

Inflammatogenic and adjuvant properties of HEMA in mice

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2-Hydroxyethylmethacrylate (HEMA), a common constituent in dental materials, is known to cause hypersensitivity reactions. While the means by which this small molecule causes adverse responses has not been ascertained, we have previously demonstrated that it binds to protein and in mice induces the production of autoantibodies to HEMA-conjugated self-protein. The present study explores the inflammatory and adjuvant properties of HEMA in response to the subcutaneous injection of HEMA and a protein. Ovalbumin (OVA) was used as a 'reporter antigen', and mouse serum albumin (MSA), conjugated *in vitro* with HEMA (MSA_H) to a low degree (0.5 molecules of HEMA per molecule of MSA on average), was used to mimic a possible *in vivo* situation. Inflammatory responses at injection sites were scored by using an ordinal scale, and immunoglobulin (Ig)G1, IgG2a, and IgE activities to OVA or MSA were assessed by enzyme-linked immunosorbent assay (ELISA). Injections of 20 µmol HEMA induced overt inflammatory skin responses, the severity of which was influenced by the co-administered substances. A significantly higher IgG1 and IgE response to OVA was induced by the presence of HEMA. Interestingly, injections with low conjugated MSA_H only induced the production of autoantibodies if free HEMA was included at the time of immunization. These findings suggest that HEMA is an inflammatogenic substance with adjuvant properties.

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Several studies have reported the occurrence of adverse reactions to acrylates, a few in patients but the overwhelming majority in dental personnel (1–4). One substance in this group that has been reported as a frequent cause of contact dermatitis is 2-hydroxyethylmethacrylate (HEMA) (5). This is a small molecule, included in dental liners to enhance the binding of resin-composites to dentin or as a solvent or copolymer in temporary restoration resins and glass ionomer cements (6–8).

Contact dermatitis induced by HEMA presents as a skin inflammation that may be of either irritant or allergic etiology (5). A local, non-specific reaction gives rise to irritant contact dermatitis, while the specific immune system is engaged in allergic contact dermatitis, also called contact hypersensitivity (CHS). A prerequisite for CHS is that HEMA, after penetration of the skin, becomes antigenic by binding to endogenous proteins. This will lead to a delayed-type hypersensitivity reaction proceeding in two phases: a sensitization phase, followed by an elicitation phase induced by repeated exposure.

Although CHS is regarded to be the primary problem with HEMA exposure, the possible induction of autoantibodies might be a thus-far unrecognized consequence. Considering that tolerance towards self can be broken by exposure to proteins with similarities to self-proteins (e.g. viral proteins or homolog proteins from other species) (9, 10), chemically modified

endogenous proteins might retain enough self-similarity to also have this effect. Our finding, that autoantibodies are induced in mice by immunization with a self-protein conjugated *in vitro* with HEMA, supports this view (11).

Most chemical sensitizers are also known to be irritant. The pro-inflammatory signal provided by an irritant has been shown to have a profound impact on the elicitation of CHS in mice (12). The aim of that study was to dissect the dose dependency from the irritant signal and the antigenic signal. While the substance that contributed the antigenic signal could be reduced by a factor of 10³, the CHS response was lost when the dose of the irritant signal was reduced by a factor of two. Thus, the irritant property of a sensitizer seems to be of significant importance for the development of an immune response.

In this study we wanted to investigate the irritant effect of HEMA and the influence of HEMA on the subsequent immune response. To achieve this, mice were injected with ovalbumin (OVA), with or without HEMA, and the ensuing anti-OVA response was followed. In order to mimic a possible *in vivo* situation, the self-protein mouse serum albumin (MSA) was conjugated *in vitro* with HEMA to a very low degree (0.5 HEMA molecules per molecule of albumin, on average). This preparation was used, with or without free HEMA, to immunize mice.

Material and methods

Substances

Phosphate-buffered saline (PBS), pH 7.2, used in the preparation of MSA, was prepared at our laboratory; otherwise Dulbecco's PBS, pH 7.2 (Gibco, Invitrogen, Stockholm, Sweden) was used in the experiments. Sodium bicarbonate (NaHCO_3) was used at two different concentrations, 25 and 100 mM (pH 8.2 and 8.1, respectively) that were filtered through a 0.2- μm filter (Acrodisc Syringe Filter; Pall Gelman East Hills, NY, USA) before use.

Prior to immunization and incubation, HEMA (cat. no. 477028; Sigma-Aldrich, St Louis, MO, USA) was passed, by order of gravity, over a column (Aldrich cat. no. 30631-2; Sigma-Aldrich), specified by the manufacturer to remove the inhibitor hydroquinone.

