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EVALUATION OF THE DERMAL TOXICITY OF LP1846

FINAL REPORT

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Undiluted LPG was applied directly to the unabraded skin of two swine using a saturated cotton sponge. Approximately 15% of the skin surface area was covered. Two other swine had a section of battle-dress uniform fabric soaked in LPG applied to their skin for 24 hours. A second dose of LPG was applied to all the animals 24 hours after the first dose. Pigs, both the ones receiving direct application and those treated with LPG impregnated fabric, were listless and had pale mucous membranes at the end of the first 24-hour exposure period. The skin was moderately erythematous with distinct areas of edema and eruption. By eight hours after the second application, the animals were moribund, cyanotic, tachypneic and vomiting. Blood samples were collected and the animals sacrificed at that time.

Gross necropsy showed erythema, edema and subcutaneous hemorrhage in the test areas. Gastric ulceration and hemorrhage, hemorrhage in large and small intestine, and a light brown discoloration of the lungs were also noted.

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Methemoglobin levels increased as early as two hours after the first exposure continued to rise until about eight hours postexposure. After a second exposure the values further increased to approximately 65%. Heinz body incidence in red cells reached nearly 100% within 16 hours of the first exposure and remained at that level until the animals were sacrificed.

Primary irritancy tests with guinea pigs showed that LPG produced significant primary responses only at concentrations of 25% and above. In dermal sensitization studies, sensitized animals had greater responses than the non-sensitized ones.

Studies were performed to determine the genotoxicity of LPG in the CHO/HGPRT Forward Mutation Assay. It was found that doses above 1 mg/ml were acutely toxic to the cells as judged by appearance and ability to grow. Inclusion of an S9 metabolizing system in the medium appeared to enhance the toxicity. There was no clear-cut evidence of mutagenic activity for LGP.

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EXECUTIVE SUMMARY

In previous studies the liquid gun propellant, LP-1846 (LPG) had shown moderate toxicity after oral or dermal administration. We evaluated the toxicity of LPG after dermal administration to Hanford Miniature pigs. We also tested LPG for its ability to affect survival and mutation frequency in Chinese hamster ovary cells in the CHO/HGPRT Forward Mutation Assay and for its ability to induce dermal sensitivity in the guinea pig.

Undiluted LPG was applied directly to the unabraded skin of two swine using a saturated cotton sponge. Approximately 15% of the skin surface area was covered. Two other swine had a section of battle-dress uniform fabric soaked in LPG applied to their skin for 24 hours. The skin was mildly abraded by rubbing the fabric lightly over it prior to the fabric section being affixed. A second dose of LPG was applied to all the animals 24 hours after the first dose.

Pigs, both the ones receiving direct application and those treated with LPG impregnated fabric, were listless and had pale mucous membranes at the end of the first 24-hour exposure period. The skin was moderately erythematous with distinct areas of edema and eruption. By eight hours after the second application, the animals were moribund, cyanotic, tachypneic and vomiting. Blood samples were collected and the animals sacrificed at that time.

Gross necropsy showed erythema, edema and subcutaneous hemorrhage in the test areas. Gastric ulceration and hemorrhage, hemorrhage in large and small intestine, and a light brown discoloration of the lungs were also noted.

Analysis of blood showed that methemoglobin levels increased as early as two hours after the first exposure. The values continued to rise until about eight hours postexposure, at which time they remained essentially constant until application of the second dose. The values then further increased to approximately 65%. Heinz body incidence in red cells reached nearly 100% within 16 hours of the first exposure and remained at that level until the animals were sacrificed.

Both primary irritancy and dermal sensitization tests were performed with Hartley guinea pigs. Primary irritancy tests showed that LPG produced significant primary responses only at concentrations of 25% and above. In dermal sensitization studies, guinea pigs were sensitized with undiluted LPG and challenged with 12.5% LPG. The sensitized animals had greater responses than the non-sensitized ones.

Studies were performed to determine the genotoxicity of LPG in the CHO/HGPRT Forward Mutation Assay. It was found that doses above 1 mg/ml were acutely toxic to the cells as judged by appearance and ability to grow. Even doses of 0.156 - 0.625 mg/ml inhibited growth and survival. Inclusion of an S9 metabolizing system in the medium appeared to enhance the toxicity. There was no clear-cut evidence of mutagenic activity for LGP.

I. INTRODUCTION

Previous studies have evaluated the acute toxic effects of LGP given by oral administration or direct dermal application to scarified skin. This information is summarized in the Safety Data and Information on Handling of HAN-Based Liquid Propellants and Their Components provided by the Army at the beginning of the study. More detailed information may be found in several Army documents referenced in the Safety Data material and listed in the Reference section (Nos. 1-5) of this present report. Results indicated that the test article was moderately toxic. This material currently is present in a number of research facilities and may be widely used in the future in a new Army weapons system. Since it may present a health hazard to personnel, the dermal penetration characteristics and toxic potential of undiluted LGP must be identified and evaluated, in order to establish standards for protecting personnel.

II. PURPOSE AND SCOPE

The purpose of this project was to examine the toxicity of LGP using in vitro and in vivo models. Dermal/systemic toxicity and dermal sensitization studies were conducted in domestic swine and guinea pigs, respectively. Evaluation of the genotoxicity of the test article was planned in mammalian in vitro cell culture systems which were to include: 1) Chinese hamster ovary (CHO)/HGPRT assay; 2) alkaline elution assay; and 3) in vitro chromosome aberration assay.

III. TASKS

A. Dermal/Systemic Toxicity of Liquid Gun Propellant (LGP) Limit Test (Swine)

B. Dermal Sensitization Study (Guinea Pigs)

C. Genotoxicity Potential (Mammalian Cells)

IV. TASK A: DERMAL/SYSTEMIC TOXICITY OF LIQUID GUN PROPELLANT (LGP) LIMIT TEST

A. PURPOSE OF STUDY

The purpose of this task was to examine the dermal/systemic toxicity potential of LGP and its direct effect on unabraded skin. This was a limit test (worst-case exposure) study utilizing undiluted LGP.

B. MATERIALS AND METHODS

Animals

Intact male Hanford miniature swine, 16-24 weeks of age and weighing between 20.4 and 23.8 kg, were used for these studies. Food and water were provided *ad libitum* during the course of the study.

Upon receipt the shipping crates were examined for evidence of damage or conditions that might have had an adverse effect on the health of the animals. The animals were uncrated and housed individually in wire-bottom canine metabolism cages. During this time the animals were given health evaluations and examined for evidence of shipping stress. Following the two week conditioning period, the animals were determined to be in good health and in compliance with the standards required for the study. They were ear tagged, weighed, and bled for pre-exposure clinical pathology profiles.

Test Article

The test article was undiluted LGP supplied by the U.S. Army Ballistic Research Laboratory, Aberdeen Proving Ground, MD 21005-5066.

Experimental Design

Investigation of the potential for dermal/systemic toxicity of LGP was evaluated using two exposure techniques: 1) direct application of undiluted LGP; and 2) dermal application by cloth patch saturated with undiluted LGP. Two animals were assigned to each of the exposure techniques (Table 1).

Direct Dermal Application Technique

The total skin area of the test animals was determined using the formula (6):

$$\text{Body Surface Area } (cm^2) = K \times W^{2/3}$$

K = 10.0
W = Body weight in grams

A measured volume of LGP was to be applied to the skin area proportionally equivalent to the skin area below the waist of an adult man (approximately 15%) for 14 consecutive working days. A cotton sponge saturated with LGP was used to uniformly distribute the LGP over the skin area. Before and after each application, the sponge was weighed to calculate the amount of LGP used in the saturating process.

Direct Cloth Application Technique

A skin area proportionally equivalent to the skin area below the waist of an adult man (approximately 15%) was determined as described above. A

fabric swatch (consisting of battle dress uniform fabric laundered once in hot water and detergent) the same size as the calculated skin area was saturated with the same measured volume utilized in the direct dermal application technique. Following rubbing of the skin area with the swatch for 5 minutes to simulate mild skin abrasion, the swatch was applied directly to the skin and held in place for 24 hours to be repeated daily for 14 consecutive working days.

Clinical Observations

The animals were observed twice daily (am and pm) for signs of severe skin irritation or systemic toxicity. Body weights were to be obtained weekly throughout the duration of the study.

