

An immune response study of oakmoss absolute and its constituents atranol and chloroatranol

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Summary

Background. Atranol and chloroatranol are the main allergens of oakmoss absolute. However, the immune responses induced by these substances are poorly characterized.

Objectives. To characterize immune responses induced by atranol, chloroatranol and oakmoss absolute in mice.

Methods. Mice were sensitized and challenged with various concentrations of atranol, chloroatranol, and oakmoss absolute. The immune responses were analysed as B cell infiltration, T cell proliferation in the draining lymph nodes, and expression of interleukin (IL)-18, IL-1 β and tumour necrosis factor- α in skin. The cytotoxicity of atranol and chloroatranol against keratinocytes was determined.

Results. Sensitization experiments showed that atranol, chloroatranol and oakmoss induced sensitization when applied in high concentrations. Challenge experiments showed that even low concentrations of atranol and chloroatranol induced sensitization. In parallel, atranol and chloroatranol elicited challenge reactions following sensitization with oakmoss. The magnitude of the immune response to the three allergens increased in the following order: atranol, chloroatranol, and oakmoss. The expression of proinflammatory cytokines was induced by chloroatranol and oakmoss, but not by atranol. Chloroatranol was found to be more cytotoxic than atranol against keratinocytes.

Conclusions. Atranol and chloroatranol can elicit both sensitization and challenge reactions, but the mixture of allergens in oakmoss absolute is more potent than atranol and chloroatranol alone.

Key words: atranol; chloroatranol; oakmoss.

Oakmoss, a natural extract derived from the lichen *Evernia prunastri*, is used in perfumery as a natural fragrance, owing to its woody aroma and fixative properties. In

perfumes, oakmoss is used as an absolute product. By solvent extraction, oakmoss absolute is transformed from oakmoss natural into a solid mass. The solid mass is diluted with ethanol, and, when the ethanol evaporates, the resulting residue is called oakmoss absolute (1). Oakmoss absolute is a major problem for patients with fragrance allergy. A recent study on patients who had been patch tested with oakmoss from 2003 to 2010 showed that 5.71% of the patients had a positive patch test reaction (2). Oakmoss absolute is one of the eight ingredients in fragrance mix I (FMI) of the European baseline patch test series. In further testing of patients who respond positively

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to FMI, oakmoss is the leading allergen, eliciting reactions in ~30% of FMI-positive patients (3–5). Interestingly, when the sensitization capacity of oakmoss is analysed with the local lymph node assay (LLNA), oakmoss absolute appears to be a weak to moderate allergen (EC3 values of 5.6–18.47%) (6). One could therefore speculate that the high frequency of oakmoss allergies in patients responding to FMI is caused by a high degree of exposure to oakmoss absolute. However, based on labelling, an investigation of the use of oakmoss absolute in perfumed cosmetic and household products for sale in the United Kingdom showed that oakmoss is used in 4% of these products (7). Thus, the ability of oakmoss to induce an immune response needs further investigation.

Atranol and chloroatranol have been identified as the main allergens in oakmoss, and can elicit positive patch test reactions in individuals already sensitized to oakmoss (8). The sensitizing capacity of atranol and chloroatranol remains to be characterized, although one report based on the LLNA has suggested that both have a high capacity to cause skin sensitization, with EC3 values of 0.6% and 0.4%, respectively (9). In 2004, an independent advisory committee to the European Commission, the Scientific Committee on Consumer Products, therefore recommended that atranol and chloroatranol should not be present in cosmetic products (9). However, investigations of the use of atranol and chloroatranol in 22 eau de toilette/eau de perfume products purchased from the Danish retail market in 2007 showed that 59% contained atranol and 36% contained chloroatranol (10).

The immune response to contact allergens is classically described as a T cell-mediated response involving both CD4⁺ and CD8⁺ T cells (11). However, several studies have indicated that B cells might also be involved in the allergic response (12–16). Allergen-specific antibodies, which are produced during the response, have been shown to play an important role in the elicitation reaction (14, 16). Furthermore, it has been shown that B cells are recruited to the draining lymph nodes in high numbers following allergen exposure, whereas exposure to irritants does not lead to B cell recruitment (12). However, others have shown that the lack of B cells results in only a minor reduction in ear-swelling in B cell-deficient μ MT mice as compared with control mice following allergen challenge, suggesting that B cells are not required during the immune response to contact allergens (17). Interestingly, it has recently been suggested that B cells might have an anti-inflammatory role during the allergic response, as mice lacking CD19 have an exaggerated response associated with reduced interleukin (IL)-10 production following allergen exposure (14, 15). A specific regulatory B cell

subset characterized by being CD1d^{high}CD5⁺ were found to be responsible for the B cell-mediated regulation of the allergic response (15). Together, these findings suggest that B cells might have both proinflammatory and anti-inflammatory roles in responses to contact allergens. These different roles might be dependent on mouse strain, allergen, and experimental protocol. Thus, the role of B cells in contact hypersensitivity needs further investigation.