Ovalbumin (Sigma-Aldrich) was passed over a column in order to remove endotoxin (Detoxi-Gel AffinityPak Column No. 20344; Pierce, Rockford, IL, USA) according to the manufacturer's instructions. This treatment reduced the endotoxin content from 300 EU mg^{-1} before passage to 0.8 EU mg^{-1} after passage, as analyzed in the *Limulus* amoebocyte lysate assay (LAL), using a chromogenic end point, carried out by the Department of Clinical Bacteriology, Sahlgrenska Academy (Göteborg University, Göteborg, Sweden).

Ovalbumin was biotinylated in order to assess the concentration of OVA-specific immunoglobulin E (IgE) in an enzyme-linked immunosorbent assay (ELISA): 4 ml of OVA (1.5 mg ml^{-1}) dissolved in 100 mM NaHCO_3 was mixed with 9 μl of biotinamidocaproate *N*-hydroxysuccinimide ester (50 mg ml^{-1}) (B-2643; Sigma-Aldrich) dissolved in dimethylsulfoxide. The mixture was gently stirred for 4 h at room temperature (20°C). Unreacted biotin was removed by diafiltration on a filter device with a nominal molecular cut-off at 30 kDa (Centriplus, Amicon no. 4412; Millipore, Billerica, MA, USA). The retentate was washed three times with PBS and then stored at -20°C.

Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich (prod. no. L-4516). The activity was analyzed as described above and found to be 500 EU μg^{-1} .

The endotoxin content in commercially available MSA was analyzed and found to vary between 44 and 1,600 EU mg^{-1} in the LAL assay. Passing over the Detoxi-Gel column led to a substantial loss of protein. Therefore, MSA was prepared from serum obtained from BALB/c mice, at our laboratory. We used a fast protein liquid chromatography (FPLC) system with three different columns: HiPrep 16/10 Q FF (17-5190-01), HiPrep Sephacryl S-100 26/60 (17-1194-01) and HiTrap Protein G HP (17-0404-03) (Amersham Biosciences, Piscataway, NJ, USA). Ten millilitres of serum (Charles River, Sulzfeld, Germany) was mixed at a ratio of 1 : 1 (v/v) with 20 mM Tris buffer (pH 8.0), dialyzed for 24 h against 20 mM Tris, then centrifuged (4°C, 1,000 g) for 10 min. The supernatant was filtered through a sterile 0.2 μm filter and passed over the ion-exchange column in aliquots. Albumin was eluted by a salt gradient of 0–80% in 150 ml, at a flow rate of 5 ml min^{-1} ; 20 mM Tris (pH 8.0) was used as the start buffer and 20 mM Tris containing 1 M NaCl as the eluting buffer. Albumin eluted at $\approx$ 109 ml. The fractions of interest were pooled and dialyzed against PBS for 48 h, with two changes, then concentrated on a filter device with a molecular cut-off of 30 kDa before passing in fractions of 3 ml (flow rate 1 ml min^{-1}) over the gel-filtration column coupled in series with the protein G

column. The albumin peaked at $\approx$ 104 ml. The pool was concentrated and filtered through a sterile 0.2 μm filter. Analyses by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and ELISA confirmed that the protein was albumin. Aliquots were found to be negative for endotoxin content, as determined by using the LAL assay mentioned above.

Three different preparations of HEMA-conjugated MSA were prepared: MSA was dialyzed against 100 mM NaHCO_3 and then incubated at 37°C with HEMA at a molecular excess of 75, 1,000 or 2,000 HEMA : MSA, respectively. The MSA used as a control was treated in the same way, but without HEMA. The reaction was stopped after 68 h by adding PBS and then dialyzing against PBS, with one exchange. The preparations were filtered through a sterile 0.2 μm filter before storage at -20°C.

2,4,6-Trinitrobenzenesulfonic acid (TNBS) reacts readily with the lysine residues of a protein; the sulfonic group is cleaved off, leaving trinitrophenyl (TNP) covalently bound to the nitrogen. The number of HEMA monomers bound to the protein was estimated based on the level of inhibition of the TNP conjugation described in our previous report (11). It was found to be 1, 18 or 33%, respectively, in the different preparations. Assuming that HEMA binds to the lysine residues, of which MSA contains 48 (13), this suggests that the albumin molecules were carrying, on average, 0.5, 9 or 16 HEMA molecules each in the different preparations. These preparations were called MSA_{H1%}, MSA_{H18%} and MSA_{H33%}, respectively.

