Immunomodulatory Properties of 2-Hydroxyethyl Methacrylate

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Cover Illustration: “Invading organisms are attacked by the Y-shaped antibody molecules. Antibodies attach to the invaders and make it easier for them to be destroyed by the immune system. At the top, invaders (gold) are eaten by a macrophage, which processes their antigens and presents them to a helper T-cell (purple). The helper cells then activate their corresponding B-cells (blue). These divide repeatedly and become plasma cells that produce vast quantities of antibodies (orange) that attack the invader (gold).” © Russel Kightley Science Images
# Table of Contents

**Abstract**  
5

**Preface**  
7

**Abbreviations**  
9

**Introduction**  
11

Acrylates and Methacrylates  
11

Contact Dermatitis  
11

**HEMA**  
12
- Characteristics and Function in Dental Resin Materials  
12
- HEMA Leakage and Leukocytes in the Oral Cavity  
13

**Innate Immunity: Neutrophils and Monocytes**  
13
- Monocytes  
14
- Neutrophils and Their Granules  
14
- Phagocytosis and The Respiratory Burst: The Radical Armor of Monocytes and Neutrophils  
15
- Effects of HEMA on the Life of Monocytes, Macrophages and Neutrophils  
17

**Adaptive Immunity: B Lymphocytes and Antibodies**  
17
- B Cell Subsets  
17
- B Cell Activation and Maturation  
18
- Antibodies: Classes, Structure and Functions  
19
- Antigens: Immunogens and Haptens  
20
- Effects of HEMA Exposure on Antibody Production  
21

**Aims**  
23

**Materials & Methods**  
25

2-hydroxyethyl methacrylate (HEMA)  
25

Effects of HEMA on the Phagocytic and Respiratory Burst Activity of Granulocytes and Monocytes (I)  
25
- Study Subjects and Sampling  
25
- HEMA Exposure in vitro  
25
- Flow cytometric Assay of Phagocyte Immune response in Activated whole blood (FAPIA)  
25
- Cell Membrane Integrity – Propidium Iodide Staining  
26
Effect of HEMA on Human B Cell Immunoglobulin Production (II)  26
  Purification of Human CD19+ B Cells from Blood  26
  Pokeweed Stimulation and HEMA Exposure in vitro  27
  B Cell Proliferation in vitro - Measuring β-rays emitted from [3H]thymidine  27
  Human IgA, IgG1 and IgM Production in vitro – ELISA  27

Effects of HEMA Exposure in vivo (III, IV)  28
  Animal Husbandry  28
  HEMA Exposure in vivo - Osmotic Pumps (III)  28
  OVA Exposure in vivo – Subcutaneous Injections (III)  28
  HEMA and OVA Exposure in vivo – Subcutaneous Injections (IV)  29
  Splenocyte Isolation (III, IV)  29
  Production of TNF-α, IL-2 and IL-6 in vitro – ELISA (III, IV)  30
  Detoxification of OVA using Polymixin B (III)  30
  Con A and OVA Stimulation in vitro – Splenocyte Proliferation (III, IV)  31
  IgA, IgG and IgM anti-OVA Antibody Activity in Serum - ELISA (III, IV)  31

Statistical Analysis  31

Results  33

Exposing Granulocytes and Monocytes to HEMA in vitro - Consequences for Bactericidal Functions (I)  33

Exposing Human B Cells to HEMA in vitro - Consequences for Immunoglobulin Production and B Cell Proliferation (II)  34

Long-Term Exposure to HEMA in vivo - Consequences for General Health Status, Splenocyte Proliferation, Splenocyte Cytokine Production and Antibody Activity (III)  36

Subcutaneous HEMA Exposure - Consequences for the Immune System (IV)  38

General Discussion  41
Acknowledgements  47
References  49
Appendix: Study I-IV
Abstract

Immunomodulatory Properties of 2-Hydroxyethyl Methacrylate

Jennie Andersson

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Professionals working in dentistry have reported adverse effects, such as allergic contact dermatitis, following exposure to 2-hydroxyethyl methacrylate (HEMA). Furthermore unpolymerized HEMA monomers leaking from cured fillings can reach the dental pulp, where HEMA could come into contact with leukocytes.

The aims of this thesis were to study specific effects of HEMA exposure on the phagocytic and respiratory burst activity of human phagocytes (study I), human immunoglobulin production (study II), antibody production (study III, study IV), leukocyte proliferation and leukocyte cytokine production (study III, study IV).

Using fluorescently labeled *Escherichia coli* it was demonstrated that HEMA does not impair the phagocytic activity of either monocytes or neutrophils in vitro. By using dihydrorhodamine, a substrate for hydrogen peroxide, it was further shown that HEMA exposure decreases neutrophil respiratory burst activity and thus impairs the bactericidal capacity.

By exposing pokeweed stimulated human B cells to HEMA for six days in vitro it was shown that HEMA specifically increases the production of the immunoglobulin IgG1 in vitro at lower concentrations, while at higher concentrations HEMA reduces IgG1 and IgM production in vitro as well as B cell proliferation. The IgA production in vitro appeared insensitive to HEMA exposure.

The effect of long-term exposure to HEMA in vivo was analyzed by implanting osmotic pumps, filled with different concentrations of HEMA, subcutaneously in mice. Pumps were left in situ for 40 days, during which time the animals were injected with ovalbumin (OVA), dissolved in bicarbonate buffer, on two occasions. Control animals received pumps filled with saline. Mice exposed to high concentrations of HEMA had an impaired weight gain throughout the exposure period and a lower splenocyte interleukin(IL)-2 production in vitro. Mice exposed to low concentrations of HEMA had an impaired weight gain in the beginning of the exposure period and lower concanavalin A stimulated splenocyte proliferation in vitro, splenocyte IL-2 production in vitro and serum IgA anti-OVA antibody activity, compared to control mice.

The in vivo effect of HEMA was further studied by injecting mice subcutaneously with HEMA dissolved in bicarbonate buffer, in the presence or absence of OVA. Mice exposed to HEMA, on two separate occasions, had a reduced splenocyte tumor necrosis factor alpha production in vitro compared to control animals injected with only buffer. Further both baseline and concanavalin A stimulated splenocyte proliferation in vitro was higher compared to controls. Mice exposed to HEMA and OVA in bicarbonate buffer had a higher IgG anti-OVA antibody activity relative to the corresponding IgM anti-OVA antibody activity, compared to animals that were injected with only OVA in buffer.

In conclusion our results suggest that HEMA can suppress as well as enhance immunological responses, specifically affecting neutrophil bactericidal function, immunoglobulin/antibody production, cytokine production and leukocyte proliferation.

Key Words: 2-Hydroxyethyl Methacrylate; Granulocyte; Respiratory burst; Immunoglobulin; B cell; Interleukin; Mouse

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Preface

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


IV. Andersson, J. & Dahlgren, U. HEMA promotes IgG but not IgM antibody production *in vivo* in mice. Accepted for publication in *European Journal of Oral Sciences*. 
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACD</td>
<td>Allergic contact dermatitis</td>
</tr>
<tr>
<td>AID</td>
<td>Activation induced cytidine deaminase</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>B_F</td>
<td>Follicular B cells</td>
</tr>
<tr>
<td>B_M</td>
<td>Marginal zone B cells</td>
</tr>
<tr>
<td>BPI</td>
<td>Bactericidal permeability increasing protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHS</td>
<td>Contact hypersensitivity</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DHR 123</td>
<td>Dihydrorhodamine 123</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cells</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>H_2SO_4</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>H_2O_2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>[^3]H</td>
<td>Tritium</td>
</tr>
<tr>
<td>ICD</td>
<td>Irritant contact dermatitis</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>J gene</td>
<td>Joining gene</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MSA</td>
<td>Mouse serum albumin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>R 123</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>Rac2</td>
<td>Ras-related C3 botulinum toxin substrate 2</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid inducible gene</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>STAT-6</td>
<td>Signal transducer and activator of transcription-6</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>V gene</td>
<td>Variable gene</td>
</tr>
</tbody>
</table>
Introduction

Acrylates and Methacrylates

The majority of materials used for dental restorations today contain acrylate and methacrylate monomers. Acrylates and methacrylates are esters, derived from the reaction of an alcohol with either acrylic or methacrylic acid. Previous studies have reported on the adverse effects of methacrylate monomers, primarily on professionals working in dentistry. The reported prevalence of methacrylate contact allergy varies from 1.3% to 22%. Methacrylates have also been implicated in cases of asthma and conjunctivitis.

