 20.8, p = 0.5318), and 20 mg/ml (f = 10.1, p = 0.8695).

At concentrations ranging from 5 to 25 mg glyphosate/ml in the presence of 5 percent S9, the mutant frequencies x 10^{-6} varied from 5.7 (p = 0.8536) to 14.9 (p = 0.4811) compared to a control values of 7.7 x 10^{-6}.

The mutant frequency for treatment with 200 µg EMS/ml averaged 150 x 10^{-6} compared to the negative control values of 9.4 x 10^{-6}. Using 2 µg B(a)P/ml in varying amounts of S9 (expressed in percentage), the average mutant frequencies were (353 x 10^{-6}) 1 percent, (186 x 10^{-6}) (2 percent), (99 x 10^{-6}) (5 percent) and (95 x 10^{-6}) (10 percent).

DISCUSSION:

The authors concluded that glyphosate was cytotoxic to CHO cells at high concentrations, i.e, > 10 mg/ml, but that significant mutagenicity at the HGPRT gene locus was not produced.

Our assessment is that the authors have assayed the test material in an appropriate dose range without or with S9 activation at several concentrations, and their data showed no significant mutagenicity. Using 1 percent S9, however, a nonsignificant dose related increase in the mutant frequency was seen in the glyphosate dose range of 5 to 22.5 mg/ml.

CONCLUSIONS:

The test material, 98.7 percent pure glyphosate, did not produce a significant mutagenic response either with or without S9 activation under the conditions of this study.

CLASSIFICATION: Acceptable.

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EPA: 68-01-6561
TASK: 61
June 4, 1984

Caswell file No: 661A

DATA EVALUATION RECORD

GLYPHOSATE

Mutagenicity (Range-Finding Study)

CITATION: Li, A.P. Effects of glyphosate on rat bone marrow cells. An unpublished report (study no. ML-83-160) prepared for Monsanto Agricultural Products Company by Environmental Health Laboratory, Monsanto Co. St. Louis, MO. Dated October 21, 1983.

REVIEWED BY:

William McLellan, Ph.D.
Senior Scientist
Dynamac Corporation

Signature: William L. McLellan
Date: 6 June 1984

I. Cecil Felkner, Ph.D.
Mgr. Genetic Toxicology Dept.
Dynamac Corporation

Signature: Ira Cecil Felkner
Date: 6-6-84

Cipriano Cueto, Ph.D.
Department Director
Dynamac Corporation

Signature: Cipriano Cueto
Date: 6-6-84

APPROVED BY:

William Dykstra, Ph.D.
EPA Scientist

Signature: William Dykstra
Date: 6-11-84

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DATA EVALUATION RECORD

STUDY TYPE: Mutagenicity (range-finding study).

CITATION: Li, A.P. Effects of glyphosate on rat bone marrow cells. An unpublished report (study no. ML-83-160) prepared for Monsanto Agricultural Products Company by Environmental Health Laboratory, Monsanto Co. St. Louis, MO. Dated October 21, 1983.

ACCESSION NUMBER: 251737.

LABORATORY: Environmental Health Laboratory, Monsanto Co. St. Louis, MO.

QUALITY ASSURANCE STATEMENT: Present, signed and dated October 21, 1984.

TEST MATERIAL: The test material was identified as glyphosate (EHL sample No. T830044) a white powder having a purity of 98.7 percent.

MATERIAL AND METHODS:

Preparation of Test Material: A stock solution of 100 mg/ml was prepared by suspending glyphosate in Hank's balanced salt solution (HBBS) and adjusting the pH to 7.5 with sodium hydroxide. Dilutions of the stock solution in HBBS were freshly prepared to yield solutions of 20, 40, 60, and 80 mg/ml.

Controls: Hank's buffered salt solution 10 ml/kg was used as the vehicle control.

Animals: The animals used in the study were male and female Sprague-Dawley rats [CD(SD)BR] from Charles River Breeding laboratories. The animals were approximately 10 weeks old at the time of test material administration; the males weighed 264-299 g and females weighed 179-202 g. Water and Purina Laboratory Chow were provided ad libitum except for a 14-24 hours fasting period just prior to dosing. Animals were maintained in individual cages in rooms maintained at 70-74 ° F and a relative humidity of between 25 and 60 percent. The rooms had 12-hour light/dark cycles.

