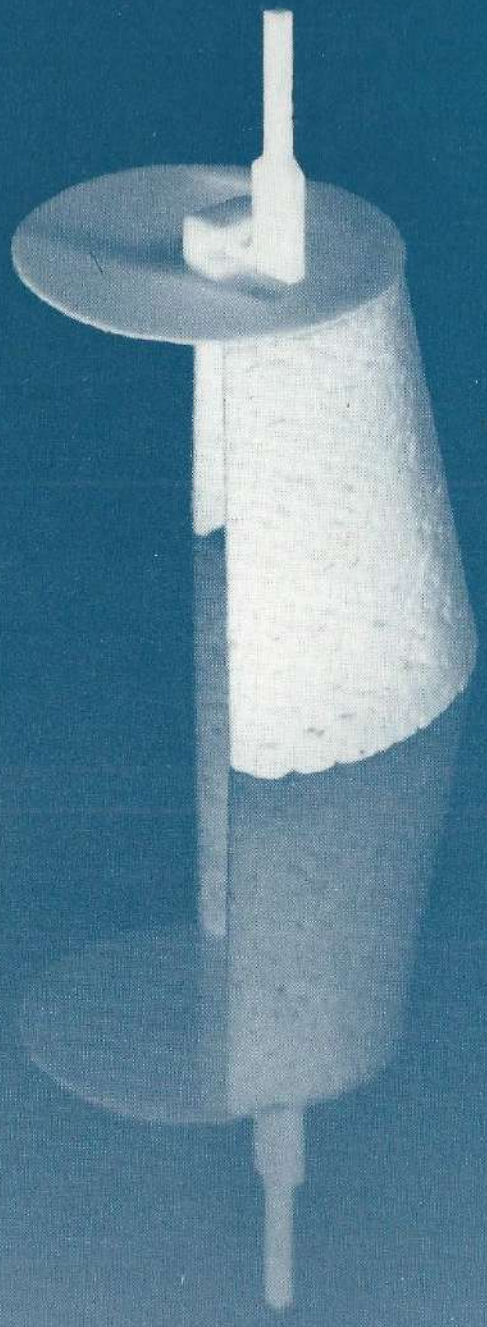
ALLOPLASTIC TYMPANIC MEMBRANE



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PROEFSCHRIFT

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. J.J.M. Beenakker,
hoogleraar in de faculteit der Wiskunde en Natuurwetenschappen,
volgens besluit van het college van dekanen te verdedigen op
donderdag 22 september 1988 te klokke 14.15 uur

door

Dirkjan Bakker

geboren te Ede in 1957

Gedrukt bij
Offsetdrukkerij Kanters
Alblasserdam

PROMOTIECOMMISSIE

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	Prof. Dr. J.P. Scherft

These investigations were supported by the Netherlands Technology Foundation (STW). Supplementary grants were received from the Heinsius Houbolt Fund. Financial support for the printing of this thesis by the Stichting 3A Honorarium Fonds is gratefully acknowledged.

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Aan mijn ouders

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Chronic otitis media is a disease of the middle ear causing hearing loss by interfering with the mechanical sound transmission system[1]. After removal of the diseased tissue, possibly including the ossicles, tympanic membrane, middle ear mucosa and bone, or the epidermis and bone of the external meatus, the function of the middle ear can be restored by tympanoplastic surgery, excised structures being replaced by an implant[2-5].

The first recorded attempt at repair of a persistant tympanic membrane perforation was made in 1640 by Bantzer[6], who used a piece of a pig's bladder mounted in an ivory tube. But it was not until 1951 that what we know as modern tympanoplasty was introduced by Wullstein[7] and Zöllner[8]. Since then, use has been made of both biologic material and alloplasts[1]. Autogenous biologic materials originate from the recipient, allogenous from a donor of the same species, and xenogenous from a different species. Alloplasts, which are made of biomaterials, are foreign to the body.

Autogenous materials have the advantage of being tolerated well by the body but are often limited in supply, must be retrieved by another operation or incision, and take longer to shape during the procedure[1]. Allogenous and xenogenous materials require special treatment[10] with a preservative to make them immunologically inert[11,12], and the degree of inertness seems to depend on the preservative used[13]. A possibly general problem associated with biological materials used to reconstruct the ossicular chain is resorption[4]. Other drawbacks of autogenous and allogenous materials are that only healthy donor tissue can be used and that autogenous and allogenous ossicles, tympanic membranes, and tympano-ossicular blocks are relatively scarce[14]. Alloplasts do not have these drawbacks, and seem to be more appropriate for cases of e.g. atelectatic ears or congenital anomalies[15]. However, alloplasts may be extruded[1,18,19] or induce strong foreign-body reactions[20].

Biologic materials (grafts) are used more often than alloplasts[1,21,25] for closing perforated tympanic membranes, although initially preference was given to artificial membranes for the treatment of such perforations[26]. Biologic materials used for this purpose include skin[27,29], vein[30], fascia[31], fat[29,32], heart valve[33], perichondrium[31], modified collagen[34-36], amnion[15], and homograft tympanic membrane[10,11,23,37,38]. Examples of non-biological materials that have been used to close perforations of the human tympanic membrane are latex rubber[39,40], moistened cotton wool[26,40,41], paper[26,42-44], Korogel[45], gold dental cylinders[26], cellophane[46], and gelatin sponge (Gelfoam^{R})[29,47]. Materials such as silicone

rubber[48], methyl-2 cyanoacrylate[49], Proplast[R;15], poly(vinyl alcohol)[50], poly(D,L-lactic acid)[51-53], poly(glycolic acid)[51-53], copoly(α-amino acid)[51-53], poly(tetrafluoroethylene)[51-53], bisphenol-A poly(carbonate)[51-53], and polyglactin 910 (Vicryl[R])[54] have been evaluated in animals. Of these, only polyglactin 910 has been used in patients but the results led the authors to conclude that Vicryl mesh is not suitable for myringoplastic surgery[54].

Biomaterials have been used more successfully for reconstruction of the ossicular chain. Besides such biological materials as autogenous and allogenous ossicles and autogenous cartilage and bone[1,55,56], a wide variety of biomaterials have been used to reconstruct the middle ear[16,57,58]. The history of biomaterials in middle ear surgery started in the early Fifties with Palavit rods[7]. Since then, a large number of non-porous alloplasts have been evaluated for reconstruction[16,57-59], i.e., Paladon[R;57], Supramid[R] (polyamide)[59-61], polyethylene[62], Teflon[R;63-65], silicone[60,61,66,67], and several acrylate glues[68]. Metals have also been used in middle ear reconstruction, i.e., stainless steel, tantalum, gold, and others[16,57,58,69]. Results obtained with solid polymeric and metallic implants were poor due to migration, extrusion, and penetration through the tympanic membrane and into the inner ear[70]. These failures were due at least partially to the absence of a firm union between tympanic membrane and implant, local pressure on the tympanic membrane, and the toxicity of some of the materials[18,19,65].

The development of porous implants made of polymers gave improvement by reducing displacement and extrusion[70-72], because tissue ingrowth led to implant stabilization[73]. Proplast[R] I (a combination of Teflon and vitreous carbon) was the first porous alloplast[18,60,70,71,74-76] and was followed by high density polyethylenes such as Plastipore[R;17,60,70,72,75-80] and Polycel[R;20], and by Proplast[R] II (a porous Teflon/aluminum oxide composite[18,81]). In the long run, however, failures with these porous implants increased[77-79]. Light and transmission electron microscopy showed e.g. severe foreign-body reactions[18,20,75,76,79,80,82]. Nevertheless, some authors are still enthusiastic about the use of Plastipore[83,85].

The next generation of alloplasts in tympanoplasty comprised bioglasses[87,88] and ceramic biomaterials[57,58,86]. The latter category can be subdivided into the aluminum oxides[89-92], the glass ceramics Macor[R;93] and Ceravital[R;55,94,95], and the calcium phosphate ceramics hydroxyapatite and tricalcium phosphate[96-100]. Carbon[101] and calcium sulfate dihydrate (plaster of Paris)[102,103] complete the list of ceramic biomaterials.

Hydroxyapatite[19,58,69,98-100,104-109], aluminum oxide[90,92], as well as

Ceravital[55,94,95,110], tricalcium phosphate[97], and Bioglass[R;88] are currently used in reconstructive middle ear surgery. Bioglass[87,111], glass ceramic[55,93,110,112], and calcium phosphate ceramics[112-114] permit a firm and direct bond with bone, although opinions differ about Macor[115]. Materials allowing bonding to bone are bioactive in this respect[116] and are frequently preferred to non-bioactive materials[117]. The bone-bonding property of these ceramics is thought to be shared with calcium phosphate dihydrate[118] but not with the aluminum oxides and carbon, in spite of the fact that aluminum oxide and carbon do allow direct contact with bone[90,101].

The calcium phosphate ceramics have a number of important properties, some of which they share with other bioactive ceramics and bioglasses. They can be produced in both porous and dense forms[119,120] and both are easily shaped by the surgeon[98,99]. Porous calcium phosphate middle ear implants allow ingrowth of fibrous tissue and bone[18,109,113,114,121-124] leading to firm implant integration[69,99,105]. Hydroxyapatite and to a lesser extent tricalcium phosphate are rather similar in composition to the mineral matrix of bone[120], which means that such implants are "less foreign" than those made of other biomaterials[98]. Studies in an animal model under infective conditions have only been done with hydroxyapatite[122,123,125] and tricalcium phosphate[124-127]. The results showed that infection did not significantly affect the biocompatibility of either material[122-125]. The limited tensile strength of calcium phosphate ceramics is a disadvantage, but for otological purposes the mechanical demands are light[14] and the mechanical properties of macroporous hydroxyapatite improve with the ingrowth of tissue[128].

Hydroxapatite has been used in reconstructive middle ear surgery to replace the bony posterior canal wall[69,99,105-107], to reconstruct the ossicular chain[69,98,105-108,129], and as bulk material in a total alloplastic middle ear prosthesis TAM[19,69,104-107].

The first reports on a total alloplastic middle ear implant date from 1980[130]. The authors defined four demands to be fulfilled, the aim being to exclude the disadvantages of the columellization technique required for ossicular replacement prostheses[131] and to include the advantages of the preserved allogenous total middle ear implant[132,133]: 1) it must approximate the normal anatomical and physiological conditions; 2) the implant sound-conducting system (ossicular chain) and its suspension (annulus and ear canal) must be implanted as a monobloc; 3) it must integrate in the host tissue the suspension system and contact points between ossicular chain and host; and it must 4) be usable in cases with defects in bony annulus and meatal wall left after safe and total eradication of the

diseased middle ear cavity.

The prototype of the TAM, the design of which was based on data obtained in temporal-bone studies, was originally made of either Teflon (non-porous) and Proplast (porous)[19,104,130] or Teflon and porous hydroxyapatite[19,104]. For reconstruction of the tympanic membrane, use was made of a fascia graft. The first TAM prototype was implanted in four patients, three of whom received Proplast[19,104,130] and the other one hydroxyapatite[19,104]. Although closure of the air/bone gap was not yet complete, all patients showed a hearing gain amounting on average to 20 decibels, which remained constant for periods of more than two years[19]. It was shown[19,109] for longer post-operative periods that hydroxyapatite gave better results than Proplast, which induced tissue necrosis. Furthermore, the hearing results would be better the more closely the mechanical properties of the prosthesis resembled those of the normal middle ear chain[134,135]. These findings led to the design of a second prototype in which hydroxyapatite was the bulk material[69,105,106].

In this second TAM the chain reconstruction consisted of two steps: attachment of an umbo made of macroporous hydroxyapatite to the canal wall via a movable hinge made of gold wire, and a piston made of solid hydroxyapatite and gold wire. The canal wall was made of macroporous hydroxyapatite[69,105-107]. A clinical follow-up study showed that none of the TAM prostheses had been extruded, but one had been removed during a second-look procedure after 1½ years[69,105,106]. Although all these patients had socially adequate hearing[105], none of them had complete air-bone gap closure and the average hearing loss was still as much as 25 decibels[106,107]. Furthermore, remodeling of the porous canal wall segment due to hydroxyapatite resorption[113] and encapsulation of the golden hinge by fibrous tissue led to loosening of the hinge and of the ossicular chain during the longer post-operative periods and this reduced the hearing gain. Work on a third prototype of the TAM was then started.

In the latest version of the total middle ear prosthesis, the canal wall will be made of macroporous hydroxyapatite and the first step of the artificial ossicular chain will consist of a hydroxyapatite umbo, but the second step of the chain will be made entirely of dense hydroxyapatite. Furthermore, an alloplastic tympanic membrane will be incorporated into the prosthesis to connect the umbo to the canal wall, thus eliminating the gold hinge. The epithelial remnants of the tympanic membrane and the ear canal will be drawn over the surface of the alloplastic tympanic membrane replacing the fascia graft.

The inclusion of a tympanic membrane in the TAM means that a complete equivalent of a normally functioning middle ear has been created[106,107]. This is

in accordance with the anatomical and physiological demands. Since the alloplastic tympanic membrane attaches the canal wall to the ossicular chain, this TAM can be considered to be a monobloc (second demand). The growth of tissue into and over the alloplastic tympanic membrane, the canal wall, the umbo, and the ossicular chain, provides integration of the suspension system in the middle ear cavity (third demand). To be suitable as alloplastic tympanic membrane a biomaterial must fulfill certain demands with respect to the following properties as well as its biocompatibility.

Physical properties

A polymer material is called a plastic or an elastomer according to the elasticity of the final material[136,137]. Polymers with elasticity are called elastomers. For sound stimulation to produce vibrations with patterns comparable to those of the tympanic membrane[138], the biomaterial must be an elastomer. In a study done with a SQUID magnetometer set up[134] the vibratory properties of the freely vibrating human tympanic membrane were compared with those of the elastomers Silastic^{R} and Dow Corning^{R} MDX-4-4210 Clean Grade Elastomer - both of which are silicones - as well as Estane 5714 F1 polyether urethane and a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) (HPOE/PBT) segmented polyether polyester copolymer[139,140]. Silastic vibration amplitudes are comparable to those of the human tympanic membrane *in vitro*, and those of the other polymers are even better. The high-frequency roll-offs of these polymers have about the same slope as that of the human tympanic membrane[139]. The same polymers and the human tympanic membrane were also assessed as to macroscopic elasticity in an *in vitro* stress-strain test according to Decraemer et al.[141], which showed[139,140] that the human tympanic membrane was the stiffest of the membranes under study, and that under the stress applied the two silicones displayed the most strain. However, all of the elastomers tested had sufficient mechanical strength for application in the TAM. To have sufficient mechanical strength, make it possible to have pores with a diameter of 100 µm, and fulfill anatomical demands, a tympanic membrane implant should have a thickness of about 100 µm and a diameter of about 1 cm. These values correspond well with those of the human tympanic membrane[142]. For sterilization of the prosthesis we usually prefer to use steam, but the temperature should neither significantly affect the implant material structure[139], nor induce chemical changes leading to unpredictable degradation behavior[143] or the formation of carcinogenic substances[144,145]. Similar changes have been reported to occur during sterilization by radiation[143,146,149] and ethylene oxide[147,150].

Biocompatibility

Tissue overgrowth

The tympanic membrane material should act as a scaffolding for tissue overgrowth. Tissue growing over and into the alloplastic tympanic membrane and the other parts of the prosthesis contributes to the connection between the ossicular chain (here the umbo) and the canal-wall segment. Evaluation of cell behavior on exposure to candidate materials is important for the assessment of an alloplastic tympanic membrane and can be done *in vitro* with cell-culture techniques as well as in animals (*in vivo*). *In vitro* techniques have the advantage that qualitative and quantitative data can be obtained relatively rapidly[151,152] and that this approach is cheaper than implantation studies. Furthermore, cell-culture methods are more sensitive for the detection of toxic substances[151,153], can be carried out on extracts of a material or its individual components[152], make it possible to evaluate interaction with isolated cells of a specific type[152], and offer the only acceptable way to study the behavior of human tissue on the experimental level[151]. However, the responses of the complete organism cannot be simulated with cell-culture methods and the results of *in vitro* techniques are difficult and sometimes even impossible to extrapolate to the clinical situation[151]. *In vitro* techniques are therefore especially suitable for preliminary screening[152].

