

Application of HEMA on intact mouse skin – effects on the immune system

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Previously, we have shown that 2-hydroxyethylmethacrylate (HEMA) can bind to protein and that autoantibodies were induced in mice by immunization with a self-protein *in vitro* conjugated with HEMA. The present study aimed to develop a model for HEMA-induced sensitivity by the application of the substance on intact skin. Female BALB/c mice were painted on the dorsum of each ear with 50% HEMA in vehicle twice a week for 6 weeks. The anti-CD3ε-stimulated lymph node production and the spontaneous spleen-cell production of the cytokines interleukin (IL)-2, IL-4, IL-6, IL-10 and interferon-γ were assessed by enzyme-linked immunosorbent assay. In another experiment, the cytokines were followed after subcutaneous HEMA injections. Animals painted with HEMA had a significantly higher IL-6 production by anti-CD3ε-stimulated lymph node cells and significantly suppressed IL-10 production by spleen cells compared to vehicle-treated mice. This correlated to some extent with the spontaneous spleen-cell production induced by subcutaneously administered HEMA. An injection of 20 μmol of HEMA induced an increased production of IL-6, while injection of 40 μmol depressed both IL-6 and IL-10 production. Although there was no sign of inflammation on the ears, findings suggest that HEMA had penetrated the skin and induced a reaction in the immune system.

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Acrylates have become widely used in dental materials, some of which have been reported to cause adverse reactions. Both irritant and allergic contact dermatitis (ICD and ACD) as well as respiratory hypersensitivity have been reported (1–5). The small-monomer 2-hydroxyethylmethacrylate (HEMA) has been acknowledged in this group as a frequent cause of ACD (5). Owing to its size and water solubility, HEMA is often included in dental bonding material where the content of HEMA can amount to be between 30% and 55% (6, 7). Another field of application is as solvent or copolymer in composite resins and glass ionomer cements. After curing, un-polymerized HEMA has been shown to be eluted from the material in amounts sufficient to be cytotoxic (8, 9).

ACD is a T-cell-mediated immune response to low-molecular-weight compounds that activates the immune system as a result of their binding to proteins in the epidermis. After an inductive sensitization phase, subsequent exposure leads to elicitation of inflammation. Recent studies suggest that interferon (IFN)-γ-producing CD8⁺

T cells initiate this response, while it is down-regulated by CD4⁺ T cells producing interleukin (IL)-4 and IL-10 (10), also reviewed in (11).

We have earlier shown that HEMA can bind to protein, thereby fulfilling one requirement for the ability to cause ACD (12). We also demonstrated that HEMA modification of a self-protein leads to the production of autoantibodies towards the carrier protein in mice (12). However, in order to study the background mechanisms for HEMA-induced ACD, a mouse model based on topical exposure of HEMA would be most useful. Contact hypersensitivity towards HEMA has been established in guinea pigs (13–15) but is to our knowledge still elusive in mice. A substance found to be cross-reactive with HEMA in guinea pig studies, 2-hydroxypropylmethacrylate (14), was found to be negative in the murine local lymph node assay (LLNA), although, due to positive patch tests, considered as an allergen in humans (16).

In the evaluation of LLNA by the Interagency Coordination Committee on the Validation of Alternative Methods (ICCVAM), this method

was recommended as a stand-alone alternative to assess contact sensitization hazard, although it was recognized that weak sensitizers might give false negative results (17). Considering that the exposure period in LLNA only last for 3 days while contact hypersensitivity towards weak allergens in humans probably arises after long-term exposure, it is possible that a longer period of exposure is necessary to identify weak allergens in mice.

The aim of the present study was to induce HEMA sensitivity by frequent 'painting' on the skin with the substance at a high dose, during an extended period of time. Subsequently, we assessed the spontaneous spleen-cell production as well as the anti-CD3 ϵ -stimulated production of the cytokines IL-2, IL-4, IL-6, IL-10 and IFN- γ from draining lymph node. The results were compared with the spontaneous spleen-cell production induced by subcutaneous HEMA injections.

Materials and Methods

Animals

Female BALB/c mice, 6 weeks old (Charles River, Sulzfeld, Germany), were kept in the animal facility according to governmental rules. The ethical committee on animal experiments approved the protocols.