Animal treatment

Groups of female BALB/c mice (Charles River), 6 wk old ($n = 8$), were immunized subcutaneously in the tail with 0.05 mg OVA, MSA or MSA_{H18%}, with or without HEMA, in a 0.05-ml solution of PBS (pH 7.2) containing 25 mM NaHCO_3 (pH 8.2) or 100 mM NaHCO_3 (pH 8.1), as outlined in Table 1. Control groups were given the solution alone or together with HEMA. Blood was drawn 2 wk after a similar booster injection given after 3 wk.

Throughout the animal experiments, HEMA was used at a final concentration of 0.4 M, meaning that the animals were exposed to 20 μmol HEMA at each injection.

To assess the influence of free HEMA on autoantibody induction, groups of animals ($n = 8$) were immunized following the same time schedule, described above, with 0.4 mg of native MSA, MSA_{H1%} or MSA_{H33%} in 100 mM NaHCO_3 , together with 0.3 μg of LPS (corresponding to 150 EU) and with or without HEMA (Table 1).

In some experiments, the spleens were aseptically removed and stored overnight at 4°C in Dulbecco's minimal essential medium (DMEM), supplemented with 5% fetal calf serum, penicillin (100 U ml^{-1}), streptomycin (100 $\mu\text{g ml}^{-1}$), and gentamicin (50 $\mu\text{g ml}^{-1}$) (DMEM + + +) (all from Gibco, Invitrogen).

Individual cell suspensions were prepared the following day (see below).

The Göteborg ethical committee on animal experiments approved the animal protocols.

Scoring of an inflammatory reaction

The animals were examined daily, for 5 d, after injection. The presence of an inflammatory reaction was assessed by scoring the color of the skin, by using an ordinal scale, in a

Table 1
Description of the various groups and their treatment

Group no.	Solution	Protein	HEMA
A			
1	PBS	OVA	X
2	PBS	OVA	–
3	PBS	–	X
4	PBS	–	–
5	25 mM NaHCO ₃	OVA	X
6	25 mM NaHCO ₃	OVA	–
7	25 mM NaHCO ₃	–	–
8	100 mM NaHCO ₃	OVA	X
9	100 mM NaHCO ₃	OVA	–
10	100 mM NaHCO ₃	MSA	X
11	100 mM NaHCO ₃	MSA	–
12	100 mM NaHCO ₃	MSA _{H18%}	X
13	100 mM NaHCO ₃	MSA _{H18%}	–
14	100 mM NaHCO ₃	–	X
15	100 mM NaHCO ₃	–	–
B			
16	100 mM NaHCO ₃ + 0.3 µg of LPS	MSA	X
17	100 mM NaHCO ₃ + 0.3 µg of LPS	MSA	–
18	100 mM NaHCO ₃ + 0.3 µg of LPS	MSA _{H1%}	X
19	100 mM NaHCO ₃ + 0.3 µg of LPS	MSA _{H1%}	–
20	100 mM NaHCO ₃ + 0.3 µg of LPS	MSA _{H33%}	X
21	100 mM NaHCO ₃ + 0.3 µg of LPS	MSA _{H33%}	–
22	100 mM NaHCO ₃ + 0.3 µg of LPS	–	–

LPS, lipopolysaccharide; MSA, mouse serum albumin; MSA_{H1%}, mouse serum albumin carrying 0.5 HEMA molecules; MSA_{H18%}, mouse serum albumin carrying 9 HEMA molecules; MSA_{H33%}, mouse serum albumin carrying 16 HEMA molecules; OVA, ovalbumin; PBS, phosphate-buffered saline. X, indicates the substance was included; –, indicates the substance was excluded.

blinded manner. The scoring system was as follows: 0, no sign of inflammation; 1, light red reaction; 2, dark red. In some cases the skin was eroded, in which case the scoring system was as follows: 0, no erosion; 1, small erosion; 2, pronounced erosion. Intermediate reactions were given half-points. The skin was healed completely after 2 wk.

Cell treatment

Spleens were squeezed through a 70-µm cell strainer (Falcon, BergmanLabora, Upplands Väsby, Sweden). The cell suspensions were collected and centrifuged on Ficoll-Paque (Amersham Biosciences) according to the manufacturer's instructions. Cells from the interface were washed twice and then suspended in DMEM + + +.