One of the other major problems associated with implantation studies is the determination of correlation between the tissue responses and biomaterial compatibility. In the first place, the processes occurring at the implantation site are inseparable from the wound reaction induced by the operation[154]. In the course of time, however, the presence of an implant affects the normal wound-healing process[154,155], and this leads to a foreign-body reaction characterized by the presence of e.g. macrophages, foreign-body giant cells, and a capsule of fibrous tissue[154-156]. The severity of the foreign-body reaction, which is considered to be a measure of biocompatibility, is determined by the nature of the interactions between the biomaterial and the host tissue and is influenced by material factors such as the chemical composition, porosity, pore size, and surface texture of the implant as well as its shape and size[156-160], but also by such experimental variables as the species of the animal, the implantation site, and the techniques used[113,114,124,161-163]. The severity of the reaction has been quantitated in terms of capsule thickness[148,162,163] and vascularity[164], the total number of macrophages and foreign-body giant cells[148,165], the over-all cell density[148], enzyme activity[156,166,167], and tissue necrosis[148,165].

Porosity

The alloplastic tympanic membrane must be porous to allow the tissue ingrowth

that provides good integration[168,169] in the middle ear. A pore size of 10 µm is too small to allow ingrowth of tissue, e.g. from the tympanic membrane[52], into polymers. In general, pores with a diameter of at least 20 µm show ingrowth of fibrous tissue and those with a diameter of 30 µm or more may contain bone tissue[170]. The total volume occupied by pores is important for the mechanical strength of the alloplastic tympanic membrane. If the pore volume is too large, the mechanical strength of the material will be reduced to the point where handling of the film will easily lead to tearing. Porosity and pore size are also known to influence the degree of the foreign-body reaction[158] as well as (see the corresponding sections in this chapter) the incidence of infection[171,172] and the risk of foreign-body carcinogenesis[173]. In view of the foreign-body reaction, implants with large porosites, i.e., those with a large pore volume relative to the polymer-volume, provoke more severe reactions after implantation than implants with smaller porosities or dense implants do[174]. This difference is attributed to the increased polymer surface exposed to the degradative activity of the organism.

Degradation

Nakazono and coworkers[175] feel that a degradable biomaterial might be more suitable for scaffolding than a non-degradable one. A degradable polymer for application as tympanic membrane has the advantage over a non-degradable material in that it can be completely replaced by tissue. Consequently, the biomaterial only affects the vibratory properties of the tympanic membrane and the hearing results temporarily[29]; for just how long depends on the specific degradation kinetics of the polymer[176]. This is important, because only membranes with structural features similar to those of the natural tympanic membrane, e.g. successfully preserved homograft tympanic membranes, are expected to have vibratory properties closely resembling those of the normal tympanic membrane[138]. However, if material degradation outruns the incorporation and replacement of the biomaterial by tympanic membrane tissue, the connection of the ossicular chain to the canal wall will be endangered. This might lead not only to substantial loss of hearing gain, but also to extrusion of the ossicular chain when it comes into contact with the tympanic membrane[70,130]. These problems are very similar to those accompanying the use of columellas[19,72,130].

Interactions between phagocytes and implants are frequently associated with implant degradation[113,114,177-180]. Hydrolysis is the main cause of polymer degradation[181-182]. The degree of polymer degradation depends on material factors, such as bonding energy, hydrophilicity, and chain composition[143,181,183], but also on components of the aqueous environment such as enzymes,

temperature, pH, salts, and lipids[113,114,146,181-190]. According to the chemical and physical properties of the polymer, a distinction can be made between degradable and non-degradable polymers[176]. However, all implants are thought to be susceptible to degradation to some degree[150,182].

Toxicity

With respect to polymer degradation, the characteristics of the released degradation products and the released proprietary additives[191] as well as of residual impurities are of importance[191,192]. Degradation products or pure polymers may be harmless or inert, as shown[148] for polylactid acid, polyglycolic acid, and polyethylene oxide, but the release of additives (e.g. UV-stabilizers, antioxidants, plasticizers) can trigger toxic, mutagenic, and carcinogenic responses[144,192-195] or have systemic effects[196,197].

Systemic effects

Systemic effects concern the influence of an implant material, breakdown products, or additives on tissues or organs remote from the site of implantation[196,197]. Systemic effects related to polymers are frequently caused by monomers released over a relatively long period, e.g. from polymethyl methacrylate[73]. The accumulation of silicone droplets in the kidney, liver, spleen, and other organs after administration in liquid form is another example[198]. Systemic immune responses to metals in dental alloys[199] and other metal implants have also been reported[200,201].

Foreign-body carcinogenesis

Besides the chemical carcinogenesis just mentioned in relation to polymer degradation, the presence of an implant may induce tumor genesis[155]. Foreign-body carcinogenesis has been shown in connection with a wide variety of implant materials e.g. certain polymers, glass, and some metals[202-206]. However, tumor frequency differs between materials[207]. Foreign-body carcinogenesis has its highest incidence with rodents[203,204,208] and is associated predominantly with smooth-surfaced materials[208,209] and less frequently with textiles and powders[209] and porous films[173]. Encapsulation of the implant by fibrous tissue[207,210], implantations lasting more than six months[208], and the formation of specific precancer cells[207,211] seem to be correlated with tumor genesis in animals. However, results of studies on foreign-body carcinogenesis in animals models are difficult to extrapolate to human health risk[208] and reports on tumor induction by implants in man[211,212] are scarce[208]. Furthermore, none of the foreign body-related tumors in man have been found to show specific precancer cells[211].

Infection

Compared with dense implants, the porous type seems to have the disadvantage of a higher risk of infection during the period before tissue ingrowth has been completed[172,213-215]. Although opinions on this point differ[171], it is generally accepted that the presence of any implant, whether porous or dense, increases the risk of infection[214,216,217]. In the presence of an implant an infection can be caused not only by organisms not usually considered pathogenic[218] but also by smaller numbers of bacteria[216]. The risk of infection is also influenced by the chemical and physical properties of the implant[217,219-221]. Because infection is a common occurrence in otology[223], and often leads to reconstructive middle ear surgery[224,225], the effects of infection are of considerable interest in an evaluation of the biocompatibility of a biomaterial to be used in that region.

Objectives

Because the failure of many middle ear alloplasts is due to incompatibility of the material[17,20,57,226], we assessed the biocompatibility of several elastomers selected as candidate materials for the alloplastic tympanic membrane in a total alloplastic middle ear (TAM). The function of the alloplastic tympanic membrane in the TAM is to promote interconnection of the canal wall with the ossicular chain. In this context we defined three major demands to be fulfilled by the biomaterial in both non-infected and infected surroundings: 1) the biomaterial must be adequate as scaffolding for tissue with a normal composition and morphology; 2) fixation of the biomaterial tissue must occur, whether by direct bonding[111,112,117] or tissue ingrowth[168,170], or, preferably, by both; 3) if degradation of the biomaterial occurs, it must not endanger scaffolding of and fixation by tissue or lead to toxic effects in either adjacent or remote tissues.

Silastic and Dow Corning MDX-4-4210 Clean Grade Elastomer silicone rubbers, Estane 5714 F1 polyether urethane[227], and 55/45 HPOE/PBT polyether polyester copolymer[228] were selected for investigation as alloplastic tympanic membrane on basis of their physical properties[129,140]. In addition, Pellethane^{R} 80A polyester urethane[229] was included because it degrades much more rapidly than Estane polyether urethane[230,231] and polypropylene oxide due to its structural properties[232,233]. Silastic is the only one of these elastomers that has been used in middle ear surgery[66,67,234,235]. All of the other polymers under study except Elastomer have been assessed in the experimental phase for use in contact with blood[232,233,236-237]. The availability of Elastomer made it possible to include a

porous silicone rubber in our experiments, because Silastic can only be obtained in the form of a dense film.

For the selection of candidate materials to be tested in implantation studies, use was made of tissue and cell-culture techniques. For this, we used tissues and cells from the rat middle ear mucosa and epithelium, respectively, cultured according to the method developed by van Blitterswijk et al.[238] for assessment of the biocompatibility of hydroxyapatite *in vitro*[239]. The *in vitro* biocompatibility was investigated quantitatively (cell proliferative activity), qualitatively (cell and tissue morphology), and with an artificial aging test according to Homsy[240]. The results are dealt with in Chapter II.

Since we are interested in polymers as alloplastic tympanic membrane materials, studies in animals should include the middle ear, and preferably the tympanic membrane, as implantation site. The middle ear of the rat was chosen because the mucosa[241-244] and tympanic membrane[245-247] are morphologically rather similar to those in man. Furthermore, numerous studies have shown that the rat middle ear is highly suitable for otosurgical experiments[18,53,101,109,113,246-251]. As already mentioned, the behavior of middle ear implants should be investigated in both non-infected and infected environments. For infection, we applied the middle ear infection model developed by Grote and van Blitterswijk[252], who used *Staphylococcus aureus* as infective organism. This pathogen, which is reported to be the micro-organism most commonly responsible for wound infection in hospitals[146,155], has also been isolated from middle ear effusions[253] and was used by many authors to evaluate the effect of infection on a large number of implant materials[122-124,146,155,171,172,213,217,254]. The morphology of the tympanic membrane in both the infected and non-infected rat middle ear is dealt with in Chapter III.

Important information about the biocompatibility of polymers *in vivo* is provided by interactions between the implants and phagocytes, fibrous tissue, and bone. Because implantation in the tympanic membrane was not likely to lead to material/bone interaction we placed candidate materials not only as underlayer in the tympanic membrane but also between the middle ear mucosa and bulla bone and between the bulla bone and adjacent muscle tissue. Implants were studied after several intervals ranging from one week up to one year. The reaction of the various tissues to the porous implant materials is discussed in Chapter IV.

The use of different implantation sites enabled us to compare material/tissue interactions as functions of those sites. This is important for assessment of the general biocompatibility, since the implantation site is one of the factors with an influence on the degree of interaction[124,162,163]. The effects of the implantation

site on the foreign-body reaction and on material degradation are dealt with in Chapter V.

The interface between polymer and tissue can also be expected to yield information on biocompatibility[113,117,211,255-257]. The reactions between biomaterials and bone can be classified according to bioactive, bioinert, and biotolerant biomaterials[14,112], that is, materials which form a bond with bone upon contact, do not form a bond with bone, or do not come into contact with bone at all, respectively. All of the polymers studied by others thus far, perhaps with the exception of the porous acrylic implants[258], are of the biotolerant type. The material/phagocyte and material/fibrous tissue interfaces too can provide information about biodegradation[113,114,177-180] and implant fixation[113,114,170], respectively. The evaluation of polymer/tissue interface characteristics is reported in Chapter VI.

As already mentioned, chronic infection often leads to the need for reconstruction of the middle ear. Since infection may follow middle ear surgery, its influence on the biocompatibility of an implant is highly relevant. For example, infection is known to influence the degree of polymer degradation[143,259], and the intensity of an infection and the middle ear response can be affected by the presence of an implant[122,129,260]. Starting three weeks after the introduction of *Staphylococcus aureus* into the middle ear and using the same time-points as those applied for non-infected ears, we assessed both the biocompatibility of the polymers under study and the influence of their presence on the middle ear mechanism. The findings are presented in Chapter VII.

The middle ear epithelium participates in the middle ear defence against infection. The epithelium covering an implant can serve as a barrier to pathogens[261], but it can also provide transport for infective organisms via the activity of the ciliated and mucous-producing epithelial cells in the mucociliary tracts[262,263]. Furthermore, covering by epithelium is the last stage of the wound-healing process and seems to play a role in implant extrusion as well[226]. These functions of the epithelia of the middle ear and tympanic membrane are discussed in Chapter VIII.

Toxicity of a material is another important cause of implant failure[136,137,193,196,264]. The composition of a polymer must be known for proper assessment of potential tissue reactions and systemic responses to the implant. Chapter IX deals with toxic risks associated with the polymers under study in relation to their composition, degradation products, and the presence of such additives as antioxidants, stabilizers, and fillers. Combined consideration of this "theoretical" information together with the findings made in the tissue-culture

experiments (Chapter II), the implantation studies (Chapters IV-VIII), and the additional toxicological experiments was expected to make it possible to draw definitive conclusions as to whether any of the candidate polymers could be acceptable as an alloplastic tympanic membrane with respect to toxicity.

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CHAPTER 2

BIOCOMPATIBILITY OF SIX ELASTOMERS *IN VITRO*

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SUMMARY

The biocompatibility of two silicone rubbers, Silastic[R] and Dow Corning[R] Elastomer, and of a polyether and a polyester urethane, a polyether polyester copolymer, and polypropylene oxide was assessed *in vitro*. These polymers were selected for assessment as a possible alloplastic tympanic membrane in a total alloplastic middle ear prosthesis. For these studies use was made of rat middle ear mucosa explants and serially cultured epithelium. The quantitative results were based on epithelial growth curves, the morphological picture was based on the findings in epithelium, and the aging of a biomaterial was simulated. Epithelium morphology was investigated by scanning and transmission electron microscopy and x-ray microanalysis.

Quantitative results showed that on Dow Corning Elastomer and polypropylene oxide, cell proliferation was significantly lower compared to normal growth curves. The morphological findings were negative for polypropylene oxide and did not discriminate between the other biomaterials under study. The artificial aging experiment indicated better biocompatibility for the polyurethanes and the polyether polyester copolymer compared with that of polypropylene oxide and both silicone rubbers. Under the simulation conditions, cells exposed to Silastic showed silicon-containing inclusions. These *in vitro* results suggest that the biocompatibility of the polyurethanes and the polyether polyester copolymer is better than that of both silicone rubbers and polypropylene oxide.

INTRODUCTION

The development of a total alloplastic middle ear prosthesis (TAM)[1] in our laboratory encounters problems associated with the need for an alloplastic tympanic membrane for attachment of the alloplastic ossicular chain to the hydroxyapatite canal wall of the prosthesis. The bulk material used for the TAM is porous hydroxyapatite[1,2]. This ceramic was found to have good biocompatibility in *in vitro* studies with rat middle ear epithelium[3] and in implantation studies in non-infected[4,5] and infected[6,7] rat middle ears. In long-term clinical studies hydroxyapatite has proven to be suitable for use in otology[8,9].

In the studies reported here, we assessed *in vitro* the biocompatibility of six biomaterials, for some of which the vibratory properties had been investigated[10,11]. The studies were done in cultured tissue; this method offered

rapid results and a basis for quantitation of biocompatibility[12]. The *in vitro* investigations comprised quantitation of proliferative activity and observation of morphological changes in rat middle ear epithelium[13] cultured on the selected biomaterials. The third component of the study was artificial aging of a given biomaterial, which was performed according to the method of Homsy[14].

MATERIALS AND METHODS

Materials

The following biomaterials were used: the Dow Corning^R silicone rubbers Silastic^R and MDX-4-4210 Clean Grade Elastomer[15], a polyether urethane[16] (Estane^R 5714 F1, B.F. Goodrich), a polyester urethane (Pellethane^R-80A, Upjohn), a poly(ethylene oxide-hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer[17] (Akzona Inc.), and polypropylene oxide[18]. For Silastic, commercially available dense films (125 µm) were used, and for the other biomaterials dense films (100-125 µm) were cast from polymer solutions. The films were air dried and kept under vacuum conditions for at least 48 h. All films were cleaned, rinsed extensively first in running tap water and then in distilled water, and air dried. Unless stated otherwise, all biomaterials were steam sterilized except polypropylene oxide, which was sterilized by UV radiation.

Explants and epithelium

For the cell morphology studies rat middle ear mucosa explants and middle ear epithelium were cultured as follows[13]. Pieces of middle ear mucosa measuring about 1*1*0.1 mm^3 were obtained from the middle ear of male Wistar rats. The pieces were allowed to attach to the substrate in a drop of Dulbecco's modification of Eagles medium and F12 (3:1) to which hydrocortisone (0.4 µg/ml), isoproterenol (10^{-6}M), penicillin (100 U/ml), and streptomycin (100 µg/ml) had been added. After that, normal amounts of medium were used, and epidermal growth factor (10 ng/ml) was added to the medium after three days of culture. All material was cultured in 10% CO_2 at 37 °C and the medium was renewed twice a week.

