Value of the Cutaneous Basophil Hypersensitivity (CBH) Response for Distinguishing Weak Contact Sensitization from Irritation Reactions in the Guinea Pig

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Numerous studies of the histology of allergic contact dermatitis reactions to potent allergens in guinea pigs and humans have indicated that there is significant tissue infiltration with basophilic leukocytes. In this study we determined whether this histologic finding could be of value in distinguishing weak sensitization reactions from primary irritation, thereby aiding in the predictive identification of weak or moderate contact allergens. Guinea pigs were sensitized by the Bueller test method. Skin reactions were graded 24, 48, and 72 h post-challenge with duplicate patch sites biopsied at the 24- or 72-h grading timepoints. The biopsies were fixed, embedded in glycol methacrylate, thin sectioned, and Giemsa stained. The number of basophils per 400 leukocytes were counted along the upper dermis just below the dermal/epidermal junction. Challenge patch sites from animals sensitized to a relatively low dose of the strong contact allergen, oxazolone, were compared with patch sites from animals challenged only with a strong irritant, sodium lauryl sulfate (SLS). Compared to normal skin (7.5 ± 1.0 basophils/400 leukocytes ± SEM) only the oxazolone patch sites showed significant basophil infiltration (36.8 ± 6.5), despite the fact that the skin reactions to the low oxazolone challenge dose were relatively weak. SLS patch sites showed no basophil infiltration above normal skin levels (4.8 ± 0.9). Subsequent blinded studies compared weak/moderate presumptive sensitization reactions (as defined by accepted visual skin grading criteria) to various chemicals (citronella, vanillin, cinnamic aldehyde, and ethylenediamine) to primary irritation reactions to the same chemicals. In each case, low-challenge-dose sensitization sites on previously treated (induced) animals showed mean basophil infiltration (range, 11.9–69.2 basophils/400 leukocytes) significantly greater than higher-dose irritant reactions (range, 1.6–13.3). The range for normal skin was 0.2–10.2 and the range for strong patch reactions to higher concentrations of oxazolone was 59.8–209.3. These data strongly indicate that light-microscopic quantitation of the CBH response can be used to distinguish relatively weak to moderate contact sensitization reactions from primary irritation reactions to the same chemicals. J Invest Dermatol 94:636–643, 1990

Guinea pig skin sensitization tests generally rely on the visual evaluation of erythematosus skin reactions for the interpretation of test results. Positive skin reactions that occur upon challenge of animals previously exposed to the test material (induced animals), in the absence of primary irritant reactions to the same test material in naive control animals, is considered to be indicative of an allergic contact sensitization response. These evaluations are relatively simple when one is dealing with moderate to strong contact allergens. However, many chemicals have marginal irritant properties at concentrations close to the threshold dose required to elicit an allergic response in previously induced animals. In such cases it is common to observe irritant reactions in control animals. Because irritant and allergic reactions are visually quite similar in guinea pigs, this can often render uninterpretable any positive reactions in the previously exposed test animals.

It would be very useful for the interpretation of predictive guinea pig skin sensitization tests to have a method available to distinguish irritation from weak to moderate sensitization reactions to chemicals. Neither standard histologic methods, nor more sophisticated immunohistochemical techniques for quantifying infiltrating lymphocyte subpopulations, have been widely successful when used to distinguish weak to moderate sensitization from irritation reactions [1,2]. However, one method we believe has such benefit is quantitative assessment of cutaneous basophil hypersensitivity (CBH) by light microscopy.

The CBH response was originally characterized by the dermal infiltration of basophilic leukocytes at sites of contact sensitization.

Abbreviations:
ACET: 100% acetone
CAL: cinnamic aldehyde
CBH: cutaneous basophil hypersensitivity
CIT: citronellal
EDA: ethylenediamine
ETOH: 100% ethanol
OXAZ: oxazolone, 4-ethoxymethylene-2-phenyloxazol-5-one
SLS: sodium lauryl sulfate
VAN: vanillin

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Reprint requests to: Michael K. Robinson, Ph.D., Human and Environmental Safety Division, The Procter & Gamble Co., Miami Valley Laboratories, Cincinnati, Ohio 45239.
reactions as well as other forms of delayed-type hypersensitivity reactions [1,3-6]. All of the work on the CBH response in contact sensitization reactions has focused on relatively strong contact allergens. There is very limited evidence that the CBH response does not occur in primary irritation reactions [1]. The CBH response has not been routinely assessed microscopically because it requires specialized tissue processing and staining for proper evaluation [4,7].

