Table 6. Estrogen agonist activities, estrogen antagonist activities, and toxicities to yeast and *Vibrio fischeri* of anthracene (ANT), photochemical reaction products of ANT, and hydroxyl-polycyclic aromatic hydrocarbons

Compound[a]	Agonist 10× (ng/L)[b]				Antagonist EC50 (ng/L)[c]				Ytox test IC50 (ng/L)[d]				VF test IC50 (ng/L)[e]	
	−S9		+S9		−S9		+S9		−S9		+S9			
ANT	—[f]		—		—		—		—		Weak[g]		—	
ATQ	—		—		—		—		—		—		—	
1-hATQ	—		—		—		4.70×10^4	(0.959)	—		3.42×10^5	(0.942)	—	
2-hATQ	1.19×10^5	(0.902)[h]	—		—		—		Weak		3.15×10^5	(0.873)	Weak	
1,4-dhATQ	—		—		—		—		Weak		1.17×10^6	(0.717)	Weak	
1,5-dhATQ	—		—		—		—		—		Weak		—	
1,8-dhATQ	—		—		—		—		6.81×10^6	(0.943)	3.53×10^5	(0.964)	—	
1,2-dhATQ	2.62×10^6	(0.868)	—		5.90×10^4	(0.983)	—		Weak		2.12×10^5	(0.960)	Weak	
1,2,4-thATQ	—		—		—		—		2.05×10^6	(0.957)	1.72×10^5	(0.968)	2.34×10^6	(0.928)
2,6-dhATQ	—		4.27×10^5	(0.858)	—		—		—		Weak		—	
2,5-dhbenzoic acid	—		—		—		—		—		—		—	
2-h-1,4-Naphthoquinone	—		—		—		—		Weak		9.08×10^5	(0.553)	—	
Salicyladehyde	—		—		—		—		—		Weak		—	
Anthrarobin	5.36×10^6	(0.818)	—		8.93×10^4	(0.987)	—		1.46×10^6	(0.931)	2.25×10^5	(0.969)	2.49×10^6	(0.905)
2-h-Dibenzofuran[i]	1.28×10^6	(0.865)	—		—		—		—		1.90×10^5	(0.917)	—	
2-h-Fluorene[i]	3.41×10^5	(0.926)	1.46×10^6	(0.958)	—		—		—		1.60×10^5	(0.870)	—	
2-h-9-Fluorenone[i]	1.86×10^5	(0.982)	—		—		—		Weak		1.99×10^5	(0.938)	Weak	
9-Phenanthrol	—		—		—		—		Weak		1.40×10^6	(0.504)	4.23×10^6	(0.898)
17β-Estradiol	2.51 × 10	(0.987)												
trans-Stilbene			8.10×10^4	(0.944)										
4-h-TF					4.16×10^5	(0.985)	2.89×10^4	(0.891)	—		—			

[a] ANT = anthracene; ATQ = anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid; 4-h-TF = 4-hydroxy-tamoxifen.
[b] Values listed are the concentration at which the chemiluminescence is 10× the controls.
[c] EC50 (effective concentration 50%); the concentration at which the chemiluminescent signal of 17β-estradiol was inhibited by 50%.
[d] IC50 (inhibitory concentration 50%): the concentration at which the luminescent signal of the yeast was inhibited by 50%.
[e] IC50 (inhibitory concentration 50%): the concentration at which the luminescent signal of the bacterium was inhibited by 50%.
[f] Nonactive.
[g] Weak was defined as a statistically significant effect at a concentration of 10 μM.
[h] Goodness of fit, r^2.
[i] Compounds reported as estrogen agonists (Schultz et al. [20]).

of organic compounds, and these compounds are present ıghout the world.

'e compared the estogenicities of ANT derivatives with : of hydroxyl-PAHs, such as 2-hydroxydibenzofuran, 2- ɔxyfluorene, and 2-hydroxy-9-fluorenone, the estrogen- of which has been reported previously [20]. These three ɔounds also showed estrogenic activity by our methods e 6). The estrogen agonist activity of 2-hATQ (1.19 × ıg/L) was slightly stronger than that of 2-hydroxy-9-flu- ɔne (1.86×10^5 ng/L), which has been reported to be the gest estrogenic compound among the estrogenic PAHs

the estrogen agonist +S9 test, 2,6-dhATQ showed es- nic activity (Table 6). Its estrogenic activity was about ıfth of that of *trans*-stilbene, the positive control in the test. This result suggested that 2,6-dhATQ is transformed trogenic compounds when it is metabolized in the liver. 2-hydroxyfluorene showed estrogenic activity in both the and the −S9 test, suggesting that it might be hard to bolize or may be transformed to other estrogenic com- ds by +S9 tests.