Topical application of HEMA

Groups of mice ($n = 8$) were painted on the dorsum of each ear with 20 μ l of 50% HEMA (i.e. 80 μ mol/ear) in vehicle (acetone : olive oil, 4 : 1) or vehicle alone twice a week for 6 weeks. The animals were killed 1 day after the last painting. Individual single-cell suspensions were prepared from draining lymph nodes and from spleens.

Subcutaneous injection of HEMA

Groups of mice ($n = 8$) were injected subcutaneously with 20 μ mol or 40 μ mol HEMA in 100 mM NaHCO₃ or with 100 mM NaHCO₃ alone as control, once a week for 3 weeks. Animals were killed 5 days after the last injection, and individual spleen-cell suspensions were prepared.

Preparation and treatment of cell suspensions

Spleen cells. Spleens were squeezed through a 70 μ m cell strainer (Falcon, Labora AB, Sollentuna, Sweden). The cell suspensions were centrifuged on Ficoll-PaqueTM (Amersham Pharmacia Biotech AB, Uppsala Sweden). Cells

from the interface were washed 2 times before they were suspended in Dulbecco's MEM, supplemented with 5% fetal calf serum, penicillin–streptomycin and gentamicin (complete DMEM) (all from Gibco BRLTM, Invitrogen, Stockholm, Sweden). Cells were seeded 5×10^5 cells/well in 0.2 ml complete DMEM in triplicates in U-shaped 96-well plates (Nunc, GTF AB, Göteborg, Sweden) and then incubated for 4 days in humid atmosphere, 5% CO₂ at 37°C before the content of the wells were transferred to tubes and centrifuged 220 g for 10 min at 4°C. The supernatant was collected and kept frozen at –20°C until analysis.

Draining lymph node cells. Lymph nodes were squeezed through a 70 μ m cell strainer. The cell suspensions were individually collected and washed 2 times. Cells were seeded 5×10^5 cells/well in 0.2 ml complete DMEM in triplicates in U-shaped 96-well plates coated with anti-CD3 ϵ antibodies. The cells were incubated in humid atmosphere, 5% CO₂ at 37°C, for 3 days before the content of the wells were transferred to tubes and centrifuged 220 g for 10 min at 4°C. The supernatant was collected and kept frozen at –20°C until analysis.

Anti-CD3 ϵ antibody stimulation. Plates were coated with 30 μ l/well of hamster anti-mouse CD3 ϵ monoclonal antibodies, 25 μ g/ml (BD Biosciences Pharmingen, Stockholm, Sweden) in phosphate-buffered saline (PBS) for 90 min in 37°C. The wells were then washed 2 times with sterile PBS before lymph node cells were seeded in triplicates 5×10^5 cells/well in 0.2 ml complete DMEM.

Assessment of cytokine production

IL-2, IL-4, IL-6, IL-10 and IFN- γ concentration in the supernatants was assessed by commercial sandwich enzyme-linked immunosorbent assay systems, 'Duosets' (R&D systems, Abingdon, UK). Plates were coated with capture antibody according to the included instructions. The next day the plates were washed 3 times with PBS–Tween-20, blocked with 1% BSA in PBS for 1 hr at room temperature before samples and standard were added. Complete DMEM was used for dilution if necessary. Plates were incubated over night at 4°C. The next day the plates were washed 4 times with PBS–Tween-20 before 2 hr of incubation with the biotinylated detection antibody at room temperature. After 4 PBS–Tween-20 washes, the plates were incubated with streptavidin–HRP for 20 min. The plates were washed anew 4 times with PBS–Tween-20

before incubation in the dark for 20 min with the substrate tetramethylbenzidine dissolved in 0.05 M phosphate citrate buffer (pH 5.0) supplemented with 30% H_2O_2 . The reaction was stopped after 20 min by the addition of 1 M H_2SO_4 . The absorbance was read on a Spectra MAX 340 at 450 nm (Molecular Devices, Sunnyvale, CA, USA).

HEMA

Prior to injections, HEMA (Sigma-Aldrich, St Louis, MO, USA; H8633) was passed over a column (Aldrich No. 30631-2, Sigma-Aldrich) to remove the inhibitor hydroquinone.