Our objective was to study the CBH response as an indicator of contact sensitization to various weak to strong contact allergens, and to determine its utility in distinguishing sensitization from irritation reactions, including primary irritation reactions to the same sensitizing chemicals. Our approach was to first compare the CBH response to both a known strong sensitizer (oxazolone) and a known irritant (sodium lauryl sulfate), and then extend the investigation to chemical sensitizers of weak to moderate sensitization potential, more relevant to occupational or consumer exposures. Our results showed consistent CBH responses to oxazolone and lesser, but significant, responses to weaker contact allergens. Irritation reactions to higher challenge doses of the same chemicals or to sodium lauryl sulfate showed no evidence of a CBH response, indicating that microscopic assessment of the CBH response can distinguish weak to moderate contact sensitization from primary irritation skin reactions in the guinea pig.

MATERIALS AND METHODS

Animals Male and female Hartley strain guinea pigs (Charles Rivers Laboratories, Wilmington, MA) were used in all experiments. The animals were housed individually, maintained on 12-h light/dark cycles, and provided food and water ad libitum. Four to ten animals were used per group in all studies.

Test Materials Test materials included sodium dodecyl sulfate (sodium lauryl sulfate [SLS]), 3,7-dimethyl-6-octenal (citronellal [CIT]), and 1,2-diaminoethane (ethylenediamine [EDA]) [all from Sigma Chemical Co., St. Louis, MO], and vanillin (VAN), 4-ethoxyhexene-2-phenyl-2-oxazolin-5-one (Oxazolone), and trans-cinnamaldehyde (cinnamic aldehyde, CAL) [all from Aldrich Chemical Company, Milwaukee, WI].

Modified Buehler Guinea Pig Sensitization Test A modification of the Buehler Method [8] was used for induction and elicitation of contact sensitization in the guinea pig. The animals received three six-h induction exposures of test materials at the same shaved site by occluded patch, once a week for three weeks. Ten to 13 d after the last induction exposure the animals were challenged at shaved naive skin sites by a 6-h occluded patch exposure to the test material. Two patches were applied at challenge, one on either side of the midline of the animal’s back. Animals were comfortably restrained during each 6-h patch exposure period.

Eighteen to 22 h after the challenge patches were removed, the test sites were depilated. The sites were scored at 24, 48, and 72 h after the challenge patches were removed from the animals. A five-point scale was used to grade the degree of erythema present at the patch sites: 0, no reaction; ±, slight, patchy erythema (i.e., barely perceptible or questionable reaction); 1, slight but confluent erythema (i.e., a slight but definite reaction at the patch site) or moderate, but patchy erythema (i.e., moderate erythema involving 50% or more of the area of the patch site); 2, moderate confluent erythema; 3, severe erythema with or without edema.

Scores presented refer to the left challenge site at 24 h and the right challenge site at 48 h and 72 h. Erythema scores of ≥ 1" were considered positive responses. The relative incidence and severity of "positive" reactions among the test and control animals were used as a basis for interpreting the test results [8].

Punch Biopsy Removal Punch biopsies were removed from the center of the test sites on the animals’ backs after scoring of the sites at 24 and 72 h. Separate sites were biopsied at the two time points (left, 24 h; right, 72 h). Punch biopsies were removed under aseptic conditions at 24 h. Immediately prior to biopsy, the animals were tranquilized by administration of 6-8 mg of Rompun (Xylazine) intramuscularly. Xylocaine was used around the periphery of the reaction site to provide local anesthesia. A 6-mm disposable biopsy punch was used to remove the tissue sample, and the biopsy sites were closed with two autoclips. The biopsies were placed in 10–15 ml of modified Karnovsky’s fixative, and post-fixed for 4 h in 10–15 ml of 0.05 M potassium phosphate buffer, pH 7.2. The 72-h biopsies were similarly removed and fixed. All biopsies were coded and not identified with any test material or treatment regimen until the microscopic evaluation was completed.

