

A Light-Emitting Mouse to Image Skin Inflammation

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Background: The mouse ear swelling test is a well-accepted method for quantitating the inflammatory response to contact irritants and sensitizing agents. However, this assay measures edema rather than the cellular component of skin inflammation.

Objective: To develop a quantitative and noninvasive assay of inflammatory cell infiltration in contact dermatitis.

Methods: We bred a transgenic bioluminescent mouse that emits light proportional to cutaneous infiltration of inflammatory cells. We characterized this model by correlating luminescence with edema and histologic analysis of affected skin. A mouse strain expressing cyclization recombinase enzyme (cre) recombinase exclusively in myeloid cells was crossed with a reporter strain containing an inactivated form of the luciferase gene. In progeny mice, cre-mediated recombination repaired the luciferase gene, causing light emission from myeloid cells. Light emission and swelling from the inflamed ear was quantitated and compared to the contralateral ear.

Results: Light intensity correlated with the inflammatory cell infiltration in the dermis. In sensitized mice challenged with squaric acid, luminescence increased about 2.2-fold while swelling increased about 1.5-fold.

Conclusion: Our model may serve as a useful screening assay for topical antiinflammatory molecules. Moreover, this approach allows real-time imaging of skin infiltration by specific inflammatory cell lineages in living animals.

ALLERGIC CONTACT DERMATITIS (ACD) is a T-cell-mediated inflammatory reaction occurring at a challenge site with a contact allergen (hapten) in sensitized animals.¹ In the mouse, the sensitization phase lasts 5 to 7 days. The hapten is taken up by Langerhans' cells and is presented to hapten-specific CD4⁺ and CD8⁺ T cells in regional lymph nodes. Challenge with the same hapten in a sensitized animal leads to the elicitation phase. Memory T lymphocytes are recruited to the dermis, resulting in erythema, edema, and inflammatory cell infiltration. In the mouse, the majority of the initial inflammatory cells are neutrophils² whereas in humans, the initial infiltrate is mixed with mostly lymphocytes and dendritic cells.³

The mouse ear swelling test (MEST) is a widely accepted animal model for quantitating delayed-contact hypersensitivity. This test has proven to be useful for

predicting human sensitization responses, in one study correctly identifying 71 of 72 tested materials as being human contact sensitizers.⁴ In 1968, Asherson and Ptak developed the technique of quantitatively measuring contact-delayed hypersensitization by measuring mouse ear swelling with a micrometer.⁵ Subsequent studies confirmed that, when the mouse ear is used as a challenge site, edema associated with the sensitization response is easily quantitated and is reliable as an endpoint.⁶ The general protocol of sensitization followed by ear challenge is as follows: (1) an agent to be tested is applied on a shaved area on the mouse's body; (2) the animal is then challenged on the ear later by applying a diluted dose of the sensitizing agent; and (3) the swelling response is measured at 24 to 48 hours post challenge. The magnitude of the swelling is assumed to correlate with the strength of the sensitizing agent.

However, the MEST does not directly measure the cellular response to ACD. Treatments for ACD involve agents such as glucocorticoids that target cellular response to contact sensitizers.⁷ We hypothesized that measuring the cellular response to contact sensitizers may have clinical utility. Thus, we developed a mouse model of ACD that measures the cellular response to a contact allergen through bioluminescent light emission from myeloid cells.

Bioluminescence is the emission of light by a living organism. Several distinct light-producing enzymes have independently evolved in marine animals, insects, and

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bacteria.⁸ The luciferase (luc) enzyme cloned from the firefly, *Photinus pyralis*, catalyzes a light-producing reaction. The reaction involves the oxidation of D-luciferin to oxyluciferin, producing one photon of light and consuming one molecule of molecular oxygen and adenosine triphosphate.

