The pH at the *S. mutans* cells–GIC interface at 90 min after glucose addition was around 5.0-5.5, significantly higher than that at the *S. mutans* cells–polymethyl methacrylate interface, regardless of immersion time. Seppä *et al.* (1992) reported that the pH in the fluid phase after sucrose fermentation by *S. mutans* cells layered on the surfaces of GIC discs for 24 hrs was higher than that on other materials, such as composite resin discs. It was also reported that the GIC eluate ceased the pH fall around pH 4.8-5.0, although *S. mutans* cells were suspended (Nakajo *et al.*, 2009). The pH change at the *S. mutans* cells–GIC interface in the present study is consistent with the results of those studies.

Nakajo *et al.* (2009) further indicated that fluoride released from GIC seemed to be the most probable reason for the inhibitory effect on acid production. The result in the present study, that a large amount of fluoride was detected only in the *S. mutans* cells at the interface with GIC, also supports a significant inhibitory effect of fluoride on bacterial acid production. Fluoride is known to have inhibitory effects on bacterial acid production by various mechanisms, such as inhibition of the glycolytic enzyme, enolase (Hüther *et al.*, 1990; Kaufmann and Bartholmes, 1992), and the proton-translocating ATPase (Bender *et al.*, 1985; Sturr and Marquis, 1990). In addition, inhibition is more effective at acidic pH, since hydrogen fluoride (HF, un-ionized fluoride) is more formed at acidic pH and penetrates bacterial cells efficiently, resulting in the enhancement of fluoride inhibition (Gutknecht and Walter, 1981).

The amounts of fluoride after bacterial fermentation were larger than those without bacterial fermentation in the present study. Al-Naimi *et al.* (2008) and Kantovitz *et al.* (2009) reported that acidic conditions led to higher fluoride release from GIC than neutral pH conditions. Moreover, the initial pH fall curves of GIC were similar to those of the other cements and polymethyl methacrylate, but the GIC curves ceased to fall gradually around pH 5.5. These findings suggest that, in addition to the effective inhibition by fluoride at acidic pH as stated above, larger amounts of fluoride are released from GIC as environmental pH becomes acidic, resulting in higher inhibitory effects on bacterial acid production. These characteristics of GIC seem to be favorable as a "smart" biomaterial inhibiting or decreasing the secondary caries activity adjacent to a restoration (Persson *et al.*, 2005; Moreau and Xu, 2010).

The acidity of GIC itself might contribute to antimicrobial activity (Hiraishi *et al.*, 2003; Vermeersch *et al.*, 2005). However, when GIC was evaluated in the experimental apparatus under the conditions of the present study, the initial pH at the bacteria–dental cement interface was shown to be around neutral (Fig. 2), probably because the acidity of GIC was neutralized during immersion in buffer solution. Similar phenomena may occur under the assumption that GIC is used as a filling material, in which GIC can be covered with oral biofilm and exposed to continuous salivary flow.

Large amounts of fluoride were released from GIC to PPB with 10 min of cement immersion, and the fluoride release rate slowed gradually and continued for 4 wks. This release curve is similar to those obtained in previous studies (De Moor *et al.*, 1996; Shaw *et al.*, 1998; Lee *et al.*, 2000). However, it was evident that the GIC immersed in PPB for 4 wks still had the ability to release fluoride and had an inhibitory effect on the pH fall.

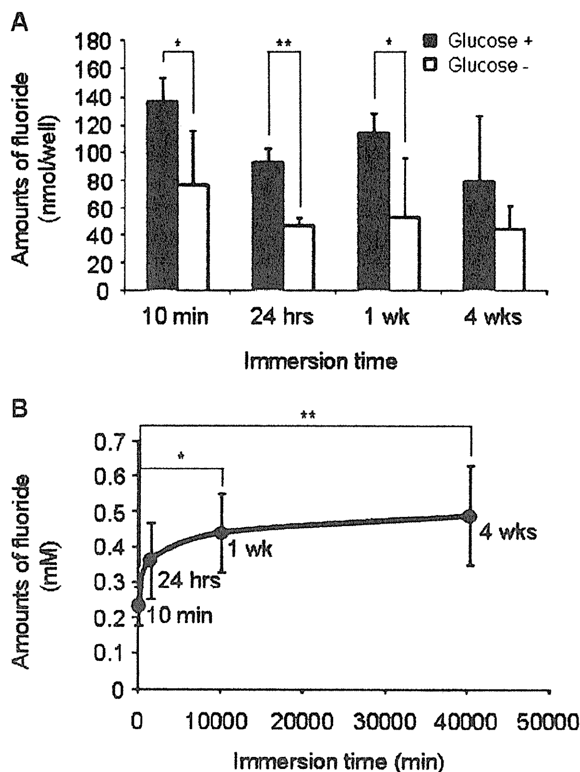

Figure 3. **(A)** The amounts of fluoride detected in the *S. mutans* NCTC 10449 cells at the interface with glass-ionomer cement at 90 min after the addition of glucose or de-ionized water and **(B)** the amounts of fluoride released into the immersion buffer from glass-ionomer cement. The data are the means of 3 and 6 independent experiments in (A) and (B), respectively. Vertical bars indicate standard deviations. $^{*}p < 0.05$, $^{**}p < 0.01$.

These results indicated that GIC inhibited the pH fall at the interface after cement immersion for a long time, suggesting that the inhibitory effect is mainly due to slow but steady release of fluoride from GIC, especially at acidic conditions, as discussed above.

The pH fall curves of ZPC and ZOE were similar to that of polymethyl methacrylate in the present study, suggesting that these cements had little inhibitory effect on bacterial acid production. Previous studies reported that ZPC and ZOE had bacterial growth-inhibitory effects (Coogan and Creaven, 1993; Boeckh *et al.*, 2002; Lewinstein *et al.*, 2005). The strong acidity of ZPC can be attributed to antibacterial activity (Lewinstein *et al.*, 2005), but the acidity of ZPC can be neutralized under our experimental conditions, and thus antibacterial activity is probably decreased. ZOE is known to disrupt the bacterial cell membrane and inhibit bacterial growth (Devi *et al.*, 2010). Although acid production activity was not inhibited under our experimental conditions, prolonged exposure of bacterial cells to ZOE might inhibit acid production.

The present study suggests that this method is useful for the assessment of the inhibitory effects of biomaterials on bacterial acid production. The advantages of this method are that the pH

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at the parasite-biomaterial interface can be measured directly, considering physiochemical interactions between bacteria and materials. This method will be able to evaluate the bacteriostatic activity of dental biomaterials, especially filling materials and replacement materials, which are exposed to the oral cavity where oral biofilm is easily formed, and will also contribute to the development of new biomaterials for caries prevention.

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Microflora Profiling of Infected Root Canal before and after Treatment Using Culture-Independent Methods

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This study aimed to profile the microflora in infected root canals before and after root canal treatment using culture-independent methods. Six infected root canals in single-rooted teeth with periapical lesions from five subjects were included. Quantification of total bacteria was performed by real-time PCR with primers targeting 16S rRNA genes. PCR products with universal 16S rRNA gene primers were cloned and partially sequenced, and bacterial identification at the species level was performed by comparative analysis with the GenBank database. The concentration of extracted DNA before treatment was higher than that after root canal treatment, although the difference was not statistically significant. Sequence analysis revealed that oral bacteria such as *Fusobacterium*, *Streptococcus*, *Olsenella*, and *Pseudoramibacter* detected in cases before root canal treatment disappeared after treatment. These results suggest that the root canal microflora are distinct before and after root canal treatment, and that treatment changes the microflora in both quantity and quality.