Preparation and Histologic Examination of Skin Biopsies Each biopsy was divided in half on a plane parallel to the hair shafts. Each half was embedded in glycol methacylate and sections from each half were cut at ~1 μm thickness. Sections ~15 μm from one another were placed on glass slides and Giemsa stained [7] at pH 5.0. Each half of the biopsy was examined microscopically; the number of basophils per 200 leukocytes through the superficial dermis was counted at 400 times magnification using a 10 × 10 mm ocular grid. The two separate counts for each half of the biopsy were then added to give the number of basophils per 400 leukocytes for each 24-h and 72-h biopsy. Figure 1 shows the typical microscopic appearance of basophils, eosinophils, and mast cells using this methodology.

Figure 1. Challenge skin reaction site from an oxazolone sensitized animal (magnification X 800, Giemsa stain). Large arrow, mast cell; medium arrow, basophil; small arrow, eosinophil.
Table I. Incidence of Patch-Test Reactions to Oxazolone (OXAZ) and Sodium Lauryl Sulfate (SLS)

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Induction</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXAZ</td>
<td>0.5% (ETOH)</td>
<td>0.01% (ACET)</td>
</tr>
<tr>
<td>SLS</td>
<td>none</td>
<td>2.5% (H2O)</td>
</tr>
<tr>
<td>Naive</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

* Scores only for those animals with minimal scabbing of the test site.

**Statistical Analysis of Data**
Statistical comparisons were made between the mean basophil counts of challenge patch sites from previously induced animals versus untreated patch sites from naive animals. The data were analyzed for statistical significance using the Student t test. A p value < 0.05 was considered statistically significant.

**RESULTS**

**Evaluation of the CBH Response to a Strong Contact Allergen and a Strong Irritant**
Our initial study was designed to determine our ability to detect a CBH response to a known strong contact sensitizer, oxazolone (OXAZ), and to determine the specificity of the response by also examining irritant skin reactions to sodium lauryl sulfate (SLS). The concentrations of OXAZ used in this study were well below the minimally irritating dose; hence, the skin reactions were relatively mild and transient (Table I). SLS produced an eschar reaction than erythema such that most sites could not be graded relative to scale. Despite the relatively mild skin reactions to OXAZ, there was significant basophil infiltration of the biopsy sites (p < 0.05 relative to normal skin sites) at both 24 and 72 h post-challenge (Fig 2). The SLS irritation reactions showed no evidence of increased basophil infiltration.

**Evaluation of the CBH Response to Strong and Weaker Contact Allergens Compared to Primary Irritation Reactions to the Same Chemicals**
The next study was designed to investigate the CBH response to two human contact allergens, citronellal and vanillin [9,10], that had given relatively weak sensitization responses [11] in prior GPSS testing. The test group of animals received induction applications of 10% citronellal (CIT) or vanillin (VAN) in ethanol and were challenged at 2.5% CIT or 0.3% VAN in acetone. Control animals for the skin-testing portion of the study were challenged with the same concentrations but were not biopsied. Instead, additional naive control animals were challenged at 10% CIT or 3% VAN in order to try and produce stronger primary irritation reactions for biopsy and CBH comparison with the respective test animals. The skin-reaction data (Table II) showed evidence of sensitization to CIT. Greater than expected irritation was observed in the low-dose challenge controls; however, the greater persistence of the reactions in the test group (see 48 h grades) was indicative of a sensitization response. The results were similar for VAN, although, in this case, both the incidence of skin reactions and their persistence were greater in the test animals than the low-dose controls. In both cases, the high-dose controls showed primary irritation reactions of comparable incidence and persistence as the test animals' reactions. OXAZ was used as a positive control in this study and was tested at higher concentrations than in the first study (Table I) in order to produce stronger skin reactions as noted in Table II.

The results of the CBH portion of this study are shown in Fig 3. In spite of the lower concentration challenges applied, animals given both induction and challenge exposures to CIT and VAN (1+1, i.e., sensitized animals) showed significantly increased (p < 0.02) CBH responses compared to animals given the higher-dose challenge concentrations (C0 only). This was observed at both 24 and 72 h post-challenge. As noted in the figure legend, the basophil infiltration in the OXAZ challenge sites were considerably higher than the prior study and were higher at 72 h (114.4 ± 14.0 basophils/400 leukocytes) relative to 24 h (59.8 ± 11.1 basophils/400 leukocytes) post-challenge, consistent with other studies using strong sensitizers [5].

