Irritancy and Allergic Responses Induced by Topical Application of *ortho*-Phthalaldehyde

Stacey E. Anderson,[1] Christina Umbright, Rajendran Sellamuthu, Kara Fluharty, Michael Kashon, Jennifer Franko,
Laurel G. Jackson, Victor J. Johnson, and Pius Joseph

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

[1] To whom correspondence should be addressed at Health Effects Laboratory Division, National Institute for Occupational Safety and Health,
1095 Willowdale Drive, Morgantown, WV 26505. Fax: (304) 285-6126. E-mail: sanderson4@cdc.gov.

Although *ortho*-phthalaldehyde (OPA) has been suggested as an alternative to glutaraldehyde for the sterilization and disinfection of hospital equipment, the toxicity has not been thoroughly investigated. The purpose of these studies was to evaluate the irritancy and sensitization potential of OPA. The EpiDerm Skin Irritation Test was used to evaluate *in vitro* irritancy potential of OPA and glutaraldehyde. Treatment with 0.4125 and 0.55% OPA induced irritation, while glutaraldehyde exposure at these concentrations did not. Consistent with the *in vitro* results, OPA induced irritancy, evaluated by ear swelling, when mice were treated with 0.75%. Initial evaluation of the sensitization potential was conducted using the local lymph node assay at concentrations ranging from 0.005 to 0.75%. A concentration-dependent increase in lymphocyte proliferation was observed with a calculated EC3 value of 0.051% compared to that of 0.089%, previously determined for glutaraldehyde. Immunoglobulin (Ig) E-inducing potential was evaluated by phenotypic analysis of draining lymph node (DLN) cells and measurement of total and specific serum IgE levels. The 0.1 and 0.75% exposed groups yielded significant increases in the IgE+B220+ cell population in the lymph nodes while the 0.75% treated group demonstrated significant increases in total IgE, OPA-specific IgE, and OPA-specific IgG$_1$. In addition, significant increases in interleukin-4 messenger RNA and protein expression in the DLNs were observed in OPA-treated groups. The results demonstrate the dermal irritancy and allergic potential of OPA and raise concern about the proposed/intended use of OPA as a safe alternative to glutaraldehyde.

Key Words: OPA; hypersensitivity; asthma; IgE.

Ortho-Phthalaldehyde (OPA) is an aromatic dialdehyde used as a high-level antimicrobial disinfectant for medical equipment which is sensitive to normal heat or steam sterilization processes. For 40 years, glutaraldehyde has been the primary choice for disinfecting heat-sensitive medical devices; however, it has been reported to induce occupational asthma and other health effects (Gannon *et al.*, 1995). For these reasons, less offensive and presumably safer alternatives to glutaralde-

hyde have been introduced. OPA, the active ingredient present in Cidex OPA, has shown superior anti-mycobactericidal activity as compared to glutaraldehyde (Lerones *et al.*, 2004), allowing for its use at lower concentrations. In addition, low volatility and no need for activation have increased the use of OPA as a more practical alternative to glutaraldehyde.

It is estimated that 3253 workers were potentially exposed to OPA compared to 376,330 for glutaraldehyde from 1981 to 1983 (NIOSH, 1990). After the selection of OPA as an alternative for glutaraldehyde, it is reasonable to assume that more than 300,000 workers could now be exposed. The estimated use of OPA in 2002 was between 10,000 and 500,000 pounds (USEPA, 2006). Along with being approved for disinfecting medical devices, OPA has also been approved for use as an indoor antimicrobial pesticide; an intermediate for the synthesis of pharmaceuticals, medicines, and other organic compounds (ChemicalLand 21, undated); as a reagent for drinking water analysis (USEPA, 2001); and as a diagnostic for urea nitrogen test system (USFDA, 2006).

Although the health effects have not been thoroughly tested, Cidex OPA has been used as a "safe" replacement for glutaraldehyde for the past 10 years. Currently, there are no regulations regarding proper use and safe exposure levels of OPA in spite of the potential of exposure for a large number of healthcare workers and their patients. Concentrations of OPA ranging from 1.0 to 13.5 ppb have been detected in air samples collected from an endoscope cleaning unit of a hospital that used OPA as its primary disinfectant (Tucker, 2008). On the other hand, in addition to the required use of hand, eye, and respiratory protection, stringent occupational exposure ceiling threshold limits have been set for glutaraldehyde (OSHA, 2006).

There are very little data available regarding toxicity for OPA with the majority of the information from case reports. The most notable case report describes four patients who experienced nine episodes of anaphylaxis after a urology practice switched from using glutaraldehyde to OPA for cystoscope disinfection (Sokol, 2004). Skin testing resulted in immediate wheal and flare reactions within 20 min and late reactions at 24 h. The authors concluded that

the immediate and late-phase skin reactions strongly suggest an immunoglobulin (Ig) E-mediated mechanism for the observed reactions. In a separate report, an anaphylactic reaction occurred in a woman receiving repeated checkups by laryngoscopy (Suzukawa *et al.*, 2007, 2006). Skin tests for a 0.55% working solution of OPA diluted at 1:1000 produced wheal and flare reactions within 15 min that lasted for 24 h. Two potential cases of occupational asthma in healthcare workers disinfecting endoscopes and similar devices with Cidex OPA have also been reported (Franchi and Franco, 2005). More recently, Fujita *et al.* (2006) investigated a case involving a female nurse who exhibited slight dyspnea and dry cough with a subsequent diagnosis of bronchial asthma and serous papules and urticaria after working with OPA. For reasons described above, OPA was nominated by National Institute for Occupational Safety and Health (NIOSH) for toxicological evaluation by the National Toxicology Program.

Animal data investigating the health effects associated with OPA exposure are limited. To date, the only published study suggests that OPA injection may act as an adjuvant in a murine ovalbumin (OVA) model (Hasegawa *et al.*, 2009). The high reactivity of OPA, suspected dermal and respiratory irritation, sensitization potential, and structural similarity to glutaraldehyde raise concerns about exposure and the need for regulation. Our laboratory has previously tested the sensitization potential of glutaraldehyde after dermal exposure in mice (Azadi *et al.*, 2004). It was identified as a sensitizer in the local lymph node assay (LLNA) with increases in local and systemic IgE levels, suggesting as IgE-mediated allergic mechanism. The purpose of these studies was to determine the irritancy and sensitization potential of dermal exposure to OPA, provide insight into the mechanism of sensitization, and then to compare these results to those previously reported for glutaraldehyde.