Contact Dermatitis

A common reaction to methacrylate monomers is contact dermatitis, which can be irritant (ICD) or allergic (ACD) in nature. ICD is caused by the innate immune system. Patients with ICD exhibit an infiltration of primarily neutrophils at the site of exposure. ACD involves a T cell-mediated, delayed-type hypersensitivity (DTH) response to low molecular weight molecules (i.e. haptens), of which acrylates and methacrylates are two examples. For ACD to occur an individual has to be sensitized. The ability of a hapten to sensitize an individual depends on the hapten’s inflammatogenic capacity, i.e. to act as a “danger signal”. The hapten also has to be capable of binding to amino acids of cutaneous proteins, thus providing new antigenic determinants. Insight into the pathological mechanisms behind ACD has been gained by using animal models. In these models the animal is first sensitized by painting the hapten on the abdomen or on the back. A few days later the animal is challenged by painting the hapten on the ear, which results in an inflammatory reaction referred to as contact hypersensitivity (CHS). The effector cells of CHS is believed to be interferon (IFN)-γ producing CD8+ T cells while it has been proposed that interleukin (IL)-10/IL-4 producing CD4+ T cells play a down-regulatory role. However CD4+ T cells can be cytotoxic against keratinocytes presenting haptenated peptides, which suggest that CD4+ T cells may cooperate with cytotoxic CD8+ T cells in damaging the tissues during CHS. B cells also play a role in CHS. Following hapten sensitization naïve B cells, located in the peritoneal cavity, are induced to migrate to the spleen where they start to produce hapten specific IgM antibodies. Upon challenge these hapten specific IgM antibodies form immune complexes when binding to the challenging hapten. The immune complexes can then activate the complement system, which leads to the generation of the complement fragment C5a. C5a in turn activates mast cells. Mast cells drive the influx of neutrophils by secreting tumor necrosis factor (TNF)-α and macrophage inflammatory protein – 2. The resolution phase of CHS is believed to be driven by CD4+ regulatory T cells and IL-10 producing mast cells.
This thesis focuses on the immunomodulating properties of 2-hydroxyethyl methacrylate (HEMA), one of the most frequently reported allergens to cause problems among dental personnel.

**HEMA**

*Characteristics and Function in Dental Resin Materials*

HEMA (CH$_2$=C(CH$_3$)COOCH$_2$CH$_2$OH) is an ester monomer found in dentin adhesives and resin glass ionomer cements. HEMA has several characteristics that make it a useful constituent of materials used for dental restorations. For instance, the HEMA monomer is relatively small (molecular weight = 130 g mol$^{-1}$) and hence has a relatively low viscosity. This confers that HEMA can function as a solvent for other larger monomers, such as bisphenol A-glycidyl methacrylate (molecular weight = 511 g mol$^{-1}$), thereby improving their polymerization rate. Further, HEMA is a polar molecule with one hydrophobic end and one hydrophilic end. The polar nature of HEMA makes it capable of functioning as a coupling agent, promoting the adhesion of a composite resin to demineralized dentin, by binding to both the hydroxyl groups on collagen fibers in demineralized dentin and to the composite resin. To be able to function as a coupling agent HEMA has to saturate the collagen fibers, which requires the displacement of surrounding water. When performing dental restorations water is displaced by solvents such as ethanol and acetone. That HEMA is soluble in ethanol and acetone is therefore another useful characteristic. Using scanning electron microscopy it has been shown that pretreatment of dentin with HEMA prior to application of resin composites and adhesives results in an increase in the thickness and the length of the hybrid layer, i.e. the layer of a dental filling that consists of a mixture of polymerized resin monomers and demineralized dentin. In other words HEMA increases the bond strength of resin composites to demineralized dentin.

HEMA is a functional monomer, which means that upon curing HEMA will form a linear polymer. Free radicals are required for polymerization to occur. In dental adhesives these radicals are generated via the decomposition of an initiator, e.g. camphoroquinone and benzoylperoxide. The initiator can be either photo or redox activated. Once a free radical has reacted with a HEMA monomer, that HEMA monomer will contain a reactive carbon atom, which in turn can react with another free HEMA monomer. In this way the polymerization of monomers will progress. In theory the polymerization terminates when all the free radicals have reacted. However, polymerization is never complete, some free monomers always remain.
**INTRODUCTION**

**HEMA Leakage and Leukocytes in the Oral Cavity**

In the oral cavity there are different areas where cells of the immune system are present. Antigen-presenting, dendritic cells called Langerhan’s cells reside in the oral epithelium. Previous studies have shown that neutrophils represent 95% - 97%, lymphocytes 1% - 2% and mononuclear cells 2% - 3% of the leukocyte population present in a normal gingival crevice. Further, there are less T cells than B cells present in the gingival crevice. Gingival crevicular fluid (GCF) flows into the gingival crevice through the junctional epithelium. GCF brings neutrophils, monocytes, B cells and T cells into the oral cavity. The superficial layers of the oral epithelium and the base of the gingival crevice are primarily populated by neutrophils, while macrophages and lymphocytes predominately reside in the basal layers of the junctional epithelium. In the dental pulp dendritic cells, T cells and macrophages can be found.

Previous studies have shown that leakage of unpolymerized HEMA monomers occurs after curing of resin-based materials. Further, HEMA monomers can penetrate through dentin. In addition HEMA can bind to proteins. Free HEMA monomers leaking from a cured filling could thus bind to proteins and the HEMA/protein conjugate could then come into contact with neutrophils, monocytes, macrophages, B cells and T cells. Contacts like these could affect the physiological functions of leukocytes belonging to both the innate branch (neutrophils, monocytes and macrophages) and the adaptive branch (B cells and T cells) of the immune system.

**Innate Immunity: Neutrophils and Monocytes**

Innate immunity represents the first line of defense against foreign invaders that have managed to penetrate the skin barrier. Innate immunity relies on the recognition of pathogen-associated molecular patterns (PAMPS), e.g. lipopolysaccharide (LPS), viral ribonucleic acid, zymosan, peptidoglycan and oxidized lipoproteins. The presence of PAMPS is sensed by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), the mannose receptor, RIG-I helicase (senses double-stranded ribonucleic acid), NOD1 and NOD2 receptors (senses peptidoglycan) and scavenger receptors. Microorganisms can also be recognized indirectly, by way of opsonins like IgG and complement fragments.

Monocytes/macrophages and granulocytes, of which the neutrophil is the most numerous representative, contribute to the innate army against infection.
IMMUNOMODULATORY PROPERTIES OF 2-HYDROXYETHYL METHACRYLATE

**Monocytes**

The monocyte is 12 µm - 20 µm in diameter and has a U-shaped nucleus. In a human adult monocytes represent 5% - 6% of the circulating leukocytes. Circulating monocytes have a half-life of about 3 days. Human monocytes are divided into subsets based on the expression of CD14, an LPS receptor, and CD16, the FcyRIII receptor. The majority (80% - 90%) of circulating monocytes are characterized by a high expression of CD14 and a lack of CD16 expression, so-called CD14^{high}CD16^{neg} monocytes. Another class of monocytes is the CD14^{low}CD16^{high} monocytes, which represent about 10% of the circulating monocyte population. CD14^{low}CD16^{high} monocytes are referred to as proinflammatory monocytes, due to an increase in cell number during acute infections and to the production of TNF-α following LPS stimulation. Furthermore, the two monocyte subsets differ in their expression of the Major Histocompatibility Complex (MHC) class II protein, CD14^{low}CD16^{high} monocytes having the higher MHC class II expression. Monocytes, not being fully differentiated upon leaving the bone marrow, can differentiate further into for instance alveolar dendritic cells, splenic dendritic cells, Langerhan's cells, lung macrophages, lymph node macrophages and splenic macrophages.

**Neutrophils and Their Granules**

The neutrophil is 12 µm - 15 µm in diameter. In a human adult neutrophils represent about 60% of the circulating leukocytes. Neutrophils have a short half-life, ranging from 6 hrs to 8 hrs, with 5-10x10^{10} neutrophils being released from the bone marrow every day. Neutrophils are referred to as polymorphonuclear granulocytes, due to their multi-lobed nucleus and the presence of several granules in their cytoplasm. There are three types of cytoplasmic granules: azurophilic granules, specific granules and gelatinase granules. In addition there are secretory vesicles, which are a source of neutrophil cell membrane receptors, e.g. CD14, CD16 and receptors for formylated bacterial peptides. The cytoplasmic granules are packed with anti-microbial proteins and proteases. Specific granules contain lactoferrin, cathelicidins and neutrophil lysozyme. Furthermore, specific granules release matrix metalloproteinases, which degrade the extracellular matrix, thereby clearing the path for incoming neutrophils. Gelatinase granules, like specific granules, contain matrix metalloproteinases and lysozyme. The azurophilic granules contribute to the arsenal of anti-microbials with myeloperoxidase (MPO), α-defensins, LPS-binding bactericidal permeability increasing protein (BPI), azurocidin and the serine proteases cathepsin G, neutrophil elastase (NE) and protease 3. The anti-microbial effects of the granule proteins involve sequestering nutrients essential to microbes, e.g. the iron binding capacity of lactoferrin, and permeabilizing microbial cell membranes, e.g. α-defensins, lysozyme, cathelicidins and BPI. Granule proteins are also involved in the formation of neutrophil extracellular traps.
Granule proteins have other functions besides acting as anti-microbials. For instance, the cathelicidins LL-37 and azurocidin promotes chemotaxis of monocytes and T cells.

Phagocytosis and The Respiratory Burst: The Radical Armor of Monocytes and Neutrophils

Neutrophils, monocytes and macrophages are capable of performing a process called phagocytosis, *i.e.* they are phagocytes (Fig. 1). Phagocytosis ensures the removal of harmful pathogens as well as apoptotic cells. Neutrophils, monocytes and macrophages have a variety of PRRs, which upon interaction with their respective PAMPs promote phagocytosis.

Following recognition of a PAMP, the plasma membrane of the phagocyte invaginates and eventually pinches off, trapping the pathogen/apoptotic host cell together with extracellular fluid inside a phagosome. The phagosome subsequently fuses with early endosomes, late endosomes and finally lysosomes, thus creating a phagolysosome. During the phagosome maturation process the environment for the trapped pathogen becomes increasingly acidic due to the presence of V-ATPases, which pumps protons into the phagosome lumen. The acidic pH hampers microbial metabolism and increases the activity of host hydrolytic enzymes. Furthermore, azurophilic and
specific granules fuse with the phagolysosome, releasing their anti-microbial proteins.