Epithelial cells were harvested by trypsinisation from the outgrowth of an explant, which was cultured for two weeks in Costar^R culture dishes (tissue culture polystyrene; TCPS). Serially cultured cells were plated at a density of 2 x 10^4 cells/cm^2 in combination with 1.2 x 10^4 lethally irradiated 3T3 feeder cells per cm^2. For the quantitation of biocompatibility and for the artificial aging, use was made of serially cultured rat middle ear epithelium.

Quantitation

Growth curves of serial cultures of rat middle ear epithelium cultured on the various biomaterials were based on cell counts performed in 35 mm Costar culture dishes after one, three, six, 10, and 14 days. Dishes with and without (control) a biofilm were UV-sterilized prior to use.

Morphology

The morphology of rat middle ear tissue cultured on the various polymer materials and TCPS (control) was established electron microscopically. The observations were performed six and 10 days after the start of the culture of (1) middle ear mucosa explants, (2) epithelium growing out of explants, and (3) serially cultured epithelium.

Artificial aging

For artificial aging of an implant equal amounts of the various biomaterials were heat-exposed to extracellular fluid (PECF) at 115°C for 48 h[14]. The PECF was a salt solution comprising most of the salts included in the nutrient medium used for tissue culture and most of the ions in the same proportions as occur in body fluid[14] (Table I). Further, aliquots of PECF from a given experiment were used to prepare the nutrient media. In addition to these simulation media, two positive control media and one negative control medium were also prepared. The positive controls consisted of PECF that was free of biomaterial during heating and PECF in which Melinex^R (a thin transparant film made of poly(ethylene terephthalate)) was exposed to heat. PECF containing polyvinyl chloride (PVC) served as negative control. On this basis nine different nutrient media were obtained for serial cultivation of rat middle ear epithelium on TCPS (Table II).

After three days of culture in routine medium, the various simulation and control media were added to the culture dishes. Three and 10 days later, the cell morphology was compared with the picture after culture in routine medium.

TABLE I. Ion concentrations (in meq./l) in body fluid* and PECF.

Ion	Body fluid	PECF
Na^+	145	154.5
K^+	5	5.4
Cl^-	113	118.5
HCO_3^-	30	44
HPO_4^{2-}	2	0

*As defined in ref. 14.

Electron microscopy.

The specimens destined for transmission electron microscopy were fixed in 1.5% glutaraldehyde in sodium cacodylate buffer (0.14 M, pH 7.4, 4°C) for 15 min. After being rinsed in phosphate-buffered saline (pH 7.4), the cells were postfixed in a 1% OsO_4 solution at room temperature for 30 min. Next, the specimens were rinsed in phosphate-buffered saline, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections were cut on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips EM 201 electron microscope. The specimens destined for scanning electron microscopy were dehydrated immediately after the glutaraldehyde and then critical point dried and gold sputtercoated. A Cambridge S180 Stereo Scan was used. Some of the material prepared for TEM was studied with a Philips EM 400 connected to a Tracor Northern (TN) 2000 x-ray microanalyser. Both single-spot analysis and x-ray mapping were performed[19].

TABLE II. Media used in the artificial aging experiment.

Medium	Polymer	Type
1	-	positive control 1
2	Melinex®	positive control 2
3	PVC	negative control
4	Silastic®	biomaterial
5	Elastomer	biomaterial
6	Estane®	biomaterial
7	Pellethane®	biomaterial
8	HPOE/PBT Copolymer	biomaterial
9	Polypropylene oxide	biomaterial

RESULTS

Quantitative analysis

Only a few middle ear epithelial cells adhered to polypropylene oxide, but these cells did not proliferate. The shape of the growth curves of the epithelial cells serially cultured on the other five biomaterials and TCPS was sigmoid (Fig. 1). Application of Wilcoxon's two-sample test ($\alpha = 0.05$) to the growth curves showed that only the epithelial cells on Dow Corning® Elastomer and polypropylene oxide proliferated at a significantly lower rate compared with those on TCPS.

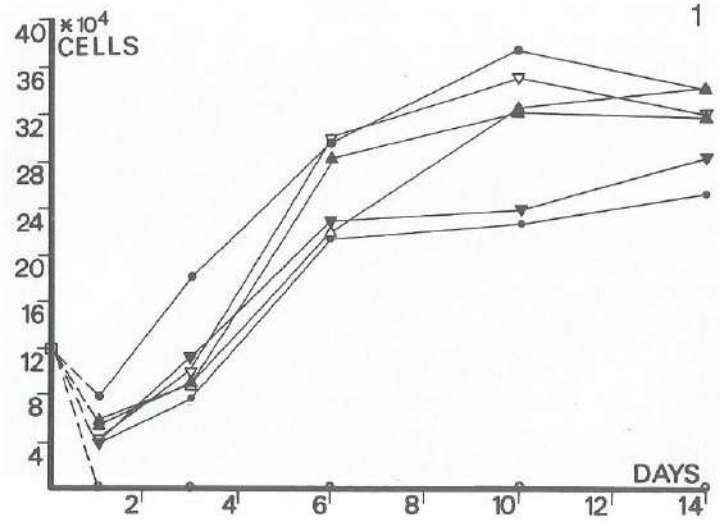
Fig. 1. Growth curves of serial cultures of middle ear epithelial cells. Tissue culture polystyrene (●), Silastic® (▽), HPOE/PBT polyether polyester copolymer (△), Estane® 5714 F1 (▲), Pellethane® 80A (▼), Dow Corning® Elastomer (·), polypropylene oxide (o).

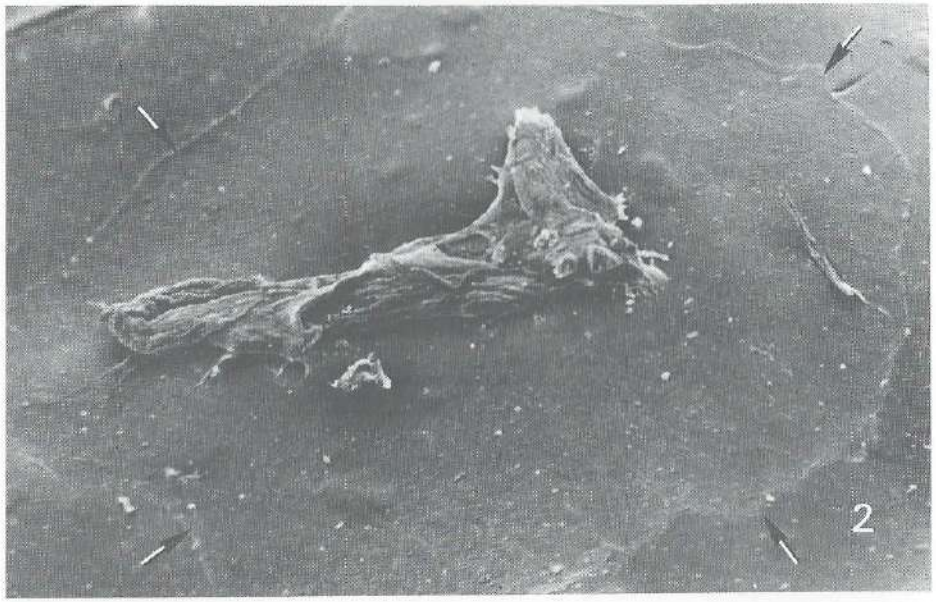
Tissue morphology

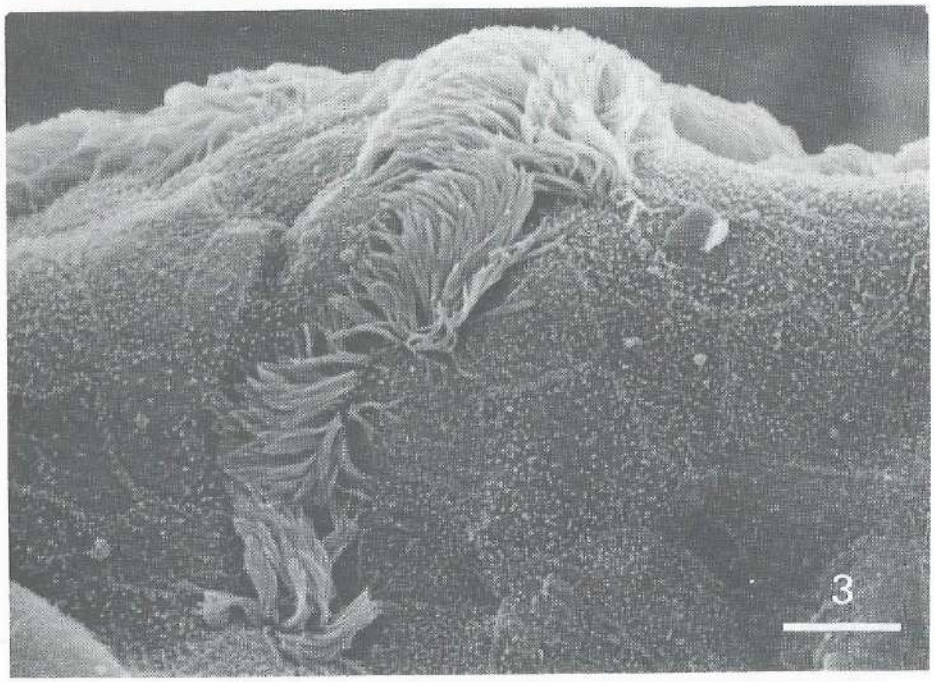
About half of the middle ear mucosa explants attached to polypropylene oxide but outgrowth of cells did not occur. More than 80% of the explants attached to the other five biomaterials and to TCPS. All of these explants showed outgrowth of a single layer of cells, which became visible three days after culture was started and was more prominent after the tenth day (Fig. 2).

Explant morphology

Scanning electron microscopy revealed that the epithelium of explants cultured on TCPS and all six biomaterials was composed of a monolayer of predominantly flat polygonal cells and a small number of ciliated cells. For all of the biomaterials studied except polypropylene oxide, the morphology of explant epithelium was comparable to that after culture on TCPS (Fig. 3). The flat polygonal cells showed variable numbers of microvilli. Transmission electron microscopy showed that the epithelium varied in thickness and lay on a basal lamina (Fig. 4). Ultrastructurally, the flat polygonal cells had a flattened nucleus, a well-developed endoplasmic reticulum, mitochondria, ribosomes, a Golgi apparatus, and an occasional primary cilium (inset Fig. 4). Cells with granules suggesting secretory activity were also present.







Outgrowth morphology

Scanning and transmission electron microscopy showed that explant outgrowth yielded a continuous monolayer of thin cells. These cells were connected by desmosomes, as was seen in transmission electron microscopy. Scanning electron microscopy revealed that most of these epithelial cells were flat and polygonal. The majority of them bore a small number of microvilli, present mainly on the cell borders. Cells with more microvilli or with a primary cilium were seen in small clusters (Fig. 5a). Scanning electron microscopy also showed that ciliated epithelium was present on the five biomaterials showing outgrowth and TCPS (Fig. 5b). Cornified envelope-like structures were found on all six materials, but in larger numbers on TCPS and Silastic^{R} (Fig. 5c). Transmission electron microscopy revealed that in general the ultrastructural morphology of the cells (Fig. 6) was comparable to that of explant epithelium. Some of the former cells had a well-developed Golgi apparatus (asterisks in Fig. 6). A small number of the outgrowth cells showed osmiophilic inclusions, probably lipid particles originating from the nutrient medium.

Serial cultivation

Rat middle ear epithelium hardly adhered to polypropylene oxide and could not be serially cultured on this material. The other biomaterials were associated with confluent cell cultures, composed of a single layer of very flat polygonal cells. The ultrastructural morphology of these cells, which tended to partially overlap neighboring cells, was comparable to that of the outgrowth cells (Fig. 7). As in the epithelial outgrowth, desmosomes occurred in areas of contact between cells (inset Fig. 7). Comparison of serially cultured and outgrowth cells showed three differences: ciliated cells were absent in serial culture, cornified envelope-like structures were more numerous in such cultures, and serially cultured cells had fewer microvilli.

Artificial aging

Comparison of the cells cultured in one of the positive control media (Table II) with those given the routine medium showed that cell confluence and morphology were similar (Fig. 8a). Cultures given the negative control medium (PVC) not only

Fig. 2. Scanning electron micrograph of middle ear mucosa explant showing epithelial outgrowth on HPOE/PBT polyether polyester copolymer. Arrows indicate the extent of the outgrowth. Length of explant: 1.8 mm.

Fig. 3. Scanning electron micrograph of ciliated and non-ciliated epithelium of explant on Silastic^{R}. Note varying numbers of microvilli. Bar = 10 µm.

had very few cells but the morphology of those cells differed from that of normal cells (Fig. 8b). Transmission electron microscopy showed the presence of pycnotic nuclei and disrupted cell membranes. The confluence and morphology of epithelium cultured in the simulation medium in the presence of the polyether and the polyester urethane (Figure 8c), as well as the polyether polyester copolymer, resembled those seen in normal serially cultured epithelium.

Cells cultured in Silastic (Fig. 8d) and Dow Corning Elastomer medium often showed a deviating morphology. Confluence did not occur in the culture dishes and a fair proportion of the cells were not flat. Transmission electron microscopy showed that many of the cells exposed of the silicone rubber media had not only morphologically normal cell organelles, but also intracellular structures not found in routinely cultured cells. Structures with prominent membranes having the appearance of dilated smooth endoplasmic reticulum (Fig. 9a, arrowheads) were seen in cells cultured in the silicone rubber media. Those exposed to Silastic medium also showed electron-dense inclusions (Fig. 9a, arrows). The largest bodies measured about 1.2*0.6 µm; the smallest no more than 0.2*0.2 µm. X-ray microanalysis showed that these inclusions contained silicon (Fig. 9b). Cells exposed to the polypropylene oxide medium did not proliferate and tended to die. The few surviving cells had an ultrastructural morphology resembling that of cells cultured with PVC medium (Fig. 8b).

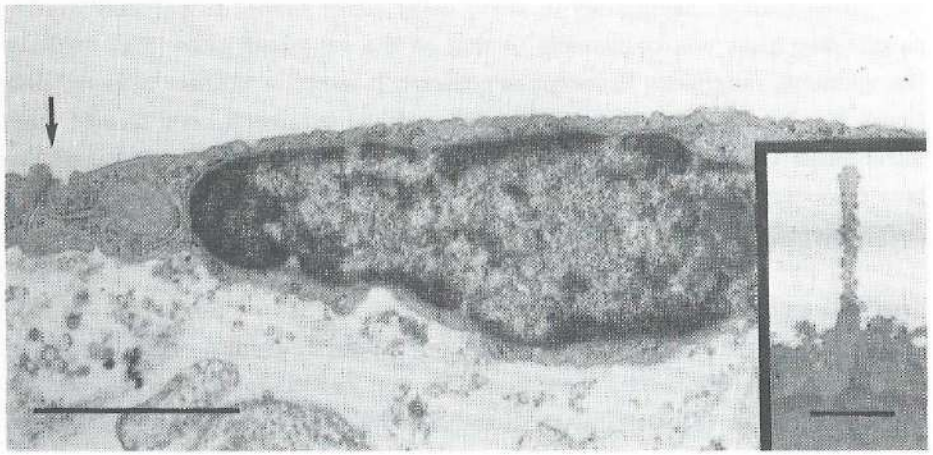


Fig. 4. Transmission electron micrograph of an explant cultured on Dow Corning^{R} Elastomer for ten days. Note the desmosome (arrow). Bar = 3 µm. Inset shows primary cilium (bar = 1.5 µm).