To create this mouse model, we employed site-specific cyclization recombinase enzyme (cre)/lox recombination. This technique is used as a tool to control gene expression. Recombination is achieved by the cyclization recombinase enzyme that removes deoxyribonucleic acid (DNA) sequences flanked by loxP sites. By breeding a cre/lox mouse, we were able to “knock in” the luciferase gene by specifically repairing it in myeloid cells. The knocking in of the luciferase gene rests on the tissue-specific expression of cre recombinase. This is achieved by the lysozyme 2 (Lyz2) promoter controlling cre expression. Mice encode two lysozyme genes: M (Lyz2), which is specific for myeloid cells, and P, which is expressed in Paneth cells.⁹ Thus, expression of cre repairs the target gene, firefly luciferase (*Luc*) in monocytes, macrophages, and granulocytes. These cells luminesce when the mice are administered the substrate D-luciferin. Thus, skin infiltration by myeloid cells can be measured by localized light emission. We are able to quantitate this light and have shown that it correlates with the density of the cellular infiltrate.

Methods

Generation of Bioluminescent Mice

Both strains of mice used to breed the bioluminescent mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in our animal care facility. The mice were subjected to normal light cycles and fed a standard diet. Bioluminescent mice were bred by crossing B6.129P2-*Lyz2^{tm1(cre)lfo}/J* mice expressing cre recombinase driven by the *Lyz2* promoter with FVB.129S6(B6)-Gt(*ROSA*)26Sor^{tm1(Luc)^{Kael}/J mice with blocked expression of the luciferase gene. Both strains were provided homozygous for the mutated genes, and their F1 progeny were used for trials in the study. The background strains of the cre and luciferase mice were C57BL/6J and FVB/NJ, respectively.}

Allergic Contact Dermatitis

Experiment Design

Animals were sensitized on a shaved area on their lower backs and challenged with squaric acid dibutyl ester

(SADBE) on both surfaces of their right ears 7 days later. The left ears were treated with vehicle on both sides and used as controls. Mouse ear luminescence and thickness was measured on the same mouse for 14 days. Mice were pre-imaged before applying the challenge dose of SADBE or vehicle to establish baseline ear luminescence and were then imaged each day after to evaluate the inflammatory response. Ear thickness was measured before application of SADBE and then each day after imaging.

We induced ACD from a modified protocol of the original optimized MEST⁴ as well as from other MEST protocols.^{1,10} The sensitizing dose of SADBE (HNP Pharmaceuticals, Torrance, CA) was based on the dose used for other mouse contact sensitizers^{4,10} and pretrials of SADBE performed on our mice. The final concentration of SADBE used for sensitization (1%) was the maximal dose determined to sensitize the mice without causing gross skin lesions. A 5% solution of SADBE was diluted with acetone (Fisher Scientific, Pittsburgh, PA) to make a 1% solution. The challenge dose was determined by performing trials with varying dilutions of the sensitizing dose. A concentration of 0.5% of SADBE gave the least gross and microscopic ear damage and was selected for use in trials.

Protocol

Four bioluminescent transgenic mice, two male and two female, were used in trials. All were brown and similar in size. A brown coat was preferred because this color minimizes light scatter during imaging. An electric clipper (WAHL, Sterling, IL) was used to shave a 4 cm² area on the lower back of each mouse. SADBE (1%, 20 μ L) was applied to the shaved area with a micropipette. The area was allowed to dry before the mice were returned to their cages. Seven days later, the challenge dose of SADBE (0.5%, 5 μ L) was applied to the dorsal and ventral surfaces of the right ear of each mouse (10 μ L). The same volume of vehicle (acetone) was applied to the contralateral ear of each mouse in the same fashion.

Mouse Ear Swelling Test

Mouse ears were measured before challenge (day 0), then post challenge after imaging each day for 14 days. Mice were anesthetized with isoflurane (Abbot, Abbott Park, IL) prior to each ear measurement to reduce error from movement. The areas just below the apex of the treated and contralateral ears were measured with a micrometer (Mitutoyo, Japan) in millimeters.

Bioluminescence Imaging

Mouse ears were imaged before challenge (day 0), and then each day post challenge for 14 days. Mice were given an intraperitoneal injection of D-luciferin potassium salt (1%, 5 μ L) (Gold Biotechnology, Inc., St. Louis, MO) with a 0.5 cc 29G syringe (EXCEL, Los Angeles, CA) 5 minutes before imaging. The animals were anesthetized with isoflurane in an oxygen environment (HME Medical Equipment, Temecula, CA). Optical imaging was performed by an IVIS imaging system (Caliper Life Sciences, Hopkinton, MA) using a deeply cooled charge-coupled device (CCD) camera. Peak ear luminescence was analyzed and quantitated with Living Image2.20 software (Caliper Life Sciences, Hopkinton, MA).