Contributing to the armor of the phagocyte is a multisubunit NADPH oxidase. When phagocytosis is initiated the NADPH oxidase subunits (gp91phox, p22phox, p40phox, p47phox, p67phox, Rac2) assemble at the emerging phagosome membrane. Once assembled, the NADPH oxidase oxidizes cytosolic NADPH, transferring two electrons from NADPH to two oxygen (O₂) molecules inside the phagolysosome, resulting in the generation of two superoxide (O₂⁻) ions. The consumption of O₂ for the production of O₂⁻ is referred to as the respiratory burst (Fig. 2). An efflux of chloride anions and a simultaneous rise in intracellular calcium levels, which occurs upon recognition of a pathogen, stimulate the respiratory burst.

O₂⁻ belongs to a group of molecules known as reactive oxygen species (ROS). Once formed by the action of NADPH oxidase, O₂⁻ can subsequently be converted to hydrogen peroxide (H₂O₂) by the action of superoxide dismutase. Neutrophil MPO potentiates the anti-microbial effect of the respiratory burst by converting H₂O₂ into hypochlorous acid, hypobromous acid or hypoiodous acid, all of which belong to the ROS family. H₂O₂ can also react with metal ions to generate highly oxidizing hydroxyl radicals. ROS released into the phagolysosome damages microbial proteins, lipids and nucleic acids.

Figure 2. The neutrophil respiratory burst is triggered by complement and Fc receptors. Figure originally published in Clinical Science. Quinn MT et al. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clin Sci. 2006; 111: 1-20. © The Biochemical Society.
INTRODUCTION

*Effects of HEMA on the Life of Monocytes, Macrophages and Neutrophils*

*In vitro* studies have revealed several effects of HEMA on monocytes/macrophages.

**Monocyte/Macrophage activities suppressed by HEMA:**

- The respiratory burst
- IL-1β and TNF-α production
- Cell proliferation
- Heat shock protein 72 expression
- Levels of the radical scavenger glutathione protein

**Monocyte/Macrophage activities promoted by HEMA:**

- Vascular endothelial growth factor expression
- Recognition of carbohydrate ligands
- Cyclooxygenase-2 expression

Further, HEMA induces DNA fragmentation in monocytes and macrophages.

*In vitro* studies on the effect of HEMA exposure on neutrophils remain to be reported.

**Adaptive Immunity: B Lymphocytes and Antibodies**

Activated B lymphocytes (plasma cells) have a diameter of up to 20 μm. B lymphocytes represent 10% - 15% of the lymphocytes found in blood and 40% - 45% of the spleen lymphocyte population. Activated B cells produce antibodies that confer antigen specificity to the immune system.

**B Cell Subsets**

B cells can be divided into B1 B cells and B2 B cells. Murine B1 B cells develop from hematopoietic stem cells in the fetal liver. B1 B cells spontaneously secrete IgM directed at polysaccharides, without an apparent immunization. They represent the first line of defense against blood-borne encapsulated bacteria.

The majority of human and murine B cells are B2 B cells. B2 B cells are derived from hematopoietic stem cells in the bone marrow. In the spleen B2 B cells mature into either marginal zone B cells (B_M) or follicular B cells (B_F) (Fig. 3). B_M reside at the marginal sinus in the murine spleen and like B1 B cells, respond rapidly to multivalent, blood-borne antigens, such as polysaccharides. B_M can also respond to glycolipids due to expression of the glycolipid receptor CD1. Following primary immunization B_M cells rapidly develop into plasmablasts, secreting large amounts of antigen specific IgM.
antibodies. B cells are referred to as recirculating B cells due to their migratory behavior, traveling via blood and lymph to lymph nodes, Peyer's patches and the spleen (Fig 3). In the lymph node, B cells respond to protein antigens with a T helper cell dependent antibody production. However B cells can also recirculate through the bone marrow where they can respond to antigen in a T cell independent manner.

**B Cell Activation and Maturation**

In the lymph node antigen peptides can be presented on the surface of macrophages, dendritic cells and B cells by MHC class I or MHC class II molecules. Antigens can also be displayed in the form of immune complexes, bound to either complement receptor 2 or Fcγ receptors on the surface of follicular dendritic cells (FDC). Furthermore, B cells can acquire soluble antigens that diffuse into the B cell follicle from the subcapsular sinuses (Fig 3).


B cells recognize antigens by way of the membrane-bound B cell receptor (BCR). The antigen specificity of the BCR is determined by gene recombinations that occur in the bone marrow during B cell development. The extensive rearrangements of variable (V), diversity-, joining (J) gene segments (heavy chain locus) and V- and J gene segments (light chain locus) that occur in the bone marrow are the basis for creating a large variety of BCR specificities and hence B cell clones.

Following antigen internalization, B cells migrate to the T cell zone, known as the paracortex in the lymph node and the periarteriolar lymphoid sheath (PALS) in the
spleen, where they are activated by interactions with T helper cells. Activated B cells migrate into primary follicles where intense proliferation ensues, forming secondary follicles and eventually germinal centers. It has been estimated that it takes about 12 hrs for a germinal center B cell to duplicate itself. In the germinal center somatic hypermutation (primarily single nucleotide exchanges) of immunoglobulin V region genes, affinity maturation and class switch recombination (CSR) take place. Finally the B cells exit the lymphoid organ as either memory B cells or antibody-producing blast cells. Memory B cells, characterized by high-affinity antigen receptors, quickly initiate the production of large amounts of antibodies upon reexposure to an antigen. Long-lived plasma cells residing in the bone marrow maintains a high titer of antigen-specific antibodies following an initial antigen exposure. Plasma cells represent 0.1% - 1% of the cells present in human bone marrow. The longevity of plasma cells in the bone marrow depends on several factors. For instance IL-6 produced by bone marrow stromal cells enhances plasma cell survival. Plasma cell survival also depends on engagement of the chemokine receptor CXCR4 by its ligand CXCL12.

**Antibodies: Classes, Structure and Functions**

Antibodies (immunoglobulins) can be divided into different classes and subclasses based on the amino acid sequence of the heavy chain constant (C) region. Humans have several classes of soluble immunoglobulins, some of which can be further divided into subclasses. These are IgA1, IgA2, IgE, IgM, IgG1, IgG2, IgG3 and IgG4. In mice, IgG are divided into the subclasses IgG1, IgG2a, IgG2b and IgG3. Like humans, mice also produce soluble IgA, IgE and IgM immunoglobulins. IgD is mainly a membrane bound immunoglobulin expressed by naïve B cells. However IgD secreting plasma cells exist, for instance in the tonsils and the nasal mucosa.

![Figure 4. Immunoglobulin architecture.](https://dreamstime.com)
Antibodies consist of two identical heavy chains and two identical light chains (kappa or lambda) giving them a Y-shaped morphology (Fig. 4) \(^{127, 131, 132}\). Heavy and light chains are linked by disulfide bonds, as are the two heavy chains. Each chain has one or more C regions and one V region \(^{133-135}\). The C- and V regions are composed of varying numbers of Ig domains. Heavy chain C regions are responsible for the biological functions of the antibody \(^{136}\). The antigen-binding site consists of six hypervariable loops, the V regions of heavy and light chains contributing with three loops each \(^{137, 138}\). The antigen-binding site is complementary to three-dimensional structures (i.e. epitopes) of a specific antigen, which is why the hypervariable loops are referred to as complementarity determining regions 1, 2 and 3 \(^{107}\). A hinge region allows for the recognition of widely spaced epitopes. The antibody repertoire of an individual may comprise more than \(10^9\), and theoretically at least \(10^{15}\), different antigen specificities.

Antibodies are involved in several immunological responses against antigens, e.g. neutralization of toxins (IgG and IgA antibodies), degranulation of Mast cells (IgE antibodies), complement-dependent cytotoxicity (IgG and IgM antibodies), opsonization and antibody-dependent-cellular cytotoxicity by way of Fc receptors (IgG antibodies) \(^{127, 139-141}\). Furthermore, one class of antibody can regulate the effector functions of another class. For instance, serum IgA antibodies can down-regulate IgG mediated phagocytosis and respiratory burst in neutrophils \(^{142, 143}\).

**Antigens: Immunogens and Haptens**

B cell antibody production can be initiated by various antigens, e.g. proteins, lipids, LPS and viruses \(^{144-146}\). These antigens are all immunogens, i.e. they can stimulate an immune response. Small chemicals like methacrylates, referred to as haptens, are only immunogenic when associated with a carrier protein \(^{147}\). It has been shown that B cell IgG and IgE anti-hapten antibody production requires the presence of T helper cells \(^{148, 149}\). Further, Palm et al. demonstrated that immunizing mice with the hapten dinitrophenyl coupled to human serum albumin (HSA), resulted in high titers of IgG1 anti-dinitrophenyl antibodies but low titers of IgG1 anti-HSA antibodies \(^{150}\). This was in contrast to the antibody response in animals immunized with HSA together with LPS, where the titer of IgG1 anti-HSA antibodies was high. The authors suggest that in contrast to PRR stimulating adjuvants (e.g. LPS) haptens primarily stimulate an antibody response that is hapten specific, as opposed to specific for the carrier protein (e.g. HSA). In addition it has been shown that hapten density affects the diversity of anti-hapten antibody specificities \(^{151}\).
Effects of HEMA Exposure on Antibody Production

Previous studies performed in mice have revealed various effects of HEMA exposure on antibody production:

- Mice injected with HEMA-conjugated mouse serum albumin (MSA) have an elevated IgG anti-MSA antibody activity in blood compared to mice injected with native MSA. That is HEMA stimulates the production of auto-antibodies [40].

