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Sublingual administration of 2-hydroxyethyl methacrylate enhances antibody responses to co-administered ovalbumin and *Streptococcus mutans*

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ABSTRACT

Objective: The oral mucosa of patients undergoing dental procedures is often exposed to residual monomers leaking from incompletely cured acrylic resins. We investigated whether 2-hydroxyethyl methacrylate (HEMA) monomers applied to the sublingual mucosa in mice modulate the antibody responses towards co-administered ovalbumin (OVA) or live oral bacteria.
Material and methods: OVA, live mouse oral commensal *Lactobacillus murinus* or live human oral commensal *Streptococcus mutans* were administered sublingually with or without HEMA to BALB/c mice on four weekly occasions. One week after the last administration, the experiment was terminated and serum antibody levels were analyzed using ELISA.
Results: Significantly increased IgG and IgE anti-OVA antibody activity was found in the sera from mice immunized with OVA together with HEMA, as compared to mice immunized with OVA alone. Likewise, *S. mutans* together with HEMA induced an IgG anti-*S. mutans* antibody response that was significantly higher than the antibody response detected after application of *S. mutans* alone. No IgG anti-*L. murinus* antibody response was detected in mice immunized with *L. murinus* together with HEMA, as compared to the background activity.
Conclusions: We report that HEMA monomers have adjuvant properties when sublingually administered in combination with OVA or *S. mutans*.

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2-Hydroxyethyl methacrylate; adjuvant; sublingual immunization; oral microflora

Introduction

Acrylic resins are common components of the composite material used in a variety of dental procedures in Sweden and many other countries, although the negative health effects of acrylates and methacrylates are well-documented [1–6]. Many of the reports describe irritant and allergic contact dermatitis in dental personnel [3,5,6]. However, allergic contact stomatitis has also been observed in some dental patients after restorative treatment, and tissue manifestations have been reported to include mild erythema in the gingiva and buccal mucosa [7], burning mouth syndrome [8,9] and lichenoid-like reactions of the lips [10]. The current consensus in the literature is that hypersensitivity reactions induced by dental resin material are caused by residual monomers leaking from dentures or recently placed fillings, although the precise etiology of these adverse reactions remains to be defined. In addition to the release of un-polymerized monomers during and within the first 24 h after the resin-curing procedure, reactive acrylate monomers diffuse into the oral environment over time as a consequence of erosion and deterioration of the material [11,12], resulting in prolonged exposure of the patient.

One of the most frequently used components in composite dental material is the highly reactive 2-hydroxyethyl methacrylate (HEMA) monomer, which is known to exert numerous modulatory effects on the immune system [13–21]. In our previous studies, we have shown that HEMA can penetrate intact skin and induce inflammation in the draining lymph nodes [18], and that it promotes immune responses to antigens, i.e. acts as an adjuvant, when administered subcutaneously together with a model antigen [19]. Whether or not HEMA can also influence immune responses towards antigens administered *via* the oral mucosa is not currently known. In addition to boosting immune responses to model antigens that are foreign to the host, HEMA has been shown to induce the production of autoantibodies when the monomer is *in vitro* conjugated to self-antigens and injected subcutaneously to BALB/c mice [16]. Although it is unknown whether HEMA spontaneously creates hapten complexes with proteins *in vivo*, these results indicate that HEMA has the ability to induce immune responses to antigens that are normally tolerated by the immune system.

The oral mucosa is exposed continuously to environmental antigens and microbes, as well as to a wide variety of resident microorganisms constituting the oral microbiome [22]. The indigenous microbiota naturally persists in homeostasis with the oral mucosal tissues and has a fundamental role in maintaining oral and systemic health. Early in the life of an individual, the immune system learns to recognize the

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normal microflora and develops regulatory mechanisms so as to avoid aberrant reactions to resident antigens (reviewed in [23–25]). Simultaneously, the immune system needs to identify and mount relevant immune responses to pathogens. Thus, the appropriate immune response to an oral antigen is crucial for the maintenance of a stable and healthy microbiome and the prevention of pathological reactions, as well as for protection against oral infections. The immune response to an antigen can be intentionally modulated for medical purposes, e.g. in the development of mucosal vaccines, where a boosted immune reaction is needed to generate protective immunity. However, un-intentional and un-controlled enhancement of the immune responses to resident antigens can result in hypersensitivity reactions and/or chronic inflammation.