Results

Spontaneous cytokine production by spleen cells after topical treatment

Mice were painted twice a week for 6 weeks with 50% HEMA in vehicle or vehicle alone on the dorsum of both ears (80 μmol HEMA/ear). Single-cell suspensions from spleen cells were grown for 4 days before the concentrations of IL-2, IL-4, IL-6, IL-10 and IFN- γ in the supernatant were assessed. While the IL-2 production was unaffected by the *in vivo* treatment, the IL-10 production was significantly suppressed (Fig. 1a,b). There were no detectable production of IL-4, IL-6 and IFN- γ .

Anti-CD3 ϵ -stimulated cytokine production by draining lymph node cells

Mice were painted twice a week for 6 weeks with 50% HEMA in vehicle or vehicle alone on the dorsum of both ears. Single-cell suspensions from draining lymph nodes were grown for 3 days on plates coated with anti-CD3 ϵ antibodies. The concentrations of IL-2, IL-4, IL-6, IL-10 and IFN- γ in the supernatants were assessed. The production of IL-6 was significantly higher in the group that had been painted with HEMA compared to those painted with vehicle (Fig. 2a). Although not significant, there was a tendency for increased production of IL-2 and IL-4 in the group painted with HEMA (Fig. 2b,c). No obvious difference in the production of IL-10 and IFN- γ could be detected (data not shown).

Spontaneous cytokine production by spleen cells after subcutaneous injection

Mice were injected subcutaneously with 20 μmol or 40 μmol HEMA in 100 mM NaHCO_3 or with 100 mM NaHCO_3 alone as control, once a week