Evaluation and Response Criteria for Skin

Skin areas were graded according to the method of Buehler (7). This scoring system ranks responses from grade 0 which indicates no response to grade 3 in which the skin exhibits marked erythema with or without edema.

Clinical Pathology

Blood samples were collected at 0, 2, 4, 8, 16, and 24 hours after dosing to determine the time-course for the development of methemoglobinemia and Heinz bodies.

C. EXPERIMENTAL PROCEDURES

Direct Dermal Application

Thirty (30) milliliters of premeasured LGP were poured from a polyethylene bottle into a 1000 milliliter beaker. A single 4 x 4 inch sterile cotton pad was weighed on a calibrated scale to determine the dry weight. The pad was then folded into quarters and clamped with a pair of stainless steel gauze forceps. A 30 x 40 cm (1200 cm^2) swatch of laundered battle-dress fabric was used as a template to define the area of skin to be painted. The approximate limits of the area on the dorsum just caudal to the shoulder blades were delineated using a non-toxic black felt tipped marker. The 30 milliliters of LGP was then uniformly painted over the skin area using the folded cotton 4 x 4 as the applicator. The test article was applied in two directions to assure the uniformity of distribution. Following the completion of the application procedure, the cotton pad was again weighed to determine its wet weight. The difference between the wet weight of the pad and the dry weight was divided by the specific gravity of the test article to determine the amount of test article retained in the pad. The difference between the retained volume of the test article and the original 30 milliliter aliquot was determined to be the actual volume of the test article applied to the skin.

Cloth Application

A 30 x 40 cm (1200 cm^2) swatch of laundered battle-dress uniform fabric was folded into quarters and placed in a 1000 milliliter beaker. Thirty (30) milliliters of premeasured LGP was poured over the cloth and into the beaker to uniformly wet the fabric. Although the cloth was not weighed, it appeared as though all of the liquid was absorbed by the cloth. The swatch was then removed from the beaker and placed on the dorsum of the animal just caudal to the shoulder blades. The cloth was lightly rubbed on the skin for 5 minutes (determined by a clock-timer) to simulate mild abrasion. At the end of the 5-minute period, the swatch was applied directly to the skin and held in place for 24 hours with a porous elastic wrap. The swatch was not weighed either before or after completion of the 24 hour application.

Dosimetry

Based on initial and adjusted volumes of the test article as a function of body surface area, it can be estimated that the average dose (expressed as ml/cm^2) to skin was 0.02 - 0.03 ml/cm^2 (Table 2). The estimated dose for the two animals with cloth patch application was slightly higher than that for the two animals with direct dermal application (Table 2); however, it is important to remember that no attempt was made to adjust the volume for cloth retention or evaporation of the test article in those two animals. Therefore the slight differences in estimated dose between the two exposure methods appear to be inconsequential, especially when the response of the skin and the level of methemoglobin are considered.

D. RESULTS

Clinical Observations

At the end of the first 24-hour exposure period, it was noted that all four animals were listless and had pale mucous membranes. They preferred to lie down and would only stand when aroused. There was evidence of emesis in the cage of one animal (Pig #30-4). The skin in the test areas of all 4 animals was moderately erythematous (Buehler grade = 2) with distinct areas of edema and eruption and distinct lines of demarcation between normal and treated skin.

Approximately eight hours following the second application (Dose-day 2) of undiluted LGP, all four animals were found be moribund, markedly cyanotic, tachypneic, and vomiting. All animals were judged to have a Buehler grade 3 response. Terminal blood samples were collected and the animals were sacrificed and necropsied.

Gross Necropsy Findings

Erythema, edema, and subcutaneous hemorrhagic areas were noted in the skin test areas of all four animals. Other major findings included gastric ulcerations and hemorrhage, hemorrhages in the large and small intestines, and a light-brown discoloration to the lungs. Per the project officer's instructions, no histopathology was performed.

Clinical Pathology

At two hours following the first exposure to the test article, it was noted that the methemoglobin concentration had increased above pre-exposure values (Figure 1). The methemoglobin concentration continued to increase until approximately 16 hours post-application and then remained nearly constant until the end of the initial 24 hour exposure period (Figure 1). Oxyhemoglobin showed only a slight decrease over the same time period (Figure 2). Heinz body concentration in red blood cells, on the other hand, reached approximately 100% within 16 hours of initial exposure and remained at that level until the animals were sacrificed (Figure 3).

Because the methemoglobin levels obtained from the first set of blood samples showed no signs of decreasing at 24 hours post-application, it was decided to reduce the sampling frequency to just two samples collected at 8 and 24 hours following the second application of the test article. Eight (8) hours after the second dermal application of undiluted LGP, the methemoglobin concentration in all four animals increased to approximately 65% while oxyhemoglobin decreased to 7% (Figures 1 and 2). Heinz body concentrations remained at 100% (Figure 3).

Conclusions

Undiluted LGP causes severe dermal irritation and systemic toxicity in swine with unabraded or slightly abraded skin under the conditions of the experiment. The toxicity is characterized by moderate to marked dermal erythema (Buehler grade 2-3), marked methemoglobin formation and marked Heinz body formation. The method of application appeared to have no effect on the degree or time-course of the dermal or systemic effects.

V. TASK B: DERMAL SENSITIZATION STUDY - (Guinea Pig)

A. INTRODUCTION

The purpose of this investigation was to examine the potential of LGP for contact sensitization in guinea pig skin. The study was divided into two major components: 1) Primary Irritancy Test and 2) Dermal Sensitization Experiment.

B. MATERIALS AND METHODS

Animals

Female Hartley guinea pigs (approximately 300 to 450 g in weight) from Charles River, Portage, MI, were used for these studies. Feed and water were provided ad libitum except during the 6-hour exposure periods as noted for each experiment. Water was supplemented with 0.264 mg/ml vitamin C. A

concentrate of the vitamin C supplement was prepared each day and dispensed into the drinking bottles. Any unused concentrate was discarded.

Upon receipt of the animals, the guinea pigs were housed individually and placed in quarantine for three weeks. During this time the health of the animals were assessed by the resident veterinarian. These tests included the random selection of representative animals for examination for endo- and ectoparasites, nasopharyngeal cultures for a battery of microorganisms, histopathology for lesions and serology for the presence of viral antibodies. Following the three week quarantine period, the animals were judged to be in good health and in compliance with the standards required for the study, ear tagged, weighed and assigned to an experiment. Starting at the end of the three week quarantine period body weights of each animal were recorded at weekly intervals until the completion of the experiment to which the guinea pigs were assigned.

Randomization of Guinea Pigs

The guinea pigs were randomly assigned to test or treatment groups within each test group on the basis of body weight using a computer software program, MIN TAB. Briefly, the guinea pigs were arranged in ascending order based on body weight taken at the end of the three week quarantine period. At this time, the animals were assigned an item number which was used to assign each guinea pig randomly to each test group and then to each treatment group.

Preparation of Solutions of Liquid Gun Propellant - LPG and 2,4-Dinitro-1-Chlorobenzene

Liquid Gun Propellant - LGP is composed of 60.8% hydroxylammonium nitrate (HAN), 19.2% triethanol-ammonium nitrate (TEAN) and 20% water. Solutions (%, v/v) of LGP were prepared each day prior to use by dilution in double distilled water. The exact percentages used were as recorded for each experiment. The positive control, 2,4-dinitro-1-chlorobenzene (DNCB, prepared daily) was dissolved in acetone and then diluted in acetone:olive oil (50%; 50%, v/v) to make working solutions. In general 2 milliliters of acetone was added to preweighed amounts of DNCB (averaging ~ 100 mg) in a glass vial. This solution was then diluted with acetone: olive oil yielding a 1% (wt/vol) stock solution. This stock solution was used for all additional dilutions of 0.01, 0.02 and 0.07% (wt/vol).

Experimental Design

Investigation of the potential for dermal sensitization by LGP was studied in four phases: 1) determination of primary irritating potential; 2) induction of sensitization; 3) rest period; 4) elicitation of response to a primary challenge.

The assignment of guinea pigs to each component of the study was based on body weight. The range in body weights was 318 grams to 444 grams. The distribution and number of guinea pigs assigned to each study were as follows:

ALLOCATION OF ANIMALS

Test	Treatment			
	LP1846	DNCB*	Solvent**	Total
Primary Irritancy	4	4	2	10
Sensitization and Challenge	20	12	6	38
Challenge only	10	6	4	20

* DNCB = 2,4-dinitro-1-chlorobenzene.
** Solvent = acetone in olive oil.