Keywords: 16S ribosomal RNA, microflora, PCR, root canals

Introduction

Through various anaerobic culturing techniques, it has been shown that the microflora associated with endodontic lesions of human teeth consist of obligate anaerobes, thus suggesting that the environment of the lesions is anaerobic and favors the growth of obligate anaerobes (Sundqvist, 1976; Ando and Hoshino, 1990; Sato *et al.*, 1993). Furthermore, molecular biological methods such as 16S rRNA sequence analysis have shown that the microflora in infected root canals is composed of various microorganisms, including obligate anaerobes (Siqueira and Rôças, 2009). Importantly, it has been reported that *Solobacterium* oral clone, *Bacteroides*-like oral clone and *Pseudoramibacter alactolyticus* are predominant before treatment, while *Streptococcus* species are predominant after treatment with a calcium hydroxide paste (Sakamoto *et al.*, 2007). However, detailed microflora profiles have not been clarified, and the influence of intracanal medicaments other than calcium hydroxide are unknown.

Therefore, in the present study, 16S rRNA sequence analysis was performed in order to profile the root canal microflora qualitatively before and after root canal treatment, using formalin guaiacol as an intracanal medicament. Quantification of total bacteria in root canals was also performed by real-time PCR.

Materials and Methods

Subjects

Subjects with apical periodontitis (two females and three males; age, 25–72 y, mean, 53.0±19.3 y) attending the Clinical Division of Endodontology of Tohoku University Hospital, Sendai, Japan, were randomly selected for this study (Table 1). Apical periodontitis was diagnosed based on clinical features, i.e., putrefactive smell, spontaneous pain, percussion pain, tenderness, pus discharge, swellings and fistula, and radiographic findings (Tronstad, 2009). Two samples (1A and 1B) were diagnosed as apical periodontitis with fistula, while the other four cases were diagnosed as chronic apical periodontitis. Selected teeth had sufficient coronal structure for adequate isolation with a rubber dam, and were free of periodontal pockets deeper than 4 mm. Based on history, all subjects were medically healthy, and received no antibiotics within the 3 months before sampling. Informed consent was obtained from all subjects, and this study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan.

Sampling and DNA extraction

At the first visit, each tooth was isolated with a rubber dam, and the surgical field was disinfected with both iodine glycerin dental disinfectants Showa (Showa Yakuhin Kako Co., Ltd., Japan) and 70% ethanol (Tronstad, 2009). A coronal access cavity was prepared with a sterilized high-speed bur under irrigation with sterile saline solution. When the pulp chamber was exposed, a sterile #15 K-file (GC, Japan) was introduced and the canal length was determined using an apex locator (Root ZX, Japan). Dentin samples were collected from an apical canal by intensive

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Table 1. Clinical features of subjects

Subjects	1		2	3	4	5
Samples	1-A	1-B	2	3	4	5
Age	72		42	25	60	66
Gender	Male		Female	Male	Male	Female
Sampling site[a]	21	22	31	22	22	41
Size of lesion[b]	8	2	4	7	4	4

[a] Tooth sampling sites are expressed using the FDI two-digit notation.
[b] Size (mm) of periapical periodontitis lesion estimated by x-ray examination.

filing with a sterile K-file suitable for the canal size.

After the first sampling, cleaning and shaping of the root canal was carried out with sterile K-files (#15 to #55; GC, Tokyo, Japan) under alternative irrigation with 3% H_2O_2 and Antiformin JP Dental (Nihon Shika Yakuhin Co., Ltd., Japan). An intracanal medicament such as Formalin Guaiacol Neo (Neo Dental Chemical Products Co., Ltd., Japan) was applied (Yamauchi *et al.*, 2010) until the next appointment, i.e., for 7 to 10 days. The coronal access cavity was sealed with temporary cement (Caviton; GC, Japan).

On the day of root canal obturation, each tooth was evaluated for clinical condition and it was confirmed that there were no clinical signs of apical periodontitis as described above. The tooth was isolated with a rubber dam, and the operative field was disinfected as described above. The temporary cement was removed and the intracanal medicament was rinsed out of the canal with sterile saline solution and a K-file. Immediately prior to root canal obturation with gutta-percha and sealer, a dentin sample was again collected. After obturation, the tooth was temporarily filled with glass ionomer cement (Fuji IX; GC, Japan).

Each file was cut with a sterilized wire cutter and was placed in 300 µl of 150 mM sterile phosphate buffered saline (pH 7.2) in a sterile tightly screw-capped tube at 4°C until use. After vortexing, dentin samples (ca. 0.1 mg) were harvested by centrifugation at 10,000×g at 4°C for 10 min and the supernatants were removed. Genomic DNA in the dentin of each sample was extracted using the GeneClean kit for Ancient DNA (Bio 101 Inc., USA) according to the manufacturer's instructions.

Quantification of total bacteria

Quantitative real-time PCR amplification was performed with universal primers for 16S rRNA genes, 357F and 907R (Lane, 1991;Yamaura *et al.*, 2005; Abiko *et al.*, 2010), and iQ SYBR Green Supermix (Bio-Rad Laboratories, USA), according to the manufacturer's instructions. Primer sequences were: 357F (5′-CTC CTA CGG GAG GCA GCA G-3′) and 907R (5′-CCG TCA ATT CMT TTR AGT TT-3′). Real-time PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 sec at 95°C for denaturation, 30 sec at 55°C for primer annealing and 30 sec at 72°C for extension. During the extension step, fluorescence emissions were monitored, and data were analyzed using iCycler iQ software (Bio-Rad Laboratories). Genomic DNA from *Enterococcus faecalis* JCM8728 was used as a standard for quantitative analysis.

16S rRNA gene sequence analysis

For cloning analysis, 16S rRNA genes were amplified by PCR with the primers 357F and 907R, as described above, and *Taq* DNA polymerase (Hot Start *Taq* Master Mix; Qiagen GmbH, Germany) according to the manufacturer's instructions. PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programed for 15 min at 95°C for initial heat activation, followed by 30 cycles of 1 min at 94°C for denaturation, 1.5 min at 55°C for primer annealing, 1.5 min at 72°C for extension and 10 min at 72°C for final extension. PCR products were separated on 1% agarose gels (Certified Low Range Ultra Agarose; Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM Borate, 1 mM EDTA, pH 8.4), stained with ethidium bromide and photographed under UV light. PCR product size (ca. 550 bp) was confirmed by comparison with molecular size markers (100-bp DNA ladder; Invitrogen Corp., USA).

PCR products were purified using the GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare Bio-Science Corp., USA), and were transformed into Qiagen EZ Competent Cells using the Qiagen PCR Cloning plus Kit (QIAGEN GmbH). Transformed competent cells were cultured on Luria-Bertani (LB) agar containing 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 0.1 mM IPTG (isopropyl-1-thio-β-D-galactoside) and 100 µg/ml ampicillin. After incubation for 16 h at 37°C, all of the white colonies obtained were transferred to LB liquid medium containing 100 µg/ml ampicillin. After overnight incubation at 37°C, plasmid DNA was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega, USA).

Plasmid DNA was sequenced (at least 530 bp) at Hokkaido System Science Co., Ltd. (Japan) using the T7 promoter primer, the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Japan Ltd., Japan) and an automated DNA sequencer (PRISM-3100; Applied Biosystems Japan Ltd.). Bacterial identification was performed using BLAST searches of the GenBank in order to identify the closest match to each of the obtained partial 16S rRNA gene sequences. Bacterial species were determined by percent sequence similarity (>98%). Rarefaction curves were generated using Analytic Rarefaction v1.3 (http://www.uga.edu/strata/software/index.html).