In addition to T and B cells, several cell types within the skin, for example keratinocytes and Langerhans cells, play important roles in the early response to allergens, in particular through their production of proinflammatory cytokines such as IL-18, IL-1 β , and tumour necrosis factor- α (TNF- α) (18–20). Allergens are sensed by these cells via pattern recognition receptors such as the toll-like receptors (TLRs). Both TLR2 and TLR4 have been shown to be important for the induction of the early inflammatory response to contact allergens (18, 19). Activation of TLRs can be either direct or indirect via allergen-induced production of endogenous TLR ligands (19–21). Recently, it was shown that the induction of the proinflammatory cytokines IL-1 β , IL-6 and interferon- γ correlates with the strength of the allergen (22).

The aim of the present study was to investigate the immune responses to atranol, chloroatranol and oakmoss with different mouse models and an *in vitro* assay.

Materials and Methods

Mice

Female CBA mice were purchased from Harlan, and housed in the specific pathogen-free animal facility at the University of Copenhagen in accordance with national animal protection guidelines (licence number 2007/501-1357). In each experiment, groups of 4 mice were used.

Chemicals

Oakmoss absolute (*E. prunastri*) was purchased from Biolandes (Le Sen, France), and contained 2.1% atranol and 0.9% chloroatranol (8). Atranol and chloroatranol were synthesized according to classic organic chemistry methods, with a purity of 98% (8).

Cells and culture conditions

The murine keratinocyte cell line PAM2.12 was kindly provided by W. L. Havran (The Scripps Research Institute, San Diego, CA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C in

5% CO₂ (Sigma, Brøndby, Denmark). DMEM was supplemented with 10% fetal bovine serum (FBS), 0.5 IU/l penicillin, 500 mg/l streptomycin, 1% L-glutamine, 2-mercaptoethanol, 12.5 ml of 25 mM HEPES, and 5 ml of 100 mM sodium pyruvate (Gibco, Invitrogen, Nærum, Denmark). The medium was changed every other day, and cells were split when 80% confluent.

Sensitization experiments

For the study of sensitization, mice were painted with 25 µl of either atranol or chloroatranol (1%, 2.5%, 5%, 10%, or 20%) or oakmoss (1%, 5%, and 20%) in olive oil/acetone (OOA) (3:1) on the dorsal side of both ears for three consecutive days (D0–D2). These concentrations were chosen on the basis of the solubility of oak moss in OOA. The thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which is incorporated in DNA of proliferating cells, was used to determine cellular proliferation (23–25). On D3, mice were given BrdU (0.8 mg/ml) in the drinking water, as previously described (23). Mice were killed on D5, and the draining auricular lymph nodes were removed.

Challenge experiments

For the study of challenge responses and cross-reactivity to the allergens, mice were painted with 25 µl of 20% oakmoss, 0.4% (26.2 mM) atranol or 0.18% (9.6 mM) chloroatranol in OOA (3:1) on the dorsal side of both ears for three consecutive days (D0–D2). These concentrations of atranol and chloroatranol were chosen because they are the concentrations in 20% oakmoss (8). After a minimum of 21 days, mice were challenged with 20% oakmoss, 0.4% atranol, 0.18% chloroatranol, or OOA as a control. To determine T cell proliferation, mice were given BrdU (0.8 mg/ml) in the drinking water on the challenge day. Mice were killed after 48 hr, and the draining auricular lymph nodes were removed.

Flow cytometry

A single-cell suspension was prepared manually from the draining lymph nodes, and the total cell number was determined. To determine the number of B cells, the cell suspensions were incubated with fluorochrome-conjugated anti-CD19 (clone ID3; BD Biosciences, Albertslund, Denmark) diluted in ice-cold phosphate-buffered saline (PBS) containing 2% FBS and 0.1% NaN₃ for 30 min on ice. After washing, cells were analysed on a FACSCalibur (BD Biosciences) with CELLQUEST software.

T cell proliferation

Proliferating cells were identified by intracellular BrdU staining according to the instructions of the manufacturer (BD Biosciences). In brief, cells were adjusted to 1×10^7 cells/ml and plated in 96-well plates (100 µl/well). Cells were stained for CD4 (RM4-5) and CD8a (53-6.7), both from BD Biosciences, as described above. After washing, cells were fixed, permeabilized, and treated with 300 µg/ml DNase for 60 min at 37°C. Finally, cells were stained intracellularly with anti-BrdU antibodies, and analysed by flow cytometry.