Skin Patch Exposure Technique (closed Patch)

The test sites were shaved with small-animal clippers before exposure to LGP and control agents. Care was used not to abrade or cut the skin with the clipper blade; only animals with intact skin were used in the study. Measured volumes (0.4 ml) of the study substances were applied to 22 mm Webril absorbent pads (Table 3). The pads were placed on the skin at designated sites on the animals' backs, covered with a 35 mm squared pad and held in place for six hours by elastic bandages wrapped around the animals. Restraining devices immobilized the animals during the exposures.

Evaluation and Response Criteria

Exposed sites were graded according to the method of Buehler (7) in which grades of 0 and 0.5 were considered to be insignificant responses, whereas those of one or greater are considered to be significant. The evaluation criteria are as follows:

0	no reaction
0.5	very faint erythema, usually nonconfluent
1	faint erythema, usually confluent
2	moderate erythema
3	strong erythema, with or without edema

Incidence and severity indices were computed for LGP exposed animals and positive and negative control animals for the sensitization study. The incidence index was the number of animals with positive response at 24 and 48 hours with respect to the total number of animals exposed. The severity index was determined for 24 and 48 hour responses by dividing the sum of the grades in a given group by the number of animals in the group.

Primary Irritancy Test

This test was used to detect the highest nonirritating concentration of the test material to be used for challenging the guinea pigs following sensitization. Four previously shaved sites on the back (two on each side of the midline) on each of four guinea pigs was exposed to 12.5, 25, 50 or 100% LGP (Table 4) or 0.004, 0.007, 0.01 or 0.02% DNCB (Table 5), in the appropriate solvent, as a positive control. Four sites on two additional animals was exposed to the solvent in which DNCB is dissolved. The various concentrations of LGP or DNCB were applied to different sites on different animals to minimize variations in responses resulting from possible differences in sensitivity of the various skin locations exposed. The responses (based on the presence or absence of erythema and edema) was graded at 24, 48 and 72 hours. The highest nonirritating concentration was defined as that which induced in half of the animals responses that are no more severe than a very faint erythema that was generally nonconfluent.

Sensitization and Primary Challenge

Sensitization was induced by exposing the guinea pigs to a single weekly 6-hour application for three consecutive weeks. Measured volumes (0.4 ml) of the highest concentration of the LGP and DNCB tolerated by the animals in the primary irritancy test (no evidence of severe irritation) was applied to a prepared site on the left shoulder of each animal. This procedure was repeated two more times at weekly intervals for a total of three 6-hour exposures (Table 3).

Following the sensitization step, a two week rest period occurred. Challenges were performed two weeks after the last sensitization exposure (five weeks after initiation of induction of sensitization). A virgin site on the animals' left back was used. In addition to the sensitized guinea pigs, previously unexposed groups of guinea pigs were exposed to LGP, DNCB and the DNCB solvent as negative controls. The challenges were accomplished employing the same technique used for induction, i.e., a 6-hour patch placed on a previously shaved skin site. The sites were graded 24, 48 and 72 hours later.

Scheduled Sacrifice

After the final evaluation of responses to the materials tested, the animals were euthanized with carbon dioxide. No formal necropsy procedure were performed.

C. RESULTS

Primary Irritancy Studies

Guinea Pigs:

As noted in Table 4 and 5, the guinea pigs were exposed to a single 6-hour application of four different concentrations of either LGP or DNCB.

Also included in the study were two additional guinea pigs exposed to the solvent in which the DNCB was dissolved. The mean body weight of each treatment group used in the study are plotted in Figure 4. Also noted in the figure was the time of challenge relative to the weighing of the animals. Body weights increased over the two week period for all groups. No major differences in mean body weights were evident among the different groups of animals, suggesting that there was no general toxicity at the concentrations used.

The primary irritancy responses of the guinea pigs exposed to DNCB were most evident at the 0.02% concentrations at 48 and 72 hours (see Table 5). There was one animal with a response grade of 1 at 24 hours and 72 hours at the 0.02% concentration. The sites treated with 0.004% DNCB exhibited only response grades of 0 or 0.5 over the entire grading period. A similar pattern of response was observed for sites treated with 0.007% DNCB and 0.010%.

Sites on the four guinea pigs treated with 12.5 to 100% LGP had demonstrable responses at 24 hours at the higher concentrations (Table 5). At 24 hours, animals treated with 25% and higher concentrations had response grades of 2 and higher in some animals. At 48 hours, the number of animals with responses higher than 2 only occurred in the 50 and 100% treated sites. At 72 hours, the responses continued to clear and there was only one animal with a response grade of 1 at the 100% treated site.

Sprague Dawley Rats

Four rats (Charles River, Portage, MI) were treated at four separate sites on each animal with neat LGP material following the same protocol as noted for guinea pigs. The responses were recorded at 24, 48 and 72 hours after treatment (Table 6). At 24 hours, three of the rats exhibited a response grade of 0 and one animal had a response grade of 0.5. At 48 and 72 hours, none of the rats exhibited any responses at any of the four dorsal sites treated with neat LGP.

Dermal Sensitization Study:

This study was divided into three steps. Animals were sensitized with the appropriate concentration of test material for three weeks, allowed to rest for two weeks and challenged with the appropriate challenge concentration.

The selection of the appropriate concentration for the induction of dermal sensitization and the appropriate challenge concentration for measuring the response for each test material was based on the data obtained in the primary irritancy test.

For the DNCB portion (positive controls) of the study, the sensitization concentration was 0.02% (w/v) and the challenge concentration was 0.007% (w/v). The challenge concentration was selection to avoid producing a primary irritancy response greater than a faint erythema in half of the animals after 6 hours. Twelve guinea pigs were sensitized with a concentration of 0.02% (w/v). Three of the guinea pigs were lost during the first sensitization

period due to restraint problems in the holding cages; they died when they became entangled in the cages. Therefore, the data reported for the DNCB sensitized and challenged animals was for the remaining nine animals. Included with the DNCB portion of the study was a challenge only group (6 animals) treated with 0.007% DNCB for one six hour challenge.

The sensitization of guinea pigs (20 animals) to LGP was with neat material. The selection of this concentration was based on the response and the recovery observed for animals in the primary irritancy test. The challenge concentration was 12% (v/v) and was selected on the same basis as noted above for DNCB.

The final group of guinea pigs were treated with the solvent {50% acetone:olive oil (v/v)} used to dilute the DNCB material. Six guinea pigs were treated according to the sensitization protocol and 4 guinea pigs were challenged only. Undiluted solvent was used in all study conditions.

The mean body weights of the sensitized and challenged animals for the three treatment groups increased during the study periods (Figure 5). Also recorded on Figure 5 were the times of sensitization and challenge. No major differences in the mean body weights of the treatment groups were observed. The body weights (Figure 6) of the challenge only animals for each treatment group were similar. No major differences in the body weights of these treatment groups were observed; thus, there was no evidence of general toxicity.

Primary sensitization results are presented in Table 7. As expected, there was a great difference in response in DNCB sensitized and challenged guinea pigs when compared to DNCB challenged only animals. The incidence index was 7 of 9 animals for the first 48 hours in the sensitized group and 0 of 6 in the unsensitized group. Also, the severity index was 0.8 to 1.2 for sensitized animals and only 0.0 to 0.2 for unsensitized animals.

Those guinea pigs treated according to the LGP sensitization protocol and challenged with 12% (v/v) LGP challenge concentration exhibited higher response grades than those animals only challenged with the 12% (v/v) LGP. The incidence and severity index for the sensitized and challenged animals were 17 of 19 and 2.4 to 1.6 for 24 and 48 hours, respectively. The same measurements for the unsensitized animals were 1 of 10 and 0.2 to 0.3, respectively.

Conclusion

Guinea pigs demonstrated a primary irritancy response to both DNCB and LGP. The response was more severe for the animals treated with LGP, but doses of LGP were higher than for the DNCB. Rats treated with neat LGP showed little primary irritancy response.

Sensitization of guinea pigs with either DNCB or LGP resulted in a significantly enhanced response to a challenge dose given two weeks after the last sensitizing dose. This was indicated both by the proportion of animals

showing a response and by the magnitude of the response. These data suggest that exposure to LGP may sensitize an individual to later exposure.