To assess the presence of OVA-specific T cells, cells were seeded, at a concentration of 5×10^5 cells/well, on a 96-well plate and left unstimulated or stimulated with detoxified OVA, at 40 µg well⁻¹, in triplicate, for 4 d in a humidified 37°C, 5% CO₂, atmosphere. Proliferation was assessed by including [methyl-³H]thymidine (1 µCi per well) (Amersham Biosciences) for the last 20 h. Cells were har-

vested on glass-fiber filters (Filtermat A 1450-421; Wallac, Turku, Finland) and radioactivity was counted in a Microbeta Trilux (PerkinElmer, Wellesley, MA, USA).

Assessing antibody activity

To assess the specific IgG activity in serum, plates (Maxisorp immuno plate; Nunc, Kamstrup, Denmark) were coated overnight at 4°C with OVA, MSA or MSA_H (0.005 mg ml⁻¹) in PBS before incubation with serum samples overnight. The following day, the plates were incubated at room temperature (20°C) for 2 h with biotin-conjugated anti-mouse IgG1 or IgG2a (BD Biosciences, Franklin Lakes, NY, USA), with extravidin-alkaline phosphatase (Sigma) for 2 h, then developed with para-nitrophenyl phosphate (Sigma) 1 mg ml⁻¹, dissolved in diethanolamine buffer, pH 9.8. The absorbance was read on a Spectra MAX 340 at 405 nm (Molecular Devices, Sunnyvale, CA, USA). A standard made of a pool of positive samples was run on each plate. Between incubations, wells were washed three to four times with PBS containing Tween 20.

Ovalbumin-specific IgE activity in serum was assessed from total IgE captured onto the plate, in order to avoid competition from IgG. Plates were coated overnight with monoclonal rat anti-mouse IgE (0.002 mg ml⁻¹) (clone R35-72; BD Biosciences) in PBS, and then blocked with 0.5% (w/v) casein for 30 min. Serum samples (starting dilution 1 : 50) and a standard made up by a pool of positive sera, were incubated overnight before incubation with biotin-conjugated OVA to assess OVA-specific serum IgE. Plates were washed with PBS containing 0.05% Tween 20 between incubations and developed with extravidin-alkaline phosphatase and paranitrophenylphosphate, as described above.

To assess the number of cells producing anti-OVA, Multiscreen HATF plates (Millipore) were coated with OVA or human serum albumin (50 µg per well), or with PBS, for 10 h at room temperature (20°C). After washing three times with PBS, the plates were blocked for 30 min with DMEM + + + before transfer of the cell suspensions in two dilutions (5×10^6 and 2.5×10^6 cells ml⁻¹) 100 µl well⁻¹. The plates were incubated overnight in a humidified 37°C, 5% CO₂ atmosphere. The next day, the plates were washed three times with PBS and twice with PBS containing 0.05% Tween 20, before a 2-h incubation with biotin-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA), diluted 1 : 2500 (v/v). The plates were then washed three times with PBS between the following incubations: 45 min with strept AB-Complex/horseradish peroxidase (DakoCytomation, Glostrup, Denmark), then 30 min with the color substrate, 3-amino-9-ethyl carbazole. Gentle washing with water ended the reaction when visible spots appeared. Spots were counted under a stereomicroscope in a blinded manner.

Statistics

The Mann–Whitney *U*-test was used in the statistical analysis.

Results

Inflammation

Only the animals that were administered HEMA developed a visible inflammatory reaction at the site of

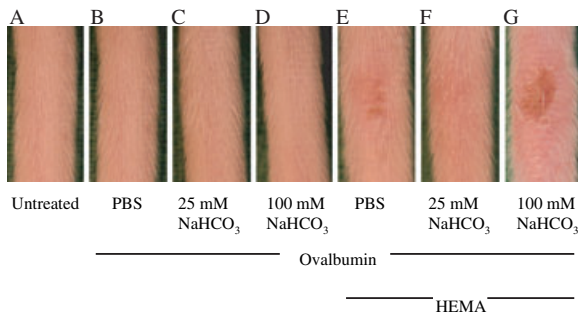


Fig. 1. Inflammatory reaction at the site of injection of the indicated substances or combination of substances. An untreated tail is shown in panel A. Groups of mice ($n = 8$) were injected subcutaneously at the base of the tail: those shown in panels B–D were given ovalbumin (OVA) only; and those shown in panels E–G were given 2-hydroxyethylmethacrylate (HEMA) + OVA. Animals shown in panels B and E were given the substances in phosphate-buffered saline (PBS), those shown in panels C and F were given the substances in 25 mM NaHCO_3 , and those shown in panels D and G were given the substances in 100 mM NaHCO_3 . A similar booster injection was given after 3 wk. Photos representative of the respective groups were taken 4 d after the booster.