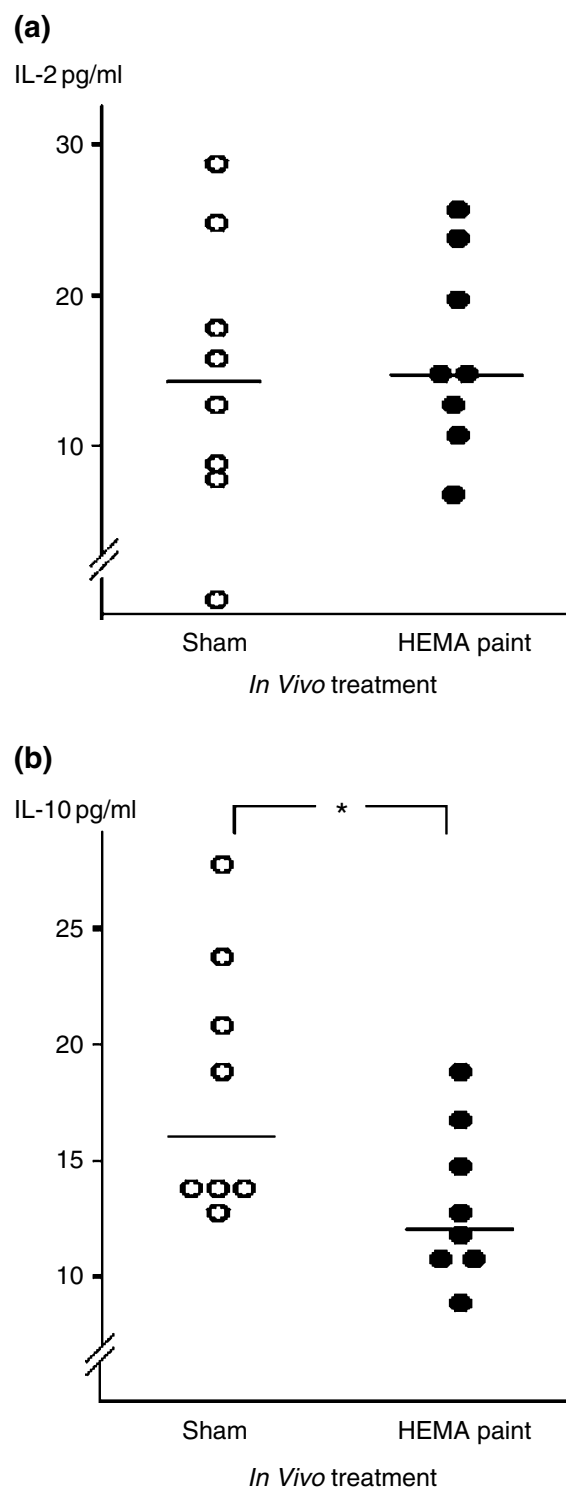


Fig. 1. Spontaneous spleen-cell production of interleukin (IL)-2 (a) and IL-10 (b). Groups of mice ($n = 8$) were painted with 50% 2-hydroxyethylmethacrylate (HEMA) in vehicle (acetone : olive oil, 4 : 1) or with vehicle alone on the dorsum of both ears, twice a week for 6 weeks. 1 day after the last painting, the animals were killed and single-cell suspensions were prepared and seeded on plates. After 4 days of incubation, the IL-2 and IL-10 concentrations of the supernatants were assessed by enzyme-linked immunosorbent assay. Dots denote 1 animal, and the horizontal bar indicates the median. Mann-Whitney *U*-test was used for statistical comparison (* $P < 0.05$).

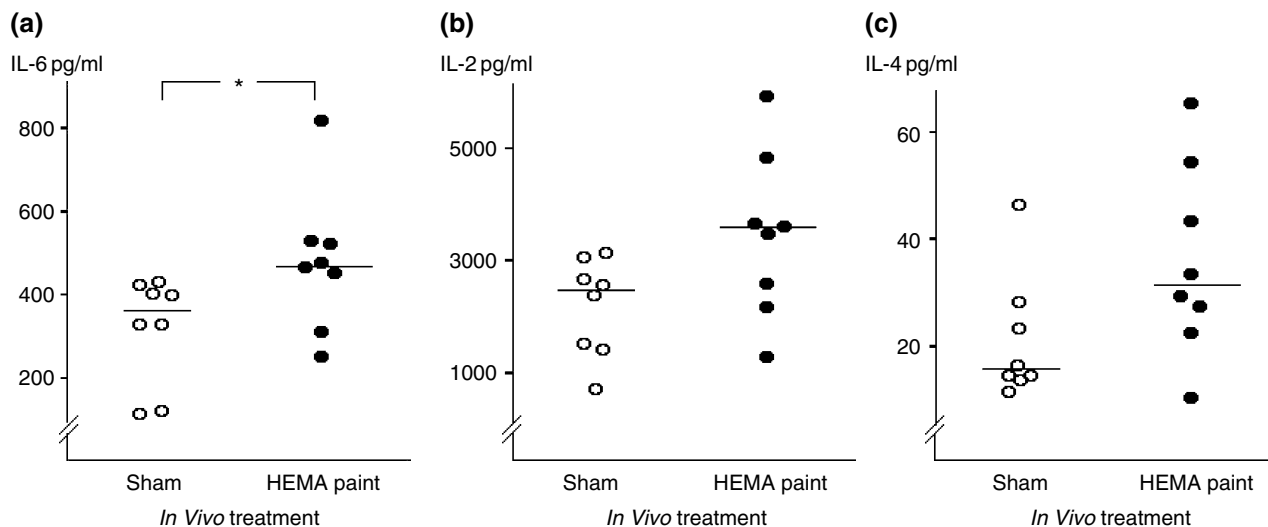


Fig. 2. Cytokine production of interleukin (IL)-6 (a), IL-2 (b) and IL-4 (c) by draining lymph node cells stimulated *in vitro* by anti-CD3 ϵ antibody. Groups of mice ($n = 8$) were painted with 50% 2-hydroxyethylmethacrylate (HEMA) in vehicle (acetone : olive oil, 4 : 1) or with vehicle alone on the dorsum of both ears, twice a week for 6 weeks. 1 day after the last painting, the animals were killed and single-cell suspensions were prepared and seeded on plates coated with anti-CD3 ϵ antibody. After 3 days of incubation, the cytokine concentration of the supernatants was assessed by enzyme-linked immunosorbent assay. Dots denote 1 animal, and the horizontal bar indicates the median. Mann–Whitney U -test was used for statistical comparison (* $P < 0.05$).

for 3 weeks. Single-cell suspensions from spleens were prepared and incubated for 4 days before the content of cytokines IL-2, IL-4, IL-6, IL-10 and IFN- γ was assessed in the supernatants. There was no detectable production of IL-4 or IFN- γ . The HEMA injections did not affect IL-2 production (data not shown). The production of IL-10 was unaffected by *in vivo* injections of 20 μ mol HEMA but significantly suppressed by injections of 40 μ mol (Fig. 3a). The production of IL-6 was significantly increased by the 20 μ mol injection but depressed by the 40 μ mol (Fig. 3b).