VI. GENOTOXICITY

A. PURPOSE OF STUDY

This study was designed to obtain data on the genotoxicity of LGP using the Chinese Hamster Ovary (CHO)/HGPRT system for measuring mutagenic activity. This assay detects mutations of the X-linked hypoxanthine-guanine phosphoribosyl transferase locus and is a widely used and well-understood assay. In wild type cells, exposure to thioguanine results in death of the cells. Mutations that cause loss or inactivation of the enzyme bestow a resistance to thioguanine on the cell and these cells will grow on thioguanine-containing medium, thus allowing the frequency of mutation to be ascertained.

B. METHODS AND RESULTS

Experiment 1. CHO cells, $5x10^4$, were dispensed into T25 flasks. After 24 hr, the cells were treated with graded levels of LGP (Table 8) in F12 medium minus serum. After 5 hours, the cells were washed and placed in medium with serum with or without S9. The cultures were observed at 3, 24, 48, and 72 hours later with an inverted phase contrast microscope.

At the higher doses of LGP, it was observed that the medium became yellow indicating that the medium had become acid. Measurement of the pH supported this conclusion as can be seen from Table 8. The pH of the cells treated with 5 mg/ml of LGP was 5.8 compared to 7.04 for the controls. Cells exposed to the two highest doses (5 and 2.5 mg/ml) of LGP for 5 hr were completely lysed when examined after they had been washed with phosphate buffered saline. The cells exposed to the lowest concentration (0.078 mg/ml) were indistinguishable from the controls and grew as well as the controls during the next three days. Doses of 0.156, 0.312, and 0.625 mg/ml did not visibly affect the cells immediately after dosing, but the cells grew more slowly during the three days following exposure.

When S9 was included during the exposure period, all of the cells appeared damaged. Even the control cells appeared less healthy than those without S9 and some came off during the washing procedure. The cells in the three highest dose groups (5.0, 2.5, and 1.25 mg/ml) appeared lysed after removal of the LGP; these groups as well as the 0.625, 0.312, and 0.156 mg/ml groups all grew slowly during the following three days. Those treated at the 0.078 mg/ml level appeared similar to the S9 controls, but were less healthy than the controls without S9. It was also noted that there was a precipitate in all S9 tubes, except for the controls and the 0.078 mg/ml dose. The appearance of damage in the S9 controls suggests that there may have been a low level of toxicity from the S9.

Experiment 2. A second experiment was performed in which the pH was adjusted after addition of LGP to the cultures to determine if the toxic effects in Experiment 1 were due to the acid conditions caused by the higher doses of LGP. Sodium hydroxide was used to adjust the pH to 7.23 before incubating the cells. The doses of LGP were the same as in Experiment 1; again one group had S9 and one did not. The cells were incubated for five hours and then washed three times with saline G. They were placed in 5 ml of F-12 with 10% FBS and the cells examined under a phase contrast microscope. Again the S9 controls did not appear as healthy as the controls without S9. However, in this experiment, there was no precipitate in any of the S9 tubes, not even in those treated with the higher levels of LGP. Overall, the S9 treated cells appeared more damaged by exposure to LGP than those that did not have S9; no cells were attached in the 5.0 and 2.5 mg/ml tubes that contained S9 but a few cells were attached in the 2.5 mg/ml level without S9. Cells treated with 0.625, 0.312, 0.156, or 0.078 mg/ml all showed attachment whether S9 was present or not. It thus appears that adjusting the pH resulted in a decreased toxicity from the LGP. However, because the Project Officer felt that addition of NaOH might represent a complicating factor, we did not neutralize samples in subsequent experiments.

Experiment 3. From the range-finding studies, we selected five dose levels (0.078, 0.156, 0.312, 0.625, and 1.25 mg/ml) to test for ability of LGP to induce mutations in the HGPRT locus. This experiment to determine mutation frequency did not include S9 because of the precipitate observed in Experiment 1 and the apparent enhanced toxicity observed in both Experiments 1 and 2. In these experiments, CHO cells were grown in liquid culture to obtain the requisite number. On the day of treatment, 5×10^5 cells were placed in tubes containing F-12 without serum. Ethyl methane sulfonate (EMS) at a concentration of one mM was used as a positive control. The cells were dosed for five hours while kept at 37°C. At the end of five hours, they were rinsed 3x with saline G and 10 ml of F-12 + 10% FBS added to each. For determination of initial survival, 10^5 cells were placed in 10 ml of F-12 with no serum. One ml of this dilution was placed into 9 ml of F-12. Aliquots were then transferred into wells on a 6-well plate and surviving colonies counted. At this time, another aliquot of 1×10^6 cells were transferred into 50 ml of F-12 medium containing 5% FBS in T150 flasks. After incubating for four days, 1×10^6 cells from each culture were transferred to new T150 flasks, again containing 50 ml of F-12 + 5% FBS. Three days later, cultures were trypsinized and 10^5 cells transferred to 10 ml of F-12. An aliquot of 0.065 ml of this dilution was placed in 38 ml of warm F-12 + 5% FBS and this, in turn, was dispensed into two 6-well plates (approximately 50 cells per well) and the cell plating efficiency determined.

For determining mutation frequency, 9.5×10^5 cells were placed in 56 ml of F-12-HX (hypoxanthine) + 5% dFBS + 30 uM 6-thioguanine. The cultures were dispensed into three 6-well plates. The number of colonies was determined three weeks later.

The initial cell survival is shown in Table 9. There appeared to be a slight decrease in initial survival for all dose levels of LGP; however, there was not a dose-response relationship. No data are presented for the 1.25 mg/ml dose because there were not enough cells to continue on after dosing.

Plating efficiencies and mutational frequencies are shown in Table 10. Plating efficiencies were not affected by any of the levels of LGP or the treatment with EMS. Mutational frequencies, however, were increased slightly by exposure to LGP, although the response was not dose related. A large increase in mutational frequency was found with EMS (approximately 30 x).

Experiment 4. This experiment was performed in a manner similar to that for Experiment 3. Initial cell survival was reduced by all levels except for the 0.078 mg/ml dose of LGP (Table 11). In this experiment, enough cells survived in the 1.25 mg/ml dose level to continue on test for mutational frequency. Again, plating efficiencies were not affected by exposure to LGP at any level (Table 11). Cells exposed to 1.25 mg/ml showed an increased mutational frequency, approximately a 9-fold increase. There was again a large increase in mutational frequency in cells exposed to EMS.

Experiment 5. A final experiment was performed to examine the effects of two dose levels (1.25 and 0.078 mg/ml) of LGP on mutational frequency and compare the response to those obtained with EMS or 6-aminochrysene (6-AC). The effect of S9 on the mutagenic response was also examined in cells treated with 6-AC or the low level of LGP. Initial cell survival was only determined on the controls, 1.25 mg/ml, and the 0.078 mg/ml + S9 (Table 12). Only the 1.25 mg/ml cells showed decreased initial survival. There was little effect of the treatments on plating efficiencies, with or without S9. It should be noted that the mutation frequency for the controls was several fold higher than in previous experiments. It is not clear why this occurred but these values are considered to be at the upper end of the control range. As expected, 6-AC by itself did not have an effect on mutation frequency. However, in the presence of S9, 6-AC was highly mutagenic, indicating that the S9 was active in converting 6-AC to an active mutagen. The 1.25 mg/ml dose of LGP, which was used without S9 being present, was not mutagenic. On the other hand, the 0.078 mg/ml dose in the presence of S9 increased the incidence of mutations approximately 14-fold.

CONCLUSIONS

In these experiments, LGP was definitely toxic at a concentration of 1.25 mg/ml as judged by initial survival studies. Although the results were not as clear, there was a tendency for reduced survival down to a concentration of 0.156 mg/ml. Plating efficiencies, on the other hand, did not seem to be much affected by any level of exposure. Most of the assays for mutagenic activity did not indicate activity for LGP. However, there was a suggestion in one experiment of increased activity for cells exposed to 1.25 mg/ml, although the effect was much lower than that seen with EMS or with 6-AC + S9 and was not consistent between experiments.

