DATA EVALUATION RECORD

TWO INSECT REPELLENT SPRAY PRODUCTS (DESIGNATED AS PRODUCT A AND PRODUCT B FOR THIS REVIEW)

STUDY TYPE: SKIN SENSITIZATION STUDIES

Prepared for
U.S. Environmental Protection Agency
Human Studies Review Board (HSRB)
April 18-20, 2007
Public Meeting

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Executive Summary

Reports on a repeated insult patch test (RIPT) in humans were submitted to confirm published reports on the dermal sensitization potential of the components of two insect repellent products. RIPT in humans is not a routinely submitted alternative to skin sensitization studies in laboratory animals for supporting registration of pesticide products such as the two insect repellents discussed here. The Agency recommends that one of three different methods should be followed for sensitization testing with animals, but also encourages measures to reduce, refine or replace the use of laboratory animals so long as an assessment of human health hazard can still be performed. Although the submitted human RIPT reported that the two tested products caused no sensitization reactions to occur, circumstances of the study and the nature of the products tested raise questions about the scientific adequacy of the study to detect weak sensitizers or to confirm results of independent animal skin sensitization studies on each of the components in the two products. Because insect repellent products are repeatedly applied directly to human skin, an appropriate dermal sensitization study is necessary to classify the pesticide products under consideration with respect to that hazard. Therefore, a data gap exists for skin sensitization testing for both insect repellent products.

I. Background

For insect repellents applied directly to human skin, the most important acute toxicity testing requirements are for acute dermal toxicity and testing for skin irritation and dermal sensitization. Other standard acute toxicity testing typically includes acute oral, acute inhalation, and eye irritation studies. These six studies together comprise the battery of six tests required to define the acute toxicity profile of any pesticide product proposed for registration. Data from these six studies provides the basis for hazard categorization and product labeling, provides a starting point for establishing a dosing regimen for subchronic and other studies, and may provide information on absorption. To accomplish these purposes, the Agency encourages test methods that address the welfare of laboratory animals in toxicity testing.

The Agency recommends several means to reduce the number of animals used to evaluate acute effects of exposure to a test substance while preserving its ability to make reasonable decisions about safety. In the case of the two insect repellent products considered here, the manufacturer has taken a weight-of-evidence approach to dermal sensitization. Human experience and animal data on each component of the two insect repellent products was the first line of analysis, and the human RIPT study was performed to confirm that the two products could be classified with respect to dermal sensitization. The sponsor notes that each component in the two insect repellent products:

- Has been characterized as a non-sensitizer in the published literature,
- Has a history of use as an intentional food additive or in cosmetic products intended for direct application to human skin, and
- Is known to the Agency.

II. Dermal Sensitization Test Methods

The Agency's guidelines for acute toxicity testing were first published in October 1982.

In 1993, as part of an analysis of reasons for Agency rejection of submitted data, EPA and industry scientists performed a guideline-by-guideline review of toxicology studies, including those on dermal sensitization. Results of this analysis were published as the *Pesticide Reregistration Rejection Rate Analysis: Toxicology*, and they showed that 38% of dermal sensitization studies submitted were rejected—the highest rate of rejection for any acute toxicity studies. The main reason for rejection was the lack of concurrent positive control data.

In 1995, representatives from the Agency met with industry representatives, Health Canada, and the California Department of Pesticide Regulation to discuss acceptable methods for the conduct of acute toxicity studies. The discussions at this meeting were incorporated into a preliminary Registration Division document titled *Conduct of Acute Toxicity Studies*.

One change in the dermal sensitization guidelines resulting from these efforts was to encourage submission of positive control data generated within six months of the submitted study. In August, 1998, the Agency encouraged the use of the guinea pig maximization test (GPMT), the Buehler procedure and other tests including the open epicutaneous test, Maurer optimization test, split adjuvant technique, Freund's complete adjuvant test, and the Draize sensitization test. In March, 2003, a further revision of the dermal sensitization test guideline was published to include guidance on the local lymph node assay (LLNA), for which the Agency still requires submission of concurrent positive control data. The Agency's dermal sensitization test guidelines have also been harmonized with international recommendations and correspond to the Organization for Economic Cooperation and Development (OECD) Test Guideline 406 for skin sensitization and Test Guideline 429 for the LLNA.