IL-1β, IL-18 and TNF-α quantitative polymerase chain reaction (qPCR)

Mice were treated on both ears with 25 µl of 20% oakmoss, 0.4% (26.2 mM) atranol or 0.18% (9.6 mM) chloroatranol or a combination of 0.4% atranol and 0.18% chloroatranol in OOA (3:1). After 24 hr, 5-mm biopsies were collected from the ears, snap-frozen in liquid nitrogen, and stored at –80°C until RNA extraction. The ears were disrupted in a PreCellys24 homogenizer (Bertin Technologies, Montigny-le Bretonneux, France), and total RNA was extracted with an RNeasy kit (Qiagen, Hilden, Germany). For reverse transcription, we used TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). IL-1β, IL-18 and TNF-α mRNA expression was detected with TaqMan Gene Expression Assays (Assay ID Mm00434228_m1, Assay ID Mm004225-m1, and Assay ID Mm0043260_g1, respectively), with the glyceraldehyde-3-phosphate dehydrogenase gene (Mm99999915_g1) as the control housekeeping gene. Real-time polymerase chain reactions (PCRs) were conducted on an Mx3005P Real-Time PCR machine (Agilent Technologies, Santa Clara, CA, USA), with the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 PCR cycles at 95°C for 15 seconds and 60°C for 1 min.

3-(4,5-dimethylthiazolyl-2)-2,5-Diphenyl tetrazolium bromide (MTT) assay

PAM2.12 cells (1×10^4 cells/well) were plated in a flat-bottomed 96-well plate 24 hr before stimulation at 37°C in 5% CO₂. Cells were stimulated with the indicated concentration of either atranol or chloroatranol for 24 hr at 37°C in 5% CO₂. As a control, cells were stimulated with acetone, which was used as the vehicle in these experiments. The supernatant was removed from the cells, and 150 µl of fresh medium was added per well. To each well, 25 µl of MTT buffer (5 mg/ml MTT in PBS, sterile filtered) was added, and samples were incubated for 4 hr at 37°C. MTT lysis buffer (195 g of sodium dodecyl

sulfate, 300 ml of *N,N*-dimethylformamide, 5 ml of 2.5 M HCl, 23 ml of 100% acetic acid, and 240 ml of H₂O) was added, and the plate was incubated overnight to dissolve formazan crystals. Absorbance at 562 nm was measured in a microplate reader (EL311; Bio-Tek Instruments, Brøndby, Denmark).

Statistical analysis

Statistical significance was calculated with a two-sample equal variance Student's *t*-test. The analysis was performed on data from a minimum of two individual experiments comparing mice (*n* = 4) exposed to the different fragrance ingredients with control mice.

Results

To determine the sensitization capacity and the immune response induced by atranol, chloroatranol, and oakmoss, we painted mice on the dorsal side of the ears with various concentrations for 3 days, and then used a modified version of the LLNA to determine the number of infiltrating B cells and proliferating T cells in the draining lymph nodes (23). Concentration-dependent infiltration of CD19⁺ B cells was found after sensitization with atranol, chloroatranol, or oakmoss (Fig. 1a–c). Likewise, for both CD4⁺ and CD8⁺ T cells, concentration-dependent proliferation was seen after sensitization with any of the three components (Fig. 1d–i). Interestingly, approximately five times more CD4⁺ BrdU⁺ T cells than CD8⁺ BrdU⁺ T cells were observed after sensitization with either atranol or chloroatranol (Fig. 1d,e,g,h). Furthermore, in mice sensitized with oakmoss, an approximately 10-fold increase in the number of proliferating CD4⁺ T cells as compared with CD8⁺ T cells was found (Fig. 1f, i). Even more interesting, keeping in mind that 20% oakmoss contains 0.4% atranol and 0.18% chloroatranol, was that, when the levels of T cell proliferation induced by the 20% dilutions of the three components were compared, oakmoss induced four-fold more proliferation of CD4⁺ T cells than atranol and chloroatranol, whereas only a minor increase in CD8⁺ T cell proliferation was seen. Thus, we could conclude that atranol, chloroatranol and oakmoss are all able to induce T cell proliferation, and that CD4⁺ T cells dominate the response.