Experimental Design: Rats (4/sex/group) were fasted overnight and then injected intraperitoneally with 10 ml of HBSS containing glyphosate. The final doses in the groups were 0, 200, 400, 600, 800, and 1,000 mg/kg. Four hours after administration of glyphosate or vehicle control, 4 mg/kg colchicine were administered ip, and two hours later the animals were sacrificed by CO_2 asphyxiation and by severance of their spinal cords.

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Preparation of Bone Marrow Cells: Bone marrow was separated from each femur into a 5 ml plastic syringe containing 2 ml HBSS. The contents were added to plastic centrifuge tubes containing 5 ml HBSS and incubated at 37° C until they were prepared for analysis.

Cell Viability Determination: An aliquot of the cell suspension was stained with acridine orange and ethidium bromide (EPL SOP L-58081-G004). Slides were prepared, and approximately 100 cells/animal at each dose level were examined by fluorescent microscopy. Since viable cells take up acridine orange and appear green and non-viable cells take up ethidium bromide and appear orange, the viable cells could be quantitated.

Determination of Mitotic Index: The cell suspensions were centrifuged, the pellet suspended in 1 ml 0.075 M KCl at 37° , and an additional 3 ml of KCl added. After 30 min incubation at 37° C, 1 ml Cornoy's fixative was added (methanol-glacial acetic acid 3/1, v/v). The cells were then pelleted, 5 ml of fresh fixative added, and the cell suspension stored at 4° C. One to 2 drops of cell suspensions were fixed on slides and stained 15-20 min with 2 percent Geimsa solution. The slides were then rinsed and air dried.

Approximately 500 cells/slide were counted to quantitate metaphase and non-metaphase cells. The mitotic index (ratio of mitotic cells to the total number of cells counted) was calculated from this data.

RESULTS:

Viability: Viability ranged from 95.8 to 98.5 percent in males and from 93.2 to 97.8 percent in females groups. Solvent control values were 96.8 percent for males and 98.5 percent for females. Hence, the author assessed that glyphosate at doses up to 1000 mg/kg, had no effect on cell viability.

Mitotic Index: The mitotic index for control males was 0.028 and for control females 0.032 (average of 4 animals). The mitotic index for males dosed at 800 mg/kg was slightly but significantly ($p = 0.05$) increased over controls (0.045). In other dose groups of males the mitotic indices were similar to controls (0.030-0.037).

In glyphosate-treated females the mitotic index was slightly lower at 400 mg/kg (0.019, $p = 0.036$) than in controls, but there were no significant differences at other dose levels (mitotic indices ranged from 0.024-0.039).

DISCUSSION:

The authors concluded that doses up to 1,000 mg/kg glyphosate could be used to score the potential cytogenetic effect in vivo in rats since there was no significant reduction in the mitotic index. However, it was noted

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that the highest dose used in the range finding study was the maximum dose that could be effectively administered based on solubility of the test compound and the volume that could be injected ip into rats.

This reviewer agrees the conclusions. The 4 percent reduction in mitotic index in 400 mg/kg females may not be compound related, since there was no dose-response relationship. Furthermore, such a slight lowering of the mitotic index would not affect the cytogenetic study. In selecting doses for in vivo cytogenicity testing, the limit should be based on solubility; the maximum dose will be inadequate if cytotoxic responses are the basis for selecting the maximum dose.

CONCLUSIONS:

Glyphosate (at dose levels between 200 - 1,000 mg/kg) did not cause any loss of viability in vivo in rat marrow cells. There was a slight decrease (4 percent) in mitotic index in females at 400 mg/kg but not at higher doses, and no effects in males. Therefore 1,000 mg/kg can be tolerated in an in vivo cytogenicity assay in rats.

CLASSIFICATION: Acceptable.

EPA: 68-01-6561
TASK: 61
June 4, 1984

Caswell # 661A

DATA EVALUATION RECORD

GLYPHOSATE

Mutagenicity
(In vivo bone marrow cytogenetics)

CITATION: Li, A.P. In vivo bone marrow cytogenetics study of glyphosate in Sprague-Dawley rats. An unpublished report (study No. ML-83-236) prepared for Monsanto Agricultural Products Company by Environmental Health Laboratory, Monsanto Co., St. Louis, MO. Dated October 20, 1983.