Immunofluorescence Assay

Peripheral blood smears were made from a luminescent transgenic mouse and a nonluminescent mouse. A small cut was made with a sterile blade on the tail of each mouse, and a drop of blood was placed on a glass slide and smeared with a coverslip. Slides were allowed to air-dry for 5 minutes. They were then fixed for 10 minutes in cold (-20°C) absolute methanol (Spectrum, Gardena, CA), then for 1 minute in cold (-20°C) acetone (Spectrum), and then washed twice in phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) for 5 minutes. A 5% solution of whole donkey serum, secondary antibody host animal, was used as a blocking agent. The primary antibody, antiluciferase (L0159, Sigma-Aldrich), was diluted to 1/500 in antibody diluent solution (Zymed, South San Francisco, CA) and was added to each slide. The slides were incubated for 60 minutes in a humidified container, then washed three times in PBS for 5 minutes. The secondary antibody, donkey antirabbit-FITC (Jackson Immuno Research Laboratories, Inc., West Grove, PA), was diluted to 1/100 in antibody diluent solution and applied to each slide. The slide preparations were incubated in a humidified dark container for 30 minutes. Slides were then washed three times for 5 minutes in PBS. 4'-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) was diluted to a concentration 1/1000 in PBS and applied to each slide for 10 minutes. The slides were washed once in PBS for 5 minutes, mounted with aqueous mounting media, and imaged on a Nikon Eclipse E400 fluorescent microscope (Nikon Instruments Inc., Melville, NY). Pictures were taken through green and blue filters with a Nikon DXM1200C digital camera mounted on the top of the microscope. Pictures were visualized with *NIS-Elements* V2.3 software (Nikon Instruments).

Histology

Ears were excised with a single-edge razor blade at predetermined time points after challenge and were fixed overnight in 10% zinc buffered formalin. After fixation, samples were given to the department of pathology at our institution and subjected to a standard protocol of dehydration, paraffin embedding, sectioning, and staining with hematoxylin and eosin. Pictures of histology sections were taken with an Olympus U-PMTUC (Olympus Tokyo, Japan) digital camera mounted on a compound light microscope (Olympus).

Electron Microscopy

Mouse ear samples were taken at predetermined time points, cut into 1 mm sections, and fixed in a 2.5% glutaraldehyde 0.1 M sodium cacodylate buffer solution for 24 hours. After fixation, samples were given to the department of pathology at the author's home institution, and a standard departmental protocol was used for embedding and staining the samples. Briefly, samples were placed in 1% OsO_4 for 1 hour, rinsed in sterile distilled water, dehydrated in ethanol, embedded in Epon capsules (Polysciences, Warrington, PA), and cured in a 60° oven overnight. Thickly cut sections were stained with toluidine blue and examined by a conventional light microscope. Areas of interest were selected and then trimmed thin for final staining and electron microscopy (EM) imaging. The selected areas were stained with prepared solutions of uranyl acetate and lead nitrate and sampled on uncoated 150-mesh thin-bar grids. A Hitachi H-600 transmission electron microscope (Chiyoda, Tokyo, Japan) using a 75 kV acceleration voltage was used to image and photograph the magnified grids.

Genotyping

Luminescent mice were screened for the presence of the cre transgene by polymerase chain reaction (PCR) amplification of DNA extracted from tail clippings as described by The Jackson Laboratory genotyping protocols for *Ly^z^{tm1(cre)Ifo}* mice. Briefly, 5 mm clippings from the tips of mice tails were dissolved in 50 μ L of DNAzol Direct (Molecular Research Center, Inc., Cincinnati, OH) and incubated overnight at room temperature. The primer sequences used to amplify the cre gene product were 5-CCC AGA AAT GCC AGA TTA CG-3 and 5-CTT GGG CTG CCA GAA TTT CTC-3. PCR products were subjected to electrophoresis to determine the presence of the cre transgene.