- The number of HEMA monomers conjugated to MSA affects the level of IgG and IgE anti-MSA autoantibody activity and the total amount of serum IgE immunoglobulin [152].

- Injecting mice with HEMA-conjugated MSA leads to the production of anti-HEMA antibodies [152].

- A bicarbonate solution containing HEMA acts as an adjuvant in mice and promotes antibody production [155].
Aims

The main objective of the present studies was to investigate effects of HEMA exposure on the innate and adaptive immune system.

The specific aims were as follows:

- To examine whether HEMA affects the phagocytic and respiratory burst activity of human peripheral blood phagocytes \textit{in vitro}.

- To examine if HEMA affects the immunoglobulin production of human B cells \textit{in vitro}.

- To study effects of long-term HEMA exposure \textit{in vivo} on the general health and the immune system of mice.

- To investigate further the specific effects of HEMA exposure \textit{in vivo} on antibody and cytokine production.
Materials & Methods

2-hydroxyethyl methacrylate (HEMA)

Before HEMA (Sigma-Aldrich, Stockholm, Sweden) was used in the studies it was passed through a column of polystyrene-co-divinylbenzene beads in order to remove the inhibitor monomethyl-ether-hydroquinone.

Effects of HEMA on the Phagocytic and Respiratory Burst Activity of Granulocytes and Monocytes (I)

Study Subjects and Sampling

Following informed consent, venous blood was collected from five, healthy female volunteers, aged 50-58 yrs.

HEMA Exposure in vitro

Blood cells were exposed to 7.5 mmol L\(^{-1}\) and 15 mmol L\(^{-1}\) HEMA, using 0.85\% NaCl as a diluent, for 2 hrs at room temperature. Blood samples left unexposed to HEMA served as controls. Cell viability following HEMA exposure was verified by trypan blue staining.

Flow cytometric Assay of Phagocyte Immune response in Activated whole blood (FAPIA)

To determine the phagocytic activity, HEMA exposed blood cells were incubated with fluorescein (FITC)-conjugated, opsonized *Escherichia coli* (*E. coli*) (1x10\(^7\) bacteria) (PHAGOTEST®, Orpegen Pharma, Heidelberg, Germany). Unconjugated, opsonized *E. coli* (1x10\(^7\) bacteria) (PHAGOBURST®, Orpegen Pharma) and dihydrorhodamine (DHR) 123 were used to determine the respiratory burst activity of HEMA exposed blood cells. DHR 123 is oxidized to fluorescent rhodamine (R) 123 by the action of ROS\(^{154, 155}\). Blood cells incubated with PBS served as matched controls. Prior to flow cytometric acquisition erythrocytes were lysed by incubating the blood samples (room temperature, dark) in a hypotonic solution (FACS lysing solution, BD Biosciences, San José, CA, USA). Erythrocytes were then discarded and remaining blood cells resuspended in sheath fluid (FACSFlow, BD Biosciences) for subsequent flow cytometric analysis.

For each blood sample 50 000 events were collected using a flow cytometer (FACSCalibur®, BD Biosciences) equipped with a 488 nm laser. Each collected event corresponds to a photon of light emitted from one fluoroscently labeled cell, as a result of being struck by a laser beam. Fluorescence emissions of FITC-*E. coli* and R 123 were detected at 530 nm. Granulocytes and monocytes were identified by their
characteristic forward and side scatter profiles (Fig. 5). Data, collected as the number of granulocytes and monocytes capable of performing phagocytosis and respiratory burst (% gated cells) and the phagocytic and respiratory burst activity (MFI), was analyzed with CellQuest Pro software (BD Biosciences).

Figure 5. A schematic dot plot of human whole blood. Each dot represents one cell detected by a flow cytometer. Illustrated are the granulocytes (green circles), the monocytes (grey circles) and the lymphocytes (black circles). FSC = Forward scatter and SSC = Side scatter.

Cell Membrane Integrity - Propidium Iodide Staining

Propidium iodide (PI), being positively charged, does not enter intact cells. Any disruption of cell membrane integrity leads to uptake of PI, which binds to nucleic acids in the cell. When excited by a laser beam, PI emits red fluorescence.

The effect of HEMA exposure on cell membrane integrity was analyzed by incubating HEMA exposed/unexposed blood cells with PI (Phagotest DNA staining solution, Orpegen Pharma). PI fluorescence emission was subsequently detected at 585 nm with a flow cytometer.

Effect of HEMA on Human B Cell Immunoglobulin Production (II)

Purification of Human CD19+ B Cells from Blood

Blood cells from eight healthy blood donors were obtained from Sahlgrenska university hospital in Gothenburg, Sweden. Only blood cells that were donated on the day of the experiment were used in the study. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Ficoll-Paque Plus, GE Healthcare)
Biosciences AB, Uppsala, Sweden). CD19+ B cells from each donor were then purified using magnetic beads coated with anti-human CD19 antibodies. CD19+ B cells from each donor were resuspended separately in enriched growth medium, i.e. D-MEM Glutamax-I (GIBCO®) supplemented with 10% heat inactivated fetal bovine serum, gentamycin (50 µg mL⁻¹), penicillin (100 Units mL⁻¹) and streptomycin (100 µg mL⁻¹).

**Pokeweed Stimulation and HEMA Exposure in vitro**

B cells were seeded in triplicate in 96-well plates at a concentration of 10⁵ cells well⁻¹. B cells were incubated (6 days, 37°C, humidified atmosphere, 5% CO₂) with 20 µg mL⁻¹ pokeweed mitogen (Lectin from *Phytolacca americana*) and various concentrations of HEMA (0 µmol L⁻¹, 15 µmol L⁻¹, 37.5 µmol L⁻¹, 75 µmol L⁻¹, 150 µmol L⁻¹, 750 µmol L⁻¹). Pokeweed is a lectin that stimulates B lymphocyte proliferation.¹⁵⁸

On the sixth day the plates were frozen.

**B Cell Proliferation in vitro – Measuring β-rays emitted from [³H]thymidine**

For proliferation studies [³H]thymidine was added on the fifth culture day to some of the plates (1 µCurie well⁻¹). Following subsequent freezing and thawing cell lysates were harvested (Harvester 96, Tomtec) onto glass fiber filters on top of which a melt-on scintillator (Meltilex® A, Wallac Oy) was applied. Incorporated [³H]thymidine was then counted (Microbeta® Trilux, PerkinElmer Sweden AB).

**Human IgA, IgG1 and IgM Production in vitro - ELISA**

After thawing the B cell cultures, the individual B cell supernatants were analyzed for the presence of IgA, IgG1 and IgM with an ELISA. MaxiSorp™ plates (Nunc A/S, Roskilde, Danmark) were treated as follows:

**IgA**
- Coated (overnight, 4 °C) with goat anti-human IgA antibodies (Mabtech AB, Nacka Strand, Sweden), diluted 1:500 in PBS, pH 7.4
- Washed 2 times with PBS
- Blocked (1 hr, room temperature) with 0.1% BSA/PBS-Tween (0.05%)
- Washed 5 times with PBS-Tween (0.05%)
- Incubated (2 hrs, room temperature) with standard (human IgA, Mabtech AB) and B cell supernatants
- Washed 5 times with PBS-Tween (0.05%)
- Incubated (1 hr, room temperature) with goat anti-human IgA antibodies conjugated to ALP (Mabtech AB), diluted 1:1000 in 0.1% BSA/PBS-Tween (0.05%)
- Washed 5 times with PBS-Tween (0.05%)
- Developed in the dark with p-Nitrophenyl-phosphate dissolved in diethanolamine buffer, pH 9.8.
Absorbance was read at 405 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices, Sunnyvale, CA, USA)

**IgG1 and IgM**

- Coated (overnight, room temperature) with mouse anti-human IgG1 antibodies (clone 8c/6-39, Sigma-Aldrich AB) diluted 1:1000 in PBS, pH 7.2 or goat anti-human IgM antibodies (Chemicon International Inc., Temecula, CA, USA) diluted 1:500 in PBS, pH 7.2
- Blocked (1 hr, room temperature) with 1% BSA/PBS
- Incubated (2 hrs, room temperature) with standard (Human IgG1 (The Binding Site Limited, Birmingham, UK) or Beadlyte® Human IgM (Upstate, Lake Placid, NY, USA)) and B cell supernatants
- Incubated (2 hrs, room temperature) with HRP conjugated antibodies, either sheep anti-human IgG (The Binding Site Limited), diluted 1:1000 in 1% BSA/PBS, or goat anti-human IgM (Chemicon International Inc.), diluted 1:5000 in 1% BSA/PBS
- Developed (in the dark) with tetramethylbenzidine dissolved in 0.05 mol L⁻¹ citrate-phosphate buffer, pH 5.0 supplemented with dimethyl sulfoxide and 30% H₂O₂
- The reaction was stopped with 1 mol L⁻¹ H₂SO₄
- Absorbance was read at 450 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices), subtracting with the background absorbance read at 540 nm
- Between each incubation the plates were washed 3 times in PBS-Tween (0.05%)

**Effects of HEMA Exposure in vivo (III, IV)**

**Animal Husbandry**

Male Balb/c mice (Charles River Laboratories, Sulzfeld, Germany) were housed under specific-pathogen-free conditions in individually ventilated cages at the Laboratory for Experimental Biomedicine at the University of Gothenburg. Food and water were provided ad libitum. Experimental protocols were approved by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden (# 380-2008).