MATERIALS AND METHODS

Test articles. OPA (more than 99%) (OPA) (CAS #643-79-8), alpha-hexylcinnamaldehyde (HCA) (CAS #101-86-0), N, N-dimethylformamide (DMF) (CAS #68-12-2), 2,4-dinitrofluorobenzene (DNFB) (CAS #70-34-8), glutaraldehyde solution (25%) (CAS #111-30-8), and potassium hydroxide (KOH) (CAS #1310-58-3) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

Tissue selection. EpiDerm (Standard EpiDerm [EPI-200], MatTek Corporation) inserts, consisting of highly differentiated epidermis tissue derived from normal, human-derived epidermal keratinocytes, were used to determine the skin irritation potential of OPA and to compare the result with that of glutaraldehyde. The *in vitro* EpiDerm Skin Irritation Test utilizes a normal, human cell–derived, metabolically active skin model that closely mimics the human epidermis both structurally and biochemically. The tissue consists of 10–15 cell layers of stratum corneum overlaying 8–12 cell layers consisting of basal, spinous, and granular cells grown at an air-liquid interface situated on 9-mm diameter, chemically modified, collagen-coated membranes with 0.4 μm pore size. This model is endorsed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Scientific Advisory Committee for its use to assess the dermal corrosion potential of chemicals (NIEHS, 2002).

EpiDerm culture and exposure. EpiDerm inserts (tissues) were preincubated in six-well plates containing 900 μl Dulbecco's Modified Eagle's Medium (DMEM) for 20 h at 37°C and 5% CO_2. Following preincubation, the tissues were treated in duplicate for 1 h with 100 μl test material. During testing, the apical surface of each tissue was exposed to test material while each tissue was fed with DMEM culture medium through the basolateral surface. The treatment doses were dissolved in water at 0.1375, 0.275, 0.4125, 0.55, and 1.1% (wt/vol) OPA; 0.1375, 0.275, 0.4125, 0.55, and 1.1% (vol/vol) glutaraldehyde; 8 N KOH; and 100% H_2O. Immediately following the incubation, EpiDerm samples were rinsed with Dulbeccos Phosphate Buffered Saline and media, and the cell viability was determined.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assay. The cell viability test was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assays according to the manufacturer's procedure (MatTek Corporation). Briefly, the EpiDerm samples were collected immediately following exposure, rinsed twice with PBS, and placed in a fresh 24-well plate containing 300 microliters per well MTT solution (1 mg/ml in MTT diluent). The cultures were then incubated for 3 h at 37°C and 5% CO_2. Next, each insert was removed carefully, the bottom was blotted with soft tissue paper, and the insert was placed in a fresh 24-well plate containing 1 milliliter per well extraction solution. The plates were stored at room temperature for 2 h in the dark. After extraction, the inserts were discarded and an additional 1 ml extraction solution per well was added. The contents of each well were mixed thoroughly, and, in triplicate, 200 μl from each well was pipetted into a 96-well plate to obtain optical density (OD) values. MTT assay results were determined by measuring the ODs of each sample at 570 nm and background corrected with OD values at 650 nm. Because the neat test articles, OPA and glutaraldehyde, result in a reduction of MTT directly (data not shown), the assay was also performed on dead EpiDerm treated identically to the live tissue samples for each chemical and concentration tested. The background-corrected ODs of the dead tissue were then subtracted from the background-corrected live tissue ODs to obtain a final OD. Chemical treatment, concentration, and their interaction were assessed for these analyses. Based on the manufacturer's recommendations, an irritant was identified as a compound resulting in a percent viability less than or equal to 50% of the H_2O control.

Species selection. Female BALB/c mice were used in this study. This mouse strain has a Th2 bias and is commonly used to evaluate IgE-mediated sensitization (Klink and Meade, 2003; Woolhiser *et al.*, 2000). The mice were purchased from Taconic (Germantown, NY) at 6–8 weeks of age. Upon arrival, the animals were allowed to acclimate for a minimum of 5 days. Each shipment of animals was randomly assigned to treatment group, weighed, and individually identified via tail marking using a permanent marker. A preliminary ANOVA on body weights was performed to insure a homogeneous distribution of animals across treatment groups. The animals were housed at a maximum of five per cage in ventilated plastic shoebox cages with hardwood chip bedding, National Institutes of Health-31 modified 6% irradiated rodent diet (Harlan Teklad), and tap water was provided from water bottles, *ad libitum*. The temperature in the animal facility was maintained between 68 and 72°F and the relative humidity between 36 and 57%. The light/dark cycle was maintained on 12-h intervals. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited NIOSH animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

Combined LLNA and irritancy assay. To determine the irritancy and sensitization potential of OPA, a combined LLNA was conducted. OPA dosing concentrations (0.005–0.75%) and vehicle (DMF) were selected based on previously published data on glutaraldehyde (Azadi *et al.*, 2004) for comparison purposes. The LLNA was performed according to the method described in the ICCVAM Peer Review Panel report (NIEHS, 1999) with minor modifications. Briefly, mice (five per group) were topically treated with DMF vehicle, increasing concentrations of OPA, or positive control (30% HCA) on the dorsal surface of each ear (25 microliters per ear) once a day for three consecutive days. DNFB was used as a positive control for the irritancy portion of the experiment. Irritancy measurements were performed as previously described (Woolhiser and Munson, 1999). The thickness of the right and left

ear pinnae of each mouse was measured using a modified engineer's micrometer (Mitutoyo Co.) before the first chemical administration and 24 h following the final exposure. The mean percentage of ear swelling was calculated based on the following equation: [(mean postchallenge ear thickness − mean prechallenge ear thickness)/mean prechallenge thickness] × 100. Animals were allowed to rest for 2 days following the last exposure. On day 6, mice were injected, intravenously, via the lateral tail vein with 20 μCi ^{3}H-thymidine (Dupont NEN; specific activity 2 Ci/mmol). Five hours after ^{3}H-thymidine injection, animals were euthanized via CO_2 inhalation, and the left and right superficial parotid draining lymph nodes (DLNs) located at the bifurcation of the jugular vein were excised and pooled for each animal. Single-cell suspensions were made and incubated overnight in 5% trichloroacetic acid, and samples were counted using a Packard Tri-Carb 2500TR liquid scintillation analyzer (Perkin Elmer). Stimulation indices (SI) were calculated by dividing the mean disintegrations per minute (DPM) per test group by the mean DPM for the vehicle control group. EC3 values (concentration of chemical required to induce a threefold increase over the vehicle control) were calculated based on the equations from Azadi *et al.* (2004) and Basketter *et al.* (1999).