DISCUSSION

We assessed the biocompatibility of several candidate tympanic membrane materials *in vitro* to obtain a basis for selection for our animal implantation studies. Besides quantitation of biocompatibility and sensitivity to toxic materials, *in vitro* tests permit rapid evaluation of the biocompatibility of a large group of biomaterials[12]. Furthermore, during the early screening the assessment of the biocompatibility with human material is by preference performed *in vitro*. Rat middle ear mucosa explants and middle ear epithelium were used for these studies. This choice was determined by four factors. First, in middle ear surgery the epithelial covering of an implant is considered to influence the success of an implant, because the epithelium is thought to play an important role in middle ear defence against the frequent infections with micro-organisms in this region. Second, biocompatibility can be studied *in vitro* in the same tissue[13]. Third, because the explants and cultured cells derive from the rat middle ear, tissue-culture results can be compared with the findings made in implantation studies in the rat middle ear. Lastly, these results can be compared with those concerning the *in vitro* biocompatibility of hydroxyapatite, which has proved to be a highly biocompatible material[3].

With respect to the proliferative activity of the cells under study, there were some interesting findings. TCPS and all of the biomaterials tested except polypropylene oxide - to which cells hardly adhered - showed sigmoid growth curves. The best substrate for cell attachment was TCPS, as indicated by the number of cells adhering to this material after one day of cultivation. Adhesion of epithelium, as indicated by cell numbers on day one, was more or less the same for all of the other biomaterials except polypropylene oxide. This seems to be in conflict with results published by Ratner et al.[20], who reported greater adhesion of chick embryo muscle cells to Pellethane^{R} 80A than to Silastic^{R}. This divergence might be due to the use of different techniques or other types of tissue[21]. Evaluation of the complete growth curves showed significantly lower levels of proliferative activity on Dow Corning^{R} Elastomer and polypropylene oxide than on TCPS. The divergent cell behavior on Dow Corning Elastomer suggested that it was not the cell/substrate adhesion that was responsible for the over-all lower level of proliferative activity but rather the behavior of the cells cultured on this biomaterial. Both cell behavior[22] and cell/substrate adhesion[21] are known to be influenced by the nature of the synthetic substrate.

In our opinion, the alterations in the morphology of explant epithelium reflected exclusively the effects of the presence of toxic substances in the nutrient medium associated with a given biomaterial, because, unlike the situation

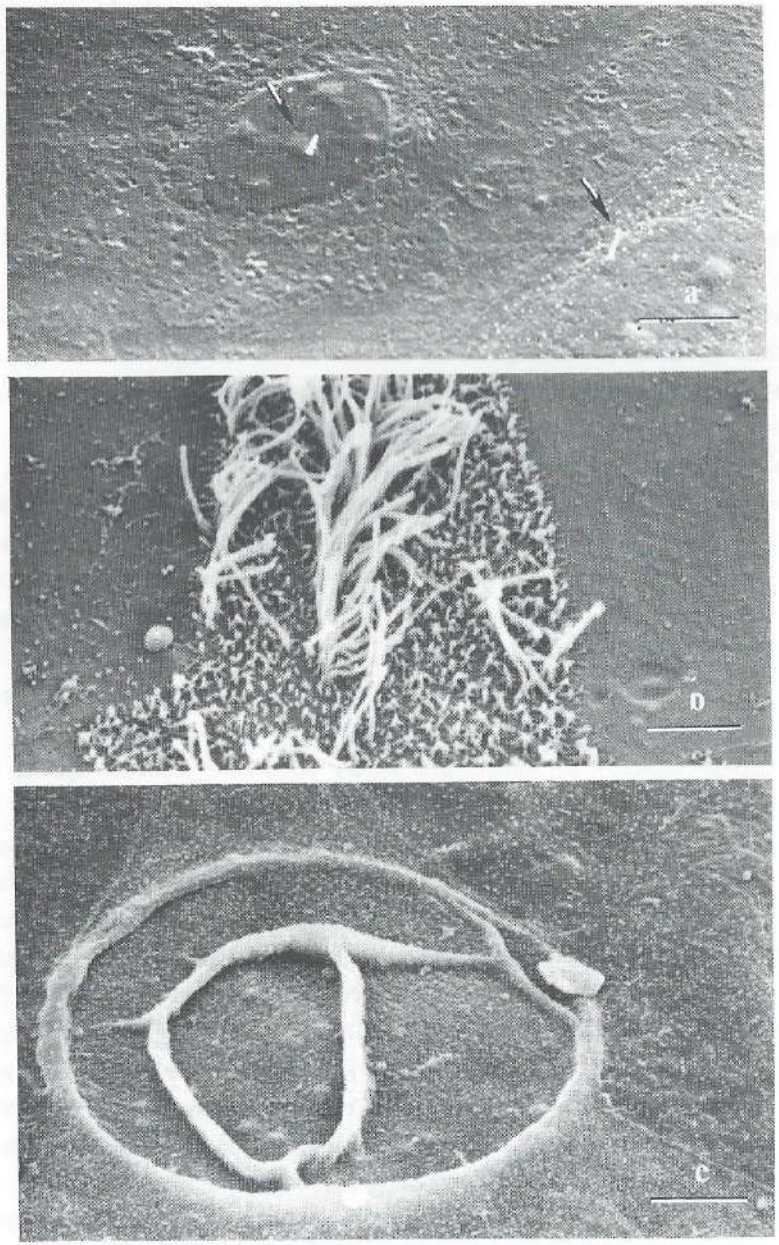


Fig. 5a-c. Scanning electron micrographs showing outgrowth cells.
a: The surface of two cells on Dow Corning^{R} Elastomer bears a primary cilium (arrows). Bar = 10 µm.
b: HPOE/PBT copolymer showing ciliated epithelium. Bar = 10 µm.
c: Epithelium on Silastic^{R}, showing a cornified envelope-like structure characteristic of terminal differentiation. Bar = 10 µm.

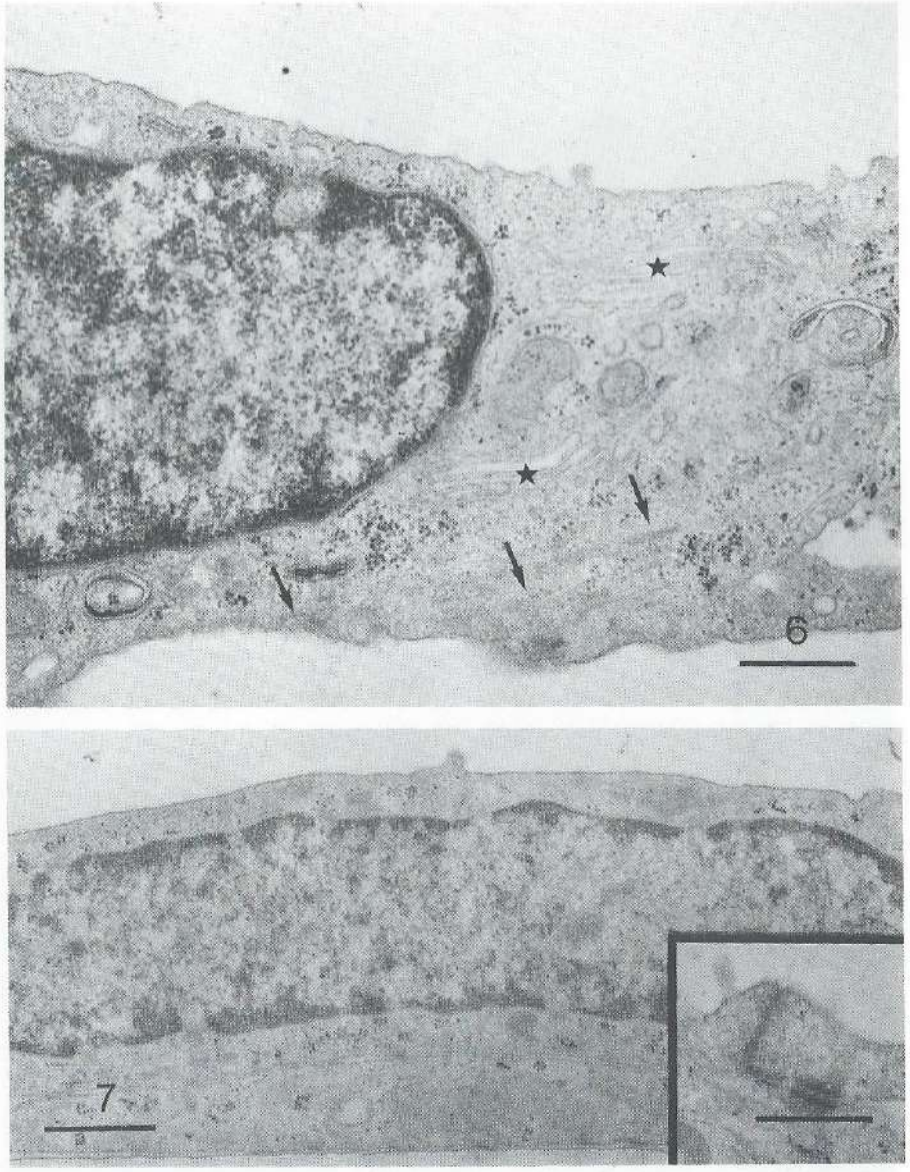
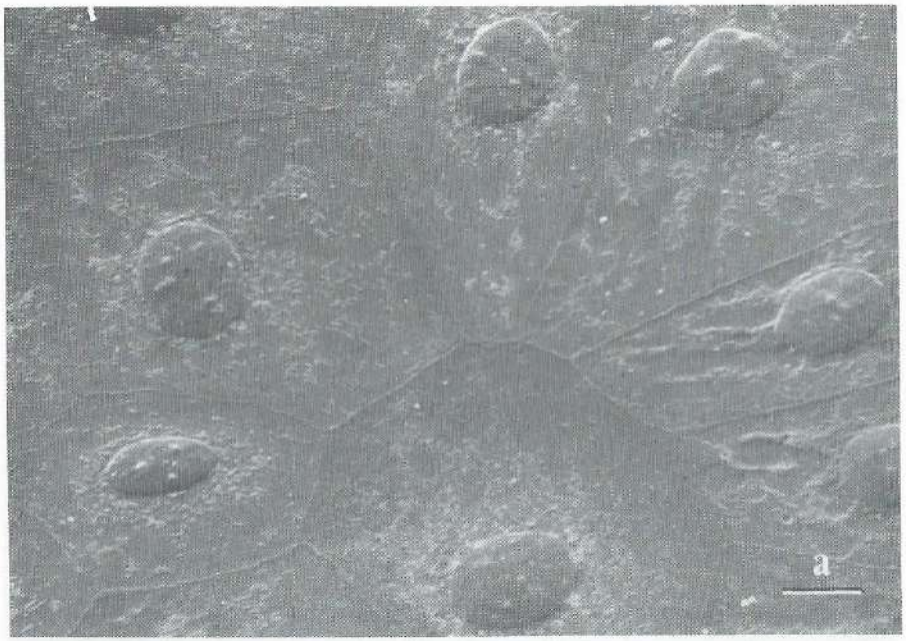


Fig. 6. Transmission electron micrograph showing outgrowth epithelium on Dow Corning^{R} Elastomer. Note the intracellular filaments adjacent to the substrate (arrows) and the well developed Golgi apparatus (asterisks). Bar = 0.6 µm.

Fig. 7. Transmission electron micrograph of serially cultured epithelium on polyether polyester copolymer. Inset: Cell junction with desmosomes and microvilli. Bars = 1 µm.



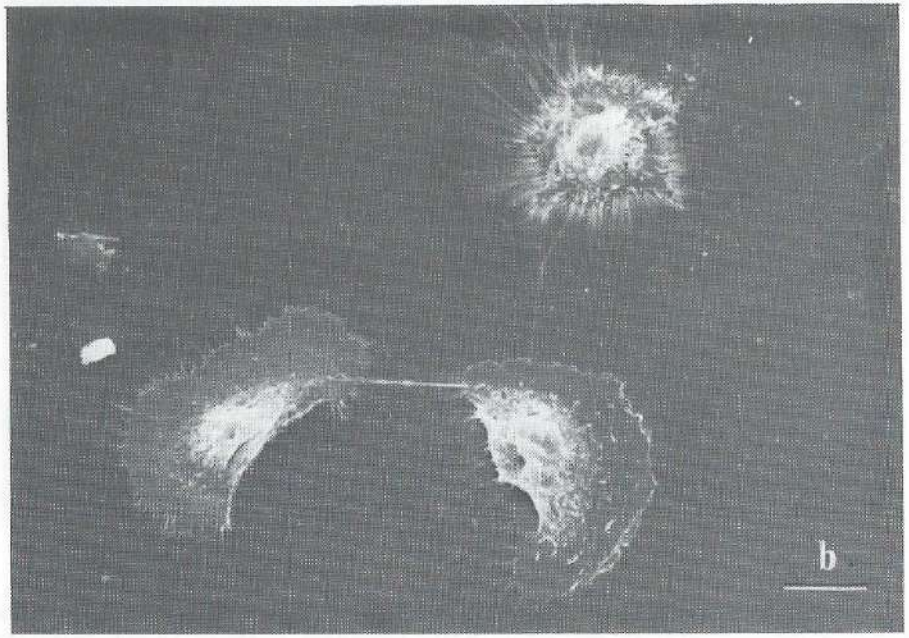
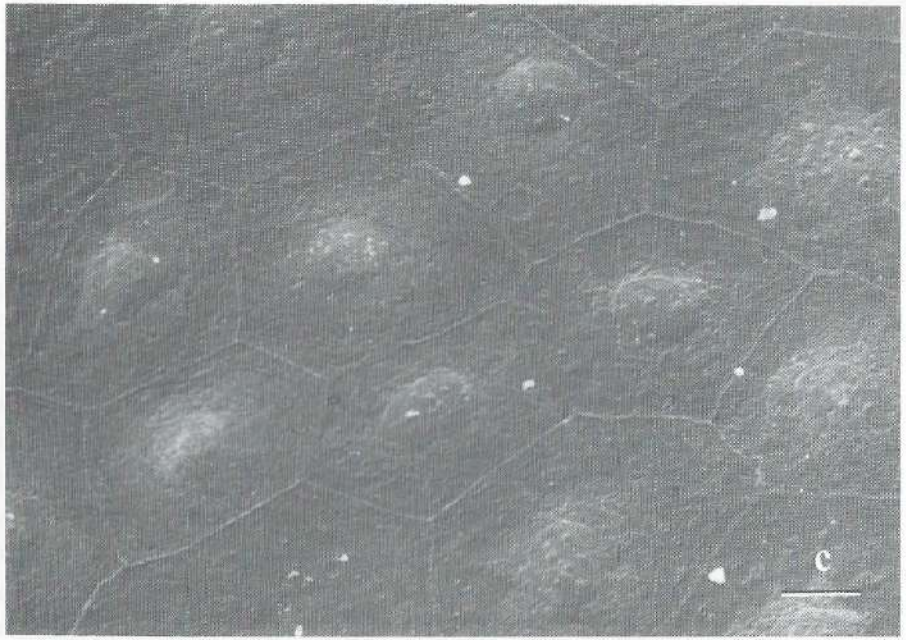
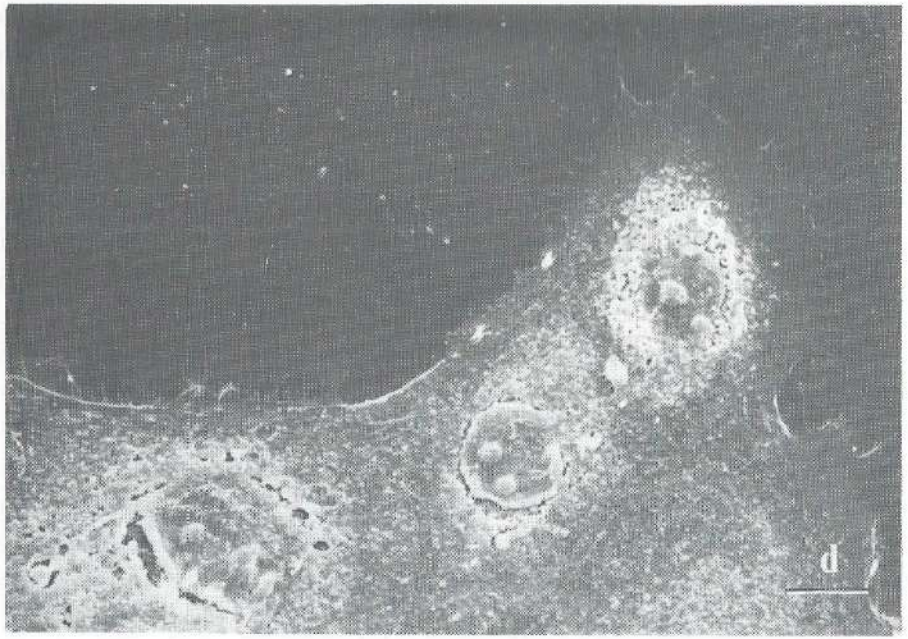


Fig. 8a-d. Series of four scanning electron micrographs showing epithelium cultured in a) routine medium; b) PVC medium (negative control); c) Estane^{R} 5714





F1 medium; d) Silastic^{R} medium. Note the close resemblances between a and c, and the divergence of b, and to a lesser extent of d. Bars = 10 µm.

for serially cultured cells or explant outgrowth, there was no direct contact between explant epithelium and the biomaterial. With respect to toxicity it must be kept in mind that relatively few explants attached to polypropylene oxide. This and the failure of both outgrowth of explants and serial cultures in the presence of polypropylene oxide suggest that toxic substances occurred in the nutrient medium in question. For example, the polypropylene oxide we used might have undergone hydrolytic degradation, resulting in the release of aldehydes. Explants cultured on TCPS and on the other five biomaterials showed cell populations resembling normal rat middle ear epithelium as to both morphologically and diversity.