**HEMA Exposure in vivo - Osmotic Pumps (III)**

Alzet ® miniature osmotic pumps (model 2006, Alzet, Cupertino, CA, USA) were loaded with either 0.9% filter-sterilized NaCl, 8.2 mol L⁻¹ HEMA or 183 μmol L⁻¹ HEMA and implanted subcutaneously at the lower back, under aseptic conditions (Fig. 6). Animals were sacrificed 40 days after pump implantation by terminal bleeding. Serum and spleens were collected.

**OVA Exposure in vivo – Subcutaneous Injections (III)**

Animals were immunized 19 days after pump implantation with ovalbumin (OVA) (Sigma-Aldrich AB) dissolved in 100 mmol L⁻¹ bicarbonate buffer. The injection consisted of 50 μg OVA in 50 μl buffer. An identical injection was given as a booster on day 34 post implantation.
Groups of mice were injected subcutaneously in the tail (50 µl/animal) with HEMA (20 µmol/animal) dissolved in bicarbonate buffer or bicarbonate buffer alone. After 3 weeks the animals received a second injection. Animals were sacrificed 6 days later, in order to study the short-term effects of HEMA exposure in vivo on cytokine production.

Protocol 2

Groups of mice were injected subcutaneously in the tail (50 µl/animal) with HEMA (20 µmol/animal) and OVA (50 µg/animal) in bicarbonate buffer, OVA (50 µg/animal) in bicarbonate buffer or bicarbonate buffer alone. After 3 weeks the animals received a booster injection. Animals were sacrificed 2 weeks later in order to study the effects of HEMA exposure in vivo on antibody production.

Splenocyte Isolation (III, IV)

Spleens were squeezed through a cell strainer with a pore size of 70 µm (BD Falcon, Bedford, MA, USA). Splenocytes were then isolated by density centrifugation, washed two times in PBS supplemented with penicillin (100 Units mL⁻¹) and streptomycin (100 µg mL⁻¹) and then resuspended in D-MEM Glutamax-I supplemented with 5% heat inactivated fetal bovine serum, gentamycin (50 µg mL⁻¹), penicillin (100 Units mL⁻¹) and streptomycin (100 µg mL⁻¹).
Mouse splenocytes were cultured for 2 days (37°C, humidified atmosphere, 5% CO₂). Supernatants were subsequently prepared by freezing and thawing. Supernatants were analyzed for the presence of TNF-α (DuoSet® # DY410, R&D Systems, Abingdon, UK), IL-2 (DuoSet® # DY402, R&D Systems) and IL-6 (DuoSet® # DY402, R&D Systems) by a sandwich ELISA. MaxiSorp™ plates (Nunc A/S) were treated as follows:

- Coated (overnight, room temperature) with goat anti-mouse TNF-α, rat anti-mouse IL-2 or rat anti-mouse IL-6 diluted 1:180 in PBS, pH 7.2
- Blocked (1 hr, room temperature) with 1% BSA/PBS
- Incubated (2 hrs, room temperature) with appropriate, recombinant standards and splenocyte supernatants
- Incubated (2 hrs, room temperature) with appropriate, biotinylated antibodies (goat anti-mouse TNF-α, goat anti-mouse IL-2, goat anti-mouse IL-6), diluted 1:180 in 0.1% BSA/PBS-Tween (0.05%), pH 7.2 (IL-2) or 1% BSA/PBS, pH 7.2 (TNF-α, IL-6)
- Incubated (20 min, room temperature, dark) with streptavidin-HRP
- Developed (20 min, room temperature, dark) with Tetramethylbenzidine dissolved in 0.05 mol L⁻¹ citrate-phosphate buffer, pH 5.0, supplemented with dimethyl sulfoxide and 30% H₂O₂
- The reaction was stopped with 1 mol L⁻¹ H₂SO₄
- Absorbance was read at 450 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices), subtracting the background absorbance read at 540 nm
- Between each incubation the plates were washed 3 times in PBS-Tween (0.05%)

**Detoxification of OVA using Polymixin B (III)**

Prior to initiating OVA stimulation experiments in vitro (see below), LPS was removed from the OVA solution by passing it twice through a column of polymixin B (DetoxiGel™AffinityPak™, Pierce, Rockford, IL, USA), according to the manufacturer's instructions. Polymixin B binds to the lipid A portion of LPS.

The LPS content of the OVA solution used for in vitro experiments was 28 endotoxin units mL⁻¹, as determined by the Limulus amebocyte lysate assay carried out at the Department of Clinical Bacteriology, the Sahlgrenska Academy. The Limulus amebocyte lysate assay relies on the activating effect of LPS on proteases participating in the coagulation cascade of the American horseshoe crab Limulus polyphemus. In the Limulus amebocyte lysate assay any LPS present in a sample activates the Limulus polyphemus proteases. The activated enzymes then react with a color substrate. Next the absorbance of the color substrate is read with a spectrophotometer.
**Con A and OVA Stimulation in vitro – Splenocyte Proliferation (III, IV)**

Concanavalin A (Con A) is a lectin that stimulates T lymphocyte proliferation by binding to carbohydrates on CD3 in the CD3/T cell receptor complex.\(^{161}\)

Splenocytes were seeded (5x10^5 cells per well) in triplicate (Nunclon Delta Surface F96 well, Nunc A/S) and then stimulated, either 4 days with 40 µg detoxified OVA (III) or 3 days with 1 µg Con A (Pharmacia AB, Uppsala, Sweden) (III, IV), at 37°C, in humidified atmosphere containing 5% CO₂. Unstimulated cells were cultured in parallel. Proliferation was analyzed by measuring [³H]thymidine incorporation (see B Cell Proliferation in vitro – Measuring β-rays emitted from [³H]thymidine).

**IgA, IgG and IgM anti-OVA Antibody Activity in Serum – ELISA (III, IV)**

Serum was analyzed for the presence of IgA, IgG and IgM anti-OVA antibody activity with an ELISA. MaxiSorp™ plates (Nunc A/S) were treated as follows:

- Coated (overnight, 4 ºC) with OVA (0.5 µg well⁻¹) dissolved in PBS, pH 7.2
- Washed 3 times with PBS-Tween (0.05%)
- Incubated (overnight, 4 ºC) with serum samples and standard (pool of serum from all animals in the study)
- Washed 3 times with PBS-Tween (0.05%)
- Incubated (2 hrs, room temperature) with biotin-conjugated antibodies, either goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:100 000 in PBS-Tween (0.05%), goat anti-mouse IgM (Southern Biotech, Birmingham, AL, USA) diluted 1:20000 in PBS-Tween (0.05%) or goat anti-mouse IgA (Southern Biotech) diluted 1:20000 in PBS-Tween (0.05%)
- Washed 4 times with PBS-Tween (0.05%)
- Incubated (2 hrs, room temperature, dark) with ExtrAvidin-ALP (Sigma-Aldrich AB)
- Washed 4 times with PBS-Tween (0.05%)
- Developed (in the dark) with p-Nitrophenyl-phosphate (Sigma-Aldrich AB) dissolved in diethanolamine buffer, pH 9.8
- Absorbance was read at 405 nm on a spectrophotometer (Spectra MAX 340, Molecular Devices)

**Statistical Analysis**

Data was analyzed statistically as follows:

- ANOVA and Tukey’s post hoc test (I)
- Related samples Wilcoxon’s signed ranks test (II)
- ANOVA together with either Tamhane’s or Bonferroni’s post hoc test, Independent samples t test (III)
- Independent samples t test (IV)

A p-value < 0.05 was considered to be statistically significant.
Results

Exposing Granulocytes and Monocytes to HEMA in vitro - Consequences for Bactericidal Functions (I)

HEMA has previously been shown to induce apoptosis by stimulating the production of ROS\(^{162}\). ROS production is important for the bactericidal function of phagocytes like neutrophils, also known as polymorphonuclear granulocytes, and monocytes/macrophages\(^{94,163}\). Neutrophils, monocytes and macrophages are present in the oral cavity where they can come into contact with unpolymerized HEMA monomers\(^{30,34}\). Our aim with study I was to examine the effect of HEMA exposure on the phagocytic and respiratory burst activity of neutrophils and monocytes. To achieve this we exposed human blood cells to 7.5 mmol L\(^{-1}\) and 15 mmol L\(^{-1}\) HEMA in vitro for 2 hrs. The HEMA concentrations that we used were chosen based on a previous study where Samuelsen et al. analyzed the effect of HEMA exposure on ROS production in rat submandibular salivary gland acinar cells\(^{162}\). Samuelsen et al. showed that 7.5 mmol L\(^{-1}\) and 15 mmol L\(^{-1}\) HEMA stimulates ROS production.

After incubating the blood cells with HEMA we examined the phagocytic and respiratory burst activity using flow cytometry, with \textit{E. coli} as bait.

\textbf{Phagocytic Activity}

The phagocytic activity of granulocytes and monocytes was detected by measuring the fluorescence of ingested FITC-conjugated \textit{E. coli}. We could not demonstrate any HEMA dependent, significant differences in the number of granulocytes or monocytes capable of performing phagocytosis. Nor were there any HEMA dependent, significant differences in granulocyte or monocyte phagocytic efficiency.