Phenotypic analysis of DLN cells. To determine if the chemicals induced a type I or type IV response, the number of IgE+B220+ cells in the DLNs was quantitated after dermal exposure to OPA using flow cytometry. For the phenotypic analysis, OPA was tested at concentrations up to 0.75%. Lymph node cell phenotypes were analyzed using flow cytometry as described by Manetz and Meade (1999). Mice were topically exposed to DMF or increasing concentrations of OPA topically on the dorsal surface of each ear (25 microliters per ear) once a day for four consecutive days. Animals were allowed to rest for 6 days after the final treatment and then euthanized on day 10 by CO_2 inhalation. Animals were weighed and examined for gross pathology at the end of the experiment. The following organs were removed, cleaned of connective tissue, and weighed: liver, spleen, kidneys, and thymus. DLNs were also collected (two nodes/animal/tube) in 2 ml PBS and were dissociated using the frosted ends of two microscope slides. Cell counts were performed using a Coulter Counter (Z2 model, Beckman Coulter), and 1×10^6 cells per sample were added to the wells of a 96-well plate. Cells were washed using staining buffer (1% bovine serum albumin/0.1% sodium azide in PBS) and then incubated with Fc block (clone 2.4G2). The cells were then incubated with anti-CD45RA/B220 (phycoerythrin, clone RA3-6B2) and anti-IgE antibodies (fluorescein isothiocyanate, clone R-35-72) or the appropriate isotype controls, diluted in staining buffer, washed, and incubated with propidium iodide (PI). All antibodies and isotype controls were purchased from BD Pharmingen. After a final wash, cells were resuspended in staining buffer and analyzed with a Becton Dickinson FACSVantage flow cytometer using a PI viability gate.

Total serum IgE. For analysis of total IgE, OPA was tested at concentrations up to 0.75%. Mice were treated with DMF, increasing concentrations of OPA, or 0.75% glutaraldehyde topically on the dorsal surface of each ear (25 microliters per ear) once a day for four consecutive days. Animals were allowed to rest for 6 days after the final treatment and were euthanized on day 10 by CO_2 inhalation. Following euthanasia of animals, blood samples were collected via cardiac puncture. Sera were separated by centrifugation and frozen at −20°C for subsequent analysis of IgE by ELISA. The standard colorimetric sandwich ELISA was performed as previously described (Butler, 2000). All antibodies and isotype controls were purchased from BD Pharmingen. In brief, 96-well flat bottom plates (Dynatec Immulon-2) were coated with (2 μg/ml in PBS) purified monoclonal rat anti-mouse IgE antibody (clone R35-72), sealed with plate sealers, and incubated overnight at 4°C. The plates were washed three times with PBS/Tween-20 and then blocked for 1 h with 2% newborn calf serum (NCS) and 0.05% sodium azide at room temperature. Initial dilutions (1:10) were made from the serum samples, and IgE control standards were prepared at 500 ng/ml. All dilutions were made in 2% NCS and 0.05% sodium azide. Serum samples and IgE control standard (mouse IgE anti-trinitrophenyl, clone C38-2) were serially diluted (1:2), added to the coated plates in a 100-μl volume, and incubated at room temperature for 1 h. The plates were washed three times with PBS/Tween-20. Biotin-conjugated rat anti-mouse IgE (clone R35-92) was added in a 100-μl

volume, and plates were incubated at room temperature for 1 h. The plates were washed three times with PBS/0.05%Tween-20. Streptavidin-alkaline phosphatase was added (100 μl of a 1:400 dilution), and plates were incubated for 1 h at room temperature. *P*-nitrophenyl phosphate (Sigma) was used as the alkaline phosphatase substrate and added to the plates in a 100-μl volume. The plates were allowed to develop for up to 30 min at room temperature or until the OD reading of the highest standard reached 3.0. Absorbance was determined using a Spectramax Vmax plate reader (Molecular Devices) at 405–605 nm. Data analysis was performed using the IBM Softmax Pro 3.1 (Molecular Devices), and the IgE concentrations for each sample were interpolated from a standard curve using multipoint analysis.

OPA-specific antibodies. Following euthanasia of animals used in the phenotypic analysis assays, blood samples were collected via cardiac puncture. Sera were separated by centrifugation and frozen at −20°C for subsequent analysis of OPA-specific IgE, IgG$_1$, and IgG$_{2a}$. Serum antibodies specific for OPA-conjugated proteins were detected using a custom indirect ELISA. Briefly, Immulon-4 microtiter plates (Nunc, Thermo Scientific) were coated overnight at 4°C with 100 μl of mouse serum albumin (MSA; 10 μg/ml in carbonate buffer, pH 9.5). Plates were washed three times with 0.05M borate buffer followed by addition of 100 μl of 0.5% OPA in distilled deionized water for 1 h at room temperature. Plates were washed three times with PBS/0.05% Tween-20 wash buffer, and nonspecific binding sites were blocked with 1% bovine serum albumin in PBS/0.05% Tween-20 (200 microliters per well) for 30 min. A twofold dilution series (1/10 to 1/5120) of serum was added (50 microliters per well) to wells coated with MSA only and wells with OPA-conjugated MSA and incubated for 2 h at 4°C. Plates were washed three times with PBS/0.05% Tween-20, and then biotin-conjugated antibodies specific for mouse IgG isotypes (IgG$_1$ and IgG$_{2a}$, BD Biosciences) were added for 1 h at 4°C. For detection of OPA-specific IgE, total IgG was removed from the sera using protein-G conjugated to Dynabeads (Invitrogen) that were coated with anti-mouse IgG antibody (BD Biosciences) according to the manufacturer's protocol. Sera were incubated with the beads for 30 min followed by bead removal with a magnet. Dilution series for the remaining sera were performed as for the IgG isotypes above. Finally, plates were washed four times, and avidin-horse radish peroxidase (50 microliters per well) was added for 30 min at room temperature followed by four washes. Tetramethylbenzidine-Turbo substrate (Pierce) was added (50 microliters per well) for 30 min followed by addition of 2M H_2SO_4 stop solution. Absorbance was read on a SpectraMax plate reader (Molecular Devices) at 650 nm during color development and at 450 nm following addition of stop solution. To confirm the presence of OPA-specific IgE antibodies, sera were heated at 56°C for 4 h and run in parallel with the nonheated sera.