injection. At first, the tail became reddish and slightly swollen; later, a hairless area evolved, sometimes accompanied with an erosion (Fig. 1). The level of inflammation was influenced by the solution in which HEMA was administered: the inflammatory reaction was scored significantly higher if HEMA was given in 100 mM NaHCO_3 than in 25 mM NaHCO_3 or PBS (Fig. 2). Co-administration of the self-protein, MSA, resulted in a significant suppression of the inflammatory response compared to that induced by HEMA alone. Co-administration of OVA did not have this effect on the HEMA-induced inflammatory response. The average inflammation score 4 d after injection, for selected groups, is shown in Fig. 2. The inflammation was resolved within 2 wk.

Cell responses

Spleen cells from animals that had been administered OVA, or OVA + HEMA, in 100 mM NaHCO_3 solution were *in vitro* stimulated with detoxified OVA. The proliferation index in cultures from animals that had been given both OVA and HEMA was significantly higher than the proliferation index in cultures from mice that only received OVA, as shown in Fig. 3. No significant differences were found in cultures from animals that were given the substances in 25 mM NaHCO_3 or in PBS (data not shown).

Antibody activity

Control animals given the solution (i.e. PBS, 25 or 100 mM NaHCO_3 or 100 mM NaHCO_3 with LPS) alone or with HEMA, but without protein, displayed no antibody activity towards OVA, MSA or MSA_H .

A significantly higher IgG1 response to OVA was induced when HEMA was co-administered with OVA in

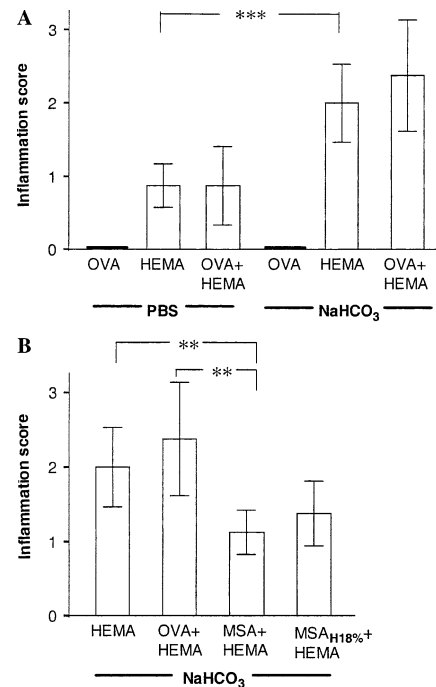


Fig. 2. Scoring of the inflammatory reaction at the site of injection of the indicated substances or combination of substances. Groups of mice ($n = 8$) were injected subcutaneously at the base of the tail with 2-hydroxyethylmethacrylate (HEMA) only, or together with ovalbumin (OVA), mouse serum albumin (MSA) or MSA *in vitro* conjugated with HEMA ($\text{MSA}_{H18\%}$) in phosphate-buffered saline (PBS) or NaHCO_3 . The scoring was performed 'blind' on the fourth day after injection. Scoring system: 0, no sign of inflammation; 1, light red reaction; 2, dark red. Scoring system in the presence of erosion was as follows: 0, no erosion; 1, small erosion; 2, pronounced erosion. Intermediate reactions were given half-points. Bars show means and the error bars show the 95% confidence interval of the mean. The Mann-Whitney *U*-test was used for statistical comparison (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$). (A) Score of the inflammatory reaction after injection of various substances in 100 mM NaHCO_3 or PBS. (B) Co-administration of MSA or OVA with HEMA in 100 mM NaHCO_3 . $\text{MSA}_{H18\%}$, mouse serum albumin carrying 9 HEMA molecules.

a bicarbonate solution, as compared to OVA given alone (Fig. 4A). This was more pronounced in 100 mM NaHCO_3 than in 25 mM NaHCO_3 . The tendency was the same, although not statistically significant, when the substances were given in PBS.

IgG2a was induced in some of the animals, mainly in those that were given both HEMA and OVA. Although not statistically significant, the tendency was that the co-administration of OVA and HEMA resulted in a higher IgG2a response to OVA (data not shown).

The co-administration of HEMA and OVA in bicarbonate solution induced a significantly higher IgE anti-OVA response than OVA given alone (Fig. 4B).

The number of IgG1 anti-OVA-producing spleen cells was assessed in animals immunized with OVA or OVA + HEMA in 100 mM NaHCO_3 . The co-administration of HEMA induced significantly more IgG anti-OVA antibody-producing cells (Fig. 5).