'gen antagonist activities of ANT and its derivatives

ıe estrogen antagonist activities of the test substances are n in Figure 4c and d and Table 6. The yeast antagonist neasured the inhibition of expression of β-galactosidase :d by competition between the test substance and 17β- diol. The positive control was 4-hydroxy-tamoxifen (4- . Toxicity to yeast was evaluated by the IC50 of the Ytox The compounds 2-hATQ, 1,4-dhATQ, 1,8-dhATQ, 1,2- 'Q, 1,2,4-thATQ, 2-h-1,4-naphthoquinone, anthrarobin, 2- .uorenone, and 9-phenanthrol showed toxicity to yeast, ɔese compounds were more toxic to yeast after they were :d with rat liver S9 than with no S9 treatment (Table 6). esults of the yeast estrogen antagonist test were evaluated ht of the results of the Ytox test. When the EC50 value : estrogen antagonist test was four times smaller than the value of Ytox test, the chemical was considered to be .ve in the antagonist test. Statistical significance was test- · means of two-way ANOVA. The result showed that the ical was positive in the antagonist test ($p < 0.0001$). :fore, we chose to define the chemical as an antagonist. ıen needed to decide whether the decrease in the chemi- ıescent signal was caused by antagonist activity or tox- to yeast. Some compounds had estrogen antagonist ac- with or without S9. In the +S9 test, 1-hATQ had estrogen onist activity. This compound was also detected in sea- · from some of the test sites (Table 4). This result suggests 1-hATQ might have estrogen antagonist activity when up by organisms.

'ity to V. fischeri

gure 4e and Table 6 show the toxicities of the test com- ds to *V. fischeri*. The VF test evaluated the acute toxicity : concentration at which the luminescent signal of these ria was inhibited to 50%; it was assessed for those chem- that, in the original samples, inhibited the luminescent l in the range 0 to 79%.

hen the tested chemical decreased the signal to a level to 80 to 89% of the control value, we regarded the icals as exhibiting weak toxicity. The 2-hATQ, 1,4- 'Q, and 1,2-dhATQ showed weak toxicity to *V. fischeri*. e target compounds, 1,2,4-thATQ was the most toxic to *V. fischeri*. These results showed that photochemical reaction of ANT produces a variety of toxic compounds, whereas the parent compound does not have these toxicities.

DISCUSSION

Little information exists on the action of hydroxyl-PAH-quinones, in particular hATQs, in the environment, though many reports have described the concentrations of intact PAHs in the atmosphere [3], marine core sediments [4], and living organisms [5]. Photochemical reaction products, such as 1-hATQ and 2-hATQ, have only tentatively been identified in previous reports on sediment pore water [15] and contaminated sediment [16]. The silyl derivatization method has been used in the measurement of low-volatility polar compounds. These polar compounds show low sensitivity and tailing in GC analysis. Highly volatile derivatives can be obtained by means of the silylation reaction. We found that analyzing hATQs using silyl derivatization of samples was successful, with high sensitivity (at the ng/L level). The BSTFA is widely used to react with hydroxyl compounds [36,37]. Silylation using BSTFA made it possible to perform highly sensitive analysis of hATQs by GC-MS. It was reported previously that the matrix-induced chromatographic enhanced detection of the chemicals was observed in the analysis by GC [38]. The matrix protects the compounds from adsorption and decomposition in the hot vaporizing injectors and thereby ensures a more complete transfer from the injector to the column compared with the results observed when standards dissolved in matrix-free solvent are used. This could be one reason why our recovery rates exceeded 100% in some cases.