**Further Evaluation of the CBH Response to Strong and Weaker Contact Allergens Compared to Primary Irritation Reactions to the Same Chemicals**
An additional study was conducted to try and confirm the observations made with one weaker sensitizer (citronellal) and extend the observations to other comparable human contact allergens, cinnamic aldehyde, and ethylenediamine [12,13]. The test group of animals received induction applications of 10% citronellal (CIT), 1% cinnamic aldehyde (CAL), or 1% ethylenediamine (EDA) in ethanol and were challenged with 1% CIT, 0.1% CAL, or 0.25% EDA in acetone. Control animals for the skin-testing portion of the study were challenged with the same concentrations but were not biopsied. Once again, additional naive control animals were challenged at higher concentrations (2.5% CIT, 0.5% CAL, or 0.5% EDA) in order to try to produce stronger primary irritation reactions for biopsy and CBH comparison with the respective test animals.

The skin-reaction data (Table III) demonstrated even greater evidence of sensitization to CIT than the prior study (even though a lower challenge concentration was tested), based on greater incidence and persistence of positive skin reactions in the induced group versus the low-concentration challenge controls. CAL-induced animals also showed evidence of sensitization based on a twofold increase in the incidence of positive animals relative to the low-concentration challenge controls. EDA-induced animals clearly
Table II. Incidence of Patch-Test Reactions to Citronellal (CIT), Vanillin (VAN), and Oxazolone (OXAZ)

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Induction</th>
<th>Challenge</th>
<th>Skin Reaction Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 ± 1 2 3</td>
<td>0 ± 1 2 3</td>
</tr>
<tr>
<td>CIT</td>
<td>10% (ETOH)</td>
<td>2.5% (ACET)</td>
<td>7 3 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5% (ACET)</td>
<td>1 6 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% (ACET)</td>
<td>4 6 3</td>
</tr>
<tr>
<td>VAN</td>
<td>10% (ETOH)</td>
<td>0.3% (ACET)</td>
<td>5 5 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3% (ACET)</td>
<td>7 3 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3% (ACET)</td>
<td>7 3 5</td>
</tr>
<tr>
<td>OXAZ</td>
<td>1% (ETOH)</td>
<td>0.1% (ACET)</td>
<td>3 3</td>
</tr>
<tr>
<td>ACETONE</td>
<td>100%</td>
<td></td>
<td>3 1 2</td>
</tr>
</tbody>
</table>

* Not treated at induction.

Figure 3. Guinea pigs were given three induction applications (in ethanol) of 10% citronellal (CIT), 10% vanillin (VAN), or 1% oxazolone (OXAZ) and were challenged with 2.5% CIT, 0.3% VAN, or 0.1% OXAZ in acetone, respectively. Vehicle-control animals were untreated during induction and were challenged with 100% acetone. Control animals for the skin-testing portion of the study were challenged with the same concentrations but were not biopsied. Instead, additional naive control animals were challenged at 10% CIT or 3% VAN for CBH comparison with the respective test animals. Skin-reaction scores are shown in Table II. The high-dose challenge groups (positive irritation control) for CIT and VAN were biopsied along with the induced test animals, the naive acetone (ACET) -challenged animals, and the OXAZ-sensitized animals. The CBH results for OXAZ-sensitized animals were 59.8 ± 11.1 (mean number of basophils/400 leukocytes ± SEM) at 24 h and 114.4 ± 14.0 at 72 h.

Table III. Incidence of Patch-Test Reactions to Citronellal (CIT), Cinnamic Aldehyde (CAL), Ethylenediamine (EDA), Oxazolone (OXAZ)

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Induction</th>
<th>Challenge</th>
<th>Skin Reaction Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 ± 1 2 3</td>
<td>0 ± 1 2 3</td>
</tr>
<tr>
<td>CIT</td>
<td>10% (ETOH)</td>
<td>1.0% (ACET)</td>
<td>4 6 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0% (ACET)</td>
<td>7 3 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5% (ACET)</td>
<td>4 6 1</td>
</tr>
<tr>
<td>CAL</td>
<td>1% (ETOH)</td>
<td>0.1% (ACET)</td>
<td>4 6 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1% (ACET)</td>
<td>6 3 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5% (ACET)</td>
<td>5 4 1</td>
</tr>
<tr>
<td>EDA</td>
<td>1% (ETOH)</td>
<td>0.25% (ACET)</td>
<td>2 7 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25% (ACET)</td>
<td>8 2 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5% (ACET)</td>
<td>2 8 1</td>
</tr>
<tr>
<td>OXAZ</td>
<td>1% (ETOH)</td>
<td>0.1% (ACET)</td>
<td>5 4 6</td>
</tr>
<tr>
<td>ACETONE</td>
<td>100%</td>
<td></td>
<td>10 4 6</td>
</tr>
</tbody>
</table>