Statistical analysis

Wilcoxon test was used to determine the statistical significance of the concentration of extracted DNA determined by quantitative real-time PCR. A p value of <0.05 was considered to be statistically significant.

Table 2. Number of clones based on 16S rRNA gene sequence analysis obtained from infected root canals at first root canal treatment and prior to root canal obturation

	GenBank Accession Numbers		Before treatment (At first root canal treatment)							After treatment (Prior to root canal obturation)						
		Samples	1A	1B	2	3	4	5	Total	1A	1B	2	3	4	5	Total
		Total	11	15	0	12	2	18	58 (100)[a]	27	25	31	6	4	3	96 (100)
Fusobacterium nucleatum	JN052091		2	1		6			9 (15.5)							0
Streptococcus mitis / oralis	JN052092							4	4 (6.9)							0
Pseudoramibacter alactolyticus	JN052093			1				3	4 (6.9)							0
Olsenella profusa	JN052094			3				1	4 (6.9)							0
Methanobrevibacter oralis	JN052095							3	3 (5.2)							0
Odoribacter denticanis	JN052096		2	1					3 (5.2)							0
Veillonella parvula	JN052097							2	2 (3.4)							0
Bacteroides sp. 22C-like	JN052098		2						2 (3.4)							0
Uncultured bacterium clone GOR_aag74h11-like	JN052099		2						2 (3.4)							0
Propionibacterium acidifaciens	JN052100			1				1	2 (3.4)							0
Deferribacteres sp. oral clone JV006	JN052101			2					2 (3.4)							0
Nevskia soli	JN052102					1			1 (1.7)							0
Sphingomonas echinoides	JN052103						1		1 (1.7)							0
Actinomyces georgiae	JN052104					1			1 (1.7)							0
Streptococcus sobrinus	JN052105							1	1 (1.7)							0
Uncultured *Olsenella* sp. clone 1+7-15	JN052106							1	1 (1.7)							0
Prevotella intermedia	JN052107			1					1 (1.7)							0
Porphyromonas endodontalis	JN052108			1					1 (1.7)							0
Treponema socranskii	JN052109		1						1 (1.7)							0
Rothia aeria	JN052110							1	1 (1.7)							0
Acholeplasma morum-like	JN052111		1						1 (1.7)							0
Eubacterium minutum	JN052112		1						1 (1.7)							0
Pyramidobacter piscolens	JN052113			1					1 (1.7)							0
Peptostreptococcus stomatis	JN052114			1					1 (1.7)							0
Adlercreutzia equolifaciens-like	JN052115			1					1 (1.7)							0
Pseudomonas putida	JN052116						1		1 (1.7)	7	8	8			1	24 (25.0)
Bradyrhizobium japonicum	JN052117					3			3 (5.2)	9	6	7		1		23 (24.0)
Methylobacterium mesophilicum	JN052118								0	8	4	3	1	1		17 (17.7)
Rhodococcus erythropolis	JN052119							1	1 (1.7)		3	2	1	1		7 (7.3)
Uncultured bacterium clone nbw572e08c1	JN052120					1			1 (1.7)	2		2			1	5 (5.2)
Sphingomonas echinoides-like	JN052121								0		1	2	1			4 (4.2)
Pseudomonas fluorescens / reactans	JN052122								0		1	1				2 (2.1)
Bacteroides-like sp. oral clone X083	JN052123			1					1 (1.7)						1	1 (1.0)
Caulobacter crescentus / vibrioides / segnis	JN052124								0				1			1 (1.0)
Uncultured bacterium clone mek64b12	JN052125								0			1				1 (1.0)
Sphingobacterium spiritivorum-like	JN052126								0			1				1 (1.0)
Propionibacterium acnes	JN052127								0				1			1 (1.0)
Methylobacterium dichloromethanicum / extorquens / thiocyanatum	JN052128								0		1					1 (1.0)
Mesorhizobium loti	JN052129								0	1						1 (1.0)
Uncultured bacterium clone nbw643g04c1	JN052130								0		1					1 (1.0)
Aquabacterium hongkongensis	JN052131								0			1				1 (1.0)
Uncultured *Acidovorax* sp. clone A19	JN052132								0			1				1 (1.0)
Massilia brevitalea	JN052133								0			1				1 (1.0)
Herbaspirillum putei	JN052134								0					1		1 (1.0)
Uncultured bacterium clone: TSCOR001_M22	JN052135								0			1				1 (1.0)
Uncultured bacterium clone G-30	JN052136								0				1			1 (1.0)

[a] Percentages are given in parentheses.

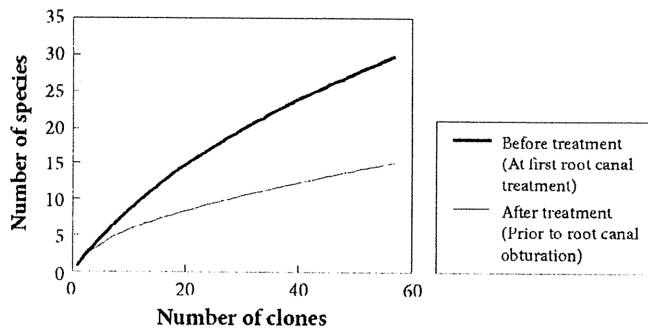

Fig. 1. Rarefaction curve analysis based on 16S rRNA gene sequence obtained from infected root canals at first root canal treatment and prior to root canal obturation.

Results

Bacterial DNA was detected in all samples, and thus bacteria were found to be present in all samples both before and after treatment. The concentration of extracted DNA before treatment (mean, 52.7 ng/ml; range, 0.36–152 ng/ml) was higher than that after treatment (mean, 10.9 ng/ml; range, 0.02–36 ng/ml), although the difference was not statistically significant ($p>0.05$).

A total of 58 and 96 clones were obtained from samples before and after treatment, respectively (Table 2). Among the 58 clones before treatment, *Fusobacterium nucleatum* (9 clones, 15.5%) was predominantly detected, followed by *Streptococcus mitis/oralis*, *P. alactolyticus*, and *Olsenella profusa* (Table 2). On the other hand, *Pseudomonas putida*, *Bradyrhizobium japonicum*, *Methylobacterium mesophilicum*, and *Rhodococcus erythropolis* were predominant among the 96 clones after treatment. Based on the rarefaction curves, the bacterial community of samples before treatment was more diverse than that after treatment (Fig. 1).

Discussion

Fusobacterium, as well as *Streptococcus*, *Olsenella*, and *Pseudoramibacter*, were predominant (39.6% in total) before root canal treatment in the present study (Tables 2 and 3), in accordance with previous studies using bacterial culturing (Le Goff *et al.*, 1997; Lana *et al.*, 2001; Vianna *et al.*, 2007) and molecular methods (Jacinto *et al.*, 2007; Blome *et al.*, 2008; Rôças and Siqueira, 2008). These four predominant genera (*Fusobacterium*, *Streptococcus*, *Olsenella*, and *Pseudoramibacter*) appeared to belong to oral bacteria, thus suggesting that these bacteria in root canals are derived from other oral sites, and that these bacteria are among the most common bacteria in root canals, being associated with periapical lesions. *Pseudoramibacter* species were reported to be predominant in a previous study based on 16S rRNA gene sequencing of clone libraries (Sakamoto *et al.*, 2007). In addition, *Pyramidobacter piscolens* and *Peptostreptococcus stomatis*, recently established bacterial species (Downes and Wade, 2006; Downes *et al.*, 2009), and *Methanobrevibacter oralis*, belonging to *Archaea* (Vianna *et al.*, 2006; Vickerman *et al.*, 2007), were detected in cases before root canal treatment in the present study (Table 2). These results were in accordance with previous studies (Downes and Wade, 2006; Vianna *et al.*, 2006; Vickerman *et al.*, 2007; Downes *et al.*, 2009) suggesting an association between microorganisms and endodontic infections.