Ours is the first report to identify and quantify eight kinds of hATQs in seawater. Our results showed high concentrations of ATQ, 2-hATQ, and 2,6-dhATQ relative to the parent compound ANT in seawater off a heavily populated area of Japan (Table 4). Of the target compounds, ATQ was detected at the highest concentration in seawater. McKinney et al. have reported the ratio of the concentration of ATQ to that of ANT in coastal marine sediments to be between 0.317 and 2.81 [14]. However, the ratios of the concentration of ATQ to that of ANT in our coastal Japanese samples were between 6.6 and 140, with the exception of the samples from station 7, where ANT was not detected. This disagreement may be related to differences in the octanol–water partition coefficients ($\log K_{ow}$) of ANT and ATQ. The $\log K_{ow}$ of ANT is 4.45, and that of ATQ is 3.34 [39]. As ANT is more hydrophobic than ATQ, a disagreement might exist between the ratios of the concentrations of ANT and ATQ in sediment and seawater.

Stability against photochemical reactions and biodegradation would also contribute to the distribution of ANT and its derivatives in the marine environment. Our data showed that ATQ was the most stable compound in seawater (Table 5). Previous studies have examined the stability of ANT and its photochemical reaction products to SSR [8,10]. The ultraviolet radiation present in the solar spectrum will photomodify parent compounds as well as contribute to chemical degradation. These data showed that the half-lives of ANT and ATQ were 2.0 and 1.8 h, respectively [8,10]. The 2-hATQ and 2,6-dhATQ are stable to SSR and are end products of the photochemical reaction of ANT [8,10]. Hydroxyl-PAH-quinones detected in the environment may be formed by photochemical reactions or by biodegradation from PAHs, or they may be discharged in waste from industrial processes.

We also determined the estrogenic activities of the pho-

tochemical reaction products of ANT. For example, 2-hATQ had estrogen agonist activity, and 1-hATQ treated with rat liver S9 had estrogen antagonist activity, although the parent compound ANT did not show these activities (Table 6). Mechanisms for the estrogenicity of various compounds have been proposed. For example, most estrogen agonists have a phenol ring with a moiety of appropriate hydrophobicity at the *para* position, and substitution of a bulky moiety at the *ortho* position reduces their activity [17].

Photomodification reactions of PAHs could result in such toxicities not shown by the parent PAHs. Because of this phenomenon, a need exists to investigate the distribution and behavior of hATQs and to estimate the fate of these compounds in the marine environment. Moreover, in this study, toxic compounds, such as hydroxyl-PAH-quinones, may exist at higher concentrations than intact PAHs in the marine environment (Table 4). The literature shows many instances of photomodified PAHs in atmospheric particulate matter [40]; further studies of the environmental existence of derivatives of other PAHs are needed because contamination by PAHs is frequently reported in the marine environment [4,5]. Seawater samples contained 2-hATQ at concentrations ranging from 1.6 to 5.5 ng/L, and this chemical showed estrogenic activity similar to that of *p*-nonylphenol. The results suggest the importance of studying the distributions of PAH derivatives as well as of PAH in the environment and of the possible environmental effects of the compounds that demonstrated estrogenicity, toxicity, and estrogen antagonism.

CONCLUSION

We observed hATQs in seawater near two populated areas of Japan. The concentrations of photochemical reaction products of ANT, such as ATQ and 2-hATQ, were generally larger than those of ANT. Both estrogen agonist activity and estrogen antagonist activity were found among the hATQs that were the products of photochemical reactions of ANT. The photochemical reaction products of ANT had estrogen agonist or antagonist activity with or without being metabolized by rat liver S9. Our data indicate the importance of monitoring not only ANT but also hATQs. Photochemical reaction products may also exist for other PAHs in the marine environment, so both intact PAHs and hydroxyl-PAH-quinones should be monitored.

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Comparison of implantation and cytotoxicity testing for initial toxic biomaterials

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Abstract:

ostract

In order to evaluate the predictive value of cytotoxicity testing, the present study compares the *in vivo* tissue responses to *in vitro* cytotoxicity before and after implantation. Zincdiethyldithiocarbamate (ZDEC) is used as a catalyst for vulcaniizing latex. ZDEC is toxic and has been used as a standard for in vitro cytotoxicity testing. Polyurethane discs with the addition of 0.5% or 1% (ZDEC) as well as non-toxic discs were inserted in the abdominal wall of rats for 1 day up to 6 weeks. After explantation the foreign body response was analyzed immunohistochemically by light microscopy. *In vitro* tests of the explanted reference materials (RMs) revealed remaining high concentrations of toxic compounds after 1-week implantation, whereas no toxicity was seen after 6 weeks implantation. This was reflected in the foreign body response where a significantly thicker capsule and more inflammatory cells were seen at 1-week for the toxic implants. Over time, with decreasing toxicity, these differences disappeared. This study also indicates that *in vivo* implantation is more effective to extract the toxic compound than *in vitro* extraction at body temperature. It is concluded that many clinically useful implant materials may be unnecessarily rejected due to the results of *in vitro* tests.