* Not treated at induction.

showed the most definitive evidence of sensitization relative to controls, based on the greater incidence, severity (including grade 2 reactions), and persistence of the skin reactions. In each case, the higher-challenge-dose control animals showed primary irritation reactions of similar incidence and persistence as the test (i.e., induced) animals' reactions. OXAZ was again used as a positive control in this study, showing similar skin reactions as in the prior study.

The results of the CBH portion of this study are shown in Fig 4. Despite the lower challenge concentrations applied, animals given both induction and challenge exposures to CIT, CAL, and EDA (i+C; i.e., sensitized animals) showed significantly increased (p < 0.05) CBH responses compared to animals given the higher-dose challenge exposures (C) only. This was observed at both 24 and 72 h post challenge. As noted in the figure legend, the basophil infiltrate in the OXAZ challenge sites was somewhat greater than the prior study (tested at the same concentrations) and was again higher at 72 h (209.3 ± 17.9 basophils/400 leukocytes) relative to 24 h (84.0 ± 37.2 basophils/400 leukocytes) post-challenge.

Because of some eosinophil infiltrates noted at oxazolone sensitization sites (see Fig 1), we also quantitated the eosinophil infiltration in this study to determine whether it showed a treatment-related increase. Unlike the situation with basophils, there was no statistically significant difference between animals given both induction and challenge exposures (I+C) to any of the test materials versus animals given higher-dose challenge exposures (C) only (Fig 5).

Assessment of the CBH Response by Quantitation of Basophils by Microscope Field Versus Differential Counting The quantitation of basophils by differential counting was a laborious procedure particularly in larger studies. The difficulty was
Figure 4. Guinea pigs were given three induction applications (in ethanol) of 10% citronellal (CIT), 1% cinnamic aldehyde (CAL), 1% ethylenediamine (EDA), or 1% oxazolone (OXAZ) and were challenged with 1% CIT, 0.1% CAL, 0.25% EDA, or 0.1% OXAZ in acetone, respectively. Vehicle-control animals were untreated during induction and were challenged with 100% acetone. Control animals for the skin testing portion of the study were challenged with the same concentrations but were not biopsied. Instead, additional naive control animals were challenged at 2.5% CIT, 0.5% CAL, or 0.5% EDA for CBH comparison with the respective test animals. Skin-reaction scores are shown in Table III. The high-dose challenge groups (positive irritation control) for CIT, CAL, and EDA were biopsied along with the induced test animals, the naive acetone (ACET) challenged animals, and the OXAZ sensitized animals. The CBH results for OXAZ sensitized animals were 84.0 ± 37.2 (mean number of basophils/400 leukocytes ± SEM) at 24 h and 209.3 ± 17.9 at 72 h.

not in visualizing the basophils but in accurately counting the various types of infiltrating leukocytes. In order to try to simplify the CBH assay, we reevaluated an earlier study (Fig 3) by counting the number of basophils in multiple microscopic fields and analyzing the data for the various treatment groups expressed as the number of basophils per 10 counting fields. These data are presented in Fig 6.

The number of basophils per 10 counting fields was generally lower than the number obtained per 400 leukocytes; however, the response pattern relative to treatment regimen was the same. Biopsies from animals induced and challenged (I+C) with CIT or VAN showed significantly greater basophil numbers than biopsies from the challenge only (C) irritant control animals (p < 0.05). Though it may be necessary to count more than 10 fields to maximize statistical differences (i.e., reduce standard errors), this practice of counting basophils by field greatly reduced the time and effort required to quantify the CBH response as compared to differential counting.