Pseudomonas, *Bradyrhizobium*, *Methylobacterium*, and *Rhodococcus* were predominant (77.2% in total) after root canal treatment in the present study (Tables 2 and 3), although the findings of the present study did not agree with a previous study reporting the predominance of *Streptococcus* species (Sakamoto *et al.*, 2007). This difference may be due to differences in the intracanal medicaments used. The isolation of *Pseudomonas* species from root canals (0.9–22%) has been reported previously (Nord *et al.*, 1972; Molander *et al.*, 1998; Cheung and Ho, 2001). *Pseudomonas*, *Bradyrhizobium*, and *Rhodococcus* species are known to be able to grow in nutrient-poor conditions such as in soils and water systems (Crist *et al.*, 1984; Ozawa and Doi, 1996; Bell *et al.*, 1998; Saito *et al.*, 1998; Minamisawa and Mitsui, 2000). This may be one of the reasons why these microorganisms survive in root canals even after root canal treatment. *Methylobacterium*, utilizing methanol and methylamine as substrates, has been detected in oral cavities (Anesti *et al.*, 2005), but this bacterium, as well as *Pseudomonas*, *Bradyrhizobium*, and *Rhodococcus*, is not reportedly related to oral diseases such as periapical periodontitis. These bacteria were detected in very small numbers before treatment (8.6% in total) in the present study, thus suggesting that they are minor components of the microflora in root canals and that they survived the treatment.

Table 3. Predominant bacterial genera[a] based on 16S rRNA gene sequence analysis of infected root canal microflora at first root canal treatment and prior to root canal obturation

	Before treatment (At first root canal treatment)		After treatment (Prior to root canal obturation)	
Fusobacterium	9	(15.5)[b]	0	(0)
Streptococcus	5	(8.6)	0	(0)
Olsenella	5	(8.6)	0	(0)
Pseudoramibacter	4	(6.9)	0	(0)
Pseudomonas	1	(1.7)	26	(27.1)
Bradyrhizobium	3	(5.2)	23	(24.0)
Methylobacterium	0	(0)	18	(18.8)
Rhodococcus	1	(1.7)	7	(7.3)

[a] Bacterial genera >5% of root canal microflora are listed.
[b] Percentages are given in parentheses.

The bacteria comprising root canal microflora were clearly different before and after root canal treatment (Table 2). More specifically, oral bacteria such as *Fusobacterium*, *Streptococcus*, *Olsenella*, and *Pseudoramibacter* detected in cases before root canal treatment completely disappeared after treatment, while *Pseudomonas*, *Bradyrhizobium*, *Methylobacterium*, and *Rhodococcus* were predominant after treatment (Table 3). In addition, the bacterial diversity of root canal microflora was different before and after root canal treatment (Fig. 1). These differences could be due to the drastic environmental changes in root canals brought about by both mechanical cleaning with dental files and topical application of dental medicaments such as formalin guaiacol.

The present study suggests that root canal treatment alters root canal microflora profiles in both quantity (bacterial amount) and quality (bacterial composition).

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世界におけるフロリデーションの現状

現在、世界中で4億500万人がフロリデーションの恩恵を受けています。そのうち少なくとも3億5000万人が浄水場でフッ化物濃度を調整した水道水を利用し、5000万人以上が天然の適正濃度の水道水を利用しています。

フロリデーションを実施している国は、オーストラリア、ニュージーランド、米国、カナダ、イギリス、アイルランド、スペイン、イスラエル、ブラジル、チリ、アルゼンチン、コロンビア、中国香港行政区、韓国、シンガポール、マレーシアなど約60か国です。

米国ではフロリデーションの普及拡大が今も継続

米国は1945年に世界で初めてフロリデーションを実施した国です。フッ化物濃度が適正に調整されている地域の人口は、1992年の62.1%から2000年には65.0%と増加し、2008年には72.4%、1億9500万人となっています。

そのうち約800万人は天然の適正濃度の水道水を利用しています。現在、フロリデーションは、米国の50大都市のうち47都市で実施されています。また、人口100万人以上の都市は、すべてフロリデーションが実施されています。

水道水以外でフロリデーションを実施する方法

スイス、フランス、オーストリア、ドイツ、ハンガリー、スロバキア、ベラルーシなど、多くのヨーロッパ諸国でフッ化物添加食塩が広く利用されています。

中央アメリカ諸国および南アメリカ諸国でも、フッ化物添加食塩は広く使用されています。

口腔保健とフッ化物利用についての情報を詳しく知りたい方は下記のホームページをご覧ください

水道水フロリデーション、フッ化物洗口、フッ化物配合歯磨剤、フッ化物歯面塗布など、フッ化物利用全般について詳しく解説されています。また、国内外の専門機関や学会などの見解もまとめてありますので、フッ化物利用について知りたい方はぜひご覧ください。

厚生労働省ホームページ「e-ヘルスネット」歯の健康
http://www.e-healthnet.mhlw.go.jp/information/teeth/h-01-005.html
（e-ヘルスネットは、厚生労働省が一般の方を対象に正しい健康情報をわかりやすく提供するために開設したサイトです。）

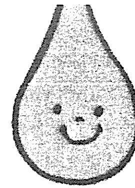
日本歯科医師会ホームページ　テーマパーク8020『口腔保健とフッ化物の応用』
http://www.jda.or.jp/park/prevent/index05.html

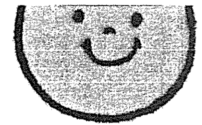
富岡甘楽歯科医師会のホームページ
http://www.tkda.jp/

公益社団法人　富岡甘楽歯科医師会
群馬県富岡市七日市640-1　TEL：0274-62-1706　E-mail：tkda@tkda.jp

富岡甘楽歯科医師会の「フロリデーションについての啓発活動」は、平成19年度から23年度まで5年連続で、
公益財団法人8020推進財団の歯科保健活動事業助成を受けています。
この度、平成22年度の事業が「他者の模範となる事業報告」に選定されました。