Author Agreement

All authors have seen and approved that the manuscript as submitted. The work is original and has not been submitted elsewhere.

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Introduction

The implantation trauma in itself initiates a local, acute host response at the site of the implanted biomaterial. The inserted biomaterial is then a persistent stimulus for a chronic reaction, the foreign body response that depends on factors related to the implant and the host tissue. It has been suggested [1] and is now widely accepted [2, 3] that the primary interactions taking place within a second, between the material's surface atoms, and the molecules of the surrounding tissue will influence the future cellular behavior and implant integration [4, 5]. According to this hypothesis, it would be devastating to for example use a material like bone cement, which is initially toxic [6, 7], and that also generates excessive heat during the curing process. Despite this, bone cement has been proven to establish an excellent clinical long-term integration in bone tissue [8].

Today, cytotoxicity testing of extracts from prospective biomaterials plays a crucial role for the initial decision, whether a proposed material is biocompatible or not. Materials intended for implant applications are regarded as biologically safe after *in vitro* cytotoxicity tests [9, 10] although additional testing to warrant their biological safety with respect to other concerns need to be performed [11]. The cytotoxicity test methods allow analysis of different cellular functions such as replication, secretion, phagocytosis, chemo taxis, chemo kinesis, surface adhesion, etc. [10]. However, since these *in vitro* tests only expose single cells in a culture, these results cannot be directly related to the outcome in such a complex biological system as living tissue [12, 13]. Therefore the predictive value

of various *in vitro* tests, like cytotoxicity, should be considered cautiously for actual implant materials.

At present, many candidate implant materials are rejected due to the results of *in vitro* tests [14, 15]. Some previous studies have shown that candidate implant materials that display a moderate in vitro cytotoxicity have an excellent biocompatibility *in vivo* relative to the controls [16]. Bone cement might for example never have reached clinical evaluation due to its adverse *in vitro* test results [17, 18].

In the present study polyurethane discs, plain or with a defined addition of the cytotoxic zincdiethyldithiocarbamate (ZDEC), an accelerator for vulcanization of latex rubber, were implanted in the abdominal wall of rats. Polyurethane was chosen due to its widespread use as an implant material, and lack of toxicity [19]. After explantation, the host response was evaluated and the explanted discs were reanalyzed with regard to *in vitro* cytotoxicity. These results were further compared to a corresponding *in vitro* extraction test of unimplanted reference materials.

Materials & Methods

Preparations of test sample

Negative reference material (RM): PU (polyurethane sheet, lot no.: 98001NP) and Positive RM: 0.5% ZDEC (zincdiethyldithiocarbamate) and 1% ZDEC PU sheets (lot no.: 96003F and P005, respectively) were used as test samples. All materials were produced as 1 mm thick sheets. These materials have been supplied worldwide as RMs for the safety test of medical devices from the Hatano Research Institute (HRI), Food and Drug Safety Center (FDSC), Kanagawa, Japan.

The sheets of materials were cut into discs using a punch. For the *in vivo* experiments discs of 5 mm diameter were used after ethylene oxide sterilization. Some of these discs were evaluated after explantation for *in vitro* cytotoxicity. These samples were immediately frozen after explantation. They were thawed and ethylene oxide sterilized again just prior to testing. The materials used for *in vitro* testing only, were made to § (right?XX) mm diameter and sterilized using Ethylene oxide (EO).

In vitro testing

All *in vitro* cytotoxicity evaluation was performed according to ISO 10993-5.