Results

Bioluminescent Mice

Bioluminescent mice with luciferase expression restricted to myeloid cells were bred. To construct this strain, a strain expressing cre recombinase driven by a myeloid-specific promoter was crossed with a luciferase reporter strain. Luciferase expression in the reporter mice was blocked by a stop codon flanked by loxP sequences. In progeny mice, specific expression of cre in myeloid cells repaired the luciferase gene by excising the stop codon. Bioluminescent progeny mice were confirmed to express the 700 bp cre gene product by PCR genotyping. LoxP mice are nonluminescent and do not express cre (Fig 1).

Immunofluorescence and Luciferase Expression in Myeloid Cells

Peripheral blood smears were made from tail veins of both luminescent and nonluminescent loxP mice. Immunofluorescence microscopy was performed after staining fixed peripheral blood smear slides with an antiluciferase antibody. A nuclear fluorescent stain, DAPI, was used as a counterstain to evaluate nuclear morphology and determine cell type (Fig 2). A peripheral blood smear from a luminescent mouse shows an early myeloid cell fluorescing green, indicating the presence of luciferase in this cell. Its curved nuclear morphology is consistent with a myeloid band cell. A peripheral blood smear from a nonluminescent loxP mouse shows two myeloid cells negative for green fluorescence, indicating the absence of luciferase expression in these cells. The DAPI counterstain highlights their nuclear morphology. The curved nuclei show signs of being pinched off to form the lobes characteristic of mature neutrophils of the myeloid cell line.



Figure 1. PCR of tail clippings from a luminescent F1 generation transgenic mouse, A, and from a nonluminescent loxP mouse, B. The migration of the amplified cyclization recombinase enzyme (cre) gene product (700 bp) is only present in the luminescent mouse.

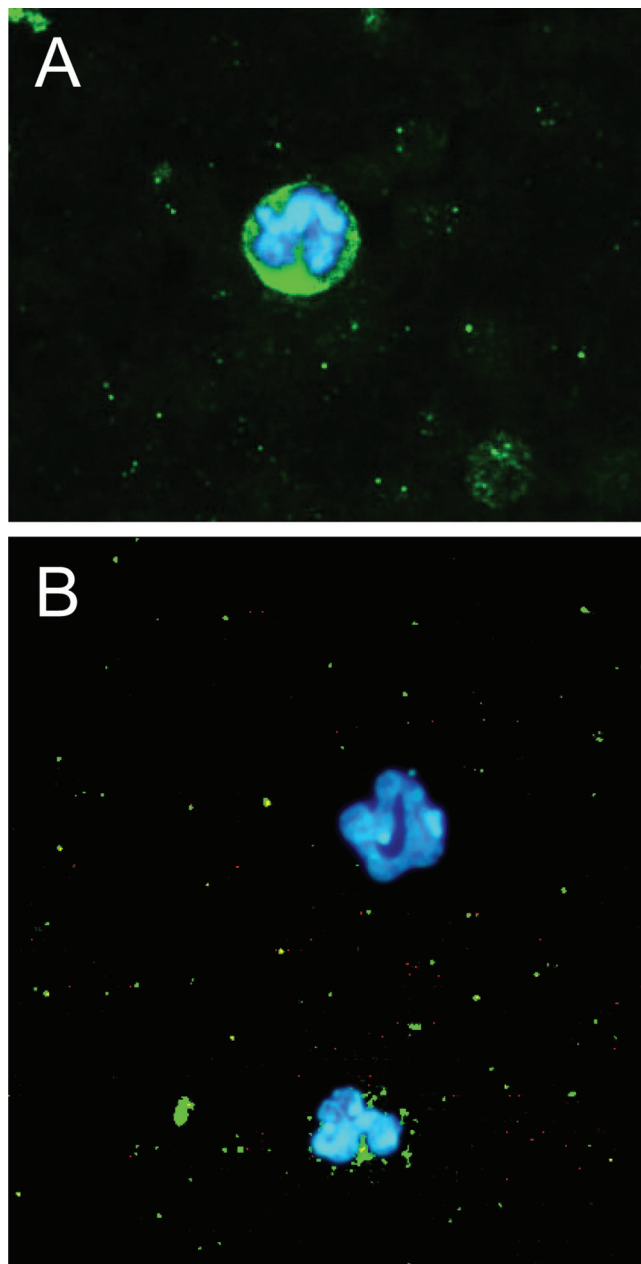


Figure 2. Immunofluorescence microscopy on peripheral blood smears from a luminescent mouse, A, and a loxP mouse, B. An immature myeloid cell is shown in A, staining with an antiluciferase antibody (green) and 4'-6-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue). Two myeloid cells are shown in B, staining only with DAPI (blue).