\textbf{Respiratory Burst Activity}

The respiratory burst activity was detected by measuring the conversion of DHR 123 to fluorescent R 123, occurring as a result of \textit{E. coli} phagocytosis. The respiratory burst activity was significantly reduced in granulocytes exposed \textit{in vitro} to 7.5 mmol L\(^{-1}\) and 15 mmol L\(^{-1}\) HEMA (MFI \pm standard deviation = 1093 \pm 262, \(p = 0.000\)) compared to unexposed granulocytes (MFI \pm standard deviation = 3338 \pm 1393) (Fig. 7). However, HEMA had no significant effect on the respiratory burst activity of monocytes. Furthermore, there were no HEMA dependent, significant effects on the number of granulocytes or monocytes capable of performing a respiratory burst.
Cell Membrane Integrity

In order to examine if the different effects of HEMA on granulocyte and monocyte respiratory burst activity was due to differences in susceptibility to HEMA cytotoxicity, we also examined granulocyte and monocyte membrane permeability by measuring PI fluorescence. We could however not demonstrate any HEMA dependent changes in granulocyte or monocyte membrane permeability.

Figure 7. Effect of HEMA on the respiratory burst activity of granulocytes and monocytes. Human whole blood was exposed in vitro to different concentrations of HEMA for 2 hrs. The production of hydrogen peroxide was assessed using DHR 123 as a substrate. The fluorescence of R 123 was detected with a flow cytometer. Data is presented as mean MFI ± standard deviation. n = 5 blood donors (0 mmol L⁻¹ HEMA, 7.5 mmol L⁻¹ HEMA) and n = 4 blood donors (15 mmol L⁻¹ HEMA). *** p< 0.001, ANOVA and Tukey’s post hoc test.

Exposing Human B cells to HEMA in vitro – Consequences for Immunoglobulin Production and B Cell Proliferation (II)

HEMA has previously been shown to affect the activity of mouse B cells. For instance, HEMA exposure in vivo leads to autoantibody production as well as an increased production of IgE antibodies in mice. The aim of study II was to examine if HEMA can affect the activity of human B cells as well. Nespoli et al. have shown that stimulating peripheral blood B cells with pokeweed in vitro enables the detection of IgG and IgM immunoglobulins in B cell culture supernatants. Further Agger et al. have reported that the maximum number of IgG+, IgM+ and IgA+ B cells is reached after 6 days of culture in the presence of pokeweed. In addition we were unable to detect IgG1, IgM and IgA immunoglobulins in preliminary experiments where B cells were cultured in the absence of pokeweed. To study the effects of HEMA exposure on human B cell activity we therefore chose to stimulate
CD19+ B cells, purified from peripheral blood, with pokeweed for 6 days in vitro, in the presence of different concentrations of HEMA, followed by analysis of the immunoglobulin production.

**IgA, IgG1 and IgM Immunoglobulins**

The presence of IgA, IgG1 and IgM immunoglobulins in the B cell supernatants was analyzed by an ELISA. B cells exposed to 15 µmol L⁻¹ or 37.5 µmol L⁻¹ HEMA produced significantly more IgG1 immunoglobulins in vitro (median for the relative concentration = 122.8%, interquartile range = 80.8%, \( p = 0.01 \) and median for the relative concentration = 155.8 %, interquartile range = 81.7%, \( p = 0.02 \) respectively) compared to unexposed B cells (Fig. 8). B cells exposed to 750 µmol L⁻¹ HEMA on the other hand had a reduced IgG1 immunoglobulin production in vitro (median for the relative concentration = 26.3%, interquartile range = 41.4%, \( p = 0.01 \)), compared to unexposed cells.

B cells exposed to 150 µmol L⁻¹ or 750 µmol L⁻¹ HEMA had a reduced IgM immunoglobulin production in vitro (median for the relative concentration = 71.2%, interquartile range = 32.7%, \( p = 0.02 \) and median for the relative concentration = 30.8%, interquartile range = 16.0%, \( p = 0.01 \) respectively), compared to unexposed cells. Furthermore, in B cell cultures containing 15 µmol L⁻¹, 37.5 µmol L⁻¹ and 75 µmol L⁻¹ HEMA, the sum of the relative concentrations of IgG1 immunoglobulins was significantly higher compared to the sum of the relative concentrations of IgM immunoglobulins (\( p = 0.01 \)). We could not demonstrate any statistically significant changes in the production of IgA immunoglobulin in the cultures containing the different HEMA concentrations, compared to unexposed cultures.

**B Cell Proliferation**

B cell proliferation, detected by measuring incorporated \(^{3}H\)thymidine, was significantly reduced in B cell cultures containing 150 µmol L⁻¹ HEMA and 750 µmol L⁻¹ HEMA (median for the relative proliferation = 74.6%, interquartile range = 6.7%, \( p = 0.02 \) and median for the relative proliferation = 39%, interquartile range = 46.8%, \( p = 0.02 \) respectively). However we could not demonstrate a HEMA dependent, significant difference in B cell proliferation in vitro, in the B cell cultures containing 15 µmol L⁻¹, 37.5 µmol L⁻¹ or 75 µmol L⁻¹ HEMA.
IMMUNOMODULATORY PROPERTIES OF 2-HYDROXYETHYL METHACRYLATE

**Figure 8.** Effect of HEMA exposure *in vitro* on B cell IgG1 production. CD19+ B cells were isolated from human blood and cultured for 6 days in the presence of different concentrations of HEMA. The presence of IgG1 immunoglobulin in the culture supernatants was then analyzed by an ELISA. Data is presented as the median and the interquartile range for the relative concentration of IgG1. n = 8 blood donors. *p < 0.05, Wilcoxon’s signed-ranks test.

**Long-Term Exposure to HEMA in vivo – Consequences for General Health Status, Splenocyte Proliferation, Splenocyte Cytokine Production and Antibody Activity (III)**

Our aim with study III was to examine how long-term exposure to minute amounts of HEMA affects the general health and the immune system of mice. We strived to mimic the effect of exposure to low levels of HEMA monomers, as occurs when recently cured restorations leak or when a person working in a dental clinic inhales the air. To accomplish this we filled Alzet® miniature osmotic pumps with different concentrations of HEMA monomers and implanted the pumps subcutaneously into the back of mice. Mice receiving pumps filled with 0.9% NaCl served as controls. The osmotic pumps were designed to deliver a dose of 0.15 µl hr⁻¹. In order to study the effects of long-term HEMA exposure *in vivo* on the immune system we immunized the mice with OVA. Spleens and serum were collected 40 days after pump implantation.

**Health Status**

Mice exposed to 161 µg of HEMA hr⁻¹ *in vivo* increased significantly less in body weight throughout the HEMA exposure period (0.000 ≤ p ≤ 0.018), compared to mice exposed to 1.35 µg of NaCl hr⁻¹ (control group). Mice exposed to 3.6 ng of HEMA hr⁻¹ *in vivo* increased significantly less in body weight in the beginning of the HEMA exposure period (0.000 ≤ p ≤ 0.037), compared to the control group.
RESULTS

Splenocyte Proliferation in vitro

The proliferation of splenocytes was measured by detecting incorporated $[^3H]$thymidine with a scintillation counter. Splenocytes from mice exposed to 3.6 ng of HEMA hr$^{-1}$ in vivo had a significantly lower Con A stimulated in vitro proliferation (mean counts per minute ± standard deviation = 2708 ± 2097) than splenocytes from both the control group (mean counts per minute ± standard deviation = 6191 ± 3701, $p = 0.036$) and the mice exposed to 161 µg of HEMA hr$^{-1}$ in vivo (mean counts per minute ± standard deviation = 6852 ± 4123, $p = 0.029$).

Production of IL-2 and TNF-α by Splenocytes in vitro

Splenocytes were cultured for 2 days. The concentration of the cytokines was then measured in splenocyte supernatants by an ELISA. Splenocytes from mice exposed to either 161 µg of HEMA hr$^{-1}$ or 3.6 ng of HEMA hr$^{-1}$ in vivo had a significantly lower IL-2 production in vitro (mean concentration of IL-2 ± standard deviation = 44.9 ± 7.2 pg mL$^{-1}$, $p = 0.019$ and mean concentration of IL-2 ± standard deviation = 33.5 ± 8.9 pg mL$^{-1}$, $p = 0.002$ respectively) compared to splenocytes from the control group (mean concentration of IL-2 ± standard deviation = 66.4 ± 19.8 pg mL$^{-1}$). We were unable to demonstrate a HEMA dependent, significant effect on TNF-α production in vitro.

IgA, IgG and IgM anti-OVA Antibody Activity

The anti-OVA antibody activity in serum was determined with an ELISA. Mice exposed to 3.6 ng of HEMA hr$^{-1}$ had a significantly lower serum IgA anti-OVA antibody activity (mean activity of anti-OVA IgA ± standard deviation = 37564 ± 3766 ELISA units, $p = 0.026$) than the control group (mean activity of anti-OVA IgA ± standard deviation = 50566 ± 13004 ELISA units) (Fig. 9). We could not demonstrate any significant differences in the activity of IgG or IgM anti-OVA antibodies in mice exposed in vivo to either 161 µg of HEMA hr$^{-1}$ or 3.6 ng of HEMA hr$^{-1}$, compared to the control group.
Figure 9. Osmotic pumps filled with different concentrations of HEMA were implanted subcutaneously into the back of mice. Control animals received pumps filled with saline. The pumps were left in situ for 40 days, during which time the animals were injected with ovalbumin (OVA) in bicarbonate buffer on two occasions. Animals were sacrificed 6 days after the last OVA injection and serum was prepared. The anti-OVA IgA antibody activity in the serum was determined with an ELISA. Data is presented as mean ELISA units ± standard deviation. n = 6 animals (161 µg of HEMA hr⁻¹), n = 8 animals (3.6 ng of HEMA hr⁻¹) and n = 8 animals (1.35 µg of NaCl hr⁻¹). * p < 0.05, Independent samples t test.