Cytokine messenger RNA analysis. To evaluate if T-helper 1 cells (Th1) and Th2 cytokines were involved in OPA sensitization, the DLNs were analyzed for messenger RNA (mRNA) expression after dermal application. The Th1 cytokines assessed were interferon-gamma (IFN-γ) and interleukin (IL)-12, and the Th2 cytokines were IL-4, IL-5, and IL-10. Mice were exposed to DMF or increasing concentrations of OPA topically on the dorsal surface of each ear (25 microliters per ear) once a day for four consecutive days. Animals were then euthanized by CO_2 inhalation 24 h after the last exposure. Animals were weighed, and the DLNs for each animal were collected in 1 ml of TRI Reagent (Molecular Research Center). RNA was isolated using TRI Reagent as specified by the manufacturer. To further purify the RNA, the RNeasy Mini Kit (QIAGEN) with optional DNase treatment was used following the manufacturer's protocol. The concentration of RNA was determined using an nanodrop-1000 spectrophotometer (Thermo Scientific Nanodrop). Reverse transcription of 1 μg RNA was performed using the Advantage RT for PCR Kit (Clontech) as directed by the manufacturer. The RNA was quantified using real-time PCR on a 7900 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) as specified in the manufacturer's protocol. Quantitative RT-PCR data are collected and expressed as relative fold increase over control, calculated by the following formula: $2^{\Delta\Delta Ct} = \Delta Ct_{sample} - \Delta Ct_{control} \times \Delta Ct = Ct_{target} - Ct_{GAPDH}$, where Ct = cycle threshold as defined by manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control.

Analysis of IL-4 production by DLN cells. To determine IL-4 protein production in the DLN, OPA was tested at concentrations up to 0.75%. DLNs were collected (two nodes/animal/tube) in 2 ml PBS from the mice analyzed for total IgE and dissociated using the frosted ends of two microscope slides. Cell counts were performed using a Coulter Counter (Z1 model, Beckman Coulter), and cells were adjusted to 1×10^6 cells per milliliter using sterile Roswell Park Memorial Institute media containing 10% fetal calf serum. Cells were added to a 48-well plate in a 500-μl volume, stimulated with α-CD3 and α-CD28 (2 μg/ml of each; BD Pharmingen), and incubated for 24 h at 37°C and 5% CO_2. Supernatants were analyzed for IL-4 production using an OptEIA ELISA kit purchased from BD Biosciences according to the manufacturer's instructions. Supernatant samples collected from each culture (two stimulated and two unstimulated for each mouse) were added to the plates in triplicate along with serial dilutions of the standards. Plates were read at 450 nm (OD values for standards ranging from 0.77 to 1.93) using a SpectraMax M2 spectrophotometer (Molecular Devices). IL-4 concentration was extrapolated from the standard curve. The final data are expressed as the mean value generated when the concentration identified for the unstimulated cultures was subtracted from the value generated from the stimulated cultures for each mouse.

Statistical analysis. To determine statistical significance for the MTT cell assay, a two-way mixed model ANOVA (SAS Proc Mixed) was utilized for the analysis, and experimental block was incorporated as a random effect. For analysis of animal studies, the data were first tested for homogeneity using the Bartlett's chi-square test. If homogeneous, a one-way ANOVA was conducted. If the ANOVA showed significance at $p < 0.05$ or less, the Dunnett's Multiple Range t-test was used to compare treatment groups with the control group. Linear trend analysis was performed to determine if OPA had exposure concentration-related effects for the specified endpoints. For analysis of OPA-specific antibodies, data were tested for outliers using Grubb's Test ($p < 0.05$), and one sample was a significant outlier for the IgE isotype and was removed from further analysis. One-way ANOVA was then used to test for overall significance within antibody isotypes, and following significant ANOVA, *post hoc* analysis using protected Student's *T*-test was performed to compare treatment groups (JMP, SAS, Cary, NC). Results were considered significant at $p < 0.05$.

RESULTS

In Vitro OPA Application Induced Dermal Irritation and Toxicity

Results of the EpiDerm skin irritation test, comparing the irritation potential of OPA with that of glutaraldehyde, are presented in Figure 1. No significant change in viability was observed at the lower concentrations (0.1375 and 0.275%) for either chemical when compared to the untreated control. Treatment with 0.4125% OPA resulted approximately in a 60% ($p < 0.01$) decrease in cell viability while 0.55 and 1.1% OPA resulted in 90–95% decreases ($p < 0.01$) in cell viability. Treatment with glutaraldehyde only resulted in 30% ($p < 0.01$) decrease in cell viability at the highest (1.1%) concentration when compared to the vehicle control. OPA was significantly more cytotoxic than glutaraldehyde ($p < 0.01$) at all concentrations tested. The positive control, 8 N KOH, decreased cell viability by 98% (data not shown).