Correlation of the Bioluminescence Response with Ear Swelling

Luminescent mice were sensitized on their lower backs with 1% SADBE and challenged 7 days later on their right ears with 0.5% SADBE. The left ears were treated with acetone vehicle. Ear thickness and luminescence were then

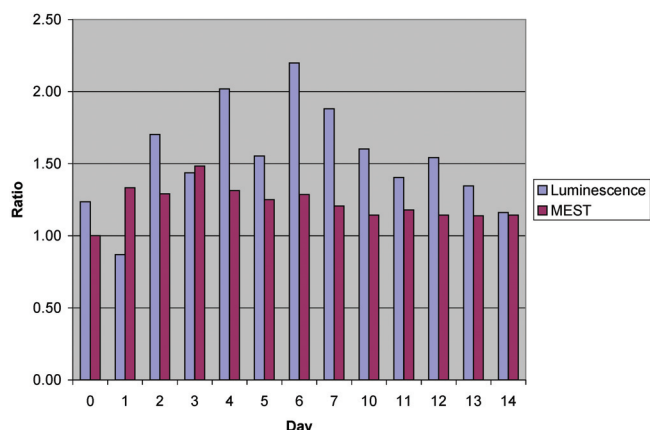


Figure 3. Luminescence and ear swelling response. Mice were sensitized with 1% squaric acid dibutyl ester (SADBE) on their lower backs. After 7 days, ears were challenged with 0.5% SADBE or acetone. The ratio is that of treated ear to control. Results are representative of numerous independent experiments. (MEST = mouse ear swelling test.)

measured daily for 14 days. Right ear luminescence was compared to left ear values to obtain a luminescence ratio at each time point. Ear swelling ratios were measured with

a micrometer. Values from both assays were plotted to compare their magnitudes and time dependence (Fig 3).

The luminescence ear ratio began to increase by 48 hours post challenge to a value of 1.7; a maximum of 2.2 was reached by day 6. The MEST ear ratio began to increase by 24 hours post challenge to a value of 1.3; a maximum of 1.5 was reached by day 3. A comparison of the maximum ratio values of each assay with respect to magnitude and time reveals that the luminescence ear ratio value is 1.5-fold greater in magnitude than the MEST and occurs 3 days later.

Correlation of Luminescence and Cellular Infiltrate

Mouse ears were excised before challenge, at day 6, and at day 14 corresponding to baseline luminescence, peak luminescence, and decline of the luminescence response. Sections were stained with hematoxylin-eosin (Fig 4). The prechallenge luminescence of the right ear was approximately equal to that of the left ear. Histology revealed a thin epidermis and dermis consisting mostly of collagen bundles, blood vessels, and a few inflammatory cells. By day 6, the right ear