The three *in vivo* animal methods for which the Agency now provides guidance include the local lymph node assay (LLNA), the GPMT, and the Buehler test, but the Agency still accepts other methods for which protocols have been reviewed and for which reported results include concurrent negative and positive control data to demonstrate the capacity of the method to detect dermal sensitization. The preferred method is the LLNA, but the Agency recognizes that this procedure is not always appropriate, and continues to accept the GPMT and Buehler methods. All of these methods include an induction treatment, followed by an "induction phase" to allow sensitization to develop, and then a challenge dose to assess the sensitization response.

A. Principles and definitions

Skin sensitization (allergic contact dermatitis) is an immunologically mediated dermal reaction to a substance. The reaction is a cellular immune response which begins with dermal exposure to a chemical hapten or incomplete allergen. The hapten is absorbed into the skin, where it forms a hapten-protein complex or antigen, recognized by Langerhans' cells as foreign protein (allergen). The Langerhans' cells then migrate to the thymus gland, where naïve T-cells become sensitized to the allergen. These sensitized T-cells proliferate and, if challenged by a subsequent dermal exposure to the hapten-protein complex, can trigger an inflammatory response. This response is delayed, since it requires an induction phase during which sensitization develops, and the response is observed only after sensitized T-cells initiate the response to a subsequent challenge exposure.

Because the inflammatory responses to dermal sensitizers and irritants are often similar (erythema, edema, etc.), irritation is an important consideration in determining the dose or

concentration of the test substance to be evaluated for sensitization. For example, the maximum concentration recommended for the LLNA is the highest achievable level that avoids overt systemic toxicity and excessive local irritation. For the Buehler test, EPA recommends a concentration at induction that is high enough to cause mild irritation, but at challenge the dose should be the highest non-irritating concentration. EPA recommendations for the GPMT are: (1) the concentration of the induction dose must be well tolerated systemically, and must be high enough to cause mild-to-moderate skin irritation; and (2) the GPMT challenge dose must use the highest non-irritating concentration.

Dosage volume for the LLNA is 25 µl/ear, and the amount of test material applied or injected in the other two test procedures is left to the investigator. Typically, dermal applications are 0.5 g/square inch (0.08 g/cm^2) for solids and 0.5 ml/ sq. in.(0.08 ml/cm^2) for liquids; intradermal injection volumes are usually 0.1 ml. The dermal applications in the GPMT are usually 0.3 ml of solution spread over a 1 x 2 inch filter paper patch (0.02 ml/cm^2) for application to the test site. For liquid products such as the insect repellents considered here, the test substance is usually applied undiluted.

B. Specific Animal Test Methods

1. The Local Lymph Node Assay (LLNA)

This method is based on the assumption that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application—in this case, the dorsal surface of the mouse ear. This proliferation is expected to be proportional to the dose applied, and can be measured objectively in terms of proliferating lymphocytes that will incorporate radioisotopes into their DNA. The LLNA assesses this proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in concurrent controls, and a positive control is added to each assay to provide an indication of appropriate assay performance.

Guidance for the LLNA calls for at least five animals per dose group. No less than three adjacent doses of the test substance should be selected from within the series 100, 50, 25, 10, 5, 2.5, 1, 0.5 and 0.1%. A solvent/vehicle control group and a positive control group are also required. A test animal will receive its treatment (test substance, vehicle/solvent control, or positive control) once each day for the first three days of the study, followed by two days without treatment. On the sixth day of the study each mouse is given the radioisotope five hours before excision of the draining auricular lymph node. The collected tissues are assayed for radioactivity and results are tabulated.