Dose-Response CBH Study with Ethylenediamine In an attempt to better determine the relationship between skin reactions in the Buehler test and the CBH response, a challenge dose-response study was conducted with EDA. EDA was selected because it produced more moderate skin reactions than CIT, VAN, or CAL in the prior studies. In this study, test animals received induction applications of 1% EDA in ethanol and were challenged with 0.2%, 0.05%, or 0.0125% EDA in acetone. In contrast to the prior two studies, a single set of control animals received the same challenge concentrations.

The skin reaction data for this experiment are shown in Table IV. There was substantial irritation observed in this study. Some was attributable to the vehicle; however, EDA elicited irritation reactions were also observed across all dose groups. Despite these irritation reactions, the EDA-induced animals showed clear evidence of sensitization based on the higher incidence, severity, and persistence of the test (induced) group reactions relative to the irritation reactions on the control animals. There was no evidence of a sensitization dose response to EDA challenge based on incidence of positive (grade ≥ "1") reactions alone. There was, however, a reduction in the overall severity of the reactions as the challenge dose was decreased. OXAZ sensitized animals again reacted strongly at challenge, whereas an OXAZ challenged control group added to this experiment as an additional control, did not react differently than the vehicle controls.

In this study, biopsies were taken only at 48 h post-challenge, just after the second skin grading timepoint. The CBH response data (recorded as number of basophils per 10 counting fields rather than by differential counting) are shown in Fig 7. For EDA, the CBH responses showed a minimal dose-response profile similar to the skin-reaction results. No statistically significant differences in CBH response were noted among any of the dosage groups, though there was a trend towards reduced mean basophil infiltration with decreasing challenge dose. For each dose tested, the CBH response in the test (induced) animals was statistically significantly greater

Figure 5. All animals and treatments were as described in Fig 4 except that eosinophil counts, rather than basophil counts, are presented. There was considerable variability in eosinophil counts and no significant differences between the mean eosinophil counts for any skin sections from animals induced and challenged (I+C) with CIT, CAL, or EDA versus the respective challenged only (C) control animals.

Figure 6. All animals and treatments were as described in Fig 3 except that basophils were counted by microscope field rather than by differential counting. Each of the slides evaluated for Fig 3 were reanalyzed by counting the number of basophils present in 8–16 fields across the specimen. The data were then normalized to number of basophils per 10 counting fields. The CBH results for OXAZ-sensitized animals were 36.4 ± 5.8 (mean number of basophils/10 counting fields ± SEM) at 24 h and 136.2 ± 13.1 at 72 h.
Table IV. Incidence of Patch-Test Reactions to Ethylenediamine (EDA) and Oxazolone (OXAZ)

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Induction</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
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<td>EDA</td>
<td>1% (ETOH)*</td>
<td>0.2% (ACET)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2% (ACET)</td>
</tr>
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<td>EDA</td>
<td>1% (ETOH)*</td>
<td>0.05% (ACET)</td>
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<td>0.05% (ACET)</td>
</tr>
<tr>
<td>EDA</td>
<td>1% (ETOH)*</td>
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<td>0.0125% (ACET)</td>
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<tr>
<td>OXAZ</td>
<td>1% (ETOH)*</td>
<td>0.1% (ACET)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1% (ACET)</td>
</tr>
<tr>
<td>VEHICLE</td>
<td>100% ETOH</td>
<td>100% (ACET)</td>
</tr>
</tbody>
</table>

*Not treated at induction.

(p < 0.002) than the respective EDA challenge control group as well as skin sites from sham-treated (ethanol induced and acetone challenged) or naive animals. The added OXAZ control animals (i.e., OXAZ challenge only) showed no increased basophil infiltration compared to skin sites from sham-treated or naive animals. The positive control OXAZ-sensitized animals again showed a strong CBH response (164.4 ± 23.6 basophils/10 counting fields).

**DISCUSSION**

The purpose of this investigation was to determine whether the cutaneous basophil hypersensitivity response could serve to distinguish sensitization from irritation responses to well-known allergens and irritants, and to extend that investigation to weaker human allergens relevant to occupational and consumer exposures. The results presented demonstrate, for the first time, a significant cutaneous basophil response at sites of elicited skin reactions to relatively weak contact allergens. In addition we observed no significant basophil infiltrate at sites of irritation reactions to a known irritant, or at sites of irritant reactions to the same, weakly sensitizing, chemicals.