When TCPS or any of the biomaterials except polypropylene oxide was used as synthetic substrate, flat polygonal cells predominated in explant outgrowths, which also showed small numbers of ciliated epithelial cells and terminally differentiated cells. The presence of terminally differentiated cells and ciliated epithelium on these biomaterials seems to be a positive phenomenon with respect to biocompatibility, since it shows that these biomaterials allow growth of cells more complex than flat polygonal epithelium. Under serial cultivation, the five biomaterials allowing tissue culture showed only flat polygonal cells with a morphology and a morphological diversity comparable to those seen for TCPS. The absence of ciliated epithelium and the morphological diversity associated with serial cultivation resembled the corresponding characteristics reported by van Blitterswijk et al.[13] Finally, the morphology of middle ear tissue cultured on all of the biomaterials studied except polypropylene oxide, was comparable to that of tissue cultured on hydroxyapatite, a biomaterial which showed good biocompatibility both in animals in experimental studies[3-7,19] and in patients when applied clinically[8,9].

The artificial aging of biomaterials was introduced by Homsy, whose experimental results[14] indicated that a relatively short exposure of a biomaterial in a medium comparable to body fluid (PECF) in combination with a high temperature mimics long-term implantation conditions with respect to biomaterial breakdown. The PECF used in our artificial aging experiments was based on the nutrient medium we used for tissue culture[13] and resembled body fluid as to ion concentration (Table I). According to Homsy[14], these ions play the most important role during *in vivo* degradation of an alloplast. PVC served as negative control. This polymer has been reported to be cytotoxic in simulation experiments comparable to ours[14] as well as in extraction experiments[23]. Rat middle ear epithelium serially cultured on TCPS with both control media (Table II) had an ultrastructural morphology comparable to that of epithelium cultured with routine

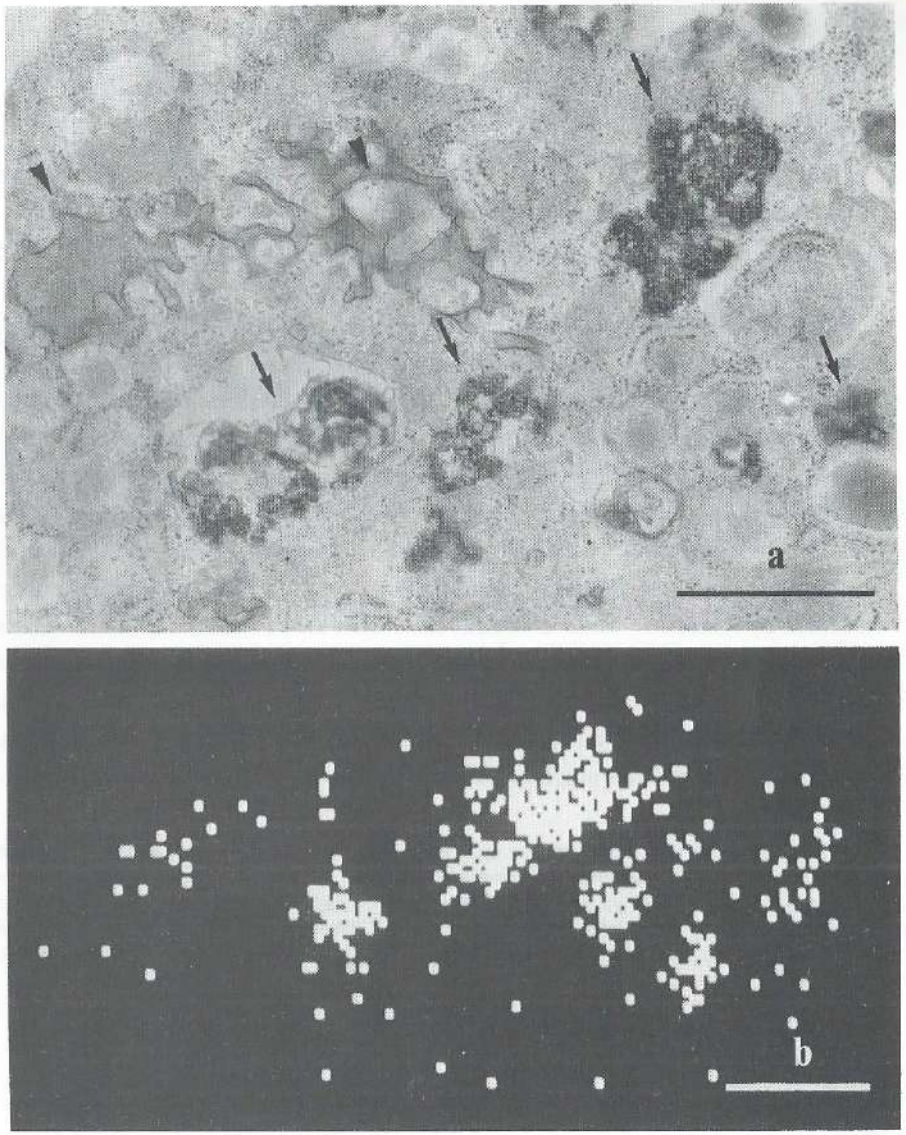


Fig. 9a and b. Transmission electron micrograph of epithelium cultured with Silastic^{R} medium.
a: Note the two types of inclusion (arrows, arrowheads). Inclusions indicated by arrows contain silicon and occurred exclusively in cells cultured with Silastic medium. Bar = 0.6 µm.
b: X-ray mapping for silicon. Cells were cultured for three days with Silastic medium. Bar = 0.6 µm.

medium, which differed from the morphology of cells cultured with the presence of PVC and polypropylene oxide medium. The latter cultures showed only a very small number of less-flat cells with deviant morphology.

Cells cultured on TCPS with Estane^{R}, Pellethane, or polyether polyester copolymer medium showed a morphology comparable to that of the positive controls. When cultures were given medium based on the PECF in which Silastic or Dow Corning Elastomer had been exposed to heat, the cells showed silicon-containing inclusions and/or structures resembling dilated endoplasmic reticulum. Furthermore, confluence of epithelium was not seen in the culture dishes. It was remarkable that inclusions containing silicon were only found after exposure to Silastic medium and not after Dow Corning Elastomer medium, because both of these biomaterials are silicone rubbers. Differences in simulation conditions can be excluded, because all simulation experiments were reproducible. The discrepancy may mean that the breakdown process is not the same for these two silicone rubbers. The intracellular presence of silicon indicates endocytosis of Silastic-derived material by the epithelium. The finding of inclusions larger than 0.2*0.2 µm suggests an accumulation process, because all media used for tissue culture were sterilized by passage through a 0.22 µm Millipore^{R} filter. On the basis of the divergent confluency and cell morphology seen in this simulation study, we consider polypropylene oxide, Dow Corning Elastomer, and Silastic to be less biocompatible than the polyurethanes and the polyether polyester copolymer.

TABLE III. Results of comparison of the six biomaterials with the positive controls in the quantitative, morphological, and aging studies *in vitro* (+ = similarity; - = dissimilarity; +/- = intermediate). The biomaterials are ranked in order of decreasing biocompatibility, according to the combined results of the three studies. In cases of equal ranking, the biomaterials are placed alphabetically.

Biomaterial	Quantitation	Morphology	Aging
HPOE/PBT Copolymer	+	+	+
Estane^{R}	+	+	+
Pellethane^{R}	+	+	+
Silastic^{R}	+	+	+/-
Elastomer	-	+	+/-
Polypropylene oxide	-	-	-

Homsy[14] did not report the presence of intracellular silicon in Silastic-treated cells, whereas other authors have described silicon in tissue. For example, Travis et al.[24] not only observed fragmentation of the silicone rubber used to isolate certain devices such as pacing catheters but also found the fragments embedded in tissue vegetations, and Landis et al.[25] found intra- and extracellular silicon in

vertebrate bone tissue. In the absence of long-term *in vivo* investigations it is impossible even to speculate about a relationship between silicon and biocompatibility.

In sum, it may be concluded on the basis of the results of the three *in vitro* studies (quantitative, morphological, and artificial aging; Table III) that the biocompatibility of the polyurethanes and HPOE/PBT polyether polyester copolymer is better than that of the silicone rubbers Silastic and Dow Corning Elastomer. Polypropylene oxide was unequivocally the least biocompatible material. Final conclusions concerning the biocompatibility of these biomaterials must await the results of *in vitro* testing of human cells and extensive implantation studies in rats now in progress.

Acknowledgements

The authors are indebted to Mr. H.K.Koerten (Laboratory for Electron Microscopy, Leiden University) for the x-ray microanalyses. The support of Dr. J.H.Kuit and Dr. J.G.F.Bots (Department of Chemical Technology, Twente University of Technology, Enschede) in the preparation of the polyurethane, polypropylene oxide, and polyether polyester copolymer biofilms is gratefully acknowledged, as is the technical assistance of Ms. P. van der Hoek, Mr. B.M.A. van der Lans, Mr. J.J.Beentjes, Mr. L.D.C.Verschragen, and Mr. S.B.Neijman.

These investigations were supported by the Netherlands Technology Foundation (STW).

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CHAPTER 3

TYMPANIC MEMBRANE STRUCTURE DURING A *STAPHYLOCOCCUS AUREUS*-INDUCED MIDDLE EAR INFECTION. A study in the rat

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SUMMARY

In response to a *Staphylococcus aureus*-induced middle ear infection the tympanic membrane showed infiltration of polymorphonuclear granulocytes, lymphocytes, and macrophages and increased areas covered by ciliary and secretory epithelium. These reactions, which were comparable to the cellular and mucociliary responses seen in the middle ear mucosa during infection, were restricted to the pars flaccida and to predominantly the annular and manubrial regions of the pars tensa. This showed that the greater part of the tympanic membrane, where the lamina propria is composed of collagenous bundles and only very thin layers of loose connective tissue, is hardly affected by or barely responds to the inflammatory stimulus.

INTRODUCTION

Most of the studies done on otitis media have been restricted to an evaluation of the events in the middle ear mucosa. Both clinical and animal experiments led to the conclusion that the middle ear response to otitis media is characterized by five events. The presence of e.g. polymorphonuclear granulocytes, macrophages, and lymphocytes indicates a cellular response[1-3] and the demonstrated synthesis of immunoglobulins A, G, and M a humoral immune response[1,4]. Further, the activity and size of the area covered by the middle ear mucociliary tracts are known to increase during an infection[3,5,6], and increased proliferative and migratory activity of fibrous tissue and a bony response have also been reported[3,5,7].

Very little attention has been paid to the course of events in the tympanic membrane during infection, with the exception of the frequent occurrence of typanosclerotic plaques in chronic otitis media[8,9] and structural changes in the pars flaccida during serous and purulent otitis[10]. The present study was performed to investigate the structural changes in the rat tympanic membrane accompanying a *Staphylococcus aureus*-induced middle ear infection. The *S.aureus*-infected rat middle ear was taken as experimental model because the mucosal responses are

characteristic for otitis media[3].

MATERIALS AND METHODS

Experimental procedures

Eighty-four tympanic membranes originating from male Wistar rats (body weight 200 gr) were divided into three groups:

Group 1: Thirty-two serving as controls.

Group 2: Thirty-two excised from infected middle ears. To establish the infection, the animals were anesthesized by an intramuscular injection of Hypnorm^{R} (100 µl/100 gr body weight), and after the bulla had been reached via a dorsal approach, a burr hole was made in it for the introduction of 0.1 ml of a suspension of *Staphylococcus aureus* in sterile physiological saline[3] containing approximately 1.4 x 10^7 bacteria.

Group 3: Twenty originating from rats given an injection of 0.1 ml sterile physiological saline in each ear. The middle ears of group 1 (the controls) were dissected and eight were processed for light microscopy (LM), 14 for transmission electron microscopy (TEM), and ten for scanning electron microscopy (SEM). Treated rats were decapitated after observation periods of one, three, seven, and 21 days and the ears were processed for the studies shown in Table I.

TABLE I. Distribution of the middle ears of the rats in groups 2 and 3 over the evalution techniques, according to the duration of the observation period (in days).

Technique	LM		TEM		SEM		Total	
Group	2	3	2	3	2	3	2	3
Period								
1	4	2	2	2	2	2	8	6
3	4	-	2	2	2	2	8	4
7	4	2	2	2	2	2	8	6
21	4	-	2	2	2	2	8	4
Total	16	4	8	8	8	8	32	20

Light microscopy

The middle ears were fixed for 2 hr in 2% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M Sörensen's buffer (pH 7.4, 4°C) followed by 14 hr in the same fixative without glutaraldehyde, and were then decalcified for 4 weeks in a 10% EDTA solution containing 2% paraformaldehyde (pH 7.4, room temperature).

After being rinsed in running tap-water for 24 hr, the specimens were routinely embedded in Paraplast^{R}. Sections (6 µm thick) were stained with hematoxylin-eosin.

Transmission and scanning electron microscopy

The ears destined for TEM were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer for 16 hr (pH 7.4, 4°C) and decalcified in 10% EDTA and 1.5% glutaraldehyde in distilled water (pH 7.4) for 4 weeks at room temperature. After being rinsed in running tap-water for 24 hr, the tympanic membranes were cut into several pieces. The pars flaccida was separated from the pars tensa, which was divided into three portions: one containing the manubrium, one taken peripherally and including the annulus, and one from the region between the annulus and manubrium. Pars flaccida and pieces of pars tensa were postfixed in 1% OsO_4 (30 min, room temperature), rinsed in phosphate-buffered saline, dehydrated in a graded alcohol series, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips 201 transmission electron microscope. For the detection of elastic fibers, part of the material was fixed in the same glutaraldehyde fixative but with 1% tannic acid added. The tympanic membranes destined for SEM were fixed in 1.5% glutaraldehyde, dehydrated, critical point dried, gold-sputtercoated, and studied in a Cambridge S180 scanning electron microscope.

RESULTS

Normal tympanic membrane morphology

The tympanic membrane of the rat was composed of a 6- to 15-µm thick slightly oval pars tensa, and a 40- to 80-µm thick pars flaccida (Fig. 1). The pars tensa, which was suspended at the annulus, had a diameter of about 3 mm and an area 4 to 5 times greater than that of the pars flaccida. Both the pars tensa (Fig. 2a) and the pars flaccida (Fig. 3a) were lined with an epidermis which was continuous with the external ear skin, a lamina propria, and an epithelium on the middle ear side.