To compare the elicitation potentials of the allergens, we next analysed the elicitation reactions induced by atranol, chloroatranol, and oakmoss absolute. The experimental setup is shown in Fig. 2a. In this setup, we used 20% oakmoss, 0.4% atranol, and 0.18% chloroatranol. These concentrations were chosen on the basis of the solubility of oakmoss, because the

highest possible oakmoss dilution that we could make and still have a homogeneous solution was 20%. The concentrations of atranol and chloroatranol correlate to the concentrations of these found in a 20% oakmoss dilution, as 2% atranol and 0.9% chloroatranol are found in pure oakmoss (8). The mice were sensitized with atranol, chloroatranol, or oakmoss absolute. Challenge with atranol induced significant infiltration of CD19⁺ B cells and proliferation of CD4⁺ T cells in mice sensitized with chloroatranol or oakmoss (Fig. 2b). However, challenge with atranol did not induce a significant responses in mice sensitized with atranol (Fig. 2b). Challenge with chloroatranol induced significant infiltration of CD19⁺ B cells in mice sensitized with chloroatranol or oakmoss absolute (Fig. 2c), and this response was increased as compared with the reaction seen after atranol challenge. In line with this, challenge with chloroatranol induced significant CD4⁺ T cell proliferation in mice sensitized with atranol, chloroatranol, and oakmoss (Fig. 2c). Challenge with oakmoss induced the strongest response for all parameters tested. Thus, oakmoss induced strong infiltration of CD19⁺ B cells and proliferation of CD4⁺ T cells in mice sensitized with atranol, chloroatranol, and oakmoss absolute (Fig. 2d). Furthermore, challenge with oakmoss induced significant proliferation of CD8⁺ T cells in mice sensitized with oakmoss (Fig. 2d). Interestingly, in mice sensitized with oakmoss, challenge with oakmoss induced fourfold and twofold higher B cell infiltration than challenge with atranol or chloroatranol, respectively. Likewise, an approximately twofold increase was seen in proliferation of CD4⁺ T cells following oakmoss challenge as compared with challenge with either atranol or chloroatranol in mice sensitized with oakmoss (Fig. 2b–d).

To further investigate the immune response to oakmoss, atranol, and chloroatranol, we examined the induction of the proinflammatory cytokines IL-18, IL-1 β and TNF- α in the skin. Mice were treated once on both ears with 0.4% atranol, 0.18% chloroatranol, 0.4% atranol and 0.18% chloroatranol, 20% oakmoss, or OOA as a control. The induction of IL-18, IL-1 β and TNF- α in allergen-exposed skin was determined after 24 hr by qPCR. Exposure to chloroatranol either alone or in combination with atranol resulted in a twofold to threefold increase in the level of IL-18 mRNA (Fig. 3a). Likewise, exposure to either chloroatranol, atranol together with chloroatranol or oakmoss resulted in increased expression of IL-1 β mRNA (Fig. 3b). Oakmoss was the most potent inducer of IL-1 β , with a fold change of approximately five. We found a small increase in the expression of TNF- α mRNA after exposure to either pure chloroatranol or chloroatranol in combination with