2. The Buehler Procedure

For the Buehler test EPA recommends topical administration of the test material via a closed patch on days 0, 6–8, and 13–15 for induction, with topical challenge at an untreated site for 6 hours on day 27–28. These sites are evaluated approximately 24 hours after removing the challenge patch, and again 24 hours after that. If the results are equivocal, the animals may be re-challenged one week later, using either the original control group or a new control group for comparison

3. The Guinea Pig Maximization Test (GPMT)

For the GPMT procedure intradermal injection with and without Freund's complete adjuvant (FCA) is used for induction, followed on days 5–8 by topical irritation/induction, followed by topical challenge for 24 hours on day 20–22. Evaluations of treated skin are made approximately 24 hours after removal of the challenge dose, and again after another 24 hours. As with the Buehler test, if the results are equivocal, the animals may be re-challenged one week later. If only 10 animals were used initially and gave equivocal results, it is strongly recommended that an additional 10 experimental and 5 control animals be used.

C. Interpretation of Test Results

Dermal sensitization studies are used to classify a product or active ingredient as either a sensitizer or a non-sensitizer. Each method used to evaluate sensitization has its own methods for interpretation of results.

1. Interpretation of LLNA Results

In the LLNA procedure, the sensitization response is determined by a stimulation index (SI), defined as the ratio of amount of radiolabeled methyl thymidine or iododeoxyuridine incorporated into test group lymph nodes to the amount in the vehicle control group. According to the LLNA test guidelines, if this ratio is 3.0 or more for at least one concentration tested (i.e., the measured radioactivity in the lymph nodes of treated animals is at least three times the radioactivity in lymph nodes from vehicle-treated controls), a substance is regarded as a skin sensitizer. Other factors to consider in evaluating the biological significance of the test outcome include the results of the SI determinations, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the solvent/vehicle and positive control responses.

2. Interpretation of Buehler Test Results

The Buehler test requires use of a sham-treated group, treated exactly like the treated test animals except that during the induction phase the test material is omitted. This helps differentiate allergic and irritation responses. Skin reactions are scored according to the following scale:

Skin Reaction	Value
Erythema and Eschar Formation:	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Necrosis (death of issue)	4
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	+N
Escher (sloughing)	+E
Edema Formation:	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure	4

The presence or absence of sensitization is determined for each animal by comparing its challenge response to its first induction treatment response and to the challenge responses of negative control animals. Any reaction observed at 48 hours after challenge that is reversed at 72 or 96 hours should be considered evidence of sensitization, so long as the response is greater than that noted in controls at the same time interval.

3. Interpreting GPMT Results

This method also includes a sham-treated group as described above for the Buehler test to ensure differentiation of sensitization and irritation responses at challenge. Skin reactions are generally scored on a 4-point scale as follows:

0 = No reaction
1 = Scattered mild redness
2 = Moderate and diffuse redness
3 = Intense redness and swelling

This scoring system is similar to that used in the human RIPT study considered below. In the GPMT the intensity and duration of the reaction are noted, but the frequency of any positive response is also important. If the vehicle controls cause no reaction, then a reaction to the test material rated 1 is just as important as one rated at 3. Results at challenge are considered in a manner similar to that described above for the Buehler test: a response stronger than the controls at 24 hours post-challenge but reversed by the 48 hour observation is considered a sensitization response, so long as those scores exceed those reported for the concurrent controls.

D. Appropriateness of Each Method

Each of the three methods described above has its advantages and disadvantages, which the Agency has attempted to accommodate in its guidance. In general, the LLNA is preferred because it demonstrates an equivalent prediction of human allergic contact dermatitis as compared to the other sensitization tests, provides quantitative data characterizing dose-response, addresses animal welfare concerns, and is suitable for testing colored substances. The LLNA

may not be appropriate for evaluating test materials such as certain metallic compounds, high molecular weight proteins, strong dermal irritants, and materials that do not sufficiently adhere to the mouse ear for an acceptable period of time during treatment. Hydrophilic materials should be incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles or test materials and runny liquids are to be avoided when using the LLNA. In situations for test materials where the LLNA is problematic, the GPMT or Buehler tests are recommended, but those methods also have advantages and disadvantages.