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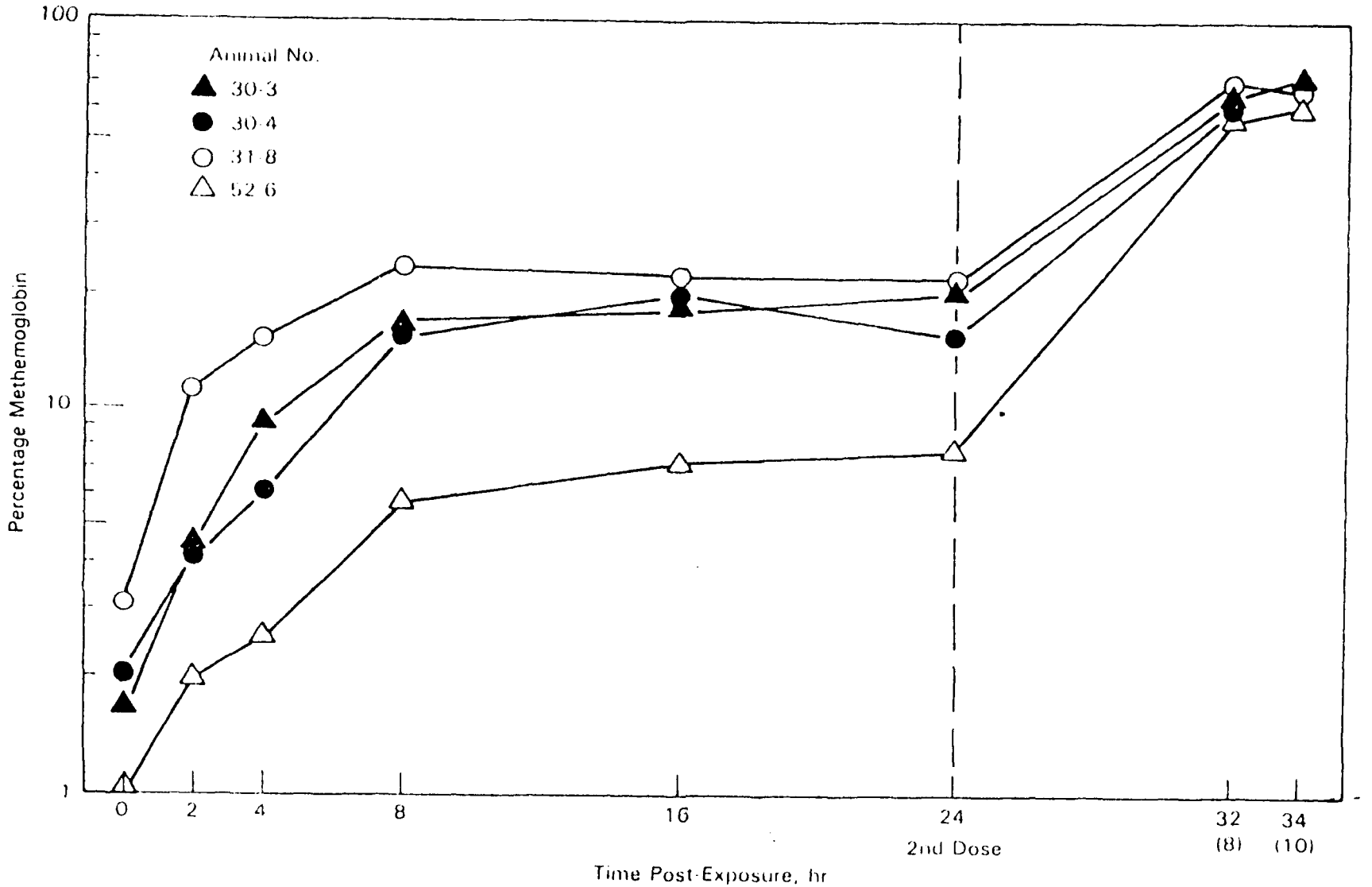

Figure 1. Methemoglobin levels in swine treated dermally with liquid gun propellant LPG-1846. Animals 30-3 and 31-8 received the test material by direct application and animals 30-4 and 52-6.

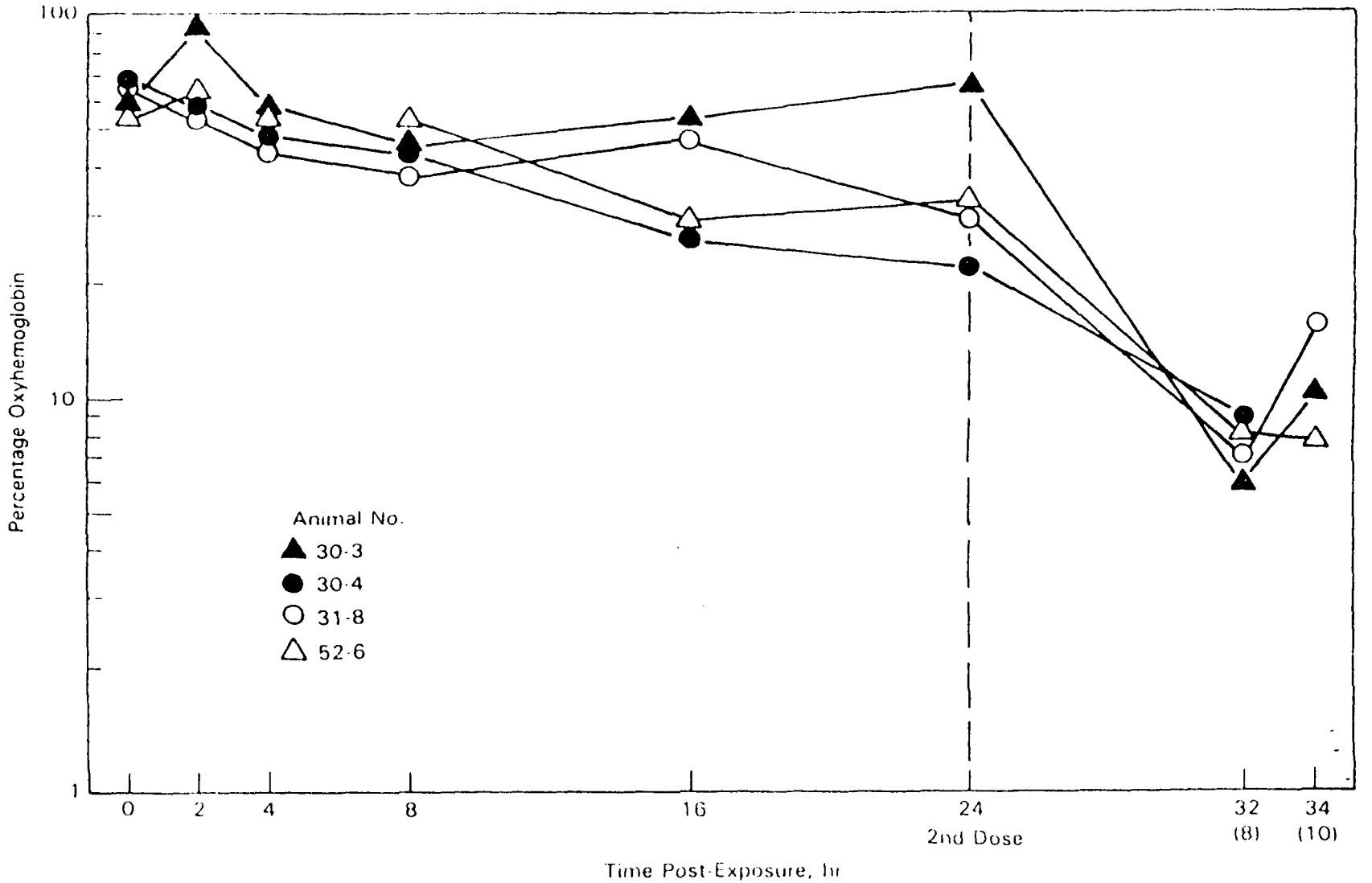

Figure 2. Oxyhemoglobin levels in swine treated dermally with liquid gun propellant LPG-1846. Animals 30-3 and 31-8 received the test material by direct application and animals 30-4 and 52-6 by cloth application.