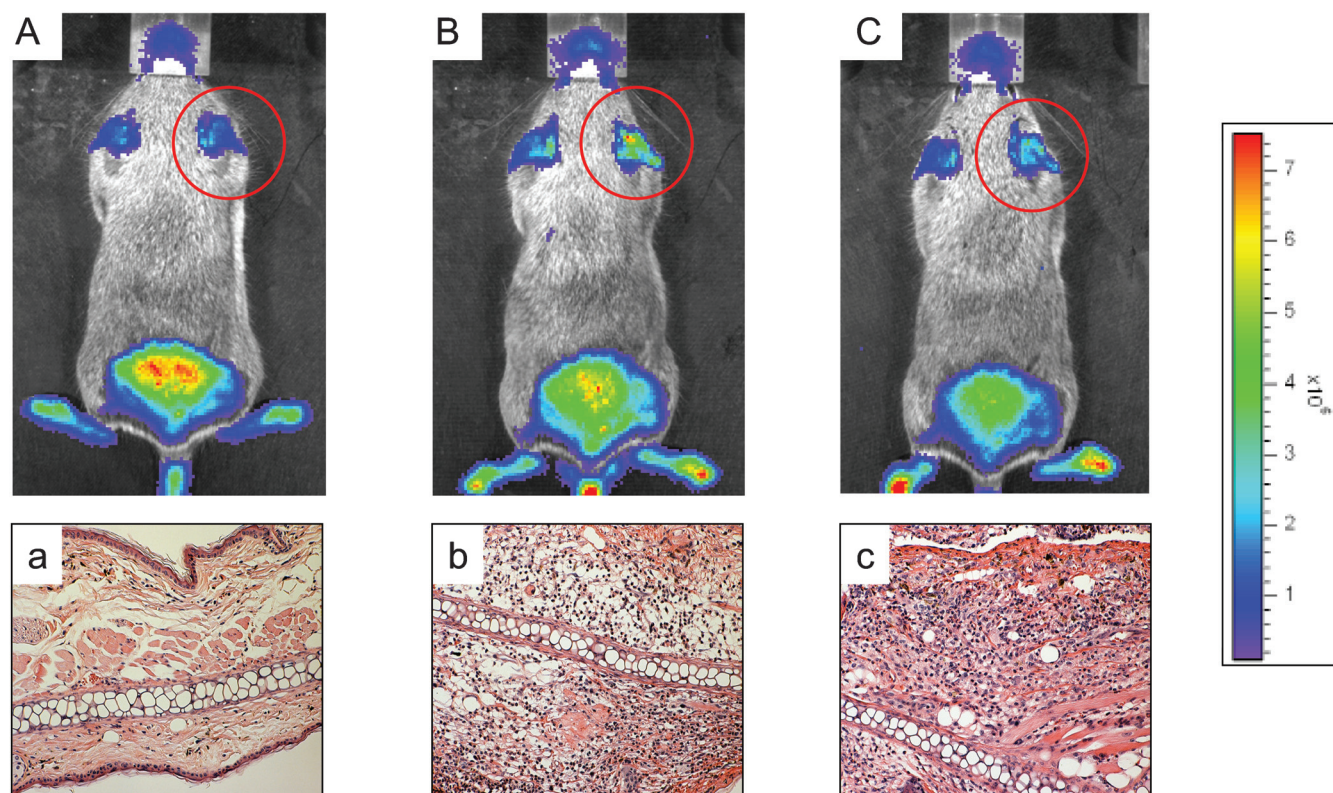


Figure 4. Representative images of mouse ear luminescence and histology, respectively. Histological sections are of corresponding mouse ears circled in the image. A and a, before challenge; B and b, day 6 post challenge; C and c, day 14 post challenge. Lower back signals are at sites of sensitization (hematoxylin and eosin staining, $\times 40$ original magnification).

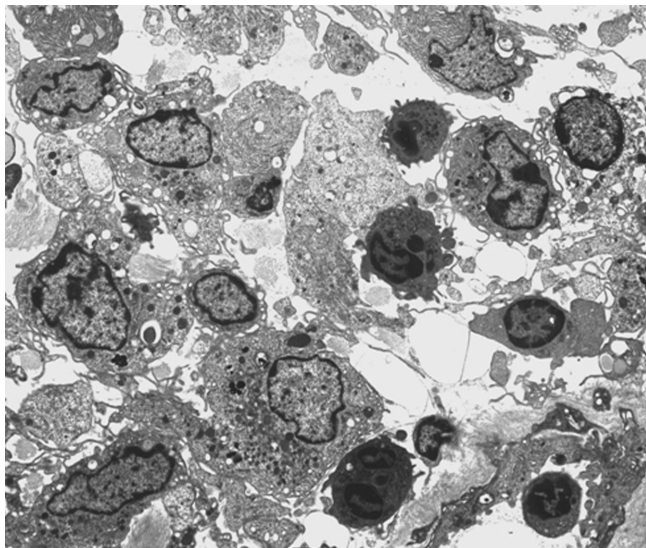


Figure 5. Electron microscopy image of a mouse post challenge. Neutrophils are the smaller and more electron-dense cells. Macrophages are larger, with increased electron density on the periphery of their nuclei (hematoxylin-eosin stain; $\times 2500$ original magnification).

luminescence signal became substantially greater than the left ear signal. Histology at day 6 showed a robust infiltrate of neutrophils. By day 14, the luminescence signal of the treated ear was only slightly greater than that of the contralateral ear. The corresponding histology shows that the dermal infiltrate consisted predominately of macrophages, fibrocytes, and a few neutrophils. An electron microscopy image of mouse ear skin post challenge (Fig 5) shows that the inflammatory infiltrate consisted of myeloid cells.