Pars tensa

The thin epidermis consisted of the inner cell layers of the stratum Malpighii, which could be subdivided into the strata basale, spinosum, and granulosum, and the outermost laminated stratum corneum. The keratinocytes of the stratum basale were connected by hemidesmosomes to a basal lamina and contained tonofilaments,

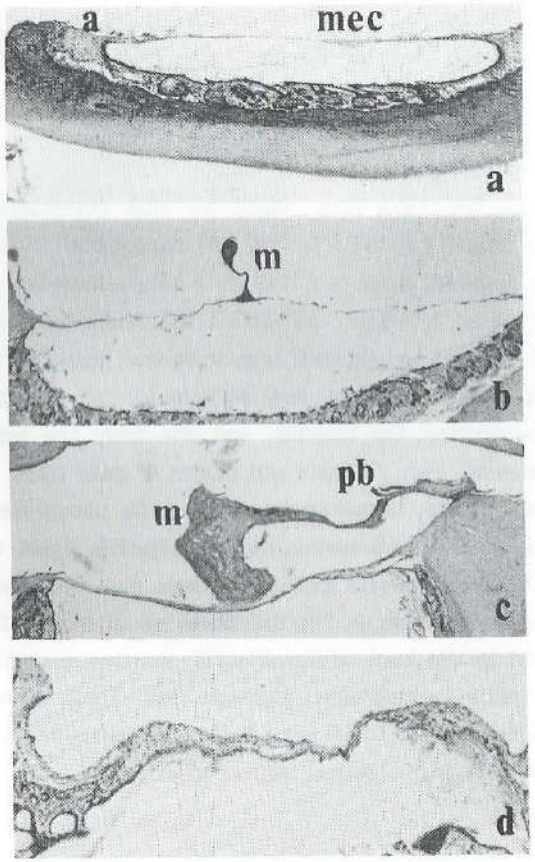


Fig. 1 a-d. Light micrographs of cross-sections of the tympanic membrane. x 20.
a: Section showing both superior quadrants of the pars tensa. A = annulus; MEC = middle ear cavity.
b: Pars tensa with manubrium (M).
c: Malleus (M) and processus brevis (PB). Note the thickness of the pars tensa.
d: Section of the pars flaccida. Note the relatively thick membrane.

like all Malpighian layers. The cells of the granular layer contained many keratohyalin granules associated with the tonofilaments. Desmosomes connected the cells of the Malpighian layer. The thickness of the stratum corneum varied between two and five layers and overlapping of cells was very prominent.

The lamina propria was composed of thin subepidermal and subepithelial layers of loose connective tissue bordering relatively thick radiate and circular collagenous bundle layers, respectively (Fig. 2a). Fibroblasts and collagen made up the connective-tissue layers. Those of the annular and manubrial portions, being the best-developed layers, also showed macrophages, lymphatics, capillaries, and mast cells. Most of the mast cells lay near nervers and capillaries (Fig. 2b).

The epithelium of the pars tensa was composed of a single layer of predominantly very flat polygonal cells. Keratohyalin granules and cornified envelopes were not seen. In the annular region the cells were less flat and sometimes had microvilli on their apical side. Small numbers of ciliated cells were seen near the annulus in the superior half of the pars tensa. Secretory cells were absent.

Pars flaccida

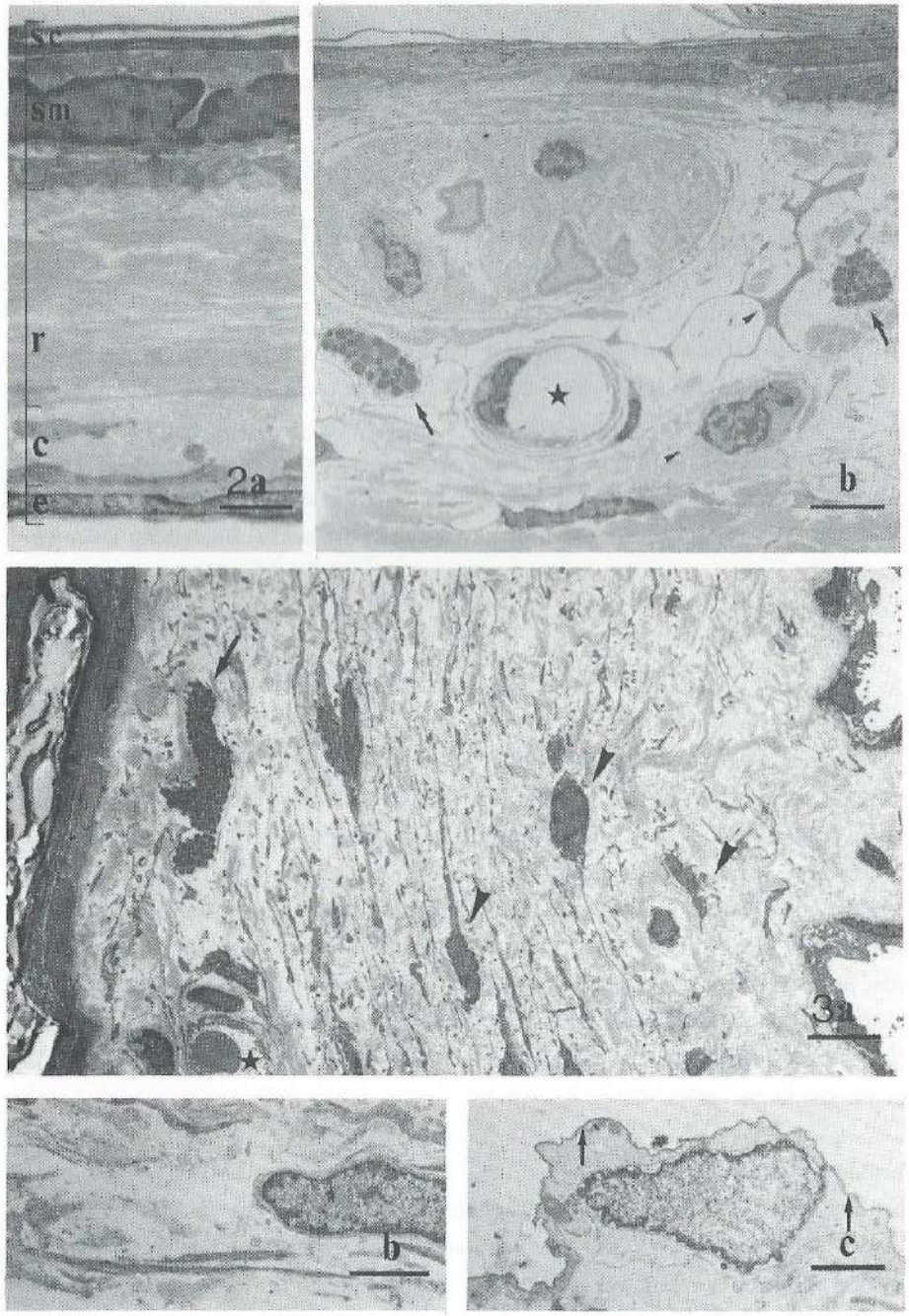
The epidermis of the pars flaccida closely resembled that of the pars tensa but the keratinocytes were not as flat.

The lamina propria of the pars flaccida was thicker than that of the pars tensa and was composed entirely of randomly organized collagen and elastic fibers (Fig. 3b). Nerve fibers, capillaries, lymphatics, mast cells, fibroblasts, and some macrophages were seen mainly close to the epidermis (Fig. 3a). The central portion of the lamina propria contained fibroblasts and an occasional blood vessel.

The epithelium was composed predominantly of a single layer of flat polygonal cells, many of them bearing microvilli and containing small numbers of tonofibrils. Pseudo-stratified epithelium, cells with secretory granules, and ciliated cells, also occurred. Some epithelial cells showed an electron-dense zone associated with the plasma membrane (Fig. 3c).

Morphology after infection

One day after the injection of *Staphylococcus aureus*, the middle ear cavity showed variable amounts of mucopurulent fluid containing large numbers of polymorphonuclear granulocytes and fewer lymphocytes and erythrocytes. The fluid frequently covered the middle ear mucosa and part of the tympanic membrane epithelium, especially the pars flaccida and the region of the annulus. The middle ear mucosa had become edematous and showed numerous dilated capilaries and



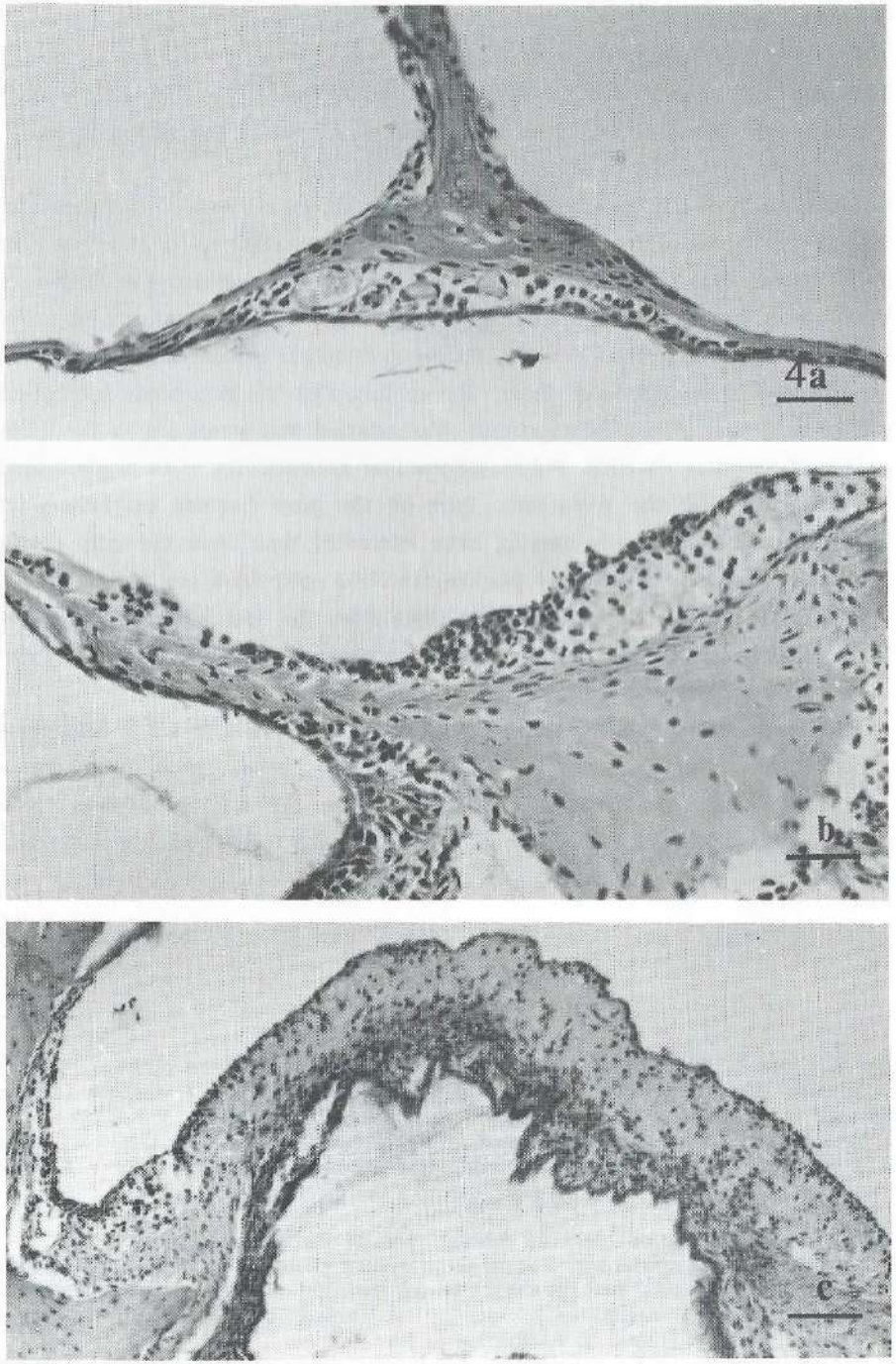
round cell infiltrates as well as ingrowth of fibrous tissue into the middle ear cavity and resorption and genesis of bulla bone. Furthermore, the middle ear epithelium had become swollen and the area occupied by the mucociliary tracts had increased. Mucosal reactions were most prominent in the region where the bulla had been penetrated for the injection of *S.aureus*.

The connective tissue of the annular and manubrial regions of the pars tensa showed edema and dilated capillaries as well as mast cells and polymorphonuclear granulocytes (Fig. 4). The epithelium covering the annulus and manubrium was sometimes more cuboidal and bore more microvilli than the epithelium of normal ears, and showed signs of damage. Polymorphonuclear granulocytes were easily distinguished in the epithelial layer. The epidermis of the pars tensa had become thicker than that of non-infected ears. Vasodilation was prominent in the lamina propria of the pars flaccida. Polymorphonuclear granulocytes were predominantly located just below the epithelium. Part of the pars flaccida epithelium was composed of cuboidal cells bearing more microvilli than were normally present (Fig. 5a). The area covered by pseudo-stratified epithelium and the number of ciliated cells were both increased. At some sites the epithelium was damaged. Degranulating mast cells (Fig. 5b) and macrophages containing microbial debris were seen regularly. The epidermis was thickened.

Middle ears injected with physiological saline showed only small amounts of mucopurulent fluid containing relatively few cells. Small numbers of polymorphonuclear granulocytes were observed in the slightly swollen lamina propriae. Vasodilation was insignificant. Some of the epithelium was cuboidal.

Fig. 2 a and b. Transmission electron micrographs of the pars tensa.
a: Section showing stratum corneum (SC) and Malpighii (SM), radiate (R) and circular (C) collagenous-bundle layers, and epithelium (E). Bar = 1.5 µm.
b: Nerve bundle in subepidermal connective tissue of the manubrium. Note mast cells (arrows), fibroblasts (arrowheads), and capillary (asterisk). Bar = 4 µm.

Fig. 3 a-c. Transmission electron micrographs of the pars flaccida.
a: Section showing epidermis, lamina propria, and epithelium. Note the dispersed fibroblasts (arrowheads) and the mast cell (arrow) and capillary (asterisk) close to the epidermis. Bar = 5 µm.
b: Elastic fibers adjacent to a fibroblast. Bar = 2 µm.
c: Epithelium with electron-dense zone (arrows) suggesting terminal differentiation. Note the absence of cell organelles. Bar = 2 µm.



After three days the number of inflammatory cells (polymorphonuclear granulocytes, lymphocytes, and macrophages) was decreased. These cells occurred chiefly near the handle of the malleus and the annulus. Both areas showed edema and dilated capillaries (Fig. 6a). The area covered by ciliated epithelium had become larger. The pars flaccida showed more severe edema and vasodilation and had larger numbers of inflammatory cells than the pars tensa. Its connective tissue layer was composed of numerous spindle-shaped fibroblasts and mast cells. The epithelium showed strongly vacuolated cells and increased numbers of cuboidal, secretory, and ciliated cells.

The structure of the tympanic membrane of rats injected with physiological saline was the same as that on day one.

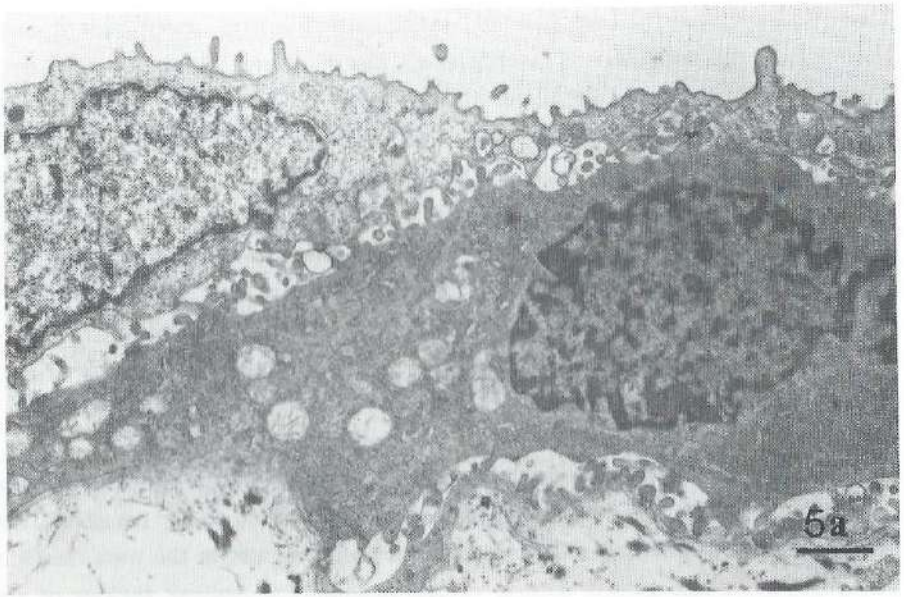
After one week, little mucopurulent fluid remained in the tympanum. The presence of inflammatory cells in the pars tensa was limited to the periphery, and vasodilation and edema had greatly diminished there but not in the pars flaccida. The thickness of the lamina propria of the annular and mallear regions of the pars tensa was almost normal and there were few mast cells. Compared with the situation on the third day, the location and number of cuboidal, ciliated, and secretory active cells were unchanged (Fig. 6b). Macrophages containing microbial debris were seen in both the pars tensa and the pars flaccida.