In Vivo Treatment with OPA Did Not Induce Local or Systemic Toxicity

There were no OPA-related animal deaths for these studies. All mice appeared clinically normal with no overt clinical
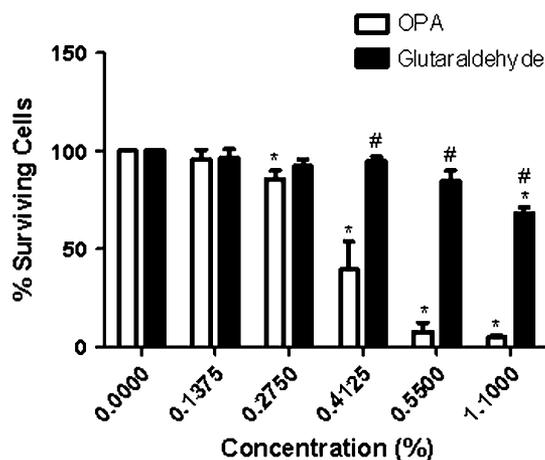

FIG. 1. Irritancy potential following treatment with OPA or glutaraldehyde. Bars represent the mean percent viability ± SE of six EpiDerm tissues determined by MTT assay. "*" Indicates significant difference ($p < 0.01$) between treatment group and untreated control. "#" Indicates significant difference ($p < 0.01$) between OPA and glutaraldehyde at indicated concentration.

toxicity (visual monitoring for appearance, ruffled fur, and discharge from eye, nose, and anus) throughout the course of these studies, and no significant loss of body weight was observed (data not shown).

In Vivo Studies Identify OPA to be an Irritant and Allergic Sensitizer

To confirm the results from the *in vitro* irritancy studies, ear swelling was evaluated in mice after dermal exposure to OPA. A dose-dependent (Linear Trend test; $p < 0.01$) increase in ear swelling was observed after OPA treatment reaching statistical significance only at the 0.75% dose (44% ear swelling, $p < 0.01$) 24 h after final chemical exposure (Fig. 2). A 0.3% DNFB was used as a positive control for irritancy studies and resulted in an average significant increase of 109% ear swelling after
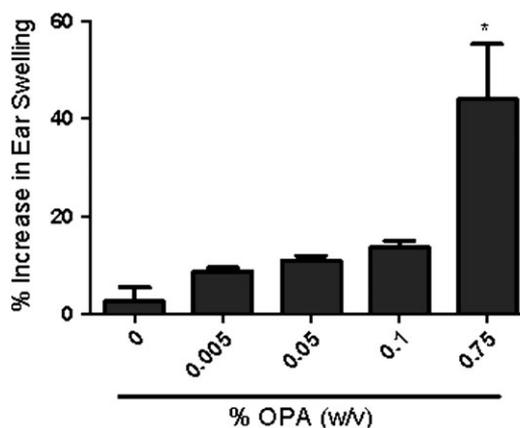
FIG. 2. Ear swelling as a result of topical application of OPA. Analysis of irritation after topical application of OPA. Bars represent mean ± SE of five mice (10 ears) per group. Levels of statistical significance are denoted as "*" ($p < 0.01$) as compared to DMF vehicle.
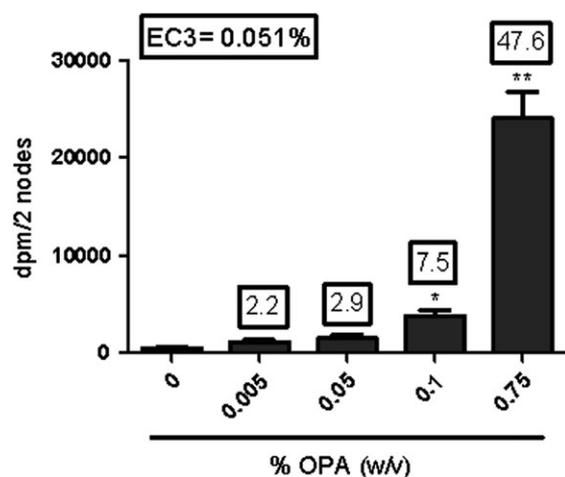
FIG. 3. Allergic sensitization potential after dermal exposure to OPA. Analysis of the allergic sensitization potential of OPA using the LLNA. ^{3}H-thymidine incorporation into DLN cells of BALB/c mice following exposure to vehicle or concentration of OPA. Numbers in boxes appearing above the bars represent the SI for each concentration tested. Bars represent mean ± SE of five mice per group. Levels of statistical significance are denoted as ''*'' ($p < 0.05$) and ''**'' ($p < 0.01$) as compared to DMF vehicle.

application (data not shown). In the LLNA, dose-dependent (Linear Trend test; $p < 0.01$) increase in DLN proliferation was identified after treatment with OPA, with counts from the 0.1 and 0.75% exposed animals significantly elevated over the vehicle control animals (Fig. 3). SI values of 2.2, 2.9, 7.5, and 47.6 were identified for the 0.005, 0.05, 0.1, and 0.75% exposure groups, respectively. An EC3 value of 0.051% (Fig. 3) was calculated. HCA (30%) was used as a positive control for these experiments and resulted in an average SI value of 8.8.

Exposure to OPA Induced an Increase in Local and Systemic IgE Levels

The mechanisms of OPA sensitization were further investigated using phenotypic analysis of B220+ and IgE+B220+ expressing cells in the DLNs. Phenotypic analysis of the DLNs of mice exposed to OPA showed dose-dependent (Linear Trend test; $p < 0.01$) increases in both the B220+ and IgE+B220+ cell populations. Consistent with the LLNA results, a statistically significant increase in percentage of B220+ cells was observed for all concentrations tested. Statistically significant increases in IgE+B220+ expressing cells were identified in the DLN of mice treated with 0.1% (percent counts) and 0.75% (percent and absolute counts) OPA (Table 1). Serum IgE is commonly used as an indicator of type I hypersensitivity to dermal sensitizers. Supporting the phenotyping results, exposure to OPA produced dose-dependent (Linear Trend test; $p < 0.01$) elevations in total serum IgE levels (Fig. 4) reaching statistical significance at the 0.5 and 0.75% treatment groups. When tested in the same study, dermal treatment with 0.75% glutaraldehyde produced 206 ± 26 ng/ml of serum IgE compared to 1081 ± 87 ng/ml for 0.75% OPA (data not shown).