REVIEWED BY:

William L. McLellan, Ph.D.
Project Scientist
Dynamac Corporation

Signature: William L. McLellan Ph.D.
Date: 6 June 1984

I. Cecil Felkner, Ph.D.
Mgr. Genetic Toxicology Dept.
Dynamac Corporation

Signature: I. Cecil Felkner
Date: 6-6-84

Cipriano Cueto, Ph.D.
Department Director
Dynamac Corporation

Signature: Cipriano Cueto
Date: 6-6-84

APPROVED BY:

W. Dykstra, Ph.D.
EPA Scientist

Signature: William Dykstra
Date: 6/11/84

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DATA EVALUATION RECORD

STUDY TYPE: Bone marrow cytogenetics in vivo (mutagenicity).

CITATION: Li, A.P. In vivo bone marrow cytogenetics study of glyphosate in Sprague-Dawley rats. An unpublished report (study No. ML-83-236) prepared for Monsanto Agricultural Products Company by Environmental Health Laboratory, Monsanto Co., St. Louis, MO. Dated October 20, 1983.

ACCESSION NUMBER: 251737.

LABORATORY: Environmental Health Laboratory. Monsanto Co., St. Louis, MO.

QUALITY ASSURANCE STATEMENT: Present and dated October 21, 1983.

TEST MATERIAL: The test material was identified as glyphosate (EHL sample No. TA830044), a white powder having a purity of 98.7 percent.

MATERIALS AND METHODS:

Preparation of Test Material: The test material was suspended in Hank's buffered salt solution (HBSS) at a concentration of 100 mg/ml and was neutralized to pH 7.0. Solutions were prepared no more than 24 hours before use. A volume of 10 ml/kg was used for ip dosing.

Controls: Cyclophosphamide, the positive control, was dissolved in HBBS (25 mg/ml). One ml/kg (25 mg/kg) was used for dosing. A volume of 10 ml/kg HBSS, the solvent control, was administered ip to control animals.

Animals: The animals used in the study were male and female Sprague-Dawley rats [CD(SD)BR] from Charles River Breeding Laboratories. which were approximately 9 weeks old at the time of dosing. Water and Purina Laboratory Chow were provided ad libitum except at the fasting period 14-24 hours prior to dosing. Animals were maintained in individual cages in rooms maintained at 70-74° F, a relative humidity of 35-60 percent and on a 12-hour light/dark cycle.

Experimental Design: Rats (18/sex/group) were fasted for 14-24 hours and then injected intraperitoneally with (i) solvent (HBBS), (ii) glyphosate (1 g/kg) or (iii) cyclophosphamide (25 mg/kg). Six animals of each sex and group (control, test group, and positive control group) were sacrificed at 6, 12, and 24 hours. Two hours before sacrifice each rat was injected ip with 2 mg/kg colchicine. Sacrifice was by CO_2 asphyxiation and spinal cord severance.

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Preparation of Bone Marrow Cells: Marrow was aspirated from each femur into a 5 ml syringe containing 2 ml HBSS. The contents were added to 5 ml of HBSS in a plastic centrifuge and maintained at 37° C until the slides were prepared.

Slide Preparation: The cells were pelleted by centrifugation (700 x g, 10 min), suspended in 1 ml of hypotonic KCl (0.075 M) and incubated at 37° C for 30 min. The cells were then fixed with an equal volume of Corney's solution (3/1, v/v methanol glacial acetic acid). The pellet was resuspended in 4 ml of fresh cold fixative and one to two drops of each suspension placed on a clean wet slide. The slides were air dried, stained for 15-20 min in a 2 percent Giemsa solution, rinsed with water, and again air dried.

Scoring of Slides: The slides were scored by 3 persons in Dr. Julian Preston's laboratory (Oak Ridge National Laboratory). Approximately 50 mitotic cells (300/treatment) were scored for chromosomal aberrations. The following data were recorded:

Number of cells scored
Number of cells with a normal chromosome numbers
Chromosome-type aberrations (dicentric, ring, deletions)
Chromatid-type aberrations (chromatid deletions, isochromatid deletions, interchanges, intrachanges)
Achromatic lesions (gaps)
Number of aneuploid cells
Location of cells with aberrations

Statistical Analysis: The student's t-test was used for data analysis, in which dosing with the test material or positive control was compared to the solvent control.