Out of the microorganisms colonizing the surfaces of the oral cavity, bacteria are the most numerous. It has been estimated that 1 mL of saliva contains over 100 million bacteria and ∼700 species or phylotypes have been identified using culture-independent techniques [22,26,27]. Although the composition of the oral microbiome differs between individuals, many bacteria are generally found across the world population. These include species under the *Streptococcus* genii, which constitutes 26.9% of the oral microbiome at the genus level stored in the Human Oral Microbiome Database (HOMD) (reviewed in [28]). *Streptococcus mutans* is an extensively studied group of *Streptococcus* species found mainly in the oral biofilm covering the teeth. In cases of dysbiosis in the oral cavity, *S. mutans* can become an opportunistic pathogen and is strongly associated with the initiation and progression of plaque formation and caries lesions.

In BALB/c mice, the gram-positive bacterium *Lactobacillus murinus* is one of the dominant commensals, constituting 38–50% of the bacteria of the oral microbiome [29–31]. *Lactobacillus murinus* is found mainly on the oral mucosa [31] and is, together with *Staphylococcus aureus* and *Staphylococcus sciuri*, one of the first bacterial species to colonize the oral cavity of BALB/c mice [30]. However, *Staphylococci* are not considered part of the normal flora, but are instead believed to be the result of repeated contamination from the environment. To our knowledge, natural occurrence of *S. mutans* in mice has not been described in the literature.

The large numbers of microbes and other antigens in the oral cavity together with the adjuvant capacity of HEMA led us to hypothesize that the un-reacted HEMA monomers released from dental filling material interact with the immune system of the oral mucosa and cause inflammation. This would affect the immune response to oral bacteria and other oral antigens, resulting in immune reactions associated with the symptoms experienced by some patients after restorative treatment. To address this question, the present study aimed to examine whether HEMA could act as an oral adjuvant and boost the immune responses to oral indigenous or previously un-encountered bacteria and other antigens.

Materials and methods

Preparation of bacteria for immunization

Lactobacillus murinus (strain 45959; Culture Collection, University of Gothenburg, Sweden), was cultured on *Lactobacilli* MRS agar plates (Acumedia Manufacturers, Lansing, MI) for 48 h in a 5% CO_2 atmosphere at 37 °C. On the day of immunization, bacterial colonies were transferred into 100 mL of sterile phosphate-buffered saline (PBS) buffer (Sigma Chemical Co., St. Louis, MO) and centrifuged at 4000*g* for 15 min at 4 °C. The supernatant fluid was discarded and the bacteria were washed twice in PBS before the optical density was measured and compared against a standard curve, to determine the bacterial concentration.

Streptococcus mutans (in-house patient isolate strain IB16) was cultured on blood agar plates (Acumedia, Lansing, MI) that contained 50 mL/L defibrinated horse blood for 24 h in a 10% CO_2 and 90% N_2 atmosphere. Just prior to immunization, the bacterial colonies were transferred to PBS buffer and prepared according to the protocol described above.

Animals

Female 6–8-week-old BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and were kept in the animal facility according to governmental rules. Experimental protocols were approved by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden (Dnr. #N186/15).

Experimental design

To mimic the leakage of un-polymerized HEMA onto the sublingual mucosa and antigen encounter, mice ($n = 7$ or 8 per group) were anaesthetized using Isoflurane (IsoFlo®vet, Abbott Laboratories Ltd, Queenborough Kent, UK) and administered HEMA together with OVA or live bacteria topically under the tongue using a micropipette on four occasions at weekly intervals. In our first experimental set-up, the mice were given 50 μg ovalbumin (OVA) grade V (Sigma-Aldrich, Saint Louis, MO) with or without 20 μmol HEMA (Sigma-Aldrich) dissolved in 10 μL of 100 mM $NaHCO_3$, and the positive control group were given 50 μg OVA together with 10 μg cholera toxin (CT) (Sigma-Aldrich). In the second experimental set-up, the mouse commensal *L. murinus* or the common human oral bacterium *S. mutans* was sublingually administered at 2.5×10^6 bacteria in 10 μL of $NaHCO_3$ per animal together with 20 μmol HEMA. Control groups were administered 20 μmol HEMA or one of the bacterial strains only. Mice-administered $NaHCO_3$ only served as negative controls. One week after the last immunization, blood was drawn from the axillary plexus and the experiments were terminated. Serum was prepared and ELISAs were performed to estimate the anti-OVA and anti-bacterial antibody responses.