Exposure to OPA-Induced Increases in OPA-Specific Antibodies

Consistent with the results for total IgE, a significant elevation in OPA-specific IgE was also observed after treatment with 0.75% OPA (Fig. 5A). Three out of the five mice exposed to 0.75% OPA had detectable levels of OPA-MSA–specific IgE antibodies in their serum. The levels were low but significantly elevated relative to all other treatment groups. Heat treatment of the serum at 56°C for 4 h abolished the signal from the ELISA, supporting that heat labile IgE was present. Mice treated with 0.75% OPA also showed increases in IgG isotypes specific for OPA-MSA. The incidence as well as level of IgG_1 (five out of five mice, Fig. 5B) antibodies were greater than those for IgG_{2a} (three out of five mice, Fig. 5C). Some of the mice treated with 0.75% OPA also showed increased serum levels of anti-MSA IgG_1 (three out of five mice, Fig. 5B) and IgE (one out of five mice, Fig. 5A), although the serum levels were lower than for OPA-MSA–specific antibodies.

Exposure to OPA Increased Expression of IL-4 mRNA and Protein Expression in the DLN

Cytokine mRNA in the DLNs was analyzed to further evaluate the effect of OPA exposure on Th1/Th2 balance. Cytokine mRNA levels analyzed included IL-4, -10, -12, and

TABLE 1
LLNA, Phenotypic, and IgE Analysis after *In Vivo* OPA Treatment

Dose group	LLNA (DPM)	IgE+B220+ (% lymphocyte population)		B220+ (% lymphocyte population)	
		%	Cells $\times 10^6$	%	Cells $\times 10^6$
DMF	515 ± 88	1.24 ± 0.43	0.16 ± 0.09	18.39 ± 0.72	1.6 ± 0.38
OPA					
0.05%	476 ± 360	1.62 ± 0.48	0.27 ± 0.14	25.55 ± 1.4*	0.42 ± 0.04
0.1%	3845 ± 582	3.51 ± 1.61**	0.52 ± 0.14	26.33 ± 2.5**	3.8 ± 1.4
0.75%	24,020 ± 4616**	23.42 ± 3.23**	1.4 ± 0.24**	31.80 ± 1.2**	15.90 ± 1.3**

Note. Levels of statistical significance are denoted as * ($p \leq 0.05$) and ** ($p \leq 0.01$) as compared to vehicle (DMF). Values present group mean ($n = 5$) ± SE.
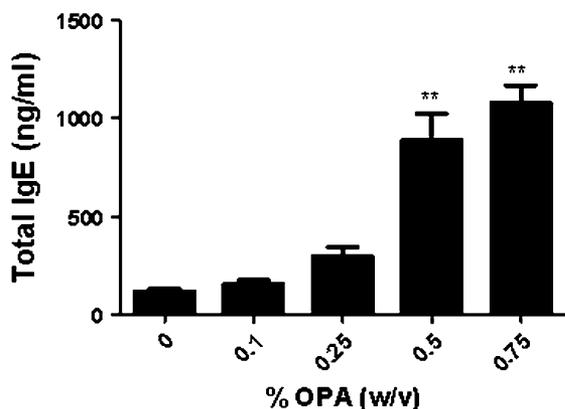
FIG. 4. Serum total IgE levels after dermal exposure to OPA. Analysis of total IgE levels in mouse sera after dermal exposure to OPA. Bars represent mean fold change ± SE of five mice per group. Levels of statistical significance are denoted as ** ($p < 0.01$) as compared to DMF vehicle. Dermal treatment with 0.75% glutaraldehyde produced 206 ± 26 ng/ml of IgE ($p < 0.05$).

IFN-γ. An increase in IL-4 mRNA expression was observed following OPA treatment reaching statistical significance ($p < 0.01$) at 0.75% (Fig. 6A). Levels of IFN-γ, IL-10, or IL-12 mRNA were not modulated following treatment with any of the OPA concentrations tested (data not shown). Consistent with the mRNA results, a dose-responsive (Linear Trend test;

$p < 0.01$) increase in IL-4 protein production by DLN was observed after dermal treatment with OPA reaching statistical significance at concentrations of 0.25% and higher (Fig. 7). When tested in the same study, dermal treatment with 0.75% glutaraldehyde only generated 217 ± 46 pg/ml of IL-4 in the DLN compared to 873 ± 129 pg/ml for the 0.75% OPA treatment group (data not shown).

DISCUSSION

Work-related asthma has become the most frequently diagnosed occupational respiratory illness, accounting for 10–25% of adult asthma with occupations in healthcare having the highest risk (Kogevinas *et al.*, 2007). Of the identified 250 substances suspected to cause occupational asthma, ~90 are low molecular weight (LMW) organic chemicals (Jarvis *et al.*, 2005). The mechanisms by which LMW chemicals cause asthma due to sensitization are believed to be different from that of high molecular weight substances and remain poorly defined (Wild and Lopez, 2003). Glutaraldehyde (100.13 MW) and OPA (134.132 MW) are dialdehydes capable of cross-linking proteins, thus functioning as effective biocides and tissue fixatives. Covalent bonding to primary amines and other protein moieties can result in the formation of hapten-carrier
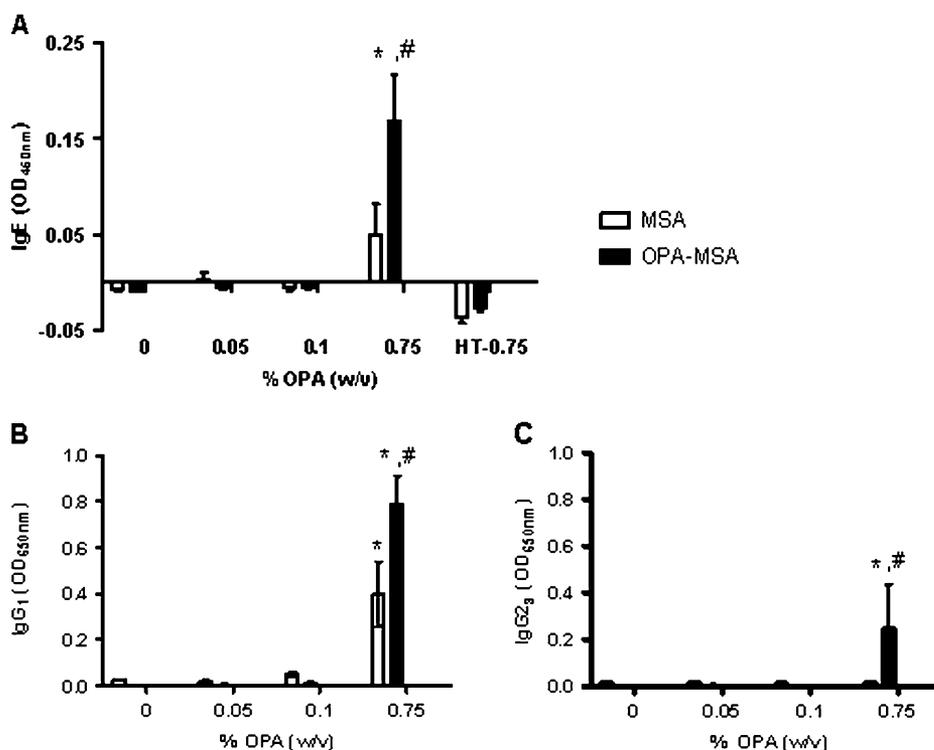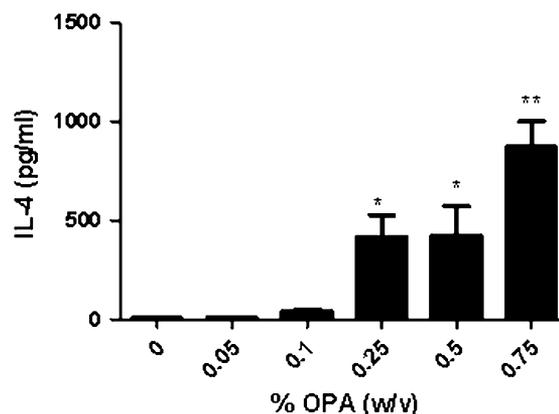