The structure of the tympanic membrane of rats given physiological saline did not differ from that in normal ears except for the presence of more cuboidal cells in the pars flaccida.

Three weeks after induction of the infection, capillary dilation and thickening of the epidermis and epithelium had almost disappeared and were restricted to the parts of the tympanic membrane most strongly affected by the infection (Fig. 7), i.e., the handle of the malleus, the annulus, and the pars flaccida.

Tympanic membranes of the physiological-saline group were very similar to those of normal ears.

Fig. 4. Light micrographs of the mallear (a) and annular (b) region of the pars tensa and of the pars flaccida (c) 3 days after onset of infection. Note vasodilation, inflammatory cells, and edema. Bars = 50 µm.



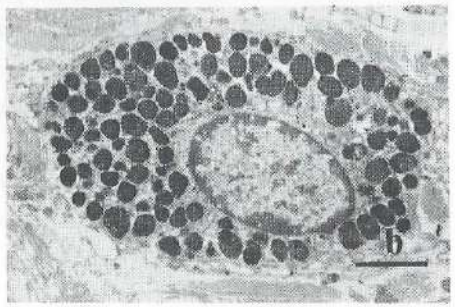
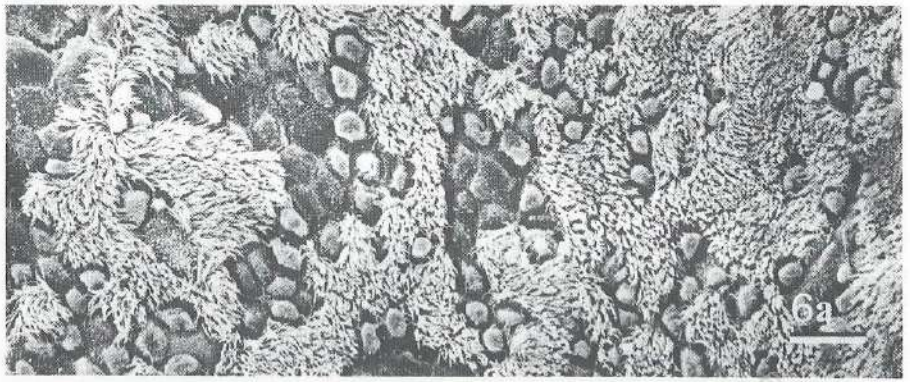
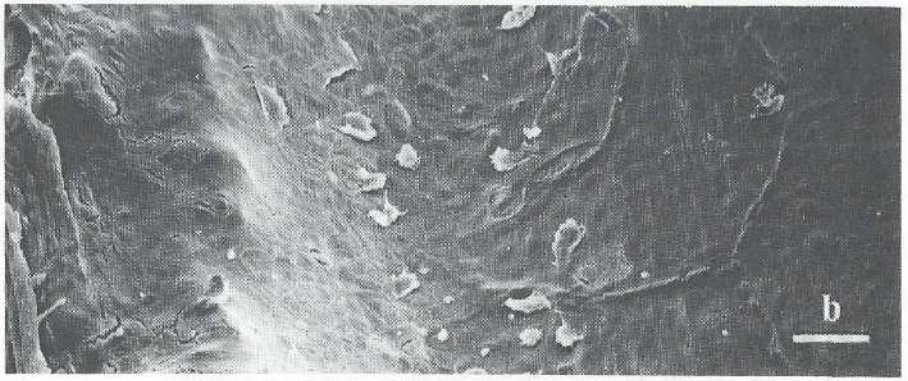


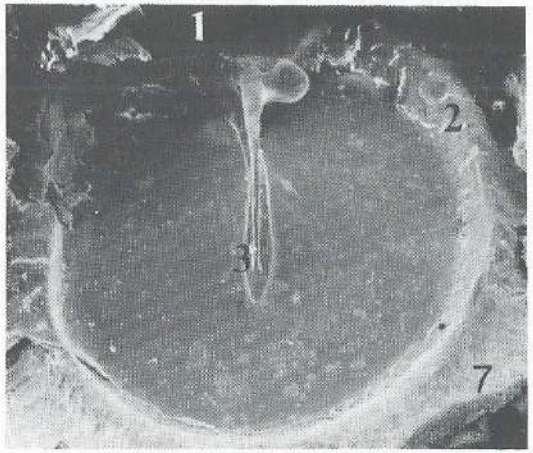
Fig. 5 a and b. Transmission electron micrographs of the pars tensa one day after the infection, showing pseudo-stratified epithelium (a) and a mast cell (b). Bars = 1 µm.

Fig. 6 a and b. Scanning electron micrographs of the annular region of the pars tensa showing vasodilation and exudate three days after infection (a) and ciliated and cuboidal cells after the seventh day (b). Bars = 30 µm.

Fig. 7. Scanning electron microscopical survey of the rat tympanic membrane showing regions most affected by infection: pars flaccida (1), and annular (2) and mallear (3) regions of the pars tensa. Tympanic membrane diameter is 2.7 mm.







DISCUSSION AND CONCLUSIONS

The structure of the rat tympanic membrane was comparable to that of man[11,12] and other animals[13-15], although small species differences exist[13]. The structure of the pars tensa deviated from that of the pars flaccida predominantly in the lamina propria and epithelium. In both cases the epidermis was rather similar and comparable to that of skin[16]. The lamina propria of the rat pars tensa commonly showed strongly orientated layers of collagenous bundles sandwiched between thin layers of connective tissue, which is the usual picture[11-15]. The connective-tissue layers were most prominent near the annulus and the manubrium and their composition was rather similar to that of the lamina propria of the pars flaccida, which was composed of loose connective tissue. As in man and other animals, however, the pars flaccida connective tissue was thicker[11-15] and had elastic fibers[12,14,15] and larger numbers of mast cells[17].

To restrict the study only to the effects of a *Staphylococcus aureus* infection on the tympanic membrane, we used the bullar route for infection instead of the route via the tympanic membrane, which was used by Grote and van Blitterswijk in a comparable study to evaluate the mucosal reactions[3]. Because the course of events in the mucosa after infection under our experimental conditions was comparable to that described by Grote and van Blitterswijk[3], the effects on the middle ear induced by both experimental procedures may be compared.

Mast cell degranulation, vasodilation, edema, infiltration by inflammatory cells, and phagocytosis of bacteria not only may take place in the infected middle ear mucosa[1-3,5,7], but after *S.aureus* infection they were also seen in the lamina propria of the rat tympanic membrane. These responses were very prominent and persisted in the annular and manubrial regions of the connective tissue of the pars tensa and in the lamina propria of the pars flaccida. Radiate and circular collagenous bundle layers of the pars tensa appeared to be unaffected.

The epithelial lining of the normal rat tympanic membrane consisted of a single layer of predominantly flat polygonal epithelium ranging from very flat (pars tensa) to pseudo-stratified (pars flaccida). Part of the epithelium of the pars flaccida contained structures suggestive of terminally differentiating epithelium[18,19] and resembled the cornified envelope of normal middle ear Eustachian tube epithelial lining[19] and cultured rat middle ear epithelium[18]. Both parts of the rat tympanic membrane showed ciliated epithelium, whereas secretory cells were restricted to the pars flaccida as in the cat[13]. However, Widemar et al.[10] did not observe ciliated cells on the rat pars flaccida, and the reports concerning the human tympanic membranes are conflicting[11,12].

After the onset of a *S.aureus* infection, the number of ciliated and secretory

cells increased first and most prominently in the pars flaccida. Increased secretory activity of tympanic membrane epithelium has been reported in patients with chronic otitis media[11], and ciliated cells on the rat pars flaccida are pathognomic for a long-standing infection[10]. These and the present findings suggest that the activity of secretory and ciliated cells on the tympanic membrane might contribute in middle ear clearance[6], as has been reported for the mucociliary tracts of the middle ear[3]. Other epithelial changes included an increase in the area covered by pseudo-stratified epithelium (as also seen during purulent otitis media[10]) and cell damage (also seen in the epithelial lining of the rat middle ear cavity after *S.aureus* infection[3]), which might have led to increased tympanic membrane permeability to *S.aureus*. Changes in the tympanic membrane structure after infection was attributed mainly to the presence of *S.aureus*, because the tympanic membranes of rats injected with sterile physiological saline were significantly less severe affected.

In sum, the structure of the rat tympanic membrane is comparable to that in man and other animals. The presence of *S.aureus* in the middle ear cavity induced cellular and epithelial responses in the rat tympanic membrane comparable to cellular and epithelial events occurring in the rat middle ear mucosa during infection. These responses were most prominent in the pars flaccida and in the annular and manubrial region of the pars tensa, i.e., where the loose connective tissue was most prominent. The greater part of the rat tympanic membrane was hardly affected by or barely responded to the induced infection.

Acknowledgements

The authors wish to thank Dr. W.Kuijpers (ENT Research Laboratory, Radboud Hospital, Nijmegen) for discussion of the manuscript, and Mr. B.M.A. van der Lans and Ms. P. van der Hoek (ENT Department), as well as Mr. L.D.C.Verschragen, Mr. S.B.Neijman, and Mr. J.J.Beentjes (Laboratory for Electron Microscopy) for technical assistance. The cooperation given by the Laboratory of Bacteriology and Parasitology and the Laboratory for Experimental Surgery of the University of Leiden is also acknowledged with gratitude.

These investigations were supported by the Netherlands Technology Foundation (STW) and by the Heinsius Houbold Fund.

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CHAPTER 4

BIOCOMPATIBILITY OF ESTANE[R] POLYURETHANE, POLYPROPYLENE OXIDE, AND HPOE/PBT (55/45) POLYETHER POLYESTER COPOLYMER. A qualitative and quantitative study in the rat middle ear.

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SUMMARY

The biocompatibility of porous implants made of Estane[R] 5714 F1 polyether urethane, polypropylene oxide, and a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer was assessed after implantation in rat middle ears for periods of up to one year. These elastomers were selected as candidates for an alloplastic tympanic membrane to serve as tissue scaffolding in a totally alloplastic middle ear to obtain permanent fixation of the ossicular chain to the canal-wall segment of this prosthesis.

Implantation of the materials led to tissue reactions initially associated with the wound-healing process and characterized by the exudate containing polymorphonuclear granulocytes, lymphocytes, macrophages, and plasma cells, increased cell-proliferative activity, and abundant collagen synthesis, whereas especially after one month not only the presence of macrophages and foreign-body giant cells surrounding the implant materials but also implant degradation were characteristic for a foreign-body reaction.

Macrophages and giant cells dominated the picture of the tissue surrounding polypropylene oxide. The altered morphology of these cells, the persistent infiltration of the implantation sites by exudate cells, and the premature death of five rats in the one-year group suggest that polypropylene oxide degradation was accompanied by the release of toxic substances. Estane and copolymer degradation did not induce tissue responses reflecting implant toxicity, and tympanic membranes given these alloplasts showed a normal healing pattern. Inclusions in the cytoplasm of macrophages associated with degradation and phagocytosis of all of the polymers under study were found to contain iron, silicon, titanium, and aluminum. This suggests that storage of polymer-derived trace elements had taken place, which underscores the importance of using only implant materials of the purest grade.

Growth of fibrous tissue and bone, the latter into Estane and HPOE/PBT copolymer implants, indicated appropriate implant fixation by tissue, which is important for adequate functioning of the alloplastic tympanic membrane, although macrophages and foreign-body giant cells were present as well. Especially the fixation of copolymer by ingrowth of bone seems promising in terms of the amount of bone in the pores and the electron-dense bone/copolymer interface. The latter is indicative for bonding osteogenesis. The HPOE/PBT copolymer is a better candidate for alloplastic tympanic membrane than Estane, and the use of polypropylene oxide cannot be recommended.

INTRODUCTION

For the reconstruction of the sound-conducting system after the eradication of diseased tissue, a total alloplastic middle ear prosthesis (TAM[1,2]) has been developed in our department. This prosthesis, which consists of an ossicular chain and a canal-wall segment to close the defect in the bony meatus[1,2], requires an alloplastic tympanic membrane for attachment of the dense hydroxyapatite ossicular chain to the macroporous hydroxyapatite canal-wall segment, the membrane acting as a scaffolding for tissue overgrowth.

Various grafts[3-5] (i.e., biological materials), have been used with varying success for closing persistent tympanic membrane perforations, whereas alloplasts have either been unsuccessfull in clinical applications (Polyglactin 910[6] and Gelfoam[7]) or only evaluated in animal experiments. Examples of experimentally used materials are polyvinyl alcohol[8], methyl-2-cyanoacrylate[9], and poly(D,L)lactic acid, polyglycolic acid, copoly(α-amino acid), polytetrafluoroethylene, and bisphenol-A polycarbonate[10,11]. Polymer failure can be attributed to the lack of scaffolding properties[8,11], e.g. because of the absence or unfavorable structure of the pores, inflammatory and foreign-body reactions[6,9-11], and early implant degradation[7,12]. The success of biological materials seems to be related to the fact that grafts have sufficient scaffolding properties[4,5] (e.g. because they include a connective-tissue layer[3]), are rapidly covered by epithelium[3], do not develop pathologic phenomena[5] such as severe inflammation[4], and stay in close contact with the perforated tympanic membrane[5,4].

In view of all this it is concluded that for successful application in the TAM, the alloplastic tympanic membrane should offer a pliable scaffolding for tissue overgrowth, be macroporous to give better implant fixation by tissue[13], and induce minimal inflammation and foreign-body reactions. Furthermore, the alloplast should become covered with epithelium, which is important for maintaining the integrity of the prosthesis[14] and decreases the risk of infection[15]. Although final degradation is not obligatory it is advantageous for optimal hearing results[5]. However, if it does occur it must not lead to the release of toxic substances[16] causing local or systemic effects[16,17]. Finally, the alloplast must be an elastomer, because otherwise it will not vibrate sufficiently upon sound stimulation[18].

The present study was performed to evaluate the response of tympanic membrane tissue, middle ear mucosa, and muscle tissue of the rat to, as well as the degradation behavior of, macroporous implants made of the elastomers Estane^{R} 5714 F1 polyether urethane, polypropylene oxide, and a poly(ethylene oxide hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer.