IgG anti-OVA antibodies

To assay IgG anti-OVA antibody activity, serum samples were added to 96-well microtitre plates (Nunc MaxiSorp; Nunc, Roskilde, Denmark) that were pre-coated with OVA grade V (Sigma-Aldrich) and incubated for 2 h to allow antibody binding. After washing off the unbound antibodies, an ALP-conjugated mouse anti-IgG antibody (Mabtech, Nacka Strand,

Sweden) was added. The antibody activity was quantified by measuring the conversion of *p*-nitrophenyl phosphate (pNPP, Sigma-Aldrich) in a spectrophotometer (Synergy 2, BioTek Instruments, Inc., Winooski, VT) at 405 nm and comparing to a standard curve.

IgE anti-OVA antibodies

The activities of IgE anti-OVA-specific antibodies were assessed using a commercial ELISA kit (BioLegend, San Diego, CA) according to the manufacturer's instructions. In brief, sera from mice were diluted 1:2 and the standard was diluted 1:2 in seven steps. The samples were added to a pre-coated microtitre plate and allowed to bind for 2 h. A washing procedure was performed and monoclonal anti-mouse IgE detection antibodies were added. After washing and adding of substrate, the reaction was developed and the absorbance was read at 450 nm. The range of detectable concentrations was 0.313–20 ng/mL.

IgG anti-L. murinus and anti-S. mutans antibodies

Lactobacillus murinus was prepared as described above and diluted to a concentration of 2×10^8 bacteria/mL in PBS. The suspension was boiled for 10 min to release the antigens and subsequently added to 96-well microtitre plates (Nunc) at 100 μL per well for overnight coating. After blocking with 0.1% BSA, 0.05% Tween in PBS, serum samples were added in duplicate in four steps (1:2 dilution series starting at 1:40). Pooled sera from immunized mice was used as the standard and were added in duplicate, diluted in seven steps starting at 1:10. Samples were incubated overnight, after which the plates were washed and goat anti-mouse IgG alkaline phosphatase (ALP)-conjugated antibodies were added. The reactions were developed using pNPP (Sigma-Aldrich) and the absorbance was read at 405 nm. The highest standard value was set at 1000 IgG units and the sample values were calculated using the standard curve.

For the IgG anti-*S. mutans* antibody assay, a boiled *S. mutans* preparation corresponding to a concentration of 2×10^8 bacteria/mL was used as the coating antigen, after which the same protocol as described for the IgG anti-*L. murinus* antibody ELISA was followed.

Statistical analysis

Analyses of differences between groups were performed using the Mann–Whitney *U*-test (GraphPad Prism 7; GraphPad Software, San Diego, CA) and a p value of $< .05$ was considered to indicate statistical significance. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$.

Results

HEMA enhances IgG and IgE anti-OVA antibody activity after sublingual application

To study the potential of HEMA to enhance the antibody response to OVA *via* the oral mucosal route, OVA-specific IgG and IgE antibody activities were measured in the sera from mice that were sublingually immunized with OVA with or without HEMA. Sera from mice immunized with OVA together with CT were used as positive controls and sera from mice-administered $NaHCO_3$ only served as negative controls. There were significantly increased IgG anti-OVA antibody activities in the sera from mice immunized with OVA together with HEMA, as compared to mice immunized with OVA only (Figure 1(a)). A similar significant difference between these groups was seen for IgE anti-OVA antibody activity (Figure 1(b)).