The Buehler test is useful when the test material cannot be prepared for intradermal injection as required by the GPMT. It provides a low rate of false positives, but is likely to produce false negative results for moderate and weak sensitizers—in short, the Buehler test is not as sensitive as the other tests. The GPMT is more sensitive to weak sensitizers, but tends to overestimate potency, and properly conducted tests do not produce many false positive results. Both these methods have extensive databases available containing results for many substances.

III. The Human Repeated-Insult Patch Test

A. Test Materials and Testing Objectives

The components of two insect repellent products were evaluated using the weight-of-evidence approach described above. Product A contains 11 ingredients (including the active ingredient) which are found in one or more of 16 previously registered products, and Product B contains 10 ingredients used in the same previously registered products. A summary of published animal dermal sensitization test results for these ingredients was provided by the sponsors:

Ingredient Number[a]	Ingredient is in Product		Test Method
	A	B	
1	Yes	Yes	Buehler
2	Yes	Yes	GPMT
3	Yes	Yes	GPMT
4	Yes	Yes	Buehler
5	Yes	Yes	No information available[b]
6	Yes	No	Not applicable
7	Yes	Yes	GPMT
8	Yes	Yes	Buehler
9	Yes	No	Not applicable[b]
10	Yes	Yes	GPMT
11	No	Yes	No information available[c]
Active ingredient	Yes	Yes	Buehler

a. Since the ingredients and nature of the products are claimed as confidential, no details are provided here.
b. The physical nature of the substance makes dermal sensitization testing inappropriate.
c. Literature indicates this substance, "…has the property of reducing skin inflammation induced by several chemicals."

All of the tested ingredients were reported to be non-sensitizers, but different variants of the Buehler and GPMT methods were used. The sponsor of the human RIPT stated that the human study was done to confirm the inference of non-sensitization from the animal evidence for each of the components.

B. Methods

1. Study participants

Male and female volunteers selected to participate in the study were at least 18 years old and in generally good health. They were free of any systemic or dermatologic disorder which would interfere with the results of the study or increase the risk of adverse events. Participants were of any skin type or race, so long as the skin pigmentation allowed discernment of erythema. Those volunteers selected for the study also completed a medical screening procedure and signed an informed consent document.

Volunteers were excluded from participation if they had any visible skin disease which would interfere with the evaluation, if they were receiving systemic or topical medication which would interfere with the study results, or if they had psoriasis or active atopic dermatitis or eczema. Those who were pregnant, planned to become pregnant during the study, or were breast-feeding were excluded as well. Finally, volunteers were excluded if they had a known sensitivity to cosmetics, skin care products, insect repellents, or topical drugs as related to the material being evaluated, or if they were participating in another study at the same time.

The study population was divided into two cohorts with the following demographics:

	Cohort 1	Cohort 2	Total
Subjects enrolled (n)	116	130	246
Age			
18 to 44 [n, (%)]	59 (50.9)	73 (56.2)	132 (53.7)
45 to 65 [n, (%)]	51 (44.0)	45 (34.6)	96 (39.0)
>65 [n, (%)]	6 (5.2)	12 (9.2)	18 (7.3)
Mean age (SD)	45.0 (12.1)	44.5 (13.9)	
Age range	18.9 to 70.3	18.2 to 69.9	
Gender			
Male [n, (%)]	27 (23.3)	25 (19.2)	52 (21.1)
Female [n, (%)]	89 (76.3)	105 (80.8)	194 (78.9)
Race			
American Indian	-	1 (0.8)	1 (0.4)
Asian [n, (%)]	-	1 (0.8)	1 (0.4)
Black [n, (%)]	8 (6.9)	2 (1.5)	10 (4.1)
Caucasian [n, (%)]	94 (81.0)	84 (64.6)	178 (72.4)
Hispanic [n, (%)]	14 (12.1)	40 (30.8)	54 (22.0)
Other [n, (%)]	-	2 (1.5)	2 (0.8)