Cell and culture conditions

V79 cells (obtained from the Japanese Cancer Research Resources Bank in September 1988, used within ten passages after thawing, passage No.5 at obtaining and passage No.14 at present) were cultured in Eagle MEM medium supplemented with 10 vol% fetal calf serum (FCS, lot no: US192415, Life Technologies), in a humidified incubator at 37°C and 5 % CO_2. The cells were certified to be mycoplasma-free.

In vitro pre-extraction test

In vitro cytotoxicity tests were applied to compare decline in cytotoxicity of materials implanted in animals to *in vitro* extraction in water solutions. Implanted materials were retrieved 1, 2, 4, 8 days and 6 weeks after implantation for cytotoxicity testing. All materials were wiped clean from tissue and kept frozen at –70°C until shipped for toxicity test. *In vitro* analysis of decline in toxicity was done by incubation of the material

in a large volume of water for 0, 4, 8 hrs and 1, 2, 4, 8 days.. The materials were then subjected to two different cytotoxicity tests.

TC-insert method (Tissue Culture) (1)

RMs were extracted for various intervals as described above and used as test sample. Extract solutions were discarded. Fifty cells suspended with 0.5 ml of culture medium were inoculated in each well. Next day, TC-inserts were set in each well. Then a test RM was put in each TC-insert (added with 0.25 ml medium). After 6 days culture, the wells were fixed, stained with Giemsa's solution and the colonies counted.

Medium extract method (2)

In this method, each RM was re-extracted again with MEM medium (1 disc/ml) for 24 hr at 37°C, in a 5% CO_2 incubator. Several doses of extracted medium were prepared by serial dilutions with fresh medium to 0.049, 0.098, 0.2, 0.39, 0.78, 1.6, 3.1, 6.3, 12.5, 25, 50 and 100 % (original extract solution). The colony formation assay was performed by plating 50 cells in each well. The day after plating, cells were treated with the extracted medium and cultured for 6 days. After fixation with methanol and staining with 10% Giemsa solution, the colonies in each well were counted.

The number of colonies at each extraction concentration was counted and expressed as the relative percentage of the negative control. Thereafter the concentration giving 50% inhibition of colony formation (IC_{50}) was calculated.

In vivo implantation

Animals

Male Sprague-Dawley rats, weighing 200-250 g, fed on standard pellets and water ad libitum, were used. The rats were anaesthetized by i.p. injections of 1.0 ml/100 g body weight of a solution containing sodium pentobarbital (60 mg/ml) and NaCl (9 mg/ml) in 1:9 volume proportions. The experimental protocol was approved by the Animal Ethics Committee in Lund, Sweden .

Implantation procedure

The test samples were implanted in rat abdominal wall. Details of the implantation procedure have been described previously [20]. In brief, the rectus abdominis muscle sheath was opened and the muscle moved laterally. Two negative and two toxic discs were inserted on either side of the linea alba, outside the peritoneum without injuring the peritoneal membrane. Some animals solely received negative implants. The rectus abdominis muscle was slipped back to cover the implant and a suture was placed in the muscle sheath to secure the position of the implant. In a first set of experiments eight animals for each implantation time period (i.e. 1 or 6 weeks) were used. In the second set, similar test samples were inserted for 1, 2, 4 and 8 days in 3(XX)animals.

Implant retrieval and tissue fixation

The animals were euthanized after 1 or 6 weeks in the first experiment and after 1,2, 4 and 8 days in the second. Implants with surrounding tissue were removed en bloc. The

specimens were washed in ice-cold phosphate buffered saline (PBS pH 7.4),embedded in Tissue Tek® O.C.T. compound (Histolab Products AB, Sweden) and snap frozen for 30 s in 2-methylbutane at -70°C. The implants were then removed by dissection of the peritoneal membrane without letting the specimen thaw. The tissues were sectioned in a cryostat (6 µm in thickness) and collected on chromium-alum treated slides and allowed to air dry. The slides were kept at -70°C until analyzed.

The explanted implant materials were analyzed at the HRI, FDSC, Kanagawa, Japan for *in vitro* cytotoxicity (ISO 10993-5) and compared to unimplanted reference materials.