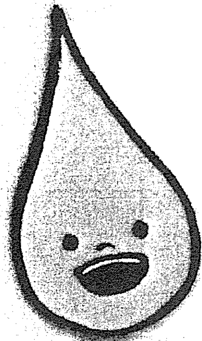
発行日：平成24年4月1日



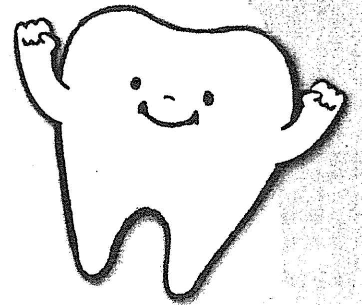


一生自分の歯で食べるために

監修　厚生労働科学研究「フッ化物応用の総合的研究班」
発行　公益社団法人 富岡甘楽歯科医師会

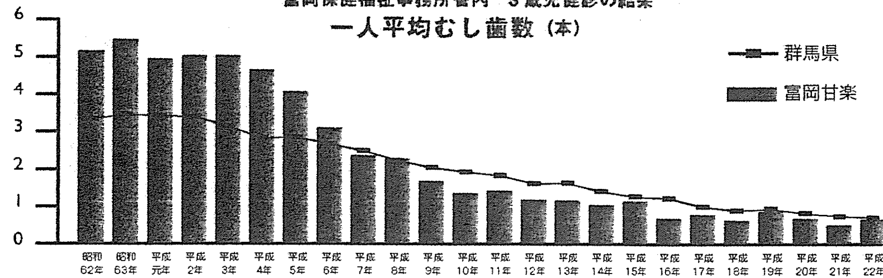


歯を失う原因の約９割は、むし歯と歯周病です。
一生自分の歯でおいしく食べるためには、むし歯と歯周病の予防が重要になりますが、そのどちらも科学的根拠に基づいた予防対策の実施により、比較的簡単に予防することができます。
むし歯予防のためには、フッ化物利用、特に公衆衛生的な対策の実施が重要です。
また、歯周病予防のためには、適切な歯みがきの励行と、かかりつけ歯科医を持ち定期的に予防管理をしていくことが大切になります。
さらに、口腔の健康増進が医療費の大幅な節約につながるということが各種の調査で認められています。
つまり、口腔の健康状況が全身の健康状態に明らかに影響を与えているということなのです。
歯科口腔保健法の公布・施行（平成２３年８月）を機に、長中期的視点で、歯科疾患の予防と健康格差の解消について考えてみましょう。



フッ化物利用で幼児のむし歯が大幅に減少

富岡甘楽地区は、約 20 年前まで乳歯のむし歯が県内で最も多い地域でした。そこで、富岡甘楽歯科医師会は、市町村と協力して健診と歯科保健指導の回数を増やし、定期的なフッ素塗布（フッ化物歯面塗布）を導入するという対策を行いました。平成 5 年度までには、管内全市町村の歯科保健事業でフッ素塗布が実施されるようになり、それにともなって家庭でのフッ化物利用も普及しました。また、富岡甘楽地区の幼稚園・保育園では、フッ化物洗口が実施されるようになりました。
その結果、3 歳児の 1 人平均のむし歯が約 5 本（平成 3 年度以前）から 0.64 本（平成 22 年度）に、むし歯のある児が約 80%（平成 3 年度以前）から 19.2%（平成 22 年度）と大幅に減少しました。平成 9 年度以降は、県内で最も幼児のむし歯が少ない地域になっています。



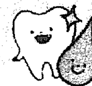
厚生労働大臣表彰を受賞

第 32 回全国歯科保健大会（平成 23 年 11 月 19 日岐阜県開催）において、富岡甘楽歯科医師会が歯科保健事業功労者表彰（厚生労働大臣表彰）を受賞しました。群馬県内の団体としては 22 年ぶりの受賞です。受賞理由は、「多年にわたり富岡甘楽地区の市町村と協力し、フッ化物応用等歯科保健事業に取組み、乳幼児のむし歯減少に貢献した功績と、高齢者や障害者の歯科保健医療の充実に尽力している」です。

また、富岡甘楽歯科医師会の入山主任歯科衛生士が、第 33 回母子保健奨励賞・毎日新聞社賞を受賞し、東宮御所において皇太子殿下からお祝いのお言葉を賜りました。受賞理由は、「歯科保健指導の充実、健診でのフッ化物歯面塗布と家庭でのフッ化物利用を組み合わせた乳歯のむし歯予防対策の推進など、乳幼児のむし歯罹患率減少に貢献した」です。歯科衛生士が毎日新聞社賞（母子保健奨励賞受賞者 15 人から 3 人を選抜）を受賞するのは今回が初めてのことです。

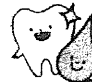
これからの むし歯予防

日本では、フッ化物洗口、フッ化物配合歯磨剤、フッ化物歯面塗布などが利用されています。しかし、先進国と比較するとフッ化物の利用が遅れているため、砂糖の消費量が少ないにもかかわらず、むし歯が多い状況が続いています。



一生涯を通したむし歯予防対策の確立のために

富岡甘楽歯科医師会は、WHO（世界保健機関）が推奨する最も優れた公衆衛生的なむし歯予防対策であるフロリデーション（水道水フッ化物濃度調整）を普及させたいと考えています。フロリデーションが実施されれば、子供から高齢者まで、給水地域で生活する住民すべてが、生涯を通じてむし歯予防の恩恵を受けることが可能になり、健康格差の解消にもつながります。
フロリデーションの実施により、むし歯が大幅に減少すれば、国が提唱する 8020 運動の実現に大きく貢献することになります。さらに、歯が残り咀嚼力が保持されることは、全身的な病気の予防や健康増進にもつながり、将来は医療費の抑制に結びつくことが確かめられています。



フッ素がむし歯予防に使われるようになった理由

20 世紀の前半に、適量のフッ素（フッ化物）が含まれる飲み水を使っている住民に、むし歯が少ないことが分かりました。この事実をもとに 1945 年から水道水にフッ素を追加して、むし歯予防に適したフッ化物濃度に調整する方法が開始されました。
逆に飲み水のフッ化物濃度が高すぎる地域では濃度を下げて調整します。これがフロリデーション（水道水フッ化物濃度調整）です。

フロリデーションがフッ化物利用の原点

むし歯予防のためのフッ化物応用法としては、フロリデーションの歴史が最も古く、米国において 1945 年から始められ、既に 67 年になりました。フロリデーションの有効性・安全性が確認される過程で、歯に直接フッ化物を作用させる局所応用（フッ化物歯面塗布、フッ化物洗口、フッ化物配合歯磨剤）の普及も進みました。半世紀以上にわたるフッ化物応用の有効性、安全性に関する研究結果に基づき、専門機関である WHO（世界保健機関）や FDI（国際歯科連盟）などが、世界各国に利用を推奨し実施を勧告しています。

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Effects on mouse immunity of long-term exposure *in vivo* to minute amounts of HEMA

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Andersson J, Dahlgren U. Effects on mouse immunity of long-term exposure in vivo *to minute amounts of HEMA.*
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2-Hydroxyethyl methacrylate (HEMA) leaks from cured restorations over time. Hence, HEMA can come into contact with cells of the immune system that are present in the oral mucosa and in the dental pulp. In this study, our aim was to develop a model of long-term exposure to minute amounts of HEMA and to record the immunological effects in mice. Osmotic pumps filled with either HEMA (8.2 M or 183 μM) or 0.9% NaCl (control) were implanted subcutaneously into the backs of mice and left *in situ* for 40 d, during which time the animals were immunized with ovalbumin (OVA). After 40 d, spleens and serum were collected. Splenocyte proliferation *in vitro* was analyzed by measuring the decomposition of [^{3}H]thymidine. Splenocyte cytokine production and serum anti-OVA IgG, IgM and IgA activity were analyzed using ELISAs. Mice exposed to both the higher and the lower HEMA concentrations gained significantly less weight and produced significantly reduced amounts of interleukin-2 (IL-2) *in vitro* compared with control mice. Mice exposed to the lower HEMA concentration had a significantly reduced concanavalin A-stimulated splenocyte proliferation *in vitro* and blood anti-OVA IgA activity. In conclusion, long-term exposure to minute amounts of HEMA *in vivo* affects the general health of mice and suppresses certain immunological functions.