RESULTS:

The frequency of chromatid-type aberrations was low in both solvent control and glyphosate treated group (Table 1).

TABLE 1. Chromatid-Type Aberrations

Time	Control	Glyphosate
6 hours	7/588[a]	6/544
12 hours	2/558	5/564
24 hours	4/555	7/479

[a] Number of aberrations/number of mitotic cells examined. Data for males and females was combined by this reviewer.

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There were no chromosomal-type aberrations in marrow cells in either solvent controls or the glyphosate group.

The positive control group was scored only at 24 hours. Because of extreme cytotoxicity only 21 cells were available for scoring in females and 256 cells in males. There was a high incidence of chromatid type aberrations (231/277 chromatid deletions, 71/231 chromatid interchanges, and 6/277 chromatid intrachanges).

DISCUSSION:

The authors concluded that glyphosate had no clastogenic effect on bone marrow cells under the conditions of the assay. Statistical analysis supported their results; however, there was a slight but non-significant increase in achromatic gaps (not considered aberrations) in the glyphosate treated group. Our assessment is that the authors' data support their conclusions. The assay sensitivity was supported by appropriate response from the positive control relative to the solvent control. The highest dose level of glyphosate used was limited to the test compound's solubility and by the volume that could be injected into a rat. A range finding study (Study No. 830082) used to set the maximum dose presented data on cytotoxicity for levels of test compound up to 1000 mg/kg. However, there was no concurrent cytotoxicity data. Moreover, only a single concentration of test compound was tested.

CONCLUSIONS:

Glyphosate did not induce significant clastogenic effects in rats under conditions of the study which was limited to the assay of a single dose level of 1000 mg/kg. Cylophosphamide at 25 mg/kg caused a high significant number of chromosomal aberrations demonstrating the sensitivity of the assay. Under the conditions of the study, glyphosate did not cause any fatalities or other signs of toxicity.

CLASSIFICATION: Unacceptable since dose-response data were not available (only a single dose was tested) and concurrent cytotoxicity data were not available.

CONFIDENTIAL BUSINESS INFORMATION
DOES NOT CONTAIN
NATIONAL SECURITY INFORMATION (EO 12065)

EPA: 68-01-6561
TASK: 61
June 4, 1984

To: Caswell File 661A

DATA EVALUATION RECORD

GLYPHOSATE JJN-1020

DNA Repair (Rat Hepatocyte)

CITATION: Williams, G.M. The hepatocyte primary culture/DNA repair assay on compound JJN-1020 (Glyphosate) using rat hepatocytes in culture. Unpublished Report No. AH-83-181 prepared by Naylor Dana Institute for Disease Prevention, American Health Foundation. Dated 10-21-83.

REVIEWED BY:

I. Cecil Felkner, Ph.D.
Mgr. Genetic Toxicology Dept.
Dynamac Corporation

Signature: Ira Cecil Felkner
Date: 6-6-84

William L. McLellan, Ph.D.
Senior Scientist
Dynamac Corporation

Signature: William L. McLellan
Date: 6 June 1984

Cipriano Cueto, Ph.D.
Department Director
Dynamac Corporation

Signature: Cipriano Cueto
Date: 6-6-84

APPROVED BY:

W. Dykstra, Ph.D.
EPA Scientist

Signature: W. Dykstra
Date: 6-11-84

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DATA EVALUATION RECORD

STUDY TYPE: DNA-repair (Rat Hepatocytes).

CITATION: Williams, G.M. The hepatocyte primary culture/DNA repair assay on compound JJN-1020 (Glyphosate) using rat hepatocytes in culture. Unpublished Report No. AH-83-181 prepared by Naylor Dana Institute for Disease Prevention, American Health Foundation. Dated 10-21-83.

ACCESSION NUMBER: 251737.

LABORATORY: Naylor Dana Institute for Disease, Prevention, American Health Foundation, 1 Dana Road, Valhalla, N.Y. 10595.