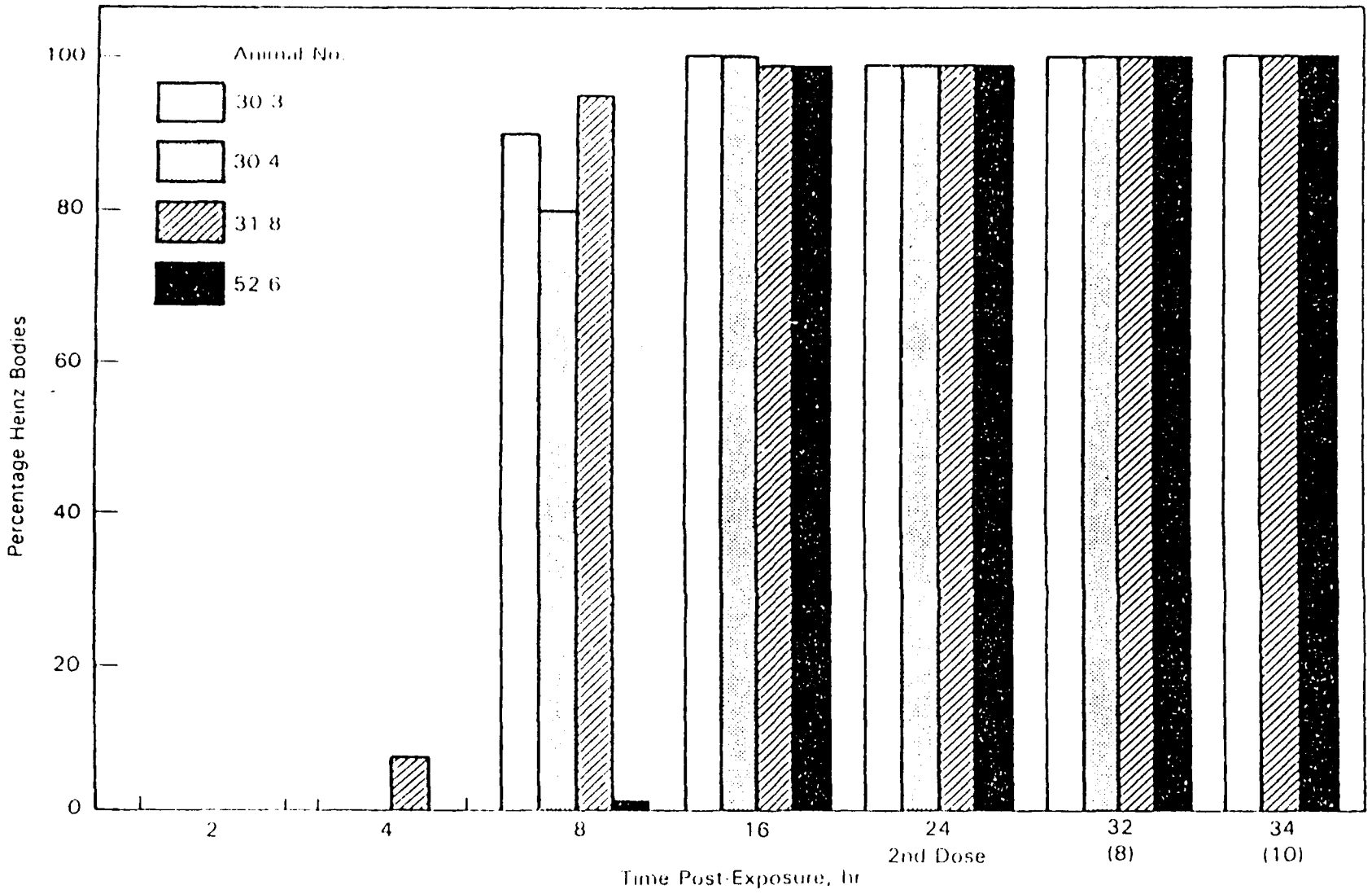

Figure 3. Percentage Heinz bodies in blood from swine treated dermally with liquid gun propellant LPG-1846. Animals 30-3 and 31-8 received the test material by direct application and animals 30-4 and 52-6 by cloth application.

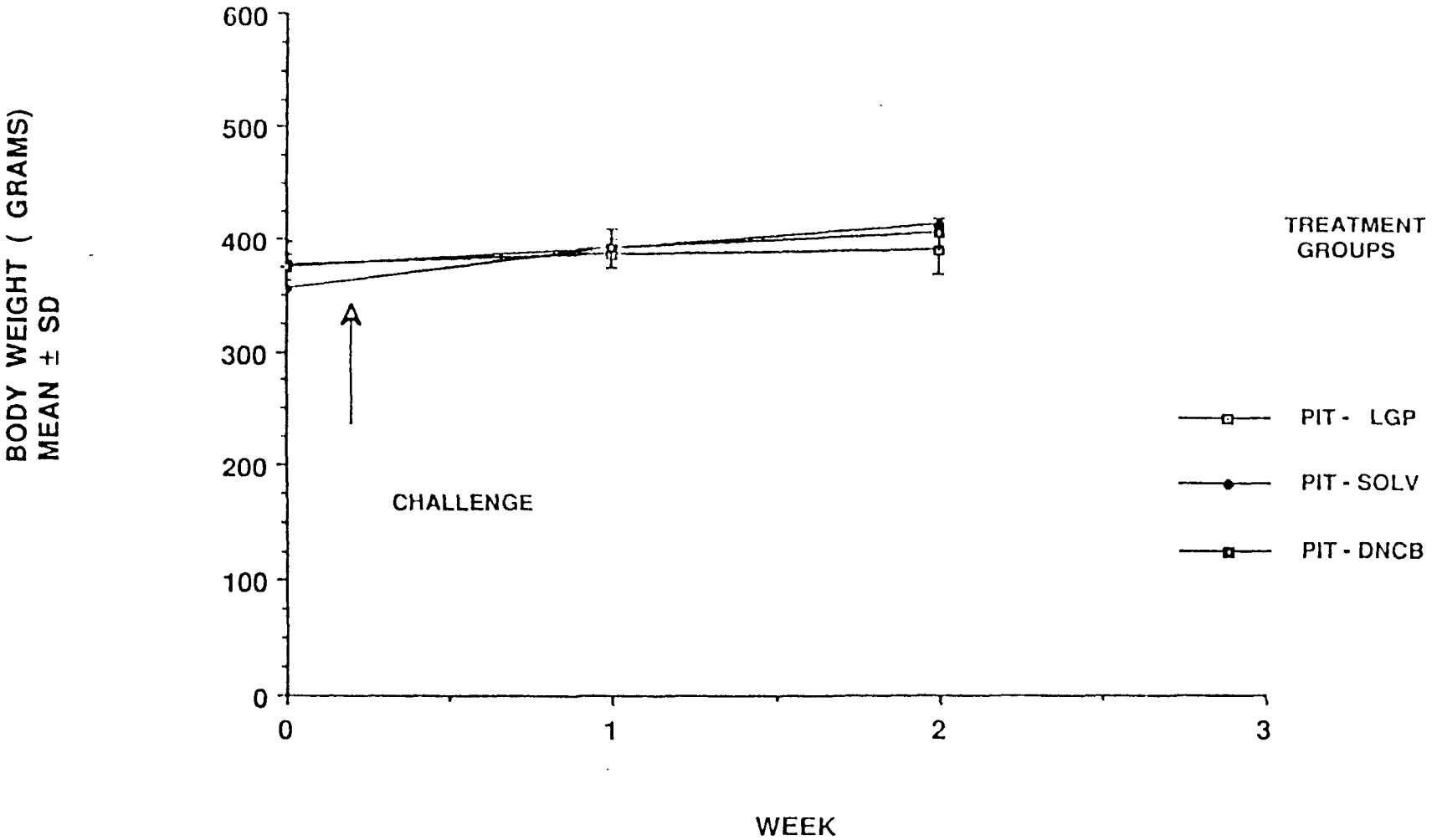

Figure 4. Body weight curves for guinea pigs treated dermally with liquid gun propellant, dinitrochlorobenzene, or vehicle solvent in a primary irritancy study.