Subcutaneous HEMA Exposure - Consequences for the Immune System (IV)

Previous studies have demonstrated the inflammatogenic and adjuvant properties of HEMA. For instance mice injected subcutaneously with HEMA dissolved in 100 mmol L⁻¹ bicarbonate buffer once a week for 3 weeks have a reduced splenocyte IL-10 production in vitro. Further the IL-6 production from draining lymph node cells, stimulated in vitro with anti-CD3 antibodies for 3 days, is higher when cells are harvested from mice painted on the ears with HEMA in acetone/olive oil, compared to control mice painted with only acetone/olive oil. Our aim with study IV was to further explore the specificity of the immunomodulatory properties of HEMA in vivo. Therefore we injected mice subcutaneously in the tail with HEMA in bicarbonate buffer or only bicarbonate buffer. The animals were sacrificed 6 days after receiving a second injection and splenocyte proliferation and inflammatory cytokine production in vitro was determined. Other groups received HEMA in bicarbonate buffer or OVA and HEMA in bicarbonate buffer. These animals were sacrificed 2 weeks after receiving a booster injection. The activity of IgG and IgM anti-OVA antibodies in blood was then determined.
**RESULTS**

*Splenocyte Proliferation in vitro*

Baseline splenocyte *in vitro* proliferation was higher for mice exposed two times to HEMA in bicarbonate buffer *in vivo* (mean counts per minute ± standard deviation = 712 ± 413, \( p = 0.001 \)), than for control mice exposed to bicarbonate buffer alone (mean counts per minute ± standard deviation = 285 ± 136).

Mice exposed two times to HEMA in bicarbonate buffer *in vivo* also had a higher Con A stimulated *in vitro* proliferation of splenocytes (mean counts per minute ± standard deviation = 24631 ± 10686, \( p = 0.016 \)), compared to control animals (mean counts per minute ± standard deviation = 12201 ± 9346).

*Production of TNF-α and IL-6 by Splenocytes in vitro*

Splenocytes from animals exposed two times *in vivo* to HEMA in bicarbonate buffer produced less TNF-α *in vitro* (mean concentration of TNF-α ± standard deviation = 233 ± 100 pg mL\(^{-1}\); \( p = 0.043 \)) than splenocytes from animals exposed to bicarbonate buffer alone (mean concentration of TNF-α ± standard deviation = 399 ± 185 pg mL\(^{-1}\)). Furthermore, the splenocyte TNF-α *in vitro* production in relation to the IL-6 *in vitro* production was significantly lower in the mice exposed to HEMA *in vivo* (mean for the ratio [TNF-α]/[IL-6] ± standard deviation = 2.6 ± 0.5, \( p = 0.000 \)), compared to the animals that received only bicarbonate buffer (mean for the ratio [TNF-α]/[IL-6] ± standard deviation = 5.8 ± 1.4).

*IgG and IgM anti-OVA Antibody Activity*

Animals immunized with OVA in the presence of HEMA had an IgG anti-OVA antibody activity in blood that was 5.7 times higher (mean anti-OVA IgG activity ± standard deviation = 17350 ± 10381 ELISA units, \( p = 0.01 \), than the IgG anti-OVA antibody activity in blood from animals immunized with OVA alone (mean anti-OVA IgG activity ± standard deviation = 3042 ± 3041 ELISA units). Furthermore, animals immunized with OVA in the presence of HEMA had a significantly higher IgG anti-OVA antibody activity relative to the corresponding IgM anti-OVA antibody activity (mean for the ratio anti-OVA IgG activity/anti-OVA IgM activity ± standard deviation = 1.7 ± 1.0, \( p = 0.012 \)), compared to animals that received only OVA (mean for the ratio anti-OVA IgG activity/anti-OVA IgM activity ± standard deviation = 0.3 ± 0.3) (Fig. 10).
Figure 10. Mice were injected two times, 3 weeks apart, with HEMA in bicarbonate buffer (HCO₃⁻), alone or together with ovalbumin (OVA). Animals were sacrificed 2 weeks after the last injection. Blood was collected and serum was prepared. The anti-OVA IgG and anti-OVA IgM antibody activity was determined with an ELISA. Data is presented as the anti-OVA IgG antibody activity relative to the anti-OVA IgM antibody activity ± standard deviation. n = 7 animals (HEMA+OVA+HCO₃⁻) and n = 8 animals (OVA+HCO₃⁻).

* $p < 0.05$, Independent samples t test.
General Discussion

In the present thesis the immunomodulatory properties of HEMA were studied. Specific points that were addressed were the effect of HEMA exposure on the bactericidal functions of neutrophils and monocytes, the production of immunoglobulins, the activity of antibodies, the production of splenocyte cytokines and splenocyte proliferation.

We have as yet not been able to determine how HEMA acts on a molecular level in vivo. The proteins, lipids and/or nucleic acids that HEMA interacts with in vivo and how such and interaction can modulate specific physiological responses remains unclear. However based on the immunological responses shown to be susceptible to HEMA exposure, different mechanisms for the immunomodulatory properties of HEMA can be proposed. In the following discussion possible mechanisms behind the effects of HEMA exposure on the immune system will be suggested.

In study I we investigated the effect of HEMA exposure on neutrophil and monocyte phagocytosis and respiratory burst activity. We found that HEMA specifically inhibited the neutrophil respiratory burst activity. The respiratory burst is driven by the multi-subunit NADPH oxidase, an enzyme whose activation depends on the assembly of several subunits at the cell membrane. It has been shown previously that HEMA decreases the formation of phosphatidic acid, a precursor of phospholipids. One possibility for the impaired neutrophil respiratory burst could therefore be that HEMA disrupts the formation of certain phospholipids and hence the integrity of the phospholipid bilayer of the plasma membrane. This could affect the proper assembly of NADPH oxidase subunits at the plasma membrane and hence the optimal function of the NADPH oxidase. To study the effects of HEMA on cell membrane integrity we incubated the blood cells with PI. However we could not demonstrate an effect of HEMA on cell membrane integrity. A cell membrane associated activity that appeared insensitive to HEMA exposure was neutrophil and monocyte phagocytosis. In other words the HEMA dependent decrease in neutrophil respiratory burst that we show in study I was not due to an impaired phagocytic capacity.

Nocca et al. have, by exposing neutrophils and monocytes to the monomers diurethane dimethacrylate and 1,4-butandiol dimethacrylate, shown that the respiratory burst of neutrophils and monocytes differs in terms of sensitivity to methacrylate exposure. It was demonstrated that both diurethane dimethacrylate and 1,4-butandiol dimethacrylate suppressed the respiratory burst of unstimulated neutrophils, however neither diurethane dimethacrylate nor 1,4-butandiol dimethacrylate was able to suppress the respiratory burst of unstimulated monocytes. In our experiments we could demonstrate an effect of HEMA exposure on neutrophil respiratory burst activity but not an effect of HEMA on monocyte respiratory burst.
activity. This suggests that HEMA might be targeting some cellular structure that is expressed by neutrophils but not expressed by monocytes. Another possibility is that monocytes somehow manage to negate the effect of HEMA on the respiratory burst. Phagocytes respond with phagocytosis and a respiratory burst when bacteria are recognized by PRRs, e.g. TLRs, expressed on the cell surface of the phagocytes. Expression of TLRs is higher in monocytes than in neutrophils. As such one possible scenario could be that HEMA binds to TLRs on both neutrophils and monocytes, thereby blocking TLR signaling. However due to the higher degree of TLR expression on the monocyte cell surface some monocyte TLRs remain unoccupied by HEMA and these “free” TLRs enable monocytes to maintain proper TLR signaling and a normal level of respiratory burst activity. However if HEMA does affect neutrophil TLR signaling, then the phagocytic activity of neutrophils would presumably be affected as well. The fact that we were unable to demonstrate an effect of HEMA on the phagocytic activity of neutrophils argues against an effect of HEMA on neutrophil TLR signaling.

In summary, even if the phagocytic capacity is unaffected by HEMA the ability of neutrophils to kill ingested pathogens is impaired.

Phagocytes are not the only cells capable of expressing the NADPH oxidase and performing a respiratory burst. B cells express all the subunits of the NADPH oxidase (gp91phox, p22phox, p40phox, p47phox, p67phox, Rac2). Furthermore ROS is produced by B cells following BCR stimulation, the ROS production of B cells being less than 1/10 of the phagocyte ROS production. Previous studies have shown that the NADPH oxidase is involved in regulating antibody production. For instance Vené et al. demonstrated that blocking the NADPH oxidase activity of LPS stimulated B cells resulted in an impaired IgM production. Further by immunizing mice with sheep red blood cells they showed that H2O2 and peroxynitrite is produced in spleen germinal centers.