2. Dosing

For the RIPT 0.2 ml of the test material was applied to a 2 x 2 cm. gauze pad attached to a non-porous plastic film adhesive bandage (occlusive patch). Volatile components of each product were allowed to evaporate from treated patches for 30 minutes prior to application to the subject's skin. Patches were secured with hypoallergenic tape to marked test sites on the infrascapular area of the back, to the right or left of the midline, or to the upper arm. The application rate on the patch was 0.05 ml/cm^2 (approximately 0.05 mg/cm^2).

3. Experimental design

The induction phase lasted three weeks, followed by a 10-15 day resting phase, followed by a challenge phase. The induction phase consisted of 9 consecutive applications (every 48 to 72 hours) at the same test site. Subjects were instructed to remove patches after 24 hours, and reactions at test sites were evaluated 48 hours after the preceding application. The next patch was applied immediately after reactions were noted. Patches applied on Fridays were removed by the subject 24 hours afterward, and the test site was not evaluated until the following Monday (72 hours post-application). Following the 9th application the subjects were dismissed for the 10-15 day rest period. In the challenge phase, patches were applied to previously unexposed test sites. Again the subjects removed the patches 24 hours after application, and skin reactions were evaluated 48 and 72 hours after application. If evidence of a sensitization response was noted, the subject was re-challenged to confirm the reaction.

4. Scoring of responses

The scoring system used in the patch study was summarized as follows:

Symbol	Definition	Value
-	No reaction	0
?	Minimal or doubtful response, slightly different from surrounding skin	0.5
+	Definite erythema, no edema	1.0
++	Definite erythema, definite edema	2.0
+++	Definite erythema, definite edema and vesiculation	3.0
D	Damage to epidermis; oozing, crusting and/or superficial erosions.	3.0

Interpretation of results was described in the report as follows:

> Sensitization is characterized by an acute allergic contact dermatitis. Typical sensitization reactions begin with an immunologic response in the dermis resulting in erythema, edema formation, and secondary epidermal damage (vesiculation), sometimes extending beyond the patch site and often accompanied by itching. Sensitization reactions tend to be delayed. The reaction typically becomes evident between 24 and 48 hours, peaks at 48-72 hours and subsequently subsides. The reaction is often greater at 72 hours than at 48 hours. The severity of the reaction is generally greater during the challenge phase of a Repeated Insult Patch Test (RIPT) than that seen during induction.

Irritant reactions are characterized as a non-immunologic, localized, superficial, exudative, inflammatory response of the skin due to an externally applied material. The typical initia1 reaction does not develop much edema or vesiculation but results in scaling, drying, cracking, oozing, crusting, and erosions. The reaction is usually sharply delineated, not spreading beyond the patch site. Irritant reactions are typically evident by 24 hours and diminish over the next 48-72 hours. Removal of the offending agent results in gradual improvement of the epidermal damage. The reaction seen at 72 hours is, therefore, less severe than that seen at 48 hours. Finally, the severity of the reaction experienced in the challenge phase is generally similar to that seen during induction,

If the results of the study indicate the likelihood of sensitization, the recommended practice is to re-challenge the subjects who have demonstrated sensitization-like reactions to confirm that these reactions arc, indeed, associated with the product. Our preferred re-challenge procedure involves the application of the product to naive sites, under both occlusive and semi-occlusive patch conditions. Use of the semi-occlusive patch condition helps to differentiate irritant and sensitization reactions. Generally speaking, if a product is a sensitizer it will produce a similar reaction under both occlusion and semi-occlusion, whereas if the product has caused an irritant reaction, the reactions will be less pronounced under the semi-occlusive condition.