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In modern dentistry, resin-modified glass ionomer cements, compomers, and bonding agents are used for restorations. A common ingredient in these materials is the monomer 2-hydroxyethyl methacrylate (HEMA) (1, 2). A cured restoration contains small amounts of unpolymerized HEMA monomers, which leak for months after curing and may reach the dental pulp as well as the oral mucosa (3, 4). 2-Hydroxyethyl methacrylate is also present in the air in clinics where resin-based materials are used (5). We have previously shown that HEMA has a multitude of effects on the immune system (6, 7). For instance, self-proteins modified by attached HEMA monomers are recognized as antigens of the immune system and evoke both anti-HEMA IgG as well as autoantibodies to the carrier (8). Furthermore, injecting HEMA subcutaneously into mice causes a decrease in the production of interleukin (IL)-6 and IL-10 from spleen cells.

Many *in vitro* studies reporting on the cytotoxic effects of HEMA have been published (9–12). In these *in vitro* studies, the longest exposure to HEMA lasted for 6 wk.

The *in vivo* biocompatibility of various composites, bonding agents, and compomers containing HEMA has been studied previously (13–15). However, only the complete products were analyzed in these studies, as opposed to the pure HEMA monomer. *In vivo* studies on the effects on the immune system of exposure, for an extended period of time, to low concentrations of pure HEMA are absent.

The objectives of the present study were to try to mimic the situation occurring when an individual is exposed to HEMA for an extended period of time and to test the hypothesis that long-term exposure to minute amounts of HEMA *in vivo* will affect the immune system.

Material and methods

Reagents and antibodies

2-Hydroxyethyl methacrylate, ovalbumin (OVA), Extr-Avidin-Alkaline Phosphatase, *p*-nitrophenylphosphate and Tween-20 were purchased from Sigma-Aldrich (Steinheim, Germany). Ficoll–Paque Plus and [methyl-^{3}H]thymidine were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). Dulbecco's modified Eagle's minimum essential medium (DMEM), Glutamax-I, heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin solution were purchased from Invitrogen (Lidingö, Sweden). Gentamycin was obtained from Serva Electrophoresis (Heidelberg, Germany). Concanavalin A (Con A) was

obtained from Pharmacia (Uppsala, Sweden). Biotinylated goat anti-mouse IgG was from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Biotinylated goat anti-mouse IgM and biotinylated goat anti-mouse IgA were from Southern Biotech (Birmingham, AL, USA).

Animal husbandry

Male BALB/c mice, 6–7 wk of age (Charles River Laboratories, Sulzfeld, Germany) were used throughout the study. The animals were housed under specific pathogen-free conditions in individually ventilated cages at the Laboratory for Experimental Biomedicine, University of Gothenburg. Food and water were provided *ad libitum*. The housing temperature was 20–23°C with a relative humidity of 40–60%. An artificial 24 h lighting-cycle of 12 h light/12 h dark was maintained. The experimental protocols were independently reviewed and approved by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden (# 380-2008).

Pretreatment of HEMA

Before use, the inhibitor monomethyl-ether-hydroquinone was removed from HEMA by passing HEMA through a column of polystyrene co-divinylbenzene beads (Sigma-Aldrich).

Preincubation and implantation of osmotic pumps

Alzet miniature osmotic pumps (model 2006, delivery rate = 0.15 μl h^{-1}; Alzet, Cupertino, CA, USA) were loaded with either 0.9% filter-sterilized NaCl (control group) or inhibitor-removed HEMA at one of two different concentrations, 8.2 M (undiluted HEMA) or 183 μM (HEMA diluted in 0.9% NaCl). Mice carrying pumps loaded with 8.2 M HEMA were thus exposed to 161 μg of HEMA h^{-1}, and mice carrying pumps loaded with 183 μM HEMA were exposed to 3.6 ng of HEMA h^{-1}. Control animals were exposed to 1.35 μg of NaCl h^{-1}. The mice were acclimatized 1 wk before the experiment. Under aseptic conditions a small incision was made on the lower back, the osmotic pump was implanted subcutaneously, and the incision was closed with metal clamps. Body weight was recorded throughout the experiment. The mice were killed 40 d after pump implantation, and spleens and serum were collected.

Immunization

The mice were immunized (primary injection) at the base of the tail with OVA dissolved in 100 mM sodium bicarbonate buffer (prepared at our laboratory), 19 d after pump implantation. An identical booster immunization was given (secondary injection) on day 34 after implantation. Primary and secondary injections both consisted of 50 μg of OVA in 50 μl of buffer.

Splenocyte isolation and supernatant preparation

Spleens were collected 40 d after implantation. Six spleens were collected from mice exposed to 161 μg of HEMA h^{-1}, while eight spleens were collected from mice exposed to 3.6 ng of HEMA h^{-1}. Eight spleens were collected from control animals. Each spleen was squeezed separately through a 70 μm cell strainer (BD Falcon, Bedford, MA, USA), and splenocytes were isolated by centrifugation over Ficoll–Paque Plus (GE Healthcare Bio-Sciences). Each spleen from each animal was cultured in triplicate wells. The median value from each of these triplicate cultures was used for the statistical evaluation. For analysis of cytokines (see below) splenocytes were seeded in plates (1×10^7 cells per well) (Sarstedt, Newton, NC, USA) in DMEM+, i.e. DMEM containing Glutamax-I supplemented with 5% heat-inactivated FBS, gentamycin (50 μg ml^{-1}), penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}), incubated for 2 d (37°C, humidified atmosphere, 5% CO_2), and then frozen until subsequent preparation of supernatants by thawing.

Quantification of cytokines in splenocyte supernatants

The concentrations of IL-2 (DuoSet DY402) and tumour necrosis factor-α (TNF-α) (DuoSet DY410) were measured in splenocyte supernatants using an ELISA, according to the manufacturer's instructions. The ELISA kits were obtained from R&D Systems (Abingdon, UK).

Ovalbumin detoxification

Before initiating OVA-stimulation experiments *in vitro* (see below), endotoxin was removed by passing OVA twice through a column (DetoxiGel AffinityPak; Pierce, Rockford, IL, USA), according to the manufacturer's instructions.

Splenocyte proliferation

From each spleen, three separate splenocyte cultures were prepared in 96-well plates at a concentration of 5×10^5 cells per well (Nunclon Δ Surface F96 well; Nunc, Roskilde, Denmark) in DMEM+. Splenocytes were then stimulated for 4 d with detoxified OVA (40 μg per well) or for 3 d with Con A (1 μg per well), at 37°C in a humidified atmosphere containing 5% CO_2. Triplicates of unstimulated cells were cultured for 4 d in parallel. [^{3}H]Thymidine was included for the final 24 h of culture (1 μCi per well), after which the cultures were frozen. Following subsequent thawing, splenocyte lysates were harvested (Harvester 96; Tomtec, Hamden, CT, USA) onto glass-fibre filters (Printed Filtermat A; Wallac, Turku, Finland) on top of which a melt-on scintillator (Meltilex A; Wallac) was applied. Decomposition of incorporated [^{3}H]thymidine was recorded as counts per minute (c.p.m.) in a Microbeta Trilux (PerkinElmer Sweden, Upplands Väsby, Sweden).