HEMA, like ROS, has previously been shown to affect antibody production in mice. We wanted to investigate if HEMA could affect the activity of human B cells as well (study II). To achieve this we purified CD19+ B cells from fresh human blood and exposed them to HEMA, at different concentrations, in the presence of pokeweed
mitogen. We found that HEMA, at certain concentrations, specifically stimulated IgG1 production \textit{in vitro}, while the IgM and IgA production \textit{in vitro} was unaffected by the same HEMA concentrations. Further we showed that the HEMA dependent increase in IgG1 production \textit{in vitro} was not due to an increase in B cell proliferation. B cells producing IgG1 have undergone the process of CSR. CSR requires the action of the enzyme Activation Induced cytidine Deaminase (AID). AID initiates an intrachromosomal recombination event between two switch regions located in the Ig heavy chain locus. The presence of a donor switch region located upstream of the exon coding for the constant region of IgM and IgD (C_\mu/C_\delta) together with acceptor switch regions located upstream of C_\alpha, C_\varepsilon and C_\gamma enables the production of the different isotypes. CSR is promoted by CD40 and TLR signaling together with cytokines secreted from T helper cells and dendritic cells. CD40/TLR signalling stimulates AID transcription, while cytokines determine which isotype will be produced due to the presence of germline promoters upstream of each acceptor switch region. The germline promoters have binding sites for cytokine activated transcription factors. For instance the germline \gamma\textsubscript{1} promoter have a binding site for the IL-4 induced transcription factor Stat-6. One possible explanation for the stimulatory effect of HEMA on IgG1 production \textit{in vitro} could be that HEMA activates the IL-4 receptor on the B cell surface, thereby initiating a signaling cascade similar to that of true IL-4. This could lead to an increase in Stat-6 activated transcription of the germline \gamma\textsubscript{1} promoter and ultimately an increased IgG1 production. Another cytokine that has been shown to stimulate IgG production by human blood B cells is IL-6. Hence the stimulatory effect of HEMA on IgG1 production could be due to an effect of HEMA on IL-6 signaling. On the other hand previous experiments performed by Burdin \textit{et al.} have shown that blocking the B cell IL-6 receptor leads to a decrease in both IgG and IgM production. We could not demonstrate a stimulatory effect of HEMA on IgM production \textit{in vitro}, which would argue against a stimulatory effect of HEMA on IL-6 signaling. However Burdin \textit{et al.} analyzed the immunoglobulin production of B cells isolated from tonsils, not the immunoglobulin production of pokeweed stimulated blood B cells. Indeed it has been shown that IL-6 promotes the IgG production of pokeweed stimulated PBMC. This finding might lend some support to the theory that an increased IL-6 signaling might be causing the HEMA stimulated increase in IgG1 production \textit{in vitro} that we observed.

In summary, the results presented in study II suggest that HEMA can stimulate the production of human immunoglobulins of a particular isotype, while leaving the production of immunoglobulins of other isotypes unaffected.

We were also interested in studying how long-term exposure to HEMA would affect the immune system. We therefore filled osmotic pumps with either concentrated HEMA or diluted HEMA. We chose to dilute HEMA to a concentration that reflects the level of HEMA to which people working in a dental clinic are exposed. Next we implanted the pumps subcutaneously into the back of mice, leaving them \textit{in situ} for 40
days. Control animals received pumps filled with saline. We show in study III that mice exposed to both high and low concentrations of HEMA initially gain less in weight than control animals, while mice exposed to concentrated HEMA have an impaired weight gain throughout the entire exposure period. A possible reason for the impaired weight gain could be the presence of an inflammation, where the inflammation affects the animal’s food intake. In humans inflammatory reactions due to HEMA exposure, such as contact dermatitis, have been reported. We could however not observe an inflammatory infiltrate in the connective tissue surrounding the outlet of the HEMA filled pumps (results not shown). However we only analyzed the tissue for inflammatory infiltrates 40 days after pump implantation. An inflammation could have been present during the first week of the experiment, an inflammation that even though it had been resolved early on had an impact on the subsequent weight gain of the animals.

Splenocytes from animals exposed in vivo to minute amounts of diluted HEMA for 40 days had a reduced proliferative response to Con A stimulation in vitro. Con A stimulates T cell proliferation by binding to carbohydrates on CD3. A possible reason for the decrease in Con A stimulated splenocyte proliferation observed following 40 days of HEMA exposure in vivo could thus be that HEMA affects the responsiveness of T cells by binding to CD3 in vivo.

We also show in study III that HEMA exposure in vivo has a negative effect on splenocyte IL-2 production in vitro. T cells proliferate and produce IL-2 when IL-2 binds to the IL-2 receptor on the T cell surface. In other words IL-2 functions in an autocrine manner. Granchi et al. has previously shown that methacrylates can have a negative effect on the expression of the IL-2 receptor. Granchi et al. exposed lymphocytes in vitro to polymethylmethacrylate, a treatment that resulted in a decreased expression of CD25, that is the α-chain of the IL-2 receptor. One possibility for the decrease in splenocyte IL-2 production in vitro following HEMA exposure in vivo could therefore be that HEMA decreases the expression of the IL-2 receptor in vivo, which would have consequences for both T cell proliferation as well as IL-2 production in vitro.

Long-term exposure to minute amounts of diluted HEMA in vivo also affected the antibody response of the animals in an isotype specific manner. That is long-term exposure to HEMA in vivo resulted in a decreased IgA anti-OVA antibody activity, while leaving the IgG and IgM anti-OVA antibody activity unaffected. IgA synthesis is regulated by the cytokine transforming growth factor (TGF)-β. TGF-β promotes transcription from the germline Igα promoter by activating the transcription factors Smad3 and Smad4. It has previously been shown that exposure in vitro to a methacrylate can negatively affect TGF-β expression. Hence a possible explanation for the HEMA dependent decrease in IgA anti-OVA antibody activity could be that HEMA binds to the TGF-β receptor in vivo, thereby hampering TGF-β signal.
transduction and hence the production of IgA anti-OVA antibodies.

In summary, the results presented in study III suggest that long-term HEMA exposure in vivo can affect the production of antibodies in an isotype specific manner as well as certain T cell activities.

In study IV we analyzed further the specific effects of HEMA exposure in vivo by injecting HEMA subcutaneously into the tail of mice. We found that Con A stimulated splenocyte proliferation was two times higher in animals exposed to HEMA dissolved in bicarbonate buffer compared to animals only exposed to the buffer. In the skin there are different metabolizing enzymes, for instance cytochrome P450. In theory HEMA that has been injected subcutaneously could be metabolized by cytochrome P450 into a product that can prevent inhibitory regulation of T cell receptor signaling, thus enhancing the effect of stimulation with a mitogen like Con A. In a previous study it was shown that mitogen activated protein kinase p38 negatively regulates the proliferation of anti-CD3 stimulated CD4+ T cells. Based on that study a possibility for the enhancing effect of HEMA on Con A stimulated splenocyte proliferation is that HEMA binds to p38, thereby preventing p38 from inhibiting Con A stimulated T cell proliferation.

Production of the cytokine TNF-α has previously been shown to be sensitive to HEMA exposure. For instance exposing human monocytes to HEMA decreases LPS stimulated TNF-α production in vitro. We show in study IV that exposing mice to HEMA in bicarbonate buffer two times results in a reduced splenocyte TNF-α production in vitro. Macrophages are a major source of TNF-α. Therefore one could argue that the reduction in splenocyte TNF-α production that we observed was due to an impairment of macrophage cytokine production as a result of previous HEMA exposure. However macrophages are also a major source of IL-6. That we were unable to demonstrate an effect of HEMA exposure in vivo on splenocyte IL-6 production in vitro might therefore argue against an impaired macrophage cytokine production. Alternatively HEMA might affect macrophage TNF-α production specifically.

The negative effect of HEMA exposure in vivo on splenocyte TNF-α production in vitro is contradictory to the observation made in study III where HEMA exposure in vivo had no demonstrable effect on splenocyte TNF-α production in vitro. One factor that differs between study III and study IV is that in the latter the mice were injected with OVA on two occasions. Furthermore LPS was not removed from the OVA solution used for immunizations. Hence the differing results might be due to a stimulatory effect of the LPS present in the OVA solution administered in study III. In other words HEMA might have had a negative effect on the TNF-α production in study III similar to that observed in study IV, but the LPS present in the OVA solution administered could have counteracted it.
We present results indicating that B cells producing antibodies of different isotypes respond differently to HEMA exposure. In study III HEMA specifically reduced IgA anti-OVA antibody production, while in study IV HEMA specifically increased IgG anti-OVA antibody production. Further in both study III and study IV the IgM anti-OVA antibody production was unaffected. In other words injecting HEMA subcutaneously on two occasions has a stimulatory effect on IgG anti-OVA antibody production while long-term exposure to minute amounts of HEMA has a negative effect on IgA anti-OVA antibody production. How HEMA can scrutinize between IgA+, IgG+ and IgM+ B cells is difficult to explain. Generally speaking protein production is regulated at different stages, from the assembly of transcription factor complexes at promoters, through transcription and translation to the stability and proper folding of a functional protein. HEMA could potentially interact with regulatory proteins at any stage in the process of antibody production. One possible explanation for the apparent scrutinizing ability of HEMA is that due to the different experimental setups in study III and study IV HEMA was degraded into different metabolites. These metabolites could have different propensities for affecting IgA or IgG anti-OVA antibody production by binding to specific amino acid sequences in the promoter region of IgA and IgG exons.

In summary studies III and IV suggest that HEMA exposure in vivo can modulate the immune system with a high degree of specificity, a specificity that is dependent on concentration and length of exposure.

In conclusion, the studies presented in this thesis demonstrate that HEMA can have suppressive as well as enhancing effects on the immune system. However the molecules with which HEMA interacts in vivo, that enable HEMA to exert its specific effects, remain to be discovered.
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