QUALITY ASSURANCE STATEMENT: Although the report stated that a quality assurance review was prepared for the study, a signed and dated report was not present.

TEST MATERIAL: The test material was identified as JJN-1020 of lot No XHJ-64, provided by Monsanto Company. Its purity was not specified and it was reported to be soluble in 0.1N NaOH.

MATERIALS AND METHODS

Hepatocyte Primary Cultures (HPC): The cells used in the study were freshly prepared hepatocytes from adult male F-344 rats. The hepatocytes were obtained by a modification of the procedure developed by Williams et al.[1] The rats were anesthetized with 50 mg/kg sodium nembutal and perfused with sterilized Solutions I and II by means of a sterile peristalic pump. Solution I contained 0.5 mM ethyleneglycol-bis-(B-aminoethyl ether) N-N'-tetracetic acid (EGTA) in calcium and magnesium free Hank's balanced salt solution, buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) adjusted to pH 7.35, using 1N NaOH. Solution II contained 100 unit/ml of type 1 collagenase in Williams' medium E (WME) buffered by 10 mM Hepes (pH 7.35).

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[1] Williams, G.M., Bermudes, E., and Scaramuzzino, D. 1977. Vitro 13:809-817.

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Perfusion was through the portal vein via a 21 gauge butterfly needle using a flow rate of 8 ml/min at 37° C for Solution I. At the start of perfusion with Solution I, the process of ligating the infrahepatic vena cava was completed and the vein severed distally so that the perfusate ran to the waste container. When the liver was uniformly blanched, a cannula was inserted into the thoracic inferior vena cava so that the perfusate could be collected by means of this return cannula; then the flow rate was increased to 40 ml/min for 2.5 min. The perfusion with Solution I was followed by perfusion with sterile Solution II at a flow rate of 20 ml/min at 37° C for 10 min. (not recirculating the return perfusate). The liver was covered with sterile gauze and warmed by a 40W light bulb.

The perfused liver was removed, trimmed of extraneous fat and connective tissue into a Petri dish with warm WME under sterile conditions. The tissue was then transferred to fresh Solution II. After opening the liver at numerous points on the inferior surface and removal of the capusule, the cells were detached by "gentle combing with a stainless steel comb and shaking off loose cells." After complete combing, the fibrous plug was discarded and 25 ml aliquots of the hepatocyte suspension were pipetted into 50 ml centrifuge tubes, adjusting the volume to 50 ml with WME, supplemented with 10 percent calf serum and 50 µg/ml gentamycin (WMES). The cell suspension was centrifuged for 2.5 min at 50 x g, and the cell pellet was resuspended in WMES. A 20-fold dilution of the cell suspension was prepared and 0.5 ml of this diluted suspension was added to 0.1 ml of 0.4 % to trypan blue so that viability (differential staining) could be assessed using a hemocytometer. The author stated that cell yields of approximately 2.0×10^8 per 100 g body weight and hepatocytes viabilities of about 90% were usually obtained.

For the HPC/DNA studies, 5×10^5 cell/ml WMES were seeded immediately onto 25 mm round coverslips in 35 mm 6 well dishes under 5% CO_2, humidified in an incubator at 37° C. The coverslips were washed with 1 ml WME two hours after seeding so that only the attached viable cells remained.

Preparation of Test Material: The test materal, JJN-1020, was solubilized in 0.1 N NaOH at a maximum solubility of 12.5 mg/ml. Serial dilutions of the stock solution were made in 0.1 N NaOH and 20 µl of the stock solutions were added to 2 ml of assay medium so that the final test concentrations were 1.25×10^{-1}, 6.25×10^{-2}, 1.25×10^{-2}, 6.25×10^{-3}, 1.25×10^{-3}, 1.25×10^{-4} and 1.25×10^{-5} mg/ml.

Controls: The positive control chemical was benzo(a)pyrene at a final concentration of 5×10^{-5} M and the negative control chemical was pyrene also at 5×10^{-5} M. Solvent controls included 1% dimethylsulfoxide (DMSO) and 1% of 0.1 N NaOH. An untreated negative control was also used.