Quantification of anti-OVA IgG, IgM, and IgA activity in serum

Serum was analyzed for the presence of anti-OVA IgG, IgM, and IgA using an ELISA, as follows. Plates (Nunc MaxiSorp; Nunc) were coated overnight with OVA (0.5 μg per well) in PBS (prepared at our laboratory) and then incubated overnight with the serum samples. A pool of sera from all animals in the experiment was used as a standard on each plate. Next, the plates were incubated for 2 h with biotin-conjugated antibodies – goat anti-mouse IgG, goat anti-mouse IgM or goat anti-mouse IgA – and then with ExtrAvidin-Alkaline Phosphatase for 2 h. Thereafter, the plates were developed using *p*-nitrophenylphosphate

dissolved in diethanolamine buffer, pH 9.8 (prepared at our laboratory). The absorbance was measured at 405 nm on a Synergy 2 spectrophotometer (Biotek, Winooski, VT, USA). The plates were washed three or four times in PBS/Tween-20 between each incubation.

Statistical analysis

One-way ANOVA, together with either Tamhane's or Bonferroni's *post hoc* test, was used to compare the change in body weight of the test groups with the control group. The type of *post hoc* test used was based on the result of Levene's test for homogeneity of variances. The proliferation and the production of TNF-α, IL-2, anti-OVA IgG, anti-OVA IgM, and anti-OVA IgA in the test groups was compared with that of the control group using an Independent Samples t-test. Statistical analyses were performed using PASW 18.0 software (SPSS, Chicago, IL, USA). Differences were considered as statistically significant when $P < 0.05$.

Results

Effects on body weight

Mice exposed to 161 μg of HEMA h^{-1} *in vivo* increased significantly less in body weight throughout the exposure period [mean increase in weight at the end of the experiment (MIW) = 4.6 g, SD = 0.8 g, $n = 6$], compared with mice exposed to 1.35 μg of NaCl h^{-1} *in vivo* (control group) (MIW = 6.6 g, SD = 1.0 g, $n = 8$) ($0.000 \leq P \leq 0.018$) (Fig. 1A,C). Furthermore, two of the mice exposed to 161 μg of HEMA h^{-1} *in vivo* had to be put down because of a massive loss in body weight.

Mice exposed to 3.6 ng of HEMA h^{-1} *in vivo* increased significantly less in body weight at the start of the exposure period, compared with the control group ($0.000 \leq P \leq 0.037$) (Fig. 1B,C). At the end of the experiment, there were no significant differences in weight gain between mice exposed to 3.6 ng of HEMA h^{-1} *in vivo* and the controls.

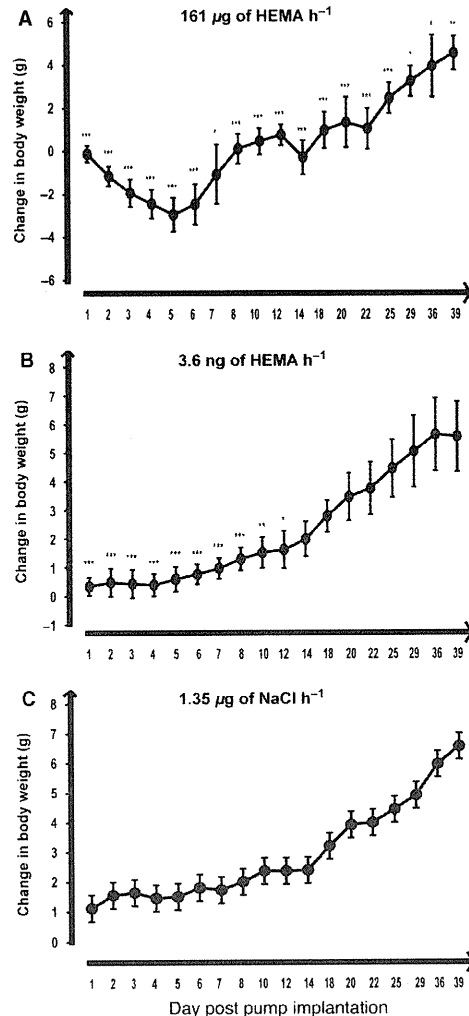

Fig. 1. Effect of long-term exposure to 2-hydroxyethyl methacrylate (HEMA) *in vivo* on body weight. The body weight of mice carrying osmotic pumps for 40 d was recorded. The pumps were prepared to ensure the delivery of 161 μg of HEMA h^{-1} (A), 3.6 ng of HEMA h^{-1} (B) or 1.35 μg of NaCl h^{-1} (C). The figure shows the mean and the SD for the change in body weight from the day of pump implantation (day 0). *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.005$, compared with the change in body weight for mice exposed to 1.35 μg of NaCl h^{-1}. The number of samples in each group was as follows: $n = 6$ (A), $n = 8$ (B), and $n = 8$ (C). The P-values were derived from one-way ANOVA together with either Tamhane's or Bonferroni's *post hoc* test.

Effects on splenocyte proliferation *in vitro*

Mice exposed to 3.6 ng of HEMA h^{-1} *in vivo* had a significantly lower Con A-stimulated *in vitro* proliferation of splenocytes than both the control group and mice exposed to 161 μg of HEMA h^{-1} *in vivo* (Fig. 2). We could not demonstrate any significant differences between the test groups and the control group with regard to OVA-stimulated *in vitro* proliferation of splenocytes.

Effects on splenocyte cytokine production

Mice exposed to either 161 μg or 3.6 ng of HEMA h^{-1} *in vivo* produced a significantly lower amount of IL-2 from splenocytes *in vitro* compared with the control group (Fig. 3). In addition, mice exposed to 3.6 ng of HEMA h^{-1} *in vivo* produced a significantly lower amount of IL-2 from splenocytes *in vitro* compared with splenocytes from mice exposed to the higher HEMA concentration *in vivo*. We could not demonstrate any significant differences between the test groups and the control group regarding the production of TNF-α from splenocytes *in vitro*.

Effects on production of anti-OVA IgG, IgM, and IgA

Mice exposed to 3.6 ng of HEMA h^{-1} had a significantly lower blood anti-OVA IgA activity than the control group at the end of the experiment (Fig. 4).

We could not demonstrate any significant differences between the test groups and the control group with regards to anti-OVA IgG or IgM activity.

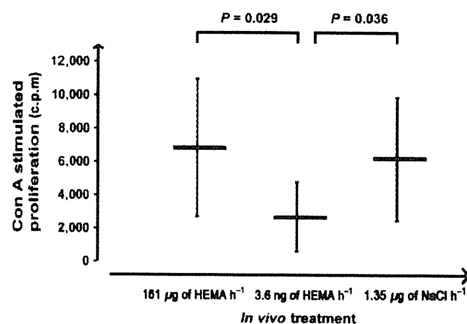

Fig. 2. Effect of long-term exposure to 2-hydroxyethyl methacrylate (HEMA) *in vivo* on concanavalin A (Con A) *in vitro*-stimulated splenocyte proliferation. Spleens were collected at day 40 from the animals described in Fig. 1. Six spleens were collected from mice exposed to 161 μg of HEMA h^{-1}, while eight spleens were collected from mice exposed to 3.6 ng of HEMA h^{-1}. Eight spleens were collected from control animals. Splenocytes were then isolated from each spleen separately by centrifugation over Ficoll. From each spleen, three separate splenocyte cultures were prepared and grown in the presence of 1 μg of Con A for 3 d. Proliferation was assessed by [^{3}H]thymidine decomposition as counts per minute (c.p.m.). The figure shows the mean and the SD of the c.p.m. for each experimental group. The number of samples in each group was as follows: n = 6 spleens (161 μg of HEMA h^{-1}), n = 8 spleens (3.6 ng of HEMA h^{-1}), and n = 8 spleens (1.35 μg of NaCl h^{-1}). The P-values were derived from an Independent Samples t-test.