Discussion

In this study, we have shown that HEMA applied topically for an extended period of time affects the immune system. The influence was recorded as decreased spontaneous production of IL-10 by spleen cells and increased IL-6 production by draining lymph node cells *in vitro* stimulated by an anti-CD3 ϵ antibody.

IL-10 is an important cytokine that has been shown to exert pleiotropic activities on the immune system, extensively reviewed in (18). In mouse models with IL-10 deficiencies, contact hypersensitivity (CHS) has been shown to be exaggerated, suggesting that IL-10 restrains irritant responses (19, 20). In the present study, we found a suppressed production of IL-10 by spleen cells *in vitro*, subsequent to *in vivo* HEMA treatment. This effect was induced both by topical

application and by subcutaneous injection. However, we could not detect any difference in the IL-10 production by draining lymph node cells stimulated with anti-CD3 ϵ antibodies *in vitro*, following topical treatment. One possible explanation could be that HEMA was not retained in the ear tissue long enough and in sufficient quantities to induce this cytokine. A study on the toxicokinetic of HEMA in guinea pigs indicated that a substantial metabolism of HEMA occurs *in vivo* because 70% of subcutaneously injected ^{14} C-labelled HEMA was exhaled as CO $_2$ after 24 hr (21). The remaining radioactivity showed a broad distribution throughout the body; 17% was found in the urine and 7.5% was found in the organs with 0.5% in the skin.

Of further interest in the current study was that the anti-CD3 ϵ -stimulated IL-6 production by draining lymph nodes cells *in vitro* was increased by the topical HEMA treatment. Stimulation with plate-bound anti-CD3 ϵ antibodies has been used in several studies to enhance *in vitro* cytokine production, for example by lymph node cells sensitized *in vivo* to dinitrofluorobenzene (10, 22, 23). IL-6 is a cytokine associated with inflammation. The secretion of IL-6 was shown to be significantly increased in allergen-activated lymph node cells as compared to vehicle-treated cells in BALB/c mice (24). The production of IL-6 does not seem to be exclusively induced by allergens, though, as mRNA for IL-6 is upregulated by topical treatment with both an irritant and an allergen in BALB/c mice (25). The increased