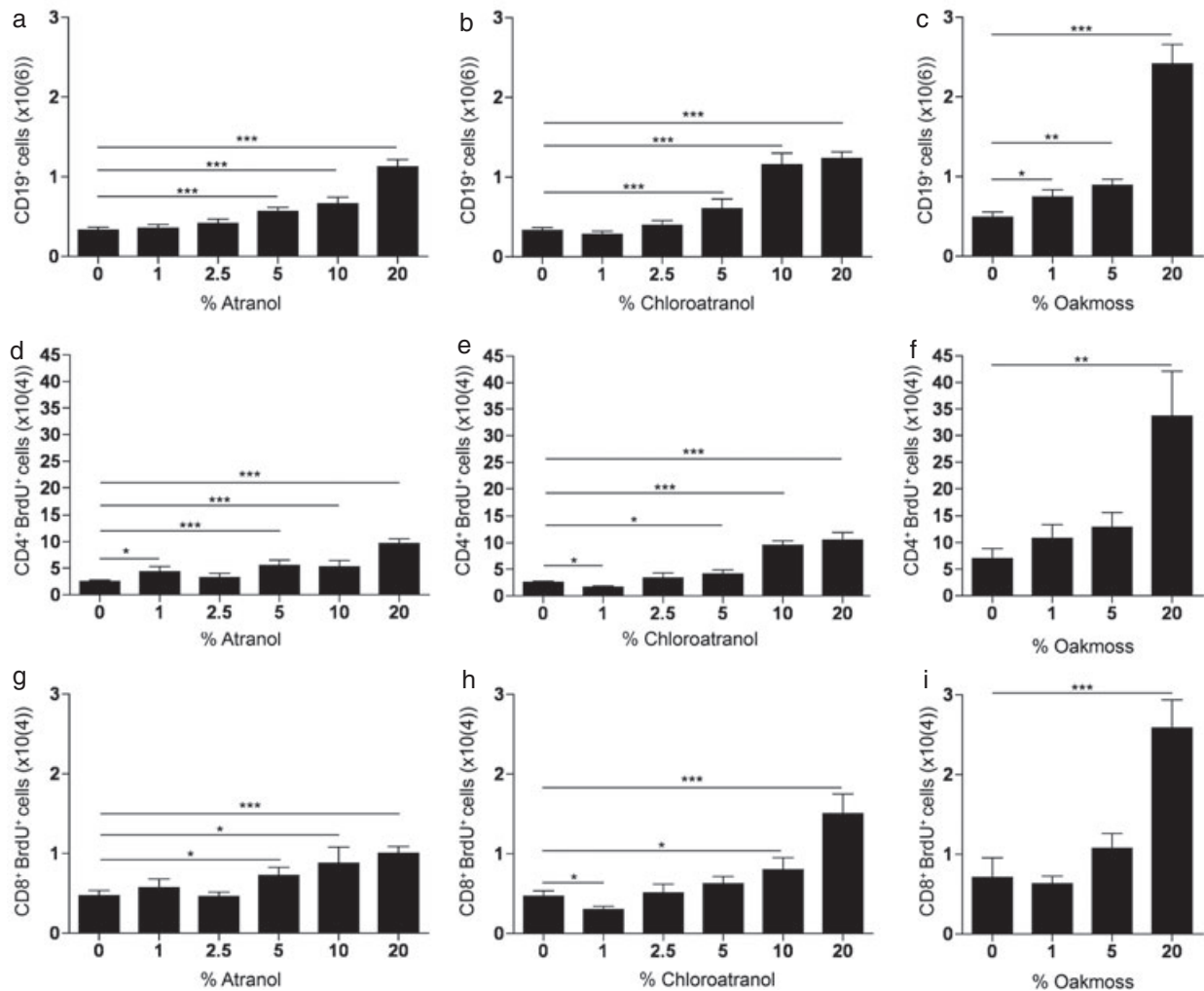


Fig. 1. Allergen dose dependency of the sensitization response. Groups of mice were exposed to different concentrations of atranol or chloroatranol (1%, 2.5%, 5%, 10%, or 20%) or oakmoss (1%, 5%, or 20%). The vehicle was olive oil/acetone. Mice were exposed to allergen for three consecutive days (D0–D2) and killed on D5. The numbers of infiltrating B cells and proliferating T cells in the draining lymph nodes were determined by flow cytometry. (a–c) Infiltration of B cells determined as CD19⁺ cells. (d–f) Proliferating CD4⁺ T cells determined as CD4⁺ BrdU⁺ cells. (g–i) Proliferating CD8⁺ T cells determined as CD8⁺ BrdU⁺ cells. Results are presented as mean ± standard error of the mean. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Data represent three individual experiments with four mice in each group/experiment. BrdU, 5-bromo-2-deoxyuridine.

atranol, and more enhanced induction after exposure to oakmoss (Fig. 3c). Interestingly, exposure to atranol did not induce expression of any of the cytokines (Fig. 3a–c). This indicated that atranol (0.4%; 26.2 mM) is a weaker allergen than chloroatranol (0.18%; 9.6 mM). To further investigate the capacity of atranol and chloroatranol to induce an immune response, we investigated the induction of death in keratinocytes by these allergens by use of an *in vitro* assay. This experiment was performed because it has been shown that components from apoptotic and dying cells can function as endogenous TLR

ligands (26). In an MTT assay, concentration-dependent induced cell death was seen following 24 hr of treatment with atranol and chloroatranol (Fig. 3d–f). Interestingly, at the same molar concentrations, chloroatranol was more cytotoxic than atranol (Fig. 3f).

Discussion

In the present study, we found that treatment with atranol, chloroatranol or oakmoss induced a concentration-dependent increase in B cell infiltration and T cell proliferation in the draining lymph nodes. Furthermore, when