Bioluminescence Response in a Nonsensitized Mouse

Luminescent mice were treated with 0.5% SADBE on their right ears. The left ears were treated with acetone vehicle.

Ear luminescence was measured daily for 14 days. Right ear luminescence was compared to left ear values to obtain a luminescence ratio at each time point. Ratio values were plotted against time (Fig 6). The luminescence ear ratio began to increase after day 4, reaching a maximum value of 4.2 on day 8. Comparison of these results with those obtained in a sensitized mouse reveals that the increase in ear luminescence was delayed by at least 2 days and that the magnitude of the maximal response was almost twofold greater.

Discussion

This study's result shows that the ear swelling response begins 24 hours post challenge and that a maximum is reached at 72 hours. This is consistent with the swelling results of other contact sensitizers.⁴ The luminescence data show that the myeloid infiltrate begins to appear 48 hours post challenge and reaches a maximum by day 6. Other studies have shown that the cellular infiltrate appears within this time frame.^{2,7} Luminescence data obtained in a nonsensitized mouse show a delay in luminescence when compared to a sensitized mouse. This result is consistent with the delayed swelling seen in primary ACD.¹ However, the magnitude of the maximal response is twofold greater than the magnitude of the maximal sensitized response. Further studies on the differences in the cellular response in sensitized and nonsensitized mice are needed to help explain this phenomenon.

For several decades, the MEST has been used as the standard method of evaluating contact sensitizers. Advances in molecular biology, however, have enabled the genetic labeling of specific cell types. By making myeloid cells luminescent, we were able to measure their density in a delayed type hypersensitivity reaction. This

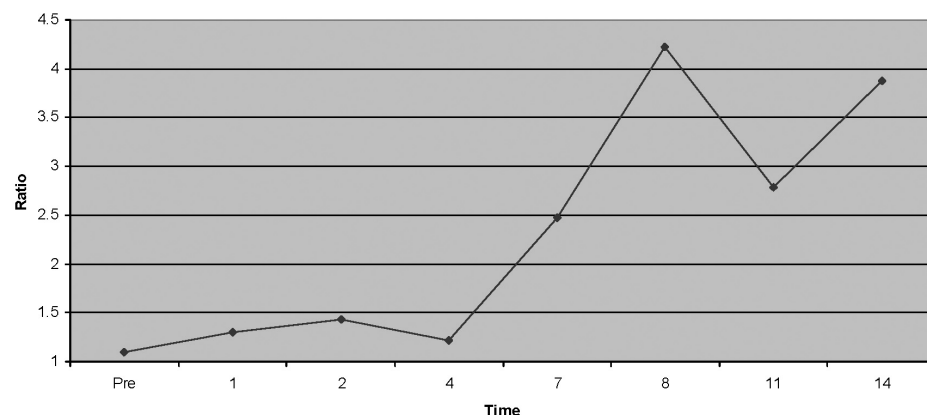


Figure 6. Luminescence in a nonsensitized mouse. 0.5% SADBE was applied to the right ear and acetone to the contralateral ear. Luminescence ratios were calculated for each time point. The results are representative of numerous independent experiments.

endpoint has pharmacologic relevance as current treatments for ACD may target the cellular response.⁷

Studies have shown that both subsets of T helper cells (Th1 and Th2) and cytotoxic T cells are recruited to the challenge site.¹¹ There may also be a role for innate immune cells and the irritant properties of sensitizers in the onset of ACD.^{7,11,12} This study's results show that the swelling response peaks earlier than the myeloid infiltrate. Also, other contact sensitizers tested by the MEST show an early swelling response.⁴ Thus, this model may measure a different aspect of the ACD response that may be more clinically relevant.

Luminescent mice may be used to screen contact sensitizers and pharmacologic agents. Furthermore, bioluminescence allows collection of real-time data regarding specific subsets of cells in vivo. This technology may be applied to other inflammatory skin diseases. Such application may add insight to the physiology of cutaneous inflammation.

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