C. Reported Results

Because the identical panel was used in a single procedure to test both products, the disposition of subjects was the same for Products A and B. Those results were as follows:

Number enrolled:		246
Number discontinued:		36
Lost to follow-up: (subjects failed to return)	26	
Voluntary withdrawal:	7	
Protocol violation: (removed patches) (exclusion medication) (on another study)	3	
Number completed:		210

Responses were reported as follows:

Product A: Panel 1												
Response	Induction Phase Readings									Make Up	Challenge	
	1	2	3	4	5	6	7	8	9		48h	72h
-	111	104	107	106	106	106	107	106	105	10	107	107
?	0	1	1	2	1	1	1	0	1	0	1	1
+	0	1	0	0	0	0	0	0	0	0	0	0
++	0	0	1	0	0	0	0	0	0	0	0	0
Total evaluated	111	106	109	108	107	107	108	106	106	10	108	108
Number absent	1	4	1	1	2	2	1	3	3		0	0
Number discontinued	4	6	6	7	7	7	7	7	7		8	8

Product A: Panel 2												
Response	Induction Phase Readings									Make Up	Challenge	
	1	2	3	4	5	6	7	8	9		48h	72h
-	119	110	111	108	110	107	101	98	98	14	102	102
Total evaluated	119	110	111	108	110	107	101	98	98	14	102	102
Number absent	1	4	2	4	1	2	4	4	4		0	0
Number discontinued	10	16	17	18	19	21	25	28	28		28	28

Product B: Panel 1												
Response	Induction Phase Readings									Make Up	Challenge	
	1	2	3	4	5	6	7	8	9		48h	72h
-	111	104	106	105	106	106	107	106	105	10	107	107
?	0	1	2	3	1	1	1	0	1	0	1	1
+	0	1	0	0	0	0	0	0	0	0	0	0
++	0	0	1	0	0	0	0	0	0	0	0	0
Total evaluated	111	106	109	108	107	107	108	106	106	10	108	108
Number absent	1	4	1	1	2	2	1	3	3		0	0
Number discontinued	4	6	6	7	7	7	7	7	7		8	8

Product B: Panel 2												
Response	Induction Phase Readings									Make Up	Challenge	
	1	2	3	4	5	6	7	8	9		48h	72h
-	119	110	111	108	110	107	101	97	97	13	102	102
?	0	0	0	0	0	0	0	1	1	1	0	0
Total evaluated	119	110	111	108	110	107	101	98	98	14	102	102
Number absent	1	4	2	4	1	2	4	4	4		0	0
Number discontinued	10	16	17	18	19	21	25	28	28		28	28

Based on these results, the investigators concluded that Products A and B were not sensitizers.

III. Discussion

A comparison of methods for animal testing and the human RIPT studies is important in determining the value of the human studies in the classification of Products A and B with respect to their potential to cause dermal sensitization. There are scientific disadvantages in doing the RIPT study:

- As the sponsor notes, there is extensive human experience with many of the components of the two insect repellent products either in cosmetics or in foods, and the subjects

participating in the patch studies may already have been exposed to the product components.

- The test items were not applied directly to the subjects' skin but to patches, and volatile components were allowed to evaporate before the patches were placed on the subjects' skin. This is inconsistent with typical use of the products. These repellent products are supposed to form a water-resistant coating on the skin, and it is unclear how this would affect absorption of the active ingredient or any of the other components of the products.

- Although the scoring system used in the RIPT study is generally similar to that used in the GPMT, in the GPMT the results at challenge are compared both to those for sham-treated control animals and to the responses seen during the initial induction phase of the study. This is probably more effective than the method described above for distinguishing irritation from sensitization responses.

- The animal data on the components suggests that any sensitization that might occur with dermal exposure to the two products would probably be weak, but the repeated patch study is not as likely as a GPMT or LLNA to detect such low grade responses. In fact, only one of the 210 subjects showed a definite response (rated as definite erythema without edema) after the second exposure to both products that did not appear at any other observation time.

Although the data support the investigators' conclusion that neither product is a sensitizer, there is uncertainty regarding the adequacy of the test.