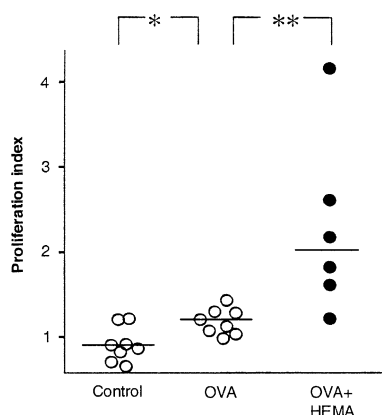


Fig. 3. Proliferation index in spleen cell suspension. Groups of mice ($n = 8$) were immunized with ovalbumin (OVA) and co-administered 2-hydroxyethylmethacrylate (HEMA), as indicated, in 100 mM NaHCO_3 . Two weeks after a similar booster injection, the animals were killed. Spleen cell suspensions, prepared and seeded at 5×10^5 cells/well on a 96-well plate, were stimulated with detoxified OVA or left unstimulated. [Methyl- ^3H]thymidine incorporation was allowed during the last 20 h before the cells were harvested on the fourth day. Proliferation was assessed from the [^3H]thymidine decomposition, measured in a β -scintillator, as counts per minute (c.p.m.). The proliferation index was calculated as follows: c.p.m. for cells stimulated with detoxified OVA $\div$ c.p.m. for unstimulated cells. During the experiment, two animals in the group given HEMA and OVA died unexpectedly. Each dot represents 1 animal and the median bar is indicated. Black circles denote animals that were co-administered HEMA. The Mann-Whitney U -test was used for statistical comparison (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$).

Immunization with $\text{MSA}_{\text{H33}\%}$, in combination with LPS, induced an autoantibody activity that was significantly higher if HEMA was co-administered. $\text{MSA}_{\text{H1}\%}$ induced an autoantibody response to MSA only if co-administered with HEMA and LPS (Fig. 6). Immunization with unmodified MSA did not induce any detectable antibody response, even in the presence of HEMA and LPS. Administration of 0.05 mg of protein per animal without LPS did not induce any detectable antibodies to MSA or $\text{MSA}_{\text{H18}\%}$ (data not shown).

Discussion

In the present study we showed that the subcutaneous injection of HEMA leads to an inflammatory reaction and that the co-administration of HEMA and a protein augment the subsequent antibody response. It was also obvious that the solution used to administer the substances, as well as the nature of the protein (i.e. if it was foreign or of self-origin) influenced the response. None of the substances induced a visible inflammatory reaction when they were given without HEMA.

While several studies have reported HEMA to be a causative agent for both irritant and allergic contact dermatitis in humans (5), attempts to develop a mouse model for the study of CHS induced by HEMA has proven unsuccessful in our hands and in others' (14).