HEMA increases the IgG anti-S. mutans antibody response but not the anti-L. murinus antibody response after sublingual application

To evaluate the effects of HEMA on the IgG anti-*L. murinus* and anti-*S. mutans* antibody activities, ELISAs were performed on the sera collected from mice that were sublingually immunized with either of the two strains together with HEMA and on sera from mice that were immunized with *L. murinus or S. mutans* or HEMA alone. Sera from mice-administered $NaHCO_3$

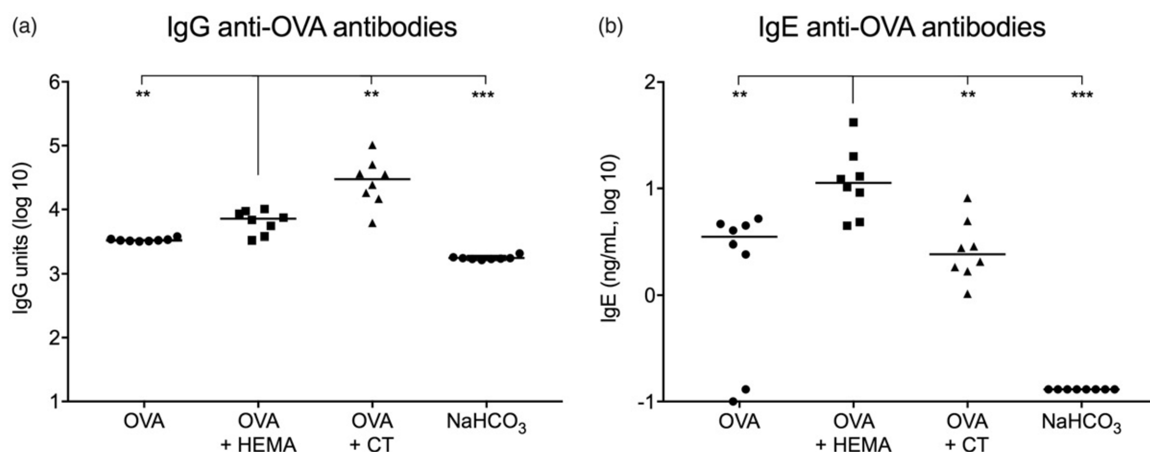

Figure 1. HEMA increases the serum IgG and IgE anti-OVA-specific antibody activities when sublingually co-administered with OVA. Mice ($n = 8$ mice/group) were sublingually immunized with OVA together with HEMA on four occasions with weekly intervals, and the experiment was terminated 1 week after the last immunization. Each data point represents an individual mouse and the data show the serum IgG (a) and IgE (b) anti-OVA antibody activities in ELISA units and ng/mL, respectively. Significant differences between the groups, as assessed by the Mann–Whitney *U*-test, are denoted as: $^{*}p < .05$ and $^{**}p < .01$.

only served as negative controls. As illustrated in Figure 2(a), no significant differences in the IgG antibody responses were detected in the sera from mice that were immunized with *L. murinus* together with HEMA, as compared to the other groups. In contrast, *S. mutans* together with HEMA induced a strong IgG antibody response that was significantly higher than the response induced by *S. mutans* only (Figure 2(b)).

Discussion

The wide-spread use of resin-based composites within dentistry spurs the need for better knowledge of their biological impacts. In this study, we hypothesized that un-cured HEMA monomers released during or subsequent to dental procedures may modulate immune responses to local antigens *via* the oral mucosa.

Previous studies using mouse models have shown that HEMA can penetrate the epidermis and affect cytokine responses in the spleen and peripheral lymph nodes [18], and that subcutaneous injection of HEMA increases the IgG and IgE antibody responses to co-administered antigens [19], i.e. HEMA has adjuvant properties. However, whether or not HEMA is able to modulate immune responses by penetrating the oral mucosa, as might occur in a clinical situation in which a patient is exposed to leaked monomers, has to this point not been clarified. In this context, our first objective was to determine whether HEMA acts as an oral mucosal adjuvant. We employed an experimental mouse model that is commonly used in vaccine studies, whereby an adjuvant and an antigen are co-administered topically under the tongue [32–34], on the basis that the sublingual mucosa is an induction site for both mucosal and systemic immune responses [33,35–37]. In humans, sublingual immunization leads to uptake by the mucosa on the ventral surface of the tongue and the floor of the mouth. However, considering that the oral cavity of a mouse is much smaller, it cannot be excluded that some of the delivered formula spills over onto other areas of the mouth and initiate additional immune responses. This resembles the situation in which HEMA is transferred to different sites in the oral cavity *via* the saliva in a dental patient. The sublingual absorption capability may also differ due to anatomical differences between mice and humans, given that the sublingual mucosa in mice is partly keratinized, whereas the human sublingual mucosa is not. Nevertheless, using this type of immunization regimen, we have been able to obtain valuable information regarding the effects on the immune system of acrylate monomers released into the oral cavity.