The ability to consistently distinguish between sensitization and irritation reactions has been a long-standing diagnostic concern and research focus of clinical and investigative dermatologists. There are certain visual criteria one can use in the differential diagnosis of human patch test reactions [14], though with certain classes of chemicals this distinction can be blurred [14,15]. In the guinea pig, both reactions are visually characterized by erythema. General histopathologic evaluation has given some indication of features to look for in making this distinction. Allergic contact dermatitis is characterized by mononuclear and polymorphonuclear leukocyte infiltration and diffuse spongiosis. Irritant dermatitis can have the same features but is also accompanied by ballooning (intracellular edema) [16]. However, routine histopathologic methods or more complex immunohistochemical evaluations (e.g., staining with monoclonal antibodies directed against particular subsets of infiltrating leukocytes) have not been able to consistently differentiate reactions to even common allergens and irritants, much less distinguish the weak sensitizers from the marginal irritant [1,2,17,18]. Recent studies have indicated that induced expression of HLA-DR antigens or the intercellular adhesion molecule-1 (ICAM-1) on epidermal keratinocytes may aid in distinguishing allergic from irritant skin reactions [17,19,20]. However, more work is needed to assess the sensitivity of this approach for diagnosing weak sensitization reactions.

We showed consistent CBH responses to the strong contact allergen, oxazolone, that were in line with those previously reported for other common allergens such as dinitrochlorobenzene [1], or dinitrofluorobenzene [5,21]. As with dinitrofluorobenzene [5], the oxazolone reactions generally increased in basophil infiltrate between 24 and 72 h after challenge. In contrast, no significant CBH response was observed for vehicle-treated skin, oxazolone challenge skin sites on naive animals, or irritant (sodium lauryl sulfate)-treated skin, relative to normal skin sites.

In guinea pig skin sensitization testing, one tries to select a highest non-irritating concentration of a chemical for challenge in order to prevent irritant reactions in the naive control animals that would otherwise confound any test (induced) group reactions. However, this can be difficult with many weak sensitizers that are also irritants, because the irritant dose response under occluded patch testing conditions can often be relatively flat (M. K. Robinson and E. R. Fletcher, unpublished data; see also Table IV). In this study, comparisons were made between skin sites on previously induced test animals that were subsequently challenged at one concentration versus skin sites on naive control animals that were challenged at higher concentrations. This was done in order to produce stronger (i.e., greater incidence, more severe, or more persistent) irritant reactions relative to those occurring on the standard skin test control animals. For each chemical tested (citronellal, vanillin, cinnamon aldehyde, and ethylenediamine), there was a significant increase in basophil...

![Figure 7](image-url)

Figure 7. Guinea pigs were given three induction applications (in ethanol) of 1% ethylenediamine (EDA), 1% oxazolone (OXAZ), or 100% ethanol (vehicle control). The EDA induced animals and naive controls were challenged with 0.2%, 0.05%, and 0.0125% EDA, in acetone, at separate patch sites. The OXAZ-induced animals, and naive controls, were challenged with 0.1% OXAZ in acetone. The ethanol-induced vehicle control animals were challenged with 100% acetone. Skin-reaction scores are shown in Table IV. All animals were biopsied after the 48-h skin grading timepoint. The basophil counts (per 10 counting fields) for animals induced and challenged with EDA were significantly greater than the counts for the respective challenge sites on control animals, the acetone-challenged control sites, and the naive skin sites (p < 0.002). The CBH results for OXAZ-sensitized animals (i.e., induced + challenged) were 164.4 ± 23.6 (mean number of basophils/10 counting fields ± SEM) at the 48-h timepoint.
infiltrate in the challenge skin reaction sites from previously induced animals relative to normal skin sites, vehicle-treated skin sites, or the irritant skin reaction sites. The basophil infiltrate at the irritant skin reaction sites was never significantly different than that of normal or vehicle-treated skin.

The conclusion that the skin reactions reported (in Tables 1-IV) for the induced and challenged animals in these studies were truly indicative of allergic sensitization is based on several factors. First of all, each of the materials tested in this study (except SLS) has been documented to be a human contact allergen [9-13,22]. Equally important and for comparison with this human data, the sensitizing potential of each chemical has previously been documented using several standard guinea pig skin sensitization testing methods, including topical application procedures [22-24].