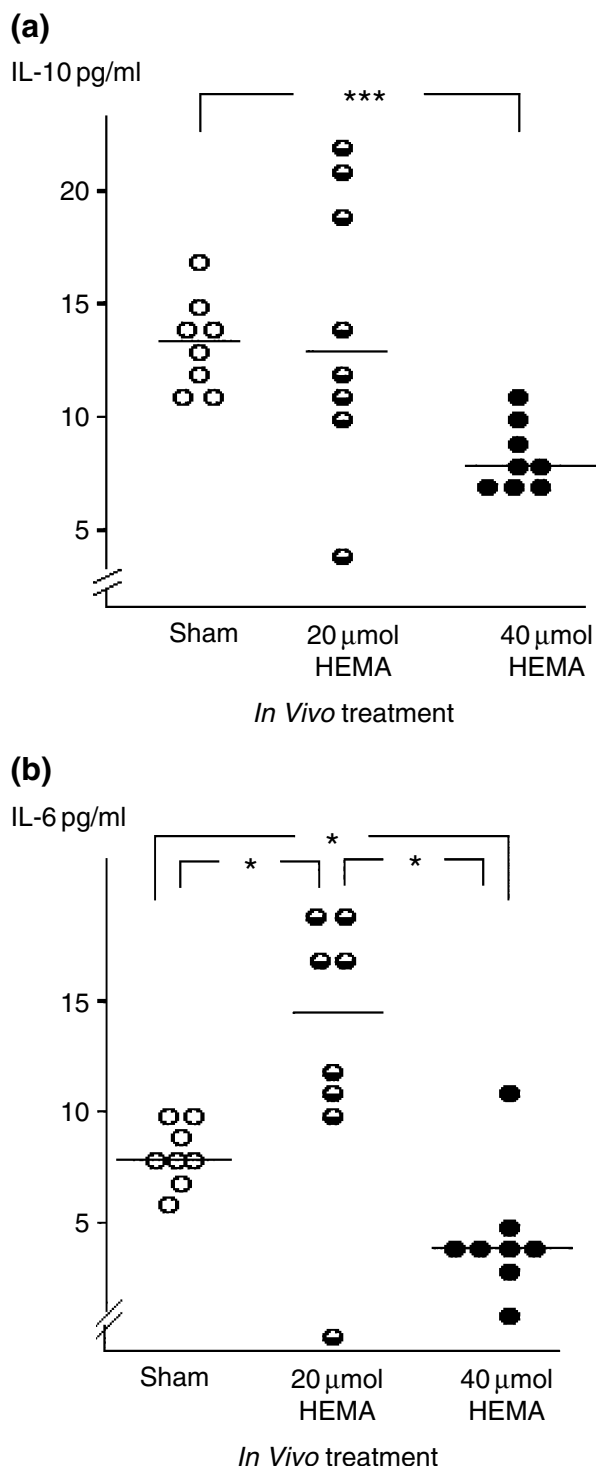


Fig. 3. Spontaneous production of interleukin (IL)-10 (a) and IL-6 (b) by spleen cells. Groups of animals ($n = 8$) received subcutaneous injections containing 20 µmol or 40 µmol HEMA in 100 mM NaHCO_3 once a week for 3 weeks. 5 days after the last injection, animals were killed and individual spleen-cell suspensions prepared. After 4 days of spontaneous growth, the cytokine concentration of the supernatants was assessed by enzyme-linked immunosorbent assay. Dots denote 1 animal, and the horizontal bar indicates the median. Mann–Whitney U -test was used for statistical comparison (* $P < 0.05$, *** $P < 0.005$).

IL-6 production provoked by the topically applied HEMA in the current study may reflect the inflammatorogenic properties of HEMA (26). An increased production of IL-6 was also found in spleen cells, following a subcutaneous injection of 20 µmol HEMA. The production was significantly depressed by injection of 40 µmol HEMA. Whether this reflects a general toxic influence of HEMA or a specific effect on IL-6 production remains to be investigated. In order to clarify if the findings in this study relate to allergenic or irritant properties of HEMA, it would be relevant to include groups treated with known strong allergens and irritants in future studies.

Several studies have demonstrated the existence of acrylate allergy in humans, and the allergenic potential of, for example, HEMA has been confirmed in studies on guinea pigs (13–15). The difficulty to develop a mouse model for HEMA-induced CHS has been addressed by others (14). Even if the frequent and long-term topical treatment applied in this study did not induce a HEMA-related CHS, the findings suggest that HEMA had actually penetrated the skin.

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References

1. Alanko K, Susitaival P, Jolanki R, Kanerva L. Occupational skin diseases among dental nurses. *Contact Dermatitis* 2004; 50: 77–82.
2. Kanerva L, Rantanen T, Aalto-Korte K et al. A multicenter study of patch test reactions with dental screening series. *Am J Contact Dermat* 2001; 12: 83–87.
3. Koutis D, Freeman S. Allergic contact stomatitis caused by acrylic monomer in a denture. *Australas J Dermatol* 2001; 42: 203–206.
4. Piirila P, Kanerva L, Keskinen H, Estlander T, Hytonen M, Tuppurainen M, Nordman H. Occupational respiratory hypersensitivity caused by preparations containing acrylates in dental personnel. *Clin Exp Allergy* 1998; 28: 1404–1411.
5. Rustemeyer T, Frosch P J. Occupational skin diseases in dental laboratory technicians. (I). Clinical picture and causative factors. *Contact Dermatitis* 1996; 34: 125–133.
6. Nakabayashi N, Takarada K. Effect of HEMA on bonding to dentin. *Dent Mater* 1992; 8: 125–130.
7. Gerzina T M, Hume W R. Diffusion of monomers from bonding resin–resin composite combinations through dentine in vitro. *J Dent* 1996; 24: 125–128.
8. Kawahara T, Nomura Y, Tanaka N, Teshima W, Okazaki M, Shintani H. Leachability of plasticizer and residual monomer from commercial temporary restorative resins. *J Dent* 2004; 32: 277–283.
9. Geurtsen W, Spahl W, Muller K, Leyhausen G. Aqueous extracts from dentin adhesives contain cytotoxic chemicals. *J Biomed Mater Res* 1999; 48: 772–777.