Immunohistochemistry

The staining procedures for the ED1 macrophage subclass were done according to Rosengren et al [21]. In brief, after removal of the endogenous peroxidase activity and blocking of unspecific binding the slides were incubated with mouse anti-rat ED1 (Serotec Ltd., Oxford, UK). Then, the specimens were incubated with a biotinylated horse anti-mouse IgG antibody followed by incubation with Vectastain ABC peroxidase standard PK-4000 kit (Vector Lab. Inc., Burlingame, CA, USA). The presence of peroxidase was detected using 3-Amino-9-Ethyl-Carbazole (Sigma Chemical Co, St. Louis, USA). The sections were counterstained in Mayers HTX. Mouse monoclonal primary antibodies directed to human cell surface antigens were used to control unspecific binding.

Image processing

All images were obtained using a RT color Spot digital camera (Diagnostic instruments, Rochester, NY) mounted on a Nikon FXA (Tokyo, Japan) microscope, using bright field

illumination. The images were retrieved and manually counted using Photoshop 5.5 software (Adobe Photoshop, Mountain View, CA, USA) on a Power Macintosh G4 computer (Apple Inc, Cupertino, CA).

Morphometry

Sections from the implants impregnated with 0.5% and 1% ZDEC-PU as well as the non-impregnated controls were evaluated for all implantation times (1 or 6 weeks; 1,2,4 or 8 days). The numbers of cells were determined by computer aided manual counting of all cell nuclei as well as cells stained for the ED1 antigen. The numbers of cell nuclei were used as a measure of the total numbers of cells, which thus includes macrophages, fibroblasts and other cells. A 10x10 square grid where each square covered a 40x40 μm large area was superimposed with one side aligned at the tissue border to the implant surface (magnification objective 20x). The thickness of the reactive capsule was determined using the grid and was defined as the distance between the tissue border to the implant and the muscle – foreign body capsule border. The numbers of cells in the measured capsule were manually counted in 5 orthogonal rows of squares from the implant surface to the border of the muscle.

Statistics

Nonparametric statistics was used (Statview 4.5 for the Macintosh, Abacus Concepts, Berkeley, CA.). Differences in tissue responses due to grade of toxicity were analyzed using the non-parametric Kruskal-Wallis test and a post hoc test was performed according to Siegel [22]. The Mann-Whitney U test was used for evaluating the effects

over time. Spearman Rank correlation test was used to analyze correlations between grade of toxicity and capsule thickness or numbers of cells.

In vitro cytotoxicity of explanted RMs

The explanted RMs were subjected to *in vitro* cytotoxicity test, using the TC-insert method (1) and Medium extract method (2), described above.

Results

Cytotoxicity of RMs extracted in vitro

TC-insert method (1)

Negative RMs showed high level of colony formation in similarity to the negative control at the present test condition. No colony formation was detected for the 0.5% and 1% ZDEC-PU film (data not shown). The positive RMs that were subject to water extraction were still toxic in all intervals of extract conditions. This implies that the water extraction is not as efficient as the actual implant situation to elute toxic materials.

Medium-extract method (2)

The colony formation was not inhibited at any conditions for the negative RM group. For the 0.5% ZDEC-PU film group, cytotoxicity decreased with increasing pre-extraction times with water (see Fig. 1). A remarkable decrease of cytotoxicity was noted after 4 and 8 days pre-extractions with water. However, the cytotoxicity did not decrease

for any of the pre-extraction times in the 1% ZDEC-PU film group. The findings indicate that the 1% ZDEC-PU film group has a different kinetic release of toxicity compared to the 0.5% ZDEC-PU film group (see Fig. 1). These results suggest that the elution of the toxic compounds depends on extraction period as well as material toxicity.

In vivo host response

After 1-week implantation the implants containing 0.5% and 1% ZDEC displayed a significantly thicker foreign body capsule compared to the non-toxic controls ($p<0008$; $p<0.0006$) (see Figs. 2 and 3a,b). This was also reflected in the number of ED1 positive macrophages as well as in the total number of cells throughout the reactive capsule, which were significantly higher at both the 0.5% ($p<0.0078$; $p<0.0113$) and the 1% implants ($p<0.0002$; $p<0.0007$) when compared to controls (see Fig. 4).