To assess the effect on serum antibody activity, we chose to use the model antigen OVA, which is a reporter immunogen that is frequently used in immunological studies. HEMA was added in the appropriate inoculums at 20 μmol dissolved in 10 μL of $NaHCO_3$, corresponding to a concentration of 2 M. This is comparable to the concentrations of HEMA used in commercial dental adhesives, although the proportion of HEMA when used clinically varies between 5 and 55% [38,39]. However, it does not simulate the amount of acrylate monomers found in saliva after dental procedures, which has been estimated by others to be in the range of 0.015–0.19 μg/mL [40]. The strategy of exaggerating the adding of a studied agent in relation to biologically relevant concentrations is favourable when using *in vivo* models, as the amplification of potential cell and tissue responses simplifies the detection of antibodies and other cell mediators. Naturally, this must be taken into consideration when interpreting the results and follow-up studies will include dose–response experiments in order to confirm the conclusions drawn from this study. On the other hand, it must be remembered that in addition to leakage from fillings, adhesives can be accidentally spilled during the dental procedure, exposing the mucosa to concentrated monomers. In such cases, the HEMA concentrations used in our study is comparable to those existing in clinical situations.

The adjuvant capacity in the sublingual zone is clearly confirmed, as the results show that co-application of HEMA and OVA under the tongue triggers a significantly stronger IgG anti-OVA antibody response than the application of OVA alone. Likewise, IgE anti-OVA antibody activity was enhanced when HEMA was co-administered with OVA, as compared to

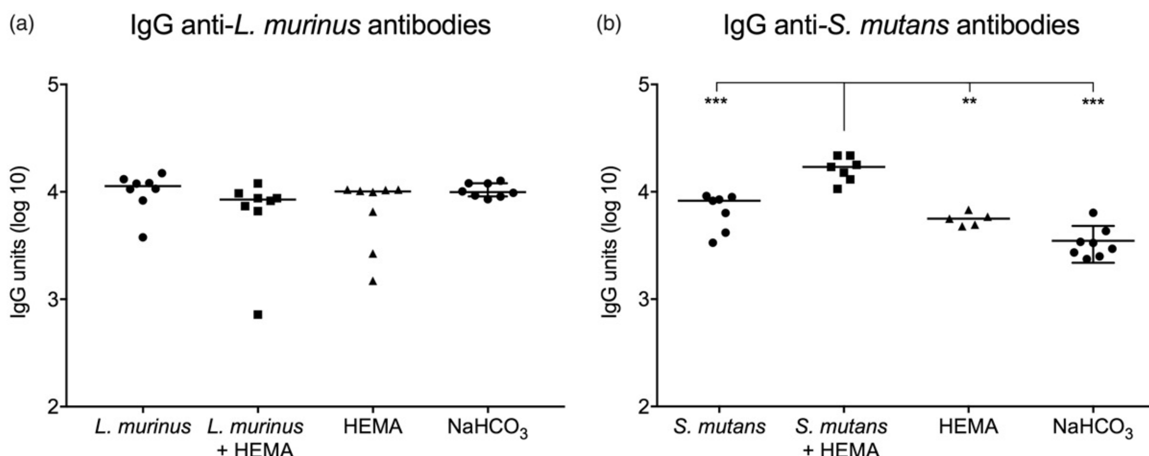

Figure 2. HEMA increases serum IgG activity when co-administered sublingually with *S. mutans* but not with *L. murinus*. Mice ($n = 5$–8 mice/group) were sublingually immunized with *S. mutans* or *L. murinus* with or without HEMA at four weekly occasions, and the experiment was terminated 1 week after the last immunization. Shown are the serum IgG anti-*L. murinus* (a) and IgG anti-*S. mutans* (b) antibody activities in ELISA units. Each data point represents an individual mouse. Significant differences between the groups, as assessed by the Mann–Whitney *U*-test, are denoted as: $^{**}p < .01$ and $^{***}p < .001$.