10. Xu H, DiIulio N A, Fairchild R L. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8⁺ T cells and interleukin (Il), 4/Il-10-producing (Th2) negative regulatory CD4⁺ T cells. *J Exp Med* 1996; 183: 1001–1012.
11. Saint-Mezard P, Berard F, Dubois B, Kaiserlian D, Nicolas J F. The role of CD4⁺ and CD8⁺ T cells in contact hypersensitivity and allergic contact dermatitis. *Eur J Dermatol* 2004; 14: 131–138.
12. Sandberg E, Bergenholtz G, Eklund C, Dahlgren U I. HEMA bound to self-protein promotes auto-antibody production in mice. *J Dent Res* 2002; 81: 633–636.
13. Clemmensen S. Sensitizing potential of 2-hydroxyethyl-methacrylate. *Contact Dermatitis* 1985; 12: 203–208.
14. Rustemeyer T, de Groot J, von Blomberg B M, Frosch P J, Scheper R J. Cross-reactivity patterns of contact-sensitizing methacrylates. *Toxicol Appl Pharmacol* 1998; 148: 83–90.
15. Rustemeyer T, de Groot J, von Blomberg B M, Frosch P J, Scheper R J. Induction of tolerance and cross-tolerance to methacrylate contact sensitizers. *Toxicol Appl Pharmacol* 2001; 176: 195–202.
16. Haneke K E, Tice R R, Carson B L, Margolin B H, Stokes W S. ICCVAM evaluation of the murine local lymph node assay. Data analyses completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. *Regul Toxicol Pharmacol* 2001; 34: 274–286.
17. Dean J H, Twerdok L E, Tice R R, Sailstad D M, Hattan D G, Stokes W S. ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. *Regul Toxicol Pharmacol* 2001; 34: 258–273.
18. Weiss E, Mamelak A J, La Morgia S, Wang B, Feliciani C, Tulli A, Sauder D N. The role of interleukin 10 in the pathogenesis and potential treatment of skin diseases. *J Am Acad Dermatol* 2004; 50: 657–675.
19. Berg D J, Leach M W, Kuhn R, Rajewsky K, Muller W, Davidson N J, Rennick D. Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J Exp Med* 1995; 182: 99–108.
20. Ferguson T A, Dube P, Griffith T S. Regulation of contact hypersensitivity by interleukin 10. *J Exp Med* 1994; 179: 1597–1604.
21. Reichl F X, Durner J, Kehe K, Manhart J, Folwaczny M, Kleinsasser N, Hume W R, Hickel R. Toxicokinetic of HEMA in guinea pigs. *J Dent* 2002; 30: 353–358.
22. Wang B, Fujisawa H, Zhuang L et al.: CD4⁺ Th1 and CD8⁺ type 1 cytotoxic T cells both play a crucial role in the full development of contact hypersensitivity. *J Immunol* 2000; 165: 6783–6790.
23. Matyszak M K, Citterio S, Rescigno M, Ricciardi-Castagnoli P. Differential effects of corticosteroids during different stages of dendritic cell maturation. *Eur J Immunol* 2000; 30: 1233–1242.
24. Hope J C, Dearman R J, Debicki R J, Kimber I, Hopkins S J. Interleukin-6 production by draining lymph node cells following primary contact sensitisation of mice: relationship to the proliferative response. *Int Arch Allergy Immunol* 1994; 103: 378–383.
25. Kondo S, Pastore S, Shivji G M, McKenzie R C, Sauder D N. Characterization of epidermal cytokine profiles in sensitization and elicitation phases of allergic contact dermatitis as well as irritant contact dermatitis in mouse skin. *Lymphokine Cytokine Res* 1994; 13: 367–375.
26. Sandberg E, Kahu H, Dahlgren U I. Inflammation and adjuvant properties of HEMA in mice. *European J Oral Sci* 2005; 113: 410–416.

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