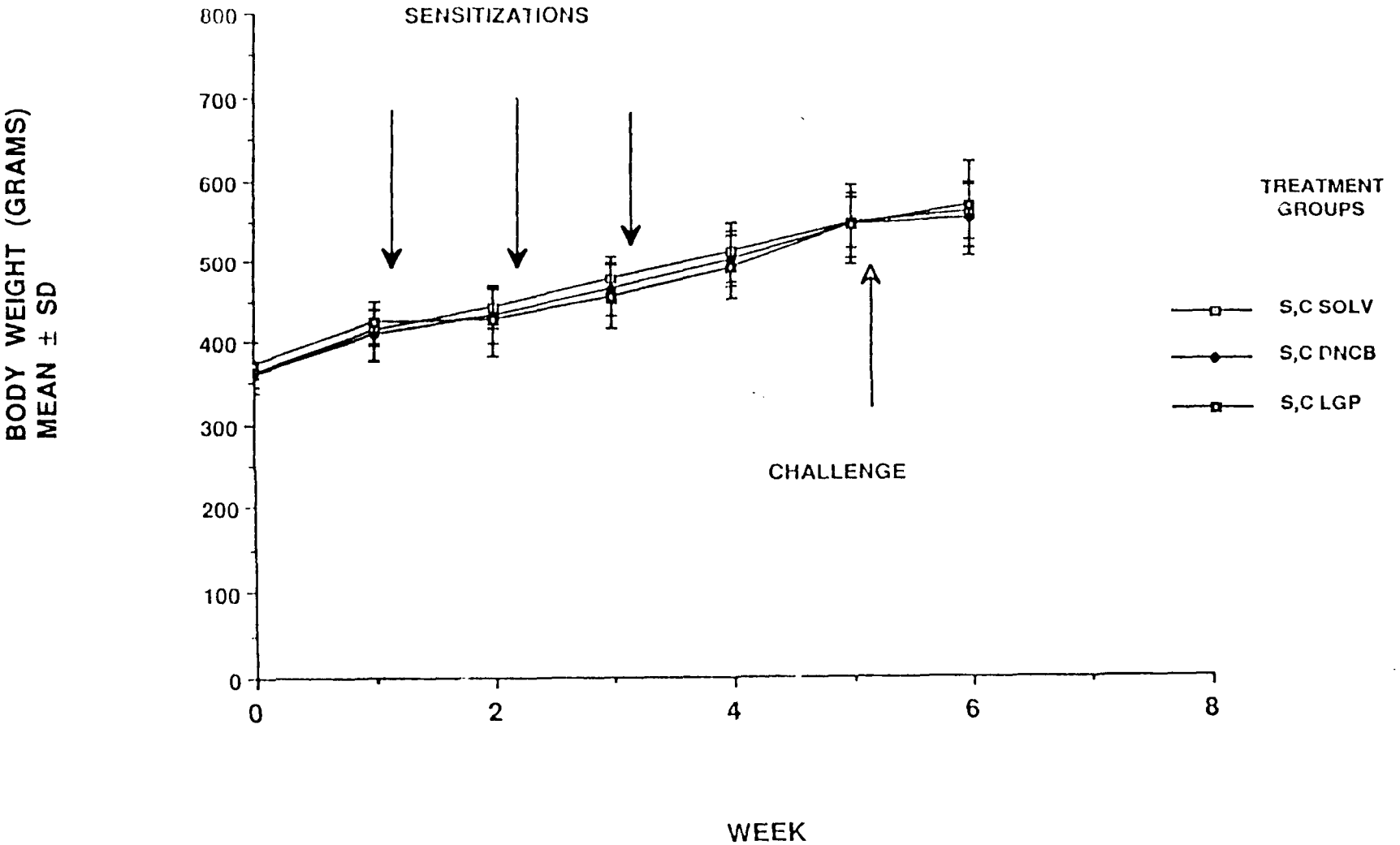

Figure 5. Body weight curves for guinea pigs sensitized and challenged with liquid gun propellant, solvent or dinitrochlorobenzene.

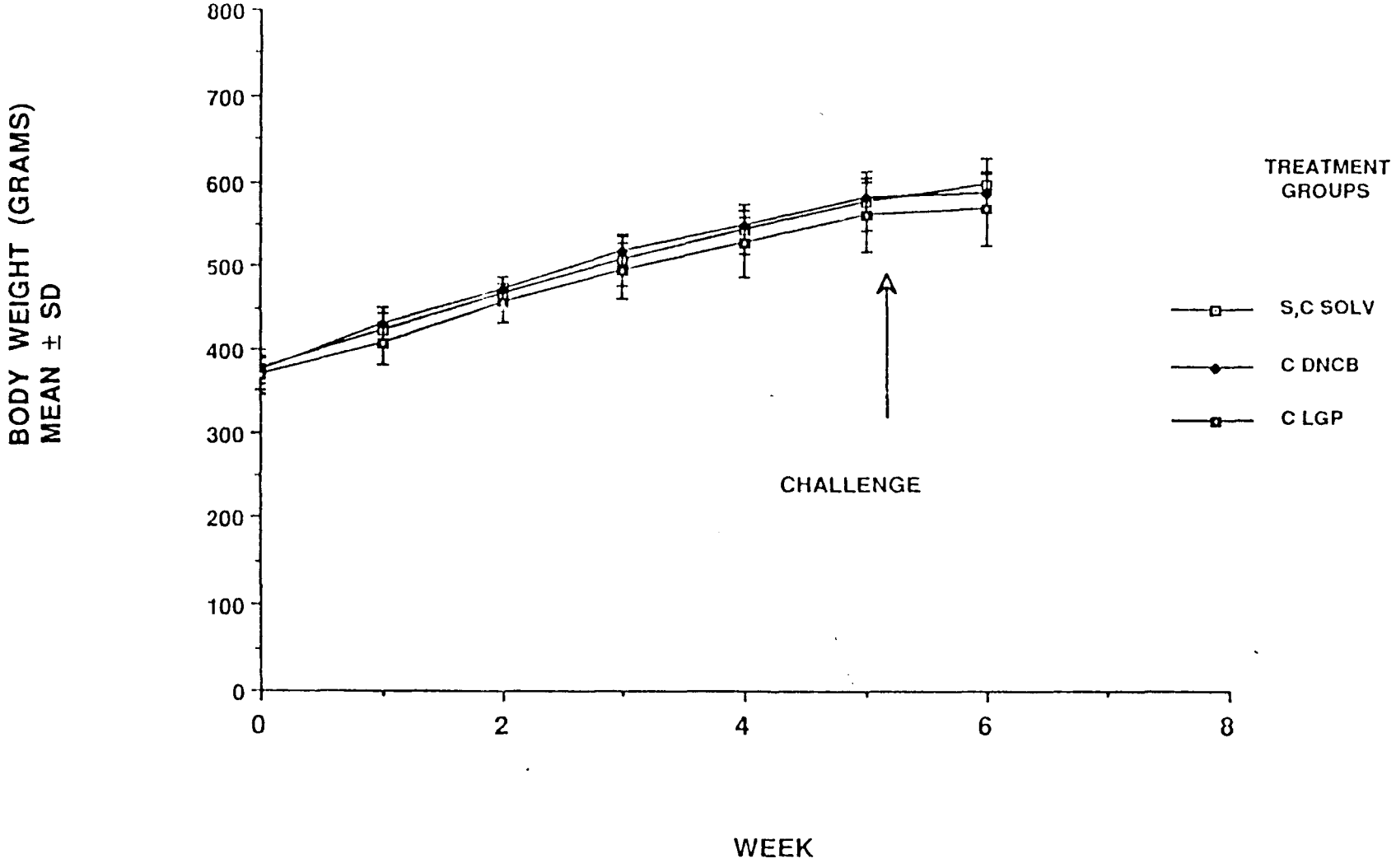

Figure 6. Body weight curves for guinea pigs challenged with liquid gun propellant, dinitrochlorobenzene, or solvent.

TABLE 1. EXPOSURE DATA-BODY SURFACE AREA (BSA)

Animal Number	Weight (kg)	Body Surface Area (cm^2)	Approximate Skin Area Exposed (cm^2)	Approximate % of BSA
30-3	23.8	8200	1200	15
31-8	23.6	8200	1200	15
30-4	23.0	8100	1200	15
52-6	20.4	7400	1200	16

BSA = $KW^{2/3}$ where W = weight in grams and K = 10.

TABLE 2. EXPOSURE DATA-VOLUME AND DOSE

	Animal Number							
	30-3		31-8		30-4		52-6	
	Dose 1	Dose 2	Dose 1	Dose 2	Dose 1	Dose 2	Dose 1	Dose 2
Volume LGP-LPG (ml)	30	30	30	30	30	30	30	30
Approximate Volume Retained in Pad (ml)	10.68	10.82	11.23	9.86	*	*	*	*
Adjusted Volume (ml)	19.32	19.18	18.77	20.14	*	*	*	*
Estimated Dose/Kg (ml/Kg)	0.81	0.81	0.80	0.85	1.30	1.30	1.30	1.30
Estimated Dose/cm^2 (ml/cm^2)	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.03

*Not measured.