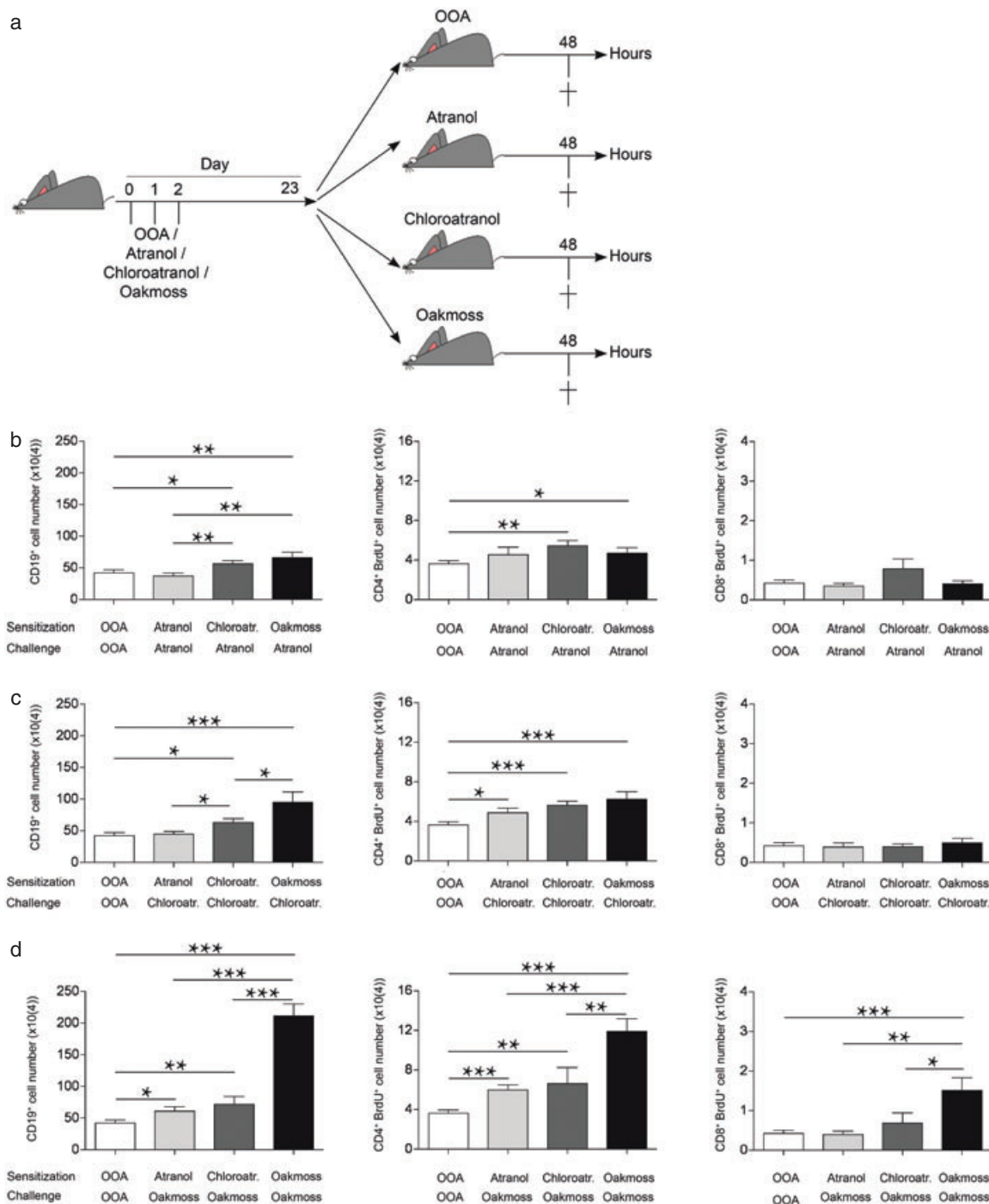


Fig. 2. Challenge responses to atranol, chloroatranol, and oakmoss. Mice were sensitized with 20% oakmoss, 0.4% atranol (26.2 mm) or 0.18% (9.6 mm) chloroatranol by exposure to the allergen for three consecutive days. After a minimum of 21 days, mice were challenged with either of the allergens. (a) The experimental setup. (b) The response after challenge with 0.4% atranol, shown as numbers of infiltrating B cells and proliferating CD4⁺ and CD8⁺ T cells. (c) The response after challenge with 0.18% chloroatranol, shown as numbers of infiltrating B cells and proliferating CD4⁺ and CD8⁺ T cells. (d) The response after challenge with 20% oakmoss, shown as numbers of infiltrating B cells and proliferating CD4⁺ and CD8⁺ T cells. Results are presented as mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represent two individual experiments with four mice in each group/experiment. BrdU, 5-bromo-2-deoxyuridine; OOA, olive oil/acetone.