Hepatocyte Primary Culture DNA Repair Assay

The HPC/DNA repair assay was performed using methods developed by Williams[2,3]. Immediately after washing with 1 ml WME, the test material and 10 µCi/ml tritiated thymidine ([^{3}H]-TdR) at 60-80 Ci/mM were added to 2 ml of the WME cell suspension. The test material was applied at 5 logarithmically decreasing concentrations on triplicate coverslips with the appropriate parallel positive and negative (untreated and solvent) controls.

After incubation for 18-24 hr in the presence of test material in [^{3}H]-TdR-WME, coverslips were removed from the wells and successively rinsed three times with 100 ml of WME. Each coverslip was then immersed for 10 min, cell surface up in 2 ml of 1 percent sodium citrate, in clean 6-well dishes, to cause nuclear swelling (permits better nuclear grain quantification), and finally fixed by three 30 min changes of glacial acetic acid (3:1), air dried and mounted on glass slides. Slides were dipped into NTB emulsion (Eastman Kodak) that had been prewarmed at 45° C for 1 hr, removed and dried in a light-tight box. Slides were wrapped in foil and stored at 4° C in cardboard slide boxes.

Ten days after storage, autoradiographs were developed for 4 min. in D19 (Eastman Kodak), placed in acidified tapwater for 30 sec, immersed in fixer (Eastman Kodak) for 10 min, and washed with tapwater for 5 min. Next, slides were stained with Harris' alum hematoxylin, counterstained with eosin, dehydrated through 100% ethanol, air dried and the coverslips sealed with Permount.

Slide Evaluation: Nuclear grains were scored with an Artek Model 880 electronic counter equipped with a microscopic attachment, using the area mode (permits distinction between discrete grains, even in aggregates). The net increase in grains induced by the test chemical or the positive control relative to the solvent control was the method used for quantification. To avoid artifacts, only cells with swollen nuclei (viable cells at fixation) and those evenly coated with emulsion were scored. From each coverslip quadrant, between 5 to 20 randomly selected cells were scored

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[2] Williams, G.M. 1977. Cancer Res. 37:1845-1851.

[3] Williams, G.M. 1980. In: Chemical Mutagens. Vol. VI eds. de Serres, F.J. and Hollaender, A. Plenum Press, N.Y. pp. 61-79.

(depending upon the nuclear/cytoplasmic grain ratio[4]). Background grain counts were assessed by counting three nuclear sized areas adjacent to the nucleus, and the net nuclear grain counts were calculated by subtracting the highest cytoplasmic count from the nuclear count.

Data Interpretation: By subtracting counts of the highest cytoplasmic background, false positive scores could be avoided. A minimum net grain count of 5 per nucleus, consistently observed in triplicate coverlsips was the criteria for a positive sample, and if the minimum was consistently observed throughout the experiment, the compound was considered positive.

If S phase cells, identified by morphology and/or high grain density in the autoradiograph, were absent then a cytotoxic response had occurred. A negative result was reported if less than 5 net nuclear grain counts were observed at the highest non-cytotoxic dose.

RESULTS:

The authors reported that cytotoxicity was not observed when the HPC cells were exposed to the highest concentration of JJN-1020 used and that none of the net grain counts/nucleus exceeded a value of 5. The highest net grain value for the test material was 1.4 ± 0.5 (1.25×10^{-1} mg JJN-1020 per ml) while the negative control values were 0.3 ± 0.5, 0.3 ± 0.1, 0.2 ± 0.3 and 0.4 ± 0.4 for DMSO, 0.1N NaOH, untreated cell culture and pyrene, respectively. The positive control, B(a)P, gave a net grain count of 22.9 ± 9.7. Hence, sensitivity of the assay was adequate.

DISCUSSION:

The author concluded that under the conditions of the HPC/DNA repair assay, no genotoxicity was induced by treatment with JJN-1020 at concentrations from 1.25×10^{-5} to 1.25×10^{-1} mg/ml.

Our assessment is that the author's evaluation of the data is correct and that all phases of the assay were well controlled.

CONCLUSIONS:

Under the conditions of the assay and as reported the test material (JJN-1020), glyphosate, did not induce DNA damage at concentrations between 1.25×10^{-5} and 1.25×10^{-1}mg/ml.

CLASSIFICATION: Acceptable.

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[4] Rogers, A.W. 1973. In: Techniques of autoradiography. Elsevier Sci. Pub. Co., p. 218.