TABLE 3.

DERMAL SENSITIZATION
(Closed Patch)

I. Primary Irritancy Study

Objective:

1. Determine sensitization concentration.
2. Determine appropriate challenge concentration for assessment of dermal sensitization.

Experimental Design:

1. Single 6-hour application.
2. Site evaluation at 24, 48 and 72 hours after exposure.

Experimental Group	Test Material Concentration %	Volume (ml)	Number of Animals	Number of Test Sites
LP-1846	12.5, 25.0, 50.0 and 100[a]	0.4	4	4
PC-DNCB	0.004, 0.007 0.01 and 0.02[b]	0.4	4	4

[a] LPG v/v% in distilled H_2O
[b] Dinitrochlorobenzene w/v in 50% acetone/olive oil (v/v)

Table 3 (continued)

DERMAL SENSITIZATION
(Closed Patch)

II. Primary Sensitization Study

Objective:

1. Determine whether LPG is a dermal sensitizing agent.

Experimental Design:

1. Single 6-hour application for three consecutive weeks.
2. Two week rest period.
3. Single 6-hour application to a non-primary irritating concentration.

Experimental Group	Test Material Concentration %	Challenge	Volume (ml)	Number of Animals
LPG	100	12.5	0.4	20
LPG Unsens.	---	12.5	0.4	10
DNCB	0.02	0.007	0.4	12
DNCB Unsens.	---	0.007	0.4	6

[a] LPG v/v% in distilled H_2O
[b] DNCB w/v in 50% acetone/olive oil (v/v)
Primary Sensitization Results (Guinea Pigs)

TABLE 4. PRIMARY IRRITANCY RESULTS (GUINEA PIGS)
Liquid Gun Propellant - LPG

	[LGP] (%)[a]	Response Grade 0	0.5	1	2	3
24 hr	0	0[b]	0	0	0	0
	12.5	3	1	0	0	0
	25.0	1	1	0	2	0
	50.0	0	1	1	0	2
	100.0	0	0	0	0	4
48 hr	0	0	0	0	0	0
	12.5	2	2	0	0	0
	25.0	1	3	0	0	0
	50.0	0	2	0	1	1
	100.0	0	0	0	1	3
72 hr	0	0	0	0	0	0
	12.5	4	0	0	0	0
	25.0	3	1	0	0	0
	50.0	2	2	0	0	0
	100.0	0	3	1	0	0

[a] LPG v/v% in distilled H_2O
[b] Number showing response

TABLE 5. PRIMARY IRRITANCY RESULTS (GUINEA PIGS)
2,4-dinitro-1-chlorobenzene

	[DNCB](%)[a]	Response Grade				
		0	0.5	1	2	3
24 hr	0.000	0[b]	0	0	0	0
	0.004	2	2	0	0	0
	0.007	4	0	0	0	0
	0.010	3	1	0	0	0
	0.020	3	0	1	0	0
48 hr	0.000	0	0	0	0	0
	0.004	3	1	0	0	0
	0.007	3	1	0	0	0
	0.010	2	2	0	0	0
	0.020	1	3	0	0	0
72 hr	0.000	0	0	0	0	0
	0.004	0	0	0	0	0
	0.007	3	1	0	0	0
	0.010	2	2	0	0	0
	0.020	0	3	1	0	0

[a] DNCB w/v% in 50% acetone:olive oil
[b] Number showing response

TABLE 6. PRIMARY IRRITANCY RESULTS (SPRAGUE-DAWLEY) RATS

	[LGP](%)[a]	Response Grade				
		0	0.5	1	2	3
24 hr	100.0	3[b]	1	0	0	0
48 hr	100.0	4	0	0	0	0
72 hr	100.0	4	0	0	0	0

[a] LPG v/v% in distilled H_2O
[b] Number showing response

TABLE 7. PRIMARY SENSITIZATION RESULTS (GUINEA PIGS)

Experiment Group		Response Grade 0	0.5	1	2	3	Incidence	Severity
LPG	24 hr	0[a]	2	1	4	12	17/19	1.6 - 2.4
Sens.	48 hr	0	2	7	7	3		
	72 hr	1	6	11	1	0		
LPG	24 hr	6	4	0	0	0	1/10	0.2 - 0.3
Unsens.	48 hr	5	4	1	0	0		
	72 hr	6	4	0	0	0		
P.C.	24 hr	1	1	7	0	0	7/9	0.8 - 1.2
DNCB	48 hr	1	1	4	3	0		
(Sens.)	72 hr	0	2	3	4	0		
P.C.	24 hr	5	1	0	0	0	0/6	0.1 - 0.2
DNCB	48 hr	3	3	0	0	0		
(Unsens.)	72 hr	4	2	0	0	0		

[a] Number of animals showing response
[b] Incidence = Number of animals exhibiting response grades $\geq$ 1 at 24 and 48 hours relative to total number of animals exposed
[c] Severity = Sum of grades at each time of evaluation divided by total number of animals.

TABLE 8. pH OF LGP IN F12 TISSUE CULTURE MEDIUM WITHOUT SERUM

LGP Concentrations mg/ml	pH
0.0	7.04
0.078	7.18
0.156	7.05
0.312	6.88
0.625	6.62
1.250	6.40
2.500	6.11
5.000	5.81

TABLE 9. INITIAL SURVIVAL OF CHO CELLS EXPOSED TO LIQUID GUN PROPELLANT (LGP) OR ETHYL METHANE SULFONATE (EMS)

Treatment	Fraction Surviving ± SD
Control	0.77 ± 0.17
EMS (1 mM)	0.66 ± 0.10
0.625 mg/ml LGP	0.55 ± 0.04
0.312 mg/ml LGP	0.58 ± 0.09
0.156 mg/ml LGP	0.59 ± 0.09
0.078 mg/ml LGP	0.57 ± 0.08

TABLE 10. PLATING EFFICIENCIES (PE) AND MUTATION FREQUENCIES (PER CELL X 10^{-5}) OF CHO CELLS EXPOSED TO LIQUID GUN PROPELLANT (LGP)

Treatment	Dose	Plating Efficiency ± SD	Mutation Frequency
Control	--	0.756 ± 0.17	9.8
Ethylmethane Sulfonate	1mM	0.760 ± 0.12	296.8
LGP	0.078 mg/ml	0.838 ± 0.09	13.9
	0.156	0.836 ± 0.12	11.8
	0.312	0.790 ± 0.12	14.3
	0.625	0.798 ± 0.13	7.0

TABLE 11. INITIAL SURVIVAL (1 DAY POST-TREATMENT), PLATING EFFICIENCIES (FRACTION FORMING COLONIES), AND MUTATION FREQUENCIES (PER VIABLE CELL X 10^{-6}) OF CHO CELLS TREATED WITH LIQUID GUN PROPELLANT

Treatment	Dose	Fraction Surviving ± SD	Plating Efficiency ± SD	Mutation Frequency
None	--	.778 ± 0.13	1.09 ± 0.19	1.8
Ethylmethane Sulfonate	1 mM	.695 ± 0.12	0.85 ± 0.08	285.6
LGP	0.078*	.857 ± 0.11	0.87 ± 0.11	6.4
	0.156	.513 ± 0.09	0.92 ± 0.15	3.2
	0.312	.623 ± 0.09	0.96 ± 0.14	2.5
	0.625	.475 ± 0.10	1.06 ± 0.17	4.5
	1.25	.399 ± 0.08	0.93 ± 0.12	16.8

*mg/ml

TABLE 12. INITIAL SURVIVAL, PLATING EFFICIENCIES AND MUTATION FREQUENCIES FOR LGP, EMS AND 6-AC

Treatment	Fraction Surviving ± SD	Plating Efficiency ± SD	Mutation Frequency
None	0.53 ± 0.11	0.86	26.0
EMS[1]	---	0.856	166.1
6-AC[2]	---	0.92	3.6
6-AC + S9	---	0.83 ± 0.13	412.0
.078 mg/ml LGP + S9	0.54 ± 0.14	0.83 ± 0.14	37.2
1.25 mg/ml LGP	0.36 ± 0.10	0.87 ± 0.09	29.5

[1] EMS = Ethylmethane sulfonate
[2] 6-AC = 6-aminochrysene