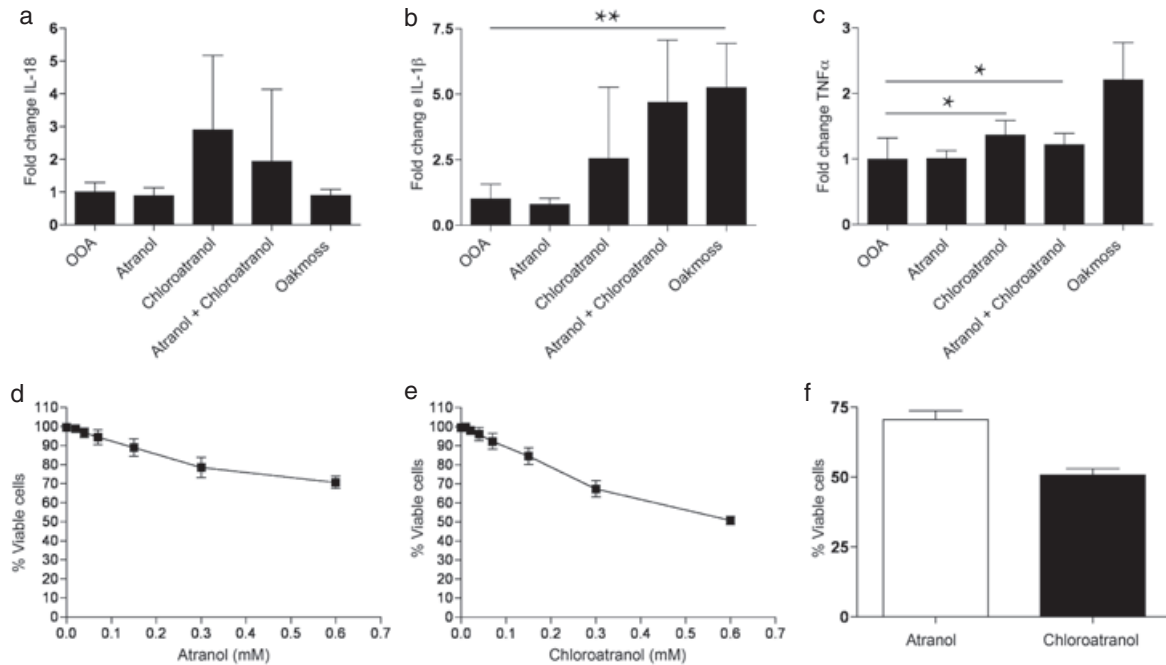


Fig. 3. Chloroatranol induces proinflammatory cytokines and more cytotoxicity than atranol. Mice were exposed to 20% oakmoss, 0.4% atranol (26.2 mM), 0.18% (9.6 mM) chloroatranol or 0.4% atranol (26.2 mM) and 0.18% (9.6 mM) chloroatranol on both ears. After 24 hr, ear biopsies were collected, and mRNA levels of interleukin (IL)-18, IL-1 β and tumour necrosis factor- α (TNF- α) were determined by quantitative polymerase chain reaction. Fold change as compared with the housekeeping gene, the glyceraldehyde-3-phosphate dehydrogenase gene, was calculated. (a–c) Fold change of IL-18 (a), fold-change of IL-1 β (b), and fold-change of TNF- α (c). Statistical analysis was performed with Student's *t*-test on Δ CT values. **p* < 0.05, ***p* < 0.01. PAM2.12 cells (keratinocytes) were stimulated for 24 hr with various concentrations of atranol or chloroatranol (0.01–0.6 mM). Following this, the viability of cells was analysed with the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide assay. (d, e) Cellular viability of PAM2.12 cells after treatment with atranol or chloroatranol, respectively. (f) Cellular viability of PAM2.12 cells after treatment with 0.6 mM atranol or chloroatranol. Cellular viability was calculated as [(absorbance)_{allergen treatment} / (absorbance)_{vehicle}] $\times$ 100%. Results are presented as mean $\pm$ standard error of the mean. Data represent three individual experiment performed in triplicate. OOA, olive oil/acetone.

comparing the challenge responses to atranol, chloroatranol and oakmoss absolute in mice sensitized with any of the three, we found that oakmoss was more potent than chloroatranol, which was more potent than atranol. In agreement with this, we found that exposure to chloroatranol alone or in combination with atranol induced the expression of IL-18, IL-1 β and TNF- α in exposed skin, whereas atranol alone failed to induce expression of any of these cytokines. Exposure to oakmoss enhanced the expression of both IL-1 β and TNF- α . Finally, by using an *in vitro* assay, we found that chloroatranol is a more potent inducer of cell death in keratinocytes than atranol.