After 6 weeks implantation the foreign body capsule reactions had subsided. Over time the capsule thickness decreased significantly for the 0.5% and 1% implants ($p<0.0287$; $p<0.0117$) to at 6 weeks be indistinguishable from the controls (see Figs. 2 and 3c,d). The ED1 positive cells and the total number of cells in the capsule displayed also a significant decrease over time ($p<0.0001$; $p<0.0004$) and at 6 weeks the number of cells became similar for all implants (see Fig.4). Above-mentioned data reflected also as a significant correlation between level of implant toxicity and capsule thickness after 1-week implantation ($p<0.0001$). The level of toxicity was also correlated to numbers of ED1 positive cells ($p<0.0001$) and to total cells ($p<0.0001$) in the foreign body capsule at the 1-

week implantation time point.

To investigate if the increase in capsule thickness at the ZDEC containing implants at 1 week was due to tissue edema the cell densities were analyzed. For all implants (both controls and toxic implants) the highest cell densities were seen at 1 week. This was significant for both the ED1 positive cells and the total numbers of cells in the foreign body capsule ($p<0.0001$; $p<0.0001$).

Some animals were solely implanted with negative implants to examine if the toxic implants influenced the tissue response to the adjacent situated negative controls. After 1-week implantation no significant differences in foreign body capsule reactions were seen for implantation with solely negative controls compared to implantation with both toxic and non-toxic implants. This indicates that the inflammatory reaction is strictly localized to the close vicinity of the implant.

In the kinetic study over day 1-8 using negative and ZDEC impregnated implants the number of test samples was insufficient for a statistical analysis. However, a trend was seen where the capsule thickness appeared to correlate to the implant toxicity. The foreign body reaction thus appeared to subside over time and also to be related to the *in vitro* measured toxicity (data not shown).

Cytotoxicity of explanted RMs

TC-insert method (1)

The explanted test samples were primarily tested using the TC-insert method. Both 0.5 and 1 % ZDEC implants were highly cytotoxic when retrieved during the

first week. However, after 8 days the 0.5% ZDEC implants showed a decreasing toxicity.

Medium-extract method (2)

To detect low levels of cytotoxicity the medium-extract method was used. This very sensitive method revealed that the positive RMs showed severe cytotoxicity at implantation. However already after 1 day in the animal the cytotoxicity of the 0.5% ZDEC implants started to subside (see Fig.5). For the 1% ZDEC implants no noticeable decrease in toxicity occurred until 8 days implantation.
After 6 weeks implantation no or very low toxicity was left in both types of ZDEC containing implants (see Fig.5).

Discussion

The implication of using materials, which have tested positive for *in vitro* cytotoxicity for implant device purpose is unclear. The host response to implanted materials with controlled toxicity has previously been reported [23-25], but the cytotoxicity after explantation was not analyzed. Often multiple samples are implanted in the same animal for testing purposes and the interference between samples becomes an issue. This study indicates that there is no interference of a toxic implant to the tissue response at a neighboring non-toxic implant. Some clinically widely used implant materials have tested positive in *in vitro* cytotoxicity tests but have proved to be successful in long term clinical

implantation. Examples of such materials are bone cements and dental filling materials [6, 25]. Some authors consider both the initial toxic effects as well as the initial thermal effects of bone cement of no importance for long-term biocompatibility [26]. For bone cements the host tissue appears to recover or be resilient to the released toxic compounds.

In the present study a significantly thicker foreign body capsule as well as higher number of cells surrounded ZDEC containing implants after 1-week implantation (see Figs.2 and 3a,b and 4). However after six weeks implantation the inflammatory response had declined, which was reflected in the thickness of the capsule and in the numbers of cells (see Figs.2 and 3c,d and 4). These results indicate that the early events in the tissue adjacent to the cytotoxic implants are not reflected in the long term tissue response. Another interesting finding was that the *in vitro* extraction with water did not elute toxic compounds to the same extent as the *in vivo* conditions.

reports on correlations between *in vitro* tests and *in vivo* results are scarce, indicating that the complex biological situation is hard to mimic with a single *in vitro* experiment. Such a comparative study was however done by Mjör et al [23], evaluating dental filling materials. The study showed that the cell culture technique (24 hours) correlated poorly with the soft tissue implantation test (7-90 days) or to the usage-test (7-45 days) in the dental pulp. Materials that displayed severe toxicity in cell culture induced small or no reactions in the implantation or in the pulp reaction tests. The opposite was also seen: very slight cell culture