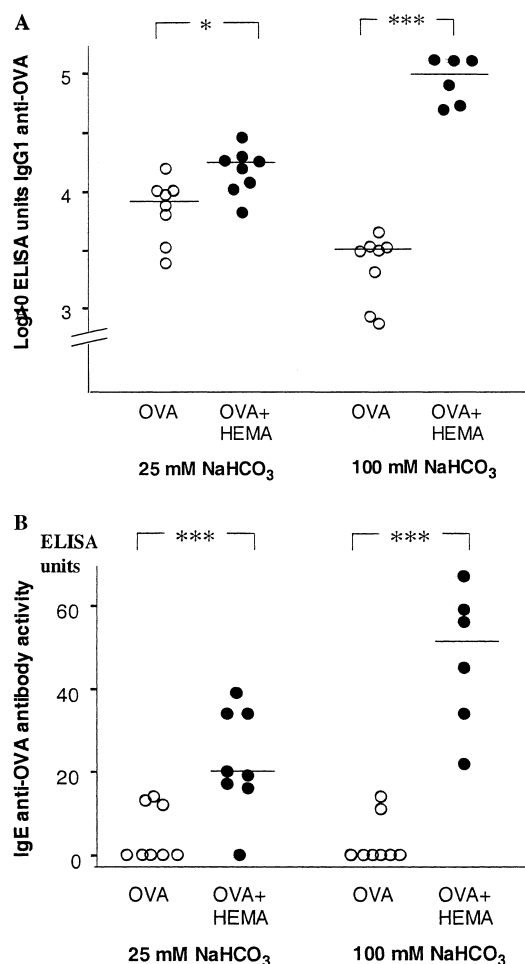


Fig. 4. Immunoglobulin (Ig)G1 and IgE activity in mice ($n = 8$) immunized with ovalbumin (OVA), with or without 2-hydroxyethylmethacrylate (HEMA), in 25 or 100 mM NaHCO_3 . The anti-OVA activity in sera was assessed 2 wk after an identical booster injection. During the experiment, 2 animals in the group given HEMA and OVA in 100 mM NaHCO_3 died unexpectedly. (A) IgG1 anti-OVA activity in blood. (B) IgE anti-OVA activity in blood. Each dot represents 1 animal and the median bar is indicated. The black circles denote animals that were co-administered HEMA. The Mann-Whitney U -test was used for statistical comparison (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$).

One explanation for this could be differences in the ability of HEMA to penetrate mouse and human skin.

In the present study, injecting HEMA subcutaneously induced an inflammatory reaction that was more pronounced if HEMA was given in 100 mM NaHCO_3 (pH 8.1) than in 25 mM NaHCO_3 (pH 8.2) or in PBS (pH 7.2). One possible explanation for this could be that the various solutions influenced the effectiveness of some of the enzymes in the skin. Hydrolases, for example, are considered to be more efficient at pH 8 than at pH 7. Hydrolases are a group of enzymes that can catalyze the cleavage of the ester bond of HEMA, yielding glycol and methacrylic acid. In a study on the toxicokinetic of HEMA in guinea pigs, it was found that $\approx 70\%$ of the subcutaneously injected HEMA was exhaled as CO_2 within 24 h, suggesting that a rather substantial meta-

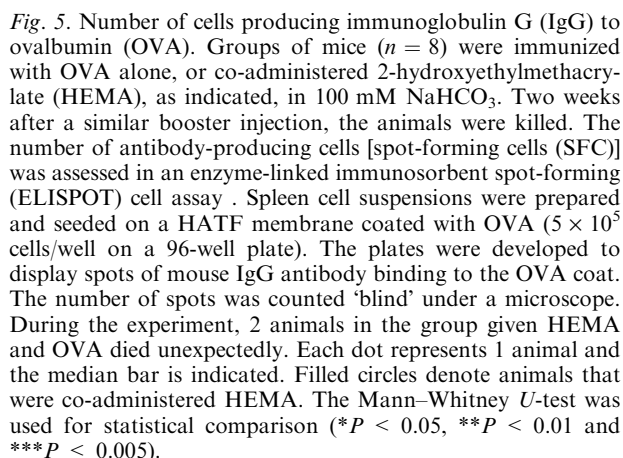


Fig. 5. Number of cells producing immunoglobulin G (IgG) to ovalbumin (OVA). Groups of mice ($n = 8$) were immunized with OVA alone, or co-administered 2-hydroxyethylmethacrylate (HEMA), as indicated, in 100 mM NaHCO_3 . Two weeks after a similar booster injection, the animals were killed. The number of antibody-producing cells [spot-forming cells (SFC)] was assessed in an enzyme-linked immunosorbent spot-forming (ELISPOT) cell assay. Spleen cell suspensions were prepared and seeded on a HATF membrane coated with OVA (5×10^5 cells/well on a 96-well plate). The plates were developed to display spots of mouse IgG antibody binding to the OVA coat. The number of spots was counted 'blind' under a microscope. During the experiment, 2 animals in the group given HEMA and OVA died unexpectedly. Each dot represents 1 animal and the median bar is indicated. Filled circles denote animals that were co-administered HEMA. The Mann-Whitney U -test was used for statistical comparison ($*P < 0.05$, $**P < 0.01$ and $***P < 0.005$).

The initial motive, to use 100 mM NaHCO₃, was to facilitate a possible conjugation of HEMA to protein *in vivo* as this solution promotes the conjugation of HEMA to protein *in vitro* (11). The lowest bicarbonate concentration was chosen as it corresponds to the HCO₃⁻ concentration of blood and stimulated saliva in humans (16). Interestingly, the character of the co-administered protein antigen also influenced the degree of inflammation. MSA, a self-protein in this model, reduced the HEMA-induced inflammation significantly, while the inflammation was largely unaffected by the 'foreign' protein OVA. The rationale could be that the inflammatory reaction was downregulated by mechanisms conferring tolerance towards 'self', attracted to the spot by the high concentration of MSA.