FIG. 5. Serum levels of antibodies specific for OPA protein adducts. Analysis of serum antibodies (A, IgE; B, IgG$_1$; and C, IgG$_{2a}$) specific for OPA-MSA conjugates determined using indirect ELISA. Samples were considered to be positive if the OD was 10 times that of the DMF control. For mice treated with 0.75% OPA, five out of five, three out of five, and three out of five mice were positive for OPA-MSA IgG$_1$, IgG$_{2a}$, and IgE, respectively. Bars represent the mean fold change ± SE of five mice per group. The heat treated-0.75 group represents serum from 0.75% OPA group that was heat treated to destroy IgE while leaving IgG intact. Significant difference at $p < 0.05$ relative to *DMF control group and ''#'' respective MSA antibody titer.
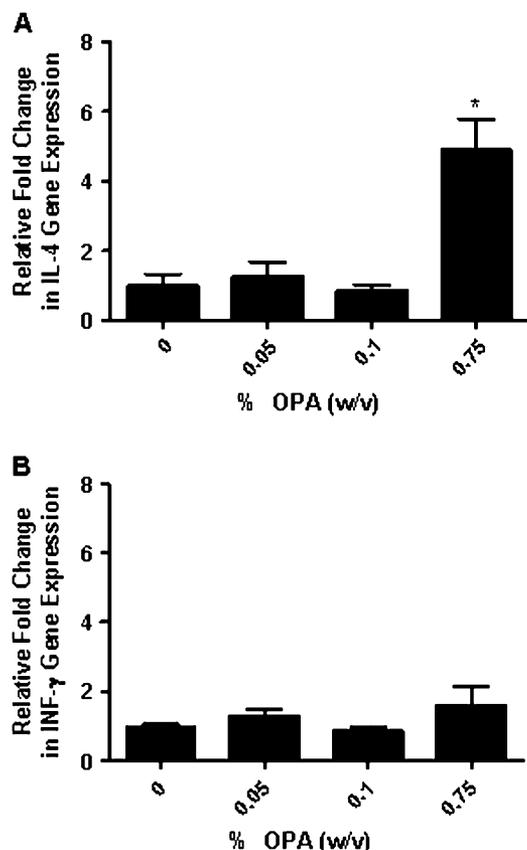
FIG. 6. DLN gene expression measured by quantitative real-time PCR. Quantitative real-time PCR analysis of IL-4 (A) or IFN-γ expression (B). Bars represent mean fold change ± SE of five mice per group. Levels of statistical significance are denoted as ''*'' ($p < 0.01$) as compared to DMF vehicle.

FIG. 7. DLN IL-4 protein expression. Analysis of IL-4 protein expression generated by stimulated DLN after dermal exposure to OPA. Bars represent mean fold change ± SE of five mice per group. Levels of statistical significance are denoted as ''*'' ($p < 0.05$) and ''**'' ($p < 0.01$) as compared to DMF vehicle. Dermal treatment with 0.75% glutaraldehyde generated 217 ± 46 pg/ml of IL-4 in the DLN ($p < 0.01$).

complexes with host proteins and may induce immunological responses. Defining the mechanism by which these chemicals induce sensitization is a critical step toward early diagnosis and prevention of work-related asthma.

While research on OPA is limited, there is extensive literature available on the adverse health effects associated with glutaraldehyde exposure (Gannon *et al.*, 1995; Rideout *et al.*, 2005; Waters *et al.*, 2003). Our laboratory has previously investigated the allergic sensitization caused by exposure to glutaraldehyde. It was identified as a moderate contact sensitizer in LLNA with an EC3 value of 0.089% (Azadi *et al.*, 2004). Although precise comparisons cannot be made because the experiments were not conducted simultaneously, the OPA studies attempted to parallel those previously described for glutaraldehyde; vehicle, mouse strain, and reagents were kept consistent between the studies. Table 2 summarizes the results from the two studies. The EC3 value for glutaraldehyde was extrapolated because the lowest concentration tested yielded an SI value greater than 3. For comparison purposes, two EC3 values are presented for OPA, one calculated based on the equation described by Basketter *et al.* (1999) and the other calculated based on the equation used to determine the EC3

value for glutaraldehyde as cited by Azadi *et al.* (2004). The EC3 values calculated for OPA, using both equations (0.079 and 0.051%), are similar to that described for glutaraldehyde (0.089%). In addition, the SI values for the 0.1 and 0.75% exposure groups were 3.5 and 12.7 for glutaraldehyde and 7.5 and 47.6 for OPA, respectively.