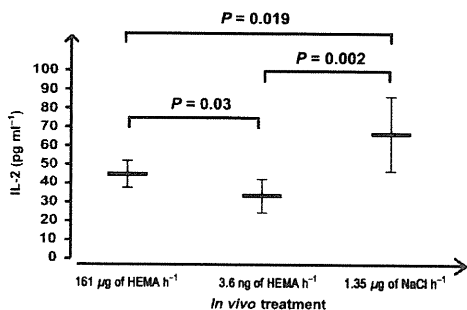

Fig. 3. Effect of long-term exposure to 2-hydroxyethyl methacrylate (HEMA) *in vivo* on splenocyte interleukin-2 (IL-2) production *in vitro*. Spleens were collected at day 40 from the animals described in Fig. 1 and splenocytes were isolated by centrifugation over Ficoll. Splenocytes were then grown for 2 d and the concentration of IL-2 was determined in the supernatants using ELISA. The figure shows the mean and the SD for the concentration of IL-2 in each experimental group. The number of samples in each group was as follows: n = 6 (161 μg of HEMA h^{-1}), n = 7 (3.6 ng of HEMA h^{-1}), and n = 8 (1.35 μg of NaCl h^{-1}). The P-values were derived from an Independent Samples t-test.

Discussion

In this study we implanted osmotic pumps, loaded with different concentrations of HEMA, subcutaneously in mice. This was undertaken to mimic the situation whereby an individual is exposed to a low concentration of HEMA for an extended period of time.

In order to estimate a possible level of HEMA exposure *in vivo*, we referred to a previous study where the

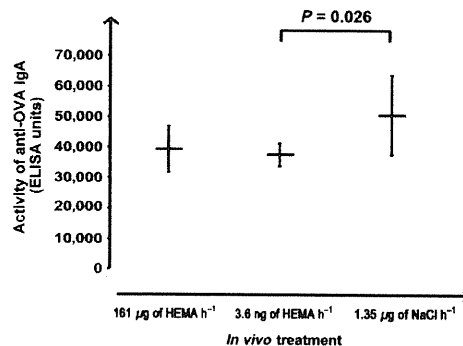

Fig. 4. Effect of long-term exposure to 2-hydroxyethyl methacrylate (HEMA) *in vivo* on serum anti-ovalbumin (OVA) IgA activity. Serum was collected at day 40 from the animals described in Fig. 1. The level of anti-OVA IgA activity in the serum was analyzed using ELISA. The figure shows the mean and the SD for anti-OVA IgA activity in serum. The number of samples in each group was as follows: n = 6 (161 μg of HEMA h^{-1}), n = 8 (3.6 ng of HEMA h^{-1}), and n = 8 (1.35 μg of NaCl h^{-1}). The P-value was derived from an Independent Samples t-test.

concentration of HEMA in the air of a dental clinic was reported to be 2.5 μg of HEMA m^{-3} air, following a whole day of working with resin-based composite materials (5). By assuming that a human adult inhales 6 l of air min^{-1}, we estimated *in vivo* HEMA exposure to be 15 ng of HEMA per kg of body weight h^{-1}, which we converted to a dosage of 3.6 ng of HEMA h^{-1} for a mouse weighing 20 g, by taking into account the differences in the weight:volume ratio between mice and humans (16). However, because there are no studies reporting on the *in vivo* concentration of HEMA in humans inhaling HEMA, we cannot, at present, determine the clinical relevance of this estimated HEMA dosage. Nevertheless, in general terms, exposure to 15 ng of HEMA h^{-1}, in humans, could be regarded as a low level of exposure.

In the current study we showed that long-term exposure to HEMA *in vivo* resulted in an impaired weight gain in mice. That is, exposure to minute amounts of HEMA over time affected the general health status of the animals. The underlying cause of this impaired weight gain could be a HEMA-induced inflammation, the inflammation in turn affecting the animal's food intake and metabolism. The inflammatory effects of exposure to HEMA *in vivo* have been described previously by our group and by others (6, 17). However, we could not see any signs of inflammation in biopsies taken close to the opening of the osmotic pumps (results not shown).

To study the possible effect of long-term exposure to HEMA on antibody production *in vivo*, the mice were immunized with OVA in sodium bicarbonate buffer. We could not demonstrate a significant difference in either anti-OVA IgG or anti-OVA IgM activity between cells from HEMA-exposed animals and control animals. In addition, we were unable to demonstrate a significant difference in OVA *in vitro*-stimulated splenocyte

proliferation. However, we have previously shown that mice immunized with a solution consisting of OVA in sodium bicarbonate buffer have a higher OVA-specific spleen cell proliferation *in vitro* and higher anti-OVA IgG activity, if HEMA is co-administered with the antigen (6). One possible explanation for these different observations could lie in the hapten quality, at physiological pH, of HEMA, which we have previously described (8). That is, free HEMA molecules released from the pumps may have been eliminated by binding to protein *in vivo*. Hence, the HEMA concentration at the site of immunization may have been too low to augment the production of anti-OVA Ig. However, we demonstrated a significantly lower anti-OVA IgA activity in mice exposed to the low concentration of HEMA. At this point we can only speculate why the IgA activity, but not the IgG or IgM activities, was affected. One possible explanation could be that owing to the effects on T cells of HEMA, the switching of B lymphocytes to IgA production was impaired, while B lymphocytes switched to IgG were present before the immunization. A selective effect on T cells is congruent with the data discussed below.

Concanavalin A is a T-cell mitogen and we found that long-term exposure to minute amounts of HEMA reduced the Con A-stimulated *in vitro* proliferation of splenocytes. The effect on T cells was also seen when we measured the *in vitro* production of IL-2. The IL-2 production by splenocytes from mice exposed to both the high and the low concentrations of HEMA *in vivo* were significantly lower than in the control cultures. A peculiar finding was that the *in vitro* IL-2 production was lower in the mice exposed to the low HEMA concentration (3.6 ng of HEMA h^{-1}) than in the mice exposed to the high HEMA concentration (161 μg of HEMA h^{-1}) (i.e. the lower HEMA concentration had a more negative effect on IL-2 *in vitro* production than the higher HEMA concentration). One possible explanation for this phenomenon could be derived from the characteristics of the osmotic pumps themselves. Upon examining the residual volumes present in the pumps after 40 d *in vivo*, we discovered that the pumps loaded with 8.2 M HEMA had dispelled all their contents while the pumps loaded with 183 μM HEMA or saline retained a small amount of their respective solutions. Hence, pumps loaded with 8.2 M (100%) HEMA appear to release their contents faster. Thus, the concentration of HEMA monomers *in vivo* at 40 d may have been lower in mice implanted with pumps containing concentrated HEMA than in the mice implanted with the pumps containing the lower HEMA concentration. This would explain why a seemingly higher HEMA concentration had a smaller inhibitory effect on the production of IL-2 *in vitro* than the lower HEMA concentration. However, an important point to make is that even though the contents of the pumps containing concentrated HEMA were released within 12 d (preliminary experiments) there was an effect on IL-2 production *in vitro* 28 d later.

The production of TNF-α was unchanged *in vitro*, indicating that the reduction in IL-2 was caused by a selective effect rather than by a general effect on the activity of the splenocytes.

Even though we have yet to discover the underlying mechanism, the important point of the present study is that exposure *in vivo* to HEMA affects the immune system, at levels that in humans translate to a very low exposure.

In conclusion, long-term exposure *in vivo* to minute amounts of HEMA can affect the general health status of mice and modulate B-cell as well as T-cell functions. These findings suggest that in individuals exposed to trace amounts of HEMA for an extended period of time, the normal function of the immune system might be comprised.

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