when the mice were given OVA without HEMA. These findings are in concordance with the results of the above-mentioned studies and corroborate that, in addition to affecting immune responses by penetrating the epidermis, HEMA can modulate specific systemic immune responses *via* the oral mucosal tissues. The production of IgE antibodies in response to antigens, referred to as sensitization [41], could in some individuals initiate allergen-related mucositis similar to allergic contact dermatitis. However, the production of IgE antibodies to an allergen does not strictly correlate to a clinically relevant allergic disease in response to allergen re-exposure.

As HEMA has the ability to bind to endogenous protein and elicit auto-antibody production *in vivo* [16], we hypothesized that HEMA may also contribute to the initiation of immune responses towards oral indigenous bacteria that naturally persist in homeostasis with the oral mucosal tissues. Thus, we decided to study whether HEMA could augment adaptive immune responses towards *L. murinus*, which is one of the predominant commensals in BALB/c mice [29–31]. In BALB/c mice purchased from Charles River Laboratories, as were used in the current study, *L. murinus* has been shown to constitute as much as ~50% of the bacteria of the oral microbiome [29]. We found no evidence of adjuvant activity, as HEMA did not significantly enhance the IgG antibody response to *L. murinus*. These findings are noteworthy, yet not remarkable, and indicate that early in life colonization by *L. murinus* induces regulatory mechanisms that are too robust to be overturned by the inflammatogenic capacity of HEMA. The association between neonatal colonization and oral tolerance has been described by several research groups, e.g. Dahlman et al. [42], who showed that rats that were intestinally colonized from birth with OVA-producing bacteria did not respond to subsequent diet-delivered OVA with either specific antibody responses or delayed-type hypersensitivity. Furthermore, Karlsson et al. [43] have shown that rats that were neonatally colonized with OVA-producing bacteria exhibited a suppressed response when later immunized with OVA, as compared to the control group.

Next, we wished to study the ability of HEMA to enhance immune responses towards bacteria that are not part of the murine oral commensal flora. Since dental caries is among the most prevalent infectious diseases in humans [44], we chose to study the adjuvant effects of HEMA when combined with the caries-associated oral bacteria *S. mutans*. Our results clearly show that HEMA boosts the IgG antibody response to *S. mutans* following sublingual application of the bacteria together with HEMA, seeing that the IgG anti-*S. mutans* antibody response induced by application of *S. mutans* in combination with HEMA was significantly higher compared to when HEMA was excluded from the inoculum. This implies that the different forms of oral discomfort experienced by some patients may be due to their exposure to acrylate monomers that are enhancing the immune responses to non-resident local bacteria, thereby creating a situation of persistent stress and chronic inflammation. The mechanisms underlying the adjuvant effect of HEMA have recently been shown to depend on the ability of HEMA to induce formation of the NLRP3 inflammasome, thereby triggering the release of the pro-inflammatory cytokines IL-1β and IL-18 [45]. Together with other cytokines of the IL-1 family, IL-1β and IL-18 are strongly associated with innate and pro-inflammatory immune responses, as IL-1 receptor activation leads to enhanced surface expression of cellular adhesion molecules and increased expression of the genes for many pro-inflammatory mediators. The adjuvant properties of the IL-1 family of cytokines are further attributed to their abilities to affect non-specifically antigen recognition and lymphocytic functions.

In conclusion, we show that HEMA has the ability to act as an adjuvant after sublingual application, enhancing the antibody responses to the food protein OVA and the, in humans, common oral bacterium *S. mutans*. However, the adjuvant capacity of HEMA is not strong enough to overcome the immunological regulation of the response to the mouse commensal bacterium *L. murinus*. These findings may be of clinical relevance as they imply that leakage of unpolymerized HEMA onto the oral mucosa may enhance immune responses towards oral bacteria and other antigens, possibly causing problems for patients who have undergone dental restoration.

Disclosure statement

No potential conflict of interest was reported by the authors.

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