Considering that HEMA is known to cause irritative contact dermatitis, we hypothesized that it might have an adjuvant effect. Hence, mice were immunized with a protein antigen co-administered with HEMA. OVA was chosen as a 'reporter antigen' as it is a common model antigen that has been used in other studies to assess adjuvant activity, for example of phthalates, quaternary ammonium compounds or diesel exhaust particles (17–19). A significantly higher IgG1 and IgE anti-OVA

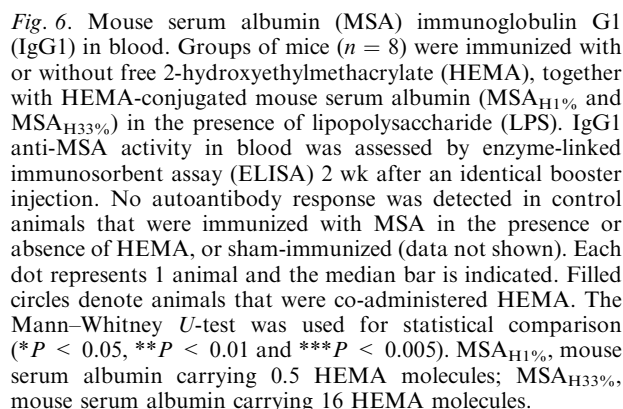


Fig. 6. Mouse serum albumin (MSA) immunoglobulin G1 (IgG1) in blood. Groups of mice ($n = 8$) were immunized with or without free 2-hydroxyethylmethacrylate (HEMA), together with HEMA-conjugated mouse serum albumin (MSA_{H1%} and MSA_{H33%}) in the presence of lipopolysaccharide (LPS). IgG1 anti-MSA activity in blood was assessed by enzyme-linked immunosorbent assay (ELISA) 2 wk after an identical booster injection. No autoantibody response was detected in control animals that were immunized with MSA in the presence or absence of HEMA, or sham-immunized (data not shown). Each dot represents 1 animal and the median bar is indicated. Filled circles denote animals that were co-administered HEMA. The Mann-Whitney U -test was used for statistical comparison ($*P < 0.05$, $**P < 0.01$ and $***P < 0.005$). MSA_{H1%}, mouse serum albumin carrying 0.5 HEMA molecules; MSA_{H33%}, mouse serum albumin carrying 16 HEMA molecules.

response, also mirrored by an elevated number of IgG anti-OVA-producing cells, was induced when HEMA was included in the mixture used for immunization. This suggests that HEMA has the potential to enhance an immune response.

Our next question was if the co-administration of free HEMA would influence the antibody response to a HEMA-modified self-protein. We have previously demonstrated, in a mouse model, that autoantibodies to native MSA are induced by immunization with a self-protein, MSA, conjugated *in vitro* with HEMA (11). The highest response was induced by the preparation having the lowest degree of conjugation; on average, three HEMA molecules per molecule of albumin (20). We also demonstrated that the binding rate of HEMA to protein depends on factors such as concentration, pH, and time. At physiological pH, the reaction occurs rather slowly, indicating that the degree of conjugation would be low under *in vivo* conditions (11). Thus, in the present study we used an MSA preparation carrying, on average, only 0.5 HEMA molecules per molecule of albumin, in an attempt to mimic a possible *in vivo* situation. In our previous experiments with HEMA-conjugated MSA, Freund's incomplete adjuvant (FICA) was included. Here we decided to introduce a small quantity of LPS as a danger signal. In agreement with our previous result, which used FICA as adjuvant, immunization with MSA_{H33%} led to the production of autoantibodies to MSA in the presence of LPS. The autoantibody activity was significantly higher if free HEMA was co-administered. Immunization with the MSA_{H1%} preparation led

to the production of autoantibodies only in the presence of free HEMA and LPS, meaning that the danger signal contributed by free HEMA was strong enough to overcome the lower immunogenicity of this preparation.

The results reported here are based on acute experiments. However, the low conjugation ratio of HEMA to protein, used in these experiments, may correspond to a situation where a person working with acrylates could be exposed to minute amounts over a long time period. Exposure to water and other chemicals could contribute to making the skin more susceptible to a HEMA-related contact dermatitis. In this study we have shown that in the presence of additional danger signals, such as free HEMA and LPS, an autoantibody response develops to a self-protein conjugated with HEMA to an extremely low degree. Considering that the co-administration of HEMA augmented the antibody response to OVA, it is also possible that exposure to HEMA could contribute to the development of allergy to other substances commonly present in a dental clinic (e.g. latex proteins from gloves).

In conclusion, our findings suggest that HEMA, besides the ability to render a self-protein immunogenic by conjugation, possesses inflammatory and adjuvant properties.

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