The Buehler method as used in this study is a well-accepted sensitization testing method [11,25] and has a long history of successful utility in predictive sensitization testing, including the detection of weak human contact allergens [8,11,26]. However, the Buehler method does not use highly exaggerative exposure methods such as intradermal injection with Freund's adjuvant. Because it utilizes only occluded topical application, any excessive skin reactions in induced and challenged animals (i.e., incidence, severity, and/or persistence), relative to reactions observed in naive control animals challenged at the same concentration, should be considered evidence of allergic contact sensitization [8,11]. Persistence of skin reactions \( \geq 1^+ \) were clearly noted in the induced and challenge (test) animals (relative to the controls challenged at the same dose) in each experiment reported in this study. In most cases, the incidence of skin reactions \( \geq 1^+ \) was also increased in the test animals.

In addition, we have conducted challenge/rechallenge Buehler tests on each of these test materials. In each case, we have been able to reproduce positive skin reactions observed at challenge by rechallenging the guinea pigs at a new, previously unexposed skin sites. These evaluations have included dose response and/or open rechallenge studies in which positive rechallenge reactions were again elicited in the test animals at concentrations below the irritation threshold, i.e., at concentrations that produced no positive irritation reactions on new naive rechallenge control animals. Thus, for example, the EDA-treated animals presented in Table IV were later rechallenged in a dose-response evaluation by open application. At EDA concentrations of 3%, 1%, and 0.3%, 17 of 19, nine of 19, and three of 19 animals, respectively, showed positive (grade \( 1^+ \) and \( 2^+ \)) skin reactions. In contrast, none of 10 naive rechallenge control animals showed positive reactions at these concentrations. These open rechallenge data provide strong evidence that the EDA-induced test animals were, in fact, sensitized.

Finally, the association of the basophil response with weak skin reactions was particularly evident with oxazolone. There are no doubts regarding the allergenic properties of this chemical, yet we clearly showed that weak skin reactions resulting from low induction and challenge exposures to oxazolone (Table I) were still associated with significant basophil infiltration (Fig 2). All of these observations support the interpretation that the induced and challenged animals reported here were, in fact, weakly sensitized. It was the purpose of the CBH histologic analysis to provide additional supportive data for that conclusion without the need for repetitive skin testing.

We were not able to make any direct correlation between the skin-reaction grade assigned to individual animals and the subsequently determined basophil number. This may reflect the technical limitation of the skin-grading procedure which is, at best, a subjective, semi-quantitative evaluation. It could also have mechanistic implications. For example, a CBH response is not prerequisite for a positive skin reaction to a contact allergen; it has been shown that cyclophosphamide-treated guinea pigs demonstrate strong skin reactions to dinitrofluorobenzene in the virtual absence of any basophil infiltrate [5], at least at the time points examined. The elicitation of skin reactions in sensitized animals is a complex process involving multiple cell types (resident and recruited) and molecular mediators [27,28]. Neither endpoint (skin grade or basophil infiltrate) alone necessarily provides the definitive indicator of the "sensitized state" of the animal; however, in concert, they may increase the confidence one has in making such an interpretation. The important factor from a predictive testing standpoint is that all, or a random sampling of, test animals be examined for basophil infiltration, not just those animals with "positive" skin grades.

These studies strongly suggest that relatively weak allergic skin reactions to various weak to moderate skin sensitizers are associated with a significant basophil hypersensitivity response that does not occur in primary irritant reactions to the same chemicals. This is true even in studies in which the erythema skin grades are sufficiently weak or infrequent that no absolute judgement as to sensitization potential can be made on the basis of skin grading alone. This methodology may thus provide a more predictive approach to assessment of the skin-sensitization potential of chemicals that have both irritant and weak sensitization properties. It might also eliminate the need for repetitive animal testing often conducted in the past in order to resolve problems associated with distinguishing irritation from allergic contact sensitization.

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REFERENCES


PRELIMINARY ANNOUNCEMENT

The III International Conference on SLE will be held in the Queen Elizabeth II Conference Centre, Westminster, London on April 13–15, 1992. For further details please contact Dr. Graham Hughes or Mrs. Denzil Fletcher, Rheumatology Department, St. Thomas' Hospital, London SE1 7EH. Telephone and Fax number: 01 633 9422.