It has previously been suggested that atranol and chloroatranol are strong allergens, with EC3 values of 0.6% and 0.4%, respectively (9). It is not possible to calculate the EC3 value by using our model, but B cell infiltration and T cell proliferation were only induced after exposure to 5% of either atranol or chloroatranol. This indicated that both atranol and chloroatranol might

not be as strong allergens as previously suggested. The highest concentration of oakmoss tested (20%), which contains only 0.4% atranol and 0.18% chloroatranol, induced twofold to fourfold higher B cell infiltration and T cell proliferation than 20% atranol or 20% chloroatranol alone. Our challenge experiments correlate well with oakmoss being more potent than pure atranol or chloroatranol, as sensitization with oakmoss induced increased infiltration of B cells as compared with sensitization with either of the pure allergens. Additionally, mice that were both sensitized and challenged with oakmoss showed twofold to threefold enhanced B cell infiltration and T cell proliferation as compared with mice sensitized with either atranol or chloroatranol and then challenged with oakmoss. Taken together, these findings indicate that oakmoss constitutes a very potent cocktail of allergens. A mixture of allergens might increase skin penetration, owing to the many different substances in the mixture. If this is so, mice sensitized with oakmoss might have been

exposed to higher actual concentrations of atranol and chloroatranol than mice sensitized with a single allergen. Furthermore, atranol and chloroatranol are not the only sensitizers present in oakmoss, as their parent compounds, namely the depsides atranorin and chloroatranorin, have been reported to be sensitizers, as has methyl- β -orcinolcarboxylate. The presence of several other sensitizers might explain the increased response to oakmoss as compared with pure atranol or chloroatranol (8). However, we cannot exclude the possibility that combining atranol and chloroatranol would increase their potency.

A higher number of B cells in the draining lymph nodes was found after exposure to oakmoss. This is an interesting observation, given that contact allergy has classically been described as a T cell-mediated response (11). Nevertheless, B cells might also play important proinflammatory and anti-inflammatory roles in the response, depending on the experimental model used (12–17). Whether the infiltrating B cells found in this study play a role as antigen-presenting cells, cytokine-producing cells, antibody-producing cells or a combination of these needs further investigation. However, we find it likely that B cells play an important role in the response to contact allergens, as we consistently find massive B cell infiltration in the draining lymph nodes following allergen exposure (23, 27, 28).

In order for a contact allergen to induce an allergic reaction, it must have the ability to both induce an inflammatory response mediated by cells within the skin and activate allergen-specific T cells (11, 20, 21). It has been shown that various proinflammatory cytokines are produced in skin rapidly after allergen exposure. IL-1 β is already expressed in skin 15 min after allergen exposure (29). In this study, we examined the expression of IL-18, IL-1 β and TNF- α in the skin 24 hr after allergen exposure. IL-1 β , in particular, was induced following allergen exposure, with the highest induction being observed following oakmoss sensitization. The fold changes that we observed were low as compared with a previous study investigating the induction of IL-1 β following allergen exposure (22). However, in that study only TNCB strongly induced IL-1 β , whereas the weaker allergen *Arnica* tincture induced changes in IL-1 β comparable to

what we found in the present study (22). Finally, the kinetics might also play a role in the different responses observed, as Lass et al. measured IL-1 β production 4.5 hr after allergen exposure, whereas we measured it 24 hr after allergen exposure (22). It is therefore plausible that we might have observed a stronger IL-1 β response if we had examined cytokine expression at an earlier time point. Interestingly, and supporting the idea that chloroatranol is a more potent allergen than atranol, chloroatranol induced expression of all three cytokines, whereas no change was observed in skin treated with atranol. Even though we did not observe any change in the mRNA levels of IL-1 β , IL-18 and TNF- α in skin treated with atranol, we could not completely exclude the possibility that atranol does not upregulate these cytokines. This might simply be a question of kinetics, and further investigations are therefore needed to fully clarify this aspect.

The production of proinflammatory cytokines after allergen exposure can be induced by TLR triggering (20, 21). Components from dying cells have been shown to function as endogenous TLR ligands, stimulating both TLR2 and TLR4 (26). Both TLR2 and TLR4 are involved in sensing allergens (18, 20). We found that, at the same molar concentration, chloroatranol treatment was more cytotoxic against keratinocytes than atranol treatment. Thus, it is likely that chloroatranol is a more potent allergen than atranol, owing to its ability to induce danger signals and TLR signalling.

Because of their ability to induce elicitation reactions in patients previously sensitized to oakmoss, atranol and chloroatranol have been suggested to be the primary allergens in oakmoss absolute (8). However, the present study shows the complexity of the response to a mixture of allergens such as oakmoss, and underlines the fact that it might be difficult to remove all sensitizers to ensure that oakmoss becomes completely safe to use in cosmetic products.

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