OPA and glutaraldehyde were both shown to induce irritation; however, *in vitro* studies showed that lower concentrations of OPA (0.4125%) had increased toxicity compared to glutaraldehyde (1.1%) when both chemicals were tested simultaneously. Skin irritation and the associated inflammatory response may be an important component to the development of a robust acquired immune response and the process of sensitization. In this light, data from both *in vitro* and *in vivo* studies suggest that OPA is an irritant as evidenced by the direct cytotoxicity to primary skin

TABLE 2
Comparison of Glutaraldehyde and OPA Sensitization Potentials

	SI	EC3	Total IgE	%B220+	%IgE+B220+
DMF[a]	1	0.089%[b]	298 ± 37	21.5 ± 1.6	2.2 ± 0.9
0.1% Glutaraldehyde[a]	3.5		359 ± 11	39.7 ± 1.3	7.2 ± 0.9
0.75% Glutaraldehyde[a]	12.7		513 ± 98	47.2 ± 1.1	19.1 ± 2.9
DMF	1	0.051%[c]	117 ± 13	18.4 ± 0.7	1.2 ± 0.4
0.1% OPA	7.5	0.079%[b]	154 ± 20	26.3 ± 2.5	3.5 ± 1.6
0.75% OPA	47.6		1081 ± 87	31.8 ± 1.2	23.4 ± 3.2

[a]Data represent results published by Azadi *et al.* (2004).
[b]Calculated using equation cited by Azadi *et al.* (2004).
[c]Calculated using equation described by Basketter *et al.* (1999).

cultures and the marked increase in ear swelling observed following topical exposure in mice. Concomitant with irritation was significant elevations in total and OPA-specific IgE serum antibodies and IgE+B220+ cell population in the DLNs of OPA-exposed mice. Manetz and Meade (1999) have shown that select chemicals capable of inducing IgE-mediated allergic responses have similar peak increases in the percent IgE+B220+ and B220+ populations and tend to become significantly elevated at equivalent concentrations. A similar trend was observed after treatment with 0.75% OPA (IgE+B220+ population increased to 23.42 ± 3.23% and B220+ population increased to 31.80 ± 1.2% of total lymphocytes). Additionally, the OPA concentration that induced significant increases in the IgE+B220+ cell population (0.1%) was lower than the concentration significantly elevating total serum IgE (0.75%). The elevation in IgE+B220+ cells relates to local binding of soluble IgE to the CD23 receptor on B cells in the DLNs, which may occur before IgE elevations in the serum. These data along with the observed elevations in IL-4 expression further support the involvement of IgE in the allergic response. Similar elevations in IL-4 and IgE+B220+ cells in DLN were observed following exposure of mice to glutaraldehyde although higher concentrations were required relative to OPA (Azadi et al., 2004). IL-4 is crucial for IgE expression because it is required for increased expression of CD23 on the B cells, B-cell proliferation, isotype switching, and IgE synthesis, and its expression often supports polarization to a Th2 hypersensitivity response. Further supporting the Th2 polarization, a significant elevation in OPA-specific IgG$_1$, a Th2-driven isotype, was observed for the 0.75% treatment group compared to vehicle control (Snapper et al., 1988a). With the exception of a single mouse, this elevation was approximately five- to eightfold greater than that observed for OPA-specific IgG$_{2a}$ antibody levels. Elevations in IgG$_{2a}$ typically represent an inflammatory response and suggest polarization to a Th1 response with class switching most often caused by elevations in IFN-γ and tumor necrosis factor-α (Snapper et al., 1988b). However, no increase in IFN-γ mRNA was observed when the DLNs of these mice were analyzed, further supporting a Th2 response. These results suggest that OPA acts as a Th2 sensitizer and may have implications for respiratory allergy. It is also possible that OPA may exacerbate existing allergy by establishing a Th2-supporting immunological milieu. Hasegawa et al. (2009) showed significant increases in OVA-specific serum IgE, IL-4 mRNA, and IL-5 mRNA in animals exposed to OVA and OPA compared to OVA only. Consistent with our results, IL-4 mRNA was also enhanced when the animals were exposed to OPA without OVA. A positive result in the LLNA (EC3 = 0.051% extrapolated from current LLNA) along with significant elevations in IgE and IL-4 expression at concentrations below or similar to that designated as the working solution (0.55%) strongly suggests that OPA is a sensitizing chemical and that this chemical would be expected to cause significant activation of the immune system following skin exposure.

These are the first studies to describe immunotoxicity induced by dermal exposure to OPA. The LLNA is a test method that was developed and validated for the identification of contact sensitizers, and while LMW chemical respiratory allergens, such as toluene diisocyanate and trimellitic anhydride (TMA), induce positive responses in the LLNA, not all LLNA-positive chemicals are associated with respiratory allergy or asthma. Although it is often thought that the most common route of exposure to respiratory allergens is inhalation, published animal and human data have shown that dermal exposure may result in respiratory tract sensitization (Fukuyama et al., 2009; Herrick et al., 2002; Petsonk et al., 2000). Studies have shown that topical application is effective in sensitizing rats to TMA, resulting in airway reactivity after inhalational challenge (Zhang et al., 2004). In addition, other literature has also shown that dermal exposure of mice to natural rubber latex can induce latex-specific IgE and airway hyperreactivity upon respiratory challenge (Howell et al., 2002). Therefore, skin exposure needs to be addressed in the risk assessment for OPA.

The identification of OPA as an irritant and sensitizing chemical along with significant increases in IgE+B220+ cells in the DLNs, IL-4 mRNA and protein expression, total and specific IgE, OPA-specific IgG$_1$, and published case reports raises concern that OPA may function as an IgE-mediated sensitizer. Comparison of these data to that obtained for glutaraldehyde demonstrates that the sensitizing potential for OPA is comparable to that of glutaraldehyde, suggesting that it may not be a safe alternative. Similar to glutaraldehyde, in an effort to reduce and prevent occupational exposure and disease, regulations for the use of this chemical may need to be established.

ACKNOWLEDGMENTS

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