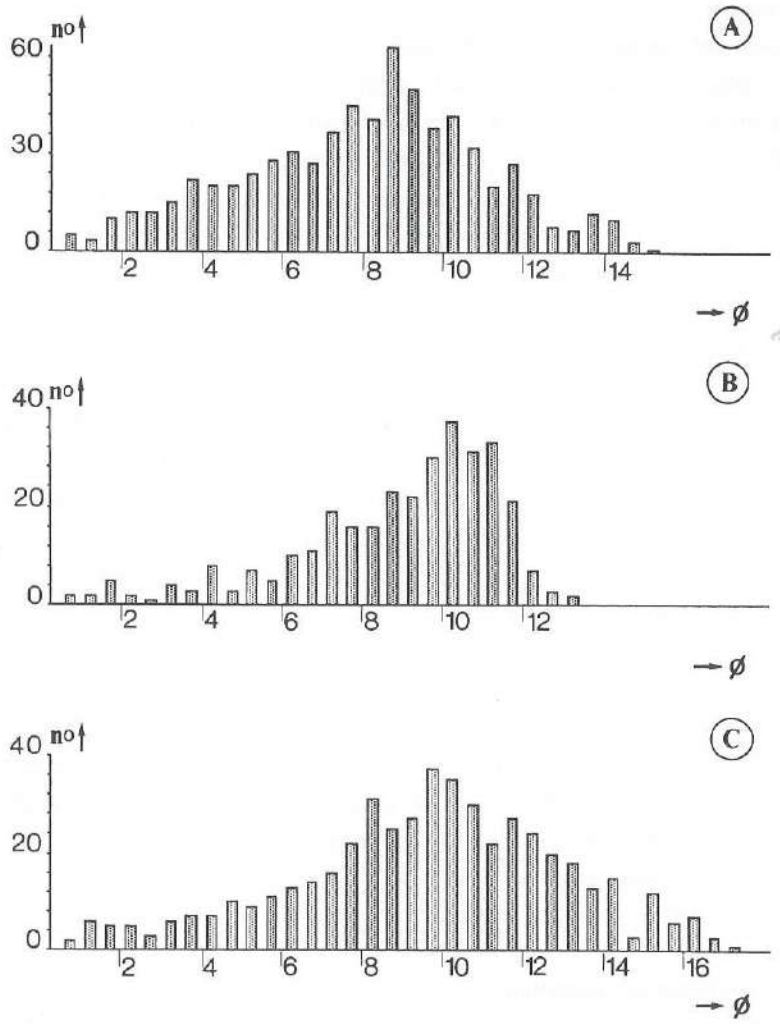


Fig. 1. Distribution of the pore diameters in the implants made of Estane^{R} polyether urethane (a), polypropylene oxide (b), and HPOE/PBT segmented polyether polyester copolymer (c).

MATERIALS AND METHODS

Implant materials

Implants made of porous films of Estane[R] 5714 F1 polyether urethane (BF Goodrich[19]), polypropylene oxide[20], and poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer (HPOE/PBT 55/45; Akzona Inc.[21]) were used. Porous films made of Estane and copolymer were prepared according to a saltcasting technique. Polypropylene oxide was liquified and fibres were spun[20] and then wound on a rotation axis to produce porous "woven" tubings which were cut lengthwise to produce the films. Scanning electron micrographs of gold sputtercoated films were evaluated on a computerized X-Y tablet to obtain information on cross-sectional porosity, the porosity of both surfaces, the average pore diameter, the average film thickness, and the distribution of the pore diameters. The data are presented in Table I and Figure 1. Polymer films were cleaned by extensive rinsing in running tapwater and distilled water. Implants originated from the same batch of each polymer, with exception of the polypropylene oxide implants used for the one-month period. Implants were prepared in three sizes determined by the implantation site (see under *Animals and implantation technique*):

1) Circular tympanic membrane implants with a diameter of 3 mm. One granule of dense hydroxapatite (called an artificial umbo and having less than 5% micropores measuring at most 3 µm in diameter and with an average radius of about 170 µm), was glued to the tympanic membrane implants with medical-grade silicone adhesive (Raumedic[R]) as model for the connection between the alloplastic ossicular chain and the alloplastic tympanic membrane.
2) Submucosal implants measuring 1 x 1 mm^2.
3) Bone/muscle implants of 2 x 2 mm^2.

Animals and implantation technique

The alloplasts were implanted at several sites to allow not only comparison of the results with those of a series of comparable experiments on calcium phosphate ceramics[22-25], but also investigation of contact between bone and implant material, which is important for the attachment of the alloplastic tympanic membrane to the canal-wall segment. The middle ears of male Wistar rats (200-220 g) were reached via a dorsal approach under Hypnorm[R] anesthesia (0.2 ml per animal), and a small hole was then made in the middle ear bulla with a diamond drill. Two submucosal implants were carefully inserted between the mucosa and the bulla bone, without damaging the mucosal layer. The defect was closed with a bone/muscle implant, which was placed between the bone and the muscle tissue

surrounding the bulla. For positioning of an implant, part of the tympanic membrane was separated from the handle of the malleus and the annulus, and the implant was placed as an underlay in the resulting defect, with the artificial umbo close to the mallear handle, after which the tympanic membrane tissue was repositioned.

Implantation periods were one and two weeks and one, three, six, and 12 months. In all, 822 implants equally distributed over the three biomaterials were studied (Table II).

Table I. Quantitative data on the implanted biofilms*.

film	porosity (%)			diameter (µm)	thickness(µm)	n
	cross-section	surface 1	surface 2			
EST	54±5	37±9	25±8	88±67	101±7	624
PPO	nd	52±5	52±5	103±65	96±5	323
COP	49±12	41±12	0	96±93	95±4	492

* Average values ± the standard deviation about the mean.
n Total number of pores.
nd Not detected.
EST, PPO or COP Estane[R], polypropylene oxide or HPOE/PBT copolymer.

Light microscopy and light-microscopical morphometry and autoradiograpy

After the animals had been killed the middle ears were dissected and fixed for 2 hr in a solution of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Sørensen's buffer (pH 7.4, 4°C) and for 14 hr in the same fixative without glutaraldehyde. Decalcification was performed for 4 weeks (10% EDTA, 2% paraformaldehyde, pH 7.4, room temperature) followed by rinsing in running tapwater for 24 hr. The specimens destined for light microscopy (LM) and morphometry (Table II) were dehydrated in a graded alcohol series and embedded in Paraplast[R]. Sections were cut 6 µm thick on a Spencer 820 microtome and stained with hematoxylin-eosin.

For light-microscopical morphometry, 4,020 micrographs of Paraplast sections were prepared at a standard magnification of 250 and a computerized X-Y tablet was used to measure the cross-sectional area occupied by biomaterial, phagocytes, fibrous tissue, and bone. Data are reported as the average plus or minus the standard deviation about the mean. The number of samples is also given.

The rats destined for autoradiography (one animal for each biomaterial and implantation time up to six months) were injected intraperitoneally with tritiated thymidine at a dose of 100 µCi/100 g animal weight (specific activity 25 mCi/mmol; Radiochemical Centre Amersham, England) one hr before decapitation.

Table II. Distribution of implants in right middle ears according to implantation time (weeks).

SUR		1	2	4	13	26	52	TOT
LM	TM	6	6	5	5	5	5	32
	SM	12	12	10	10	10	10	64
	BM	6	6	5	5	5	5	32
TEM	TM	3	3	3	3	3	3	18
	SM	6	6	6	6	6	6	36
	BM	3	3	3	3	3	3	18
SEM	TM	3	3	3	3	3	3	18
	SM	6	6	6	6	6	6	36
	BM	3	3	3	3	3	3	18

SUR Survival time.
LM Light microscopy and light-microscopical morphometry and autoradiography.
TEM Transmission electron microscopy.
SEM Scanning electron microscopy.
TM, SM, BM Tympanic membrane, submucosal or bone/muscle implant.

The method of fixation and decalcification of the middle ears was the same as was used for LM, but embedding was done in glycol methacrylate (JB-4 embedding kit, Polyscience^{R}). Sections (2 µm thick) were coated with K5 photographic emulsion, exposed for 4 weeks at 4°C, and after development were stained with methyl-green pyronin. The proliferative activity was quantitated by counting the labeled nuclei of macrophages, foreign-body giant cells, fibroblasts, and bone cells in 193 sections.

Electron microscopy

The specimens destined for transmission electron microscopy (TEM; Table II) were fixed in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer for 16 hr (pH 7.4, 4°C). After decalcification for 4 weeks (10% EDTA, 1.5% glutaraldehyde, room temperature) and rinsing for 24 hr in running tapwater, the implants with surrounding tissue were dissected, postfixed in 1% OsO_4 (phosphate-buffered, 30 min, room temperature), dehydrated in alcohol and embedded in Epon. Ultrathin sections were stained for 15 min with uranyl acetate and for 10 min with lead citrate and examined in a Philips EM 201 electron microscope. Semithin sections were stained with toluidine blue and examined with a light microscope. Single-spot x-ray microanalysis was performed for some of the TEM sections with a Philips EM 400 electron microscope connected to a Tracor Northern (TN) 2000 X-ray micro-analyser.

For scanning electron microscopy (SEM; Table II), middle ears were fixed in

1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer for 2 hr (pH 7.4, 0-4°C), rinsed in phosphate-buffered saline, dehydrated in alcohol, critical point-dried under carbon dioxide, sputter coated with gold and examined with a Cambridge S180 Stereo Scan.

RESULTS

Tissue ingrowth

Tympanic membrane implants

The combined SEM, TEM, and LM results show that initially exudate cells covered parts of the implants and filled parts of the pores. Next, proliferating and migrating fibroblasts formed a layer of fibrous tissue surrounding the exudate cells and served as a substrate for epithelium overgrowth. The situation after one week is shown in Figure 2 for copolymer. Two weeks after the implantation of Estane^{R} and polyproplene oxide the lateral (meatal) surface of the tympanic membrane was completely covered by an epidermis and the medial surface (facing the middle ear cavity) by a single layer of flat polygonal epithelium. Copolymer implants were completely covered after four weeks of implantation. The autoradiographical findings showed a peak in the proliferative activity of exudate cells and fibroblasts after one week, although considerably less tritiated thymidine was incorporated by exudate cells than by fibroblasts (Table III; Fig. 3). The proliferative activity of the fibroblasts covering copolymer implants (Table III) was much higher than for Estane and polypropylene oxide.

After two weeks, Estane pores were completely filled with either exudate cells alone or exudate cells together with fibrous tissue. Pores in polypropylene oxide and copolymer were completely filled by tissue of a similar composition but not before four weeks postoperatively. During the first week, the exudate-cell population was composed predominantly of macrophages, polymorphonuclear granulocytes, and lymphocytes, but also showed plasma cells and multinucleated cells. After two weeks, the numbers of polymorphonuclear granulocytes, lymphocytes, and plasma cells decreased and the number of multinucleated cells increased. Multinucleated cells were only found at the surface of the implants, whereas macrophages also occurred in the fibrous tissue filling the pores and under the epidermis and epithelium. The other types of exudate cell were dispersed in the fibrous tissue, but some of the lymphocytes occasionally adhered to polypropylene oxide. Both macrophages and multinucleated cells frequently showed vacuoles containing implant-derived material.

After one month, the fibrous tissue contained fibroblasts, an abundance of

randomly organized collagen, capillaries, nerves, and exudate cells. After three months, the number of fibroblasts and exudate cells had decreased in Estane and copolymer tympanic membranes, whereas the collagen had increased in amount and was packed in bundles lying parallel to the surface of the tympanic membranes (Figs. 4 and 5), and the tympanic membranes had become thinner. Fibrous tissue surrounding polypropylene oxide showed relatively large numbers of fibroblasts, macrophages, polymorphonuclear granulocytes, lymphocytes, and plasma cells from the first through the sixth postoperative month. The collagen was not bundled but randomly organized. The tympanic membranes implanted with polypropylene oxide remained rather thick for up to six months, but after one year both the composition of the fibrous tissue and the thickness of the tympanic membranes were comparable with those of specimens implanted with Estane or copolymer.

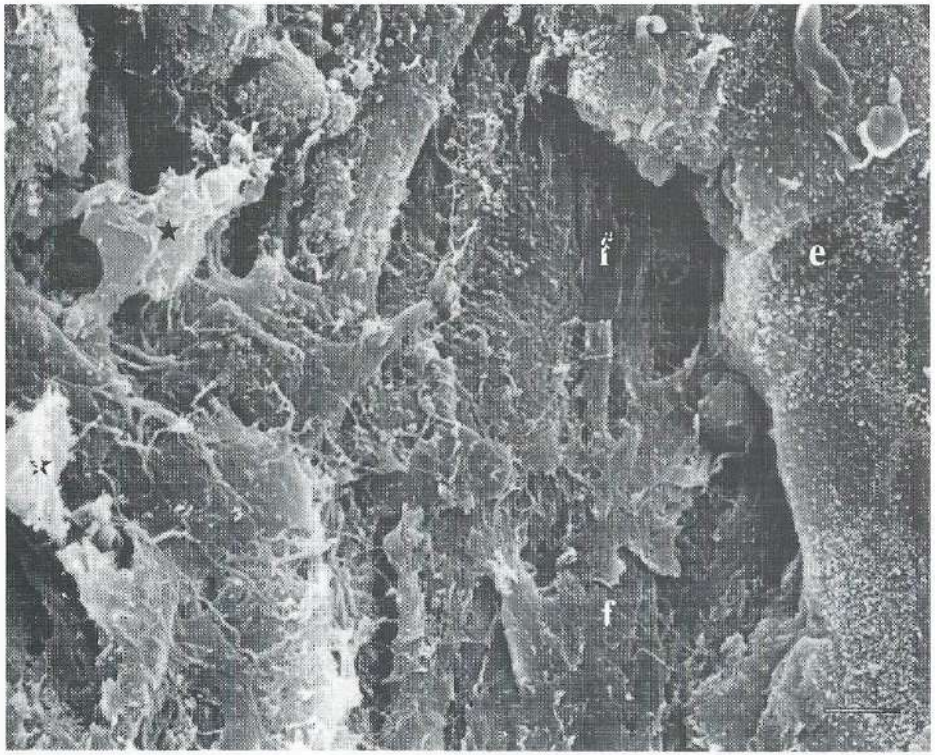


Fig. 2. Scanning electron micrograph of a 'copolymer' tympanic membrane showing fibroblasts (F) and epithelium (E) covering the region close to the handle of the malleus. Note the exudate cells (asterisks). Bar = 4.2 µm.

Table III. ^{3}H-Thymidine incorporation by exudate cells, fibrous tissue, and bone surrounding tympanic-membrane (TM), submucosal (SM), and bone/muscle (BM) implants, expressed as number of labeled nuclei per 0.1 mm^2 tissue.

HPOE/PBT copolymer

Interval	Exudate cells			Fibrous tissue			Bone*	
(weeks)	TM	SM	BM	TM	SM	BM	SM	BM
1	0.8	0.1	0.1	14	5	2.5	0.5	0.1
2	0.2	0.1	0.1	4	2.1	0.6	0.1	0
4	0.1	0.2	0.1	1.5	0.9	0.8	0.2	0
13	0.1	0.1	0	0.8	0.4	0.4	0.2	0.1
26	0	0.1	0	0.5	0.5	0.3	0.4	0

Estane^{R}

Interval	Exudate cells			Fibrous tissue			Bone*	
(weeks)	TM	SM	BM	TM	SM	BM	SM	BM
1	0.2	0.1	0	6.2	1.3	1.4	0	0
2	0.1	0.1	0	2	0.5	0.4	0	0
4	0.2	0.1	0	0.6	0.4	0.3	0	0
13	0	0	0	0.8	0.1	0	0	0
26	0	0	0	0.3	0.2	0	0	0

Polypropylene oxide

Interval	Exudate cells			Fibrous tissue			Bone*	
(weeks)	TM	SM	BM	TM	SM	BM	SM	BM
1	0.3	0.3	0.1	5.9	2	0.8	0	0
2	0.3	0.1	0	3.4	4	0.4	0	0
4	0.4	0.2	0	1.3	0.6	0.5	0	0
13	0.6	0.3	0.1	0.5	0.3	0.1	0	0
26	1	0.3	0	0.3	0.3	0	0	0

*The tympanic membrane is not a suitable site for the analysis of contact with bone.

Most of the artificial umbos made of hydroxyapatite lay close to the handle of the malleus throughout the entire observation period and were incorporated into thin layers of fibrous tissue and covered by flat polygonal epithelium (Fig. 4b). Macrophages and multinucleated cells were associated predominantly with the silicone adhesive.

Submucosal implants

On the medial side, submucosal implants were separated from the epithelium by connective tissue. The opposite side lay against the bone of the bulla. All Estane pores were completely filled with exudate cells and fibrous tissue in the first postoperative week and all pores in polypropylene oxide in the second week. Most of the pores in copolymer implants were filled with exudate cells and fibrous tissue in the second week, but some empty pores were seen in all observation periods up to one year. Proliferative activity of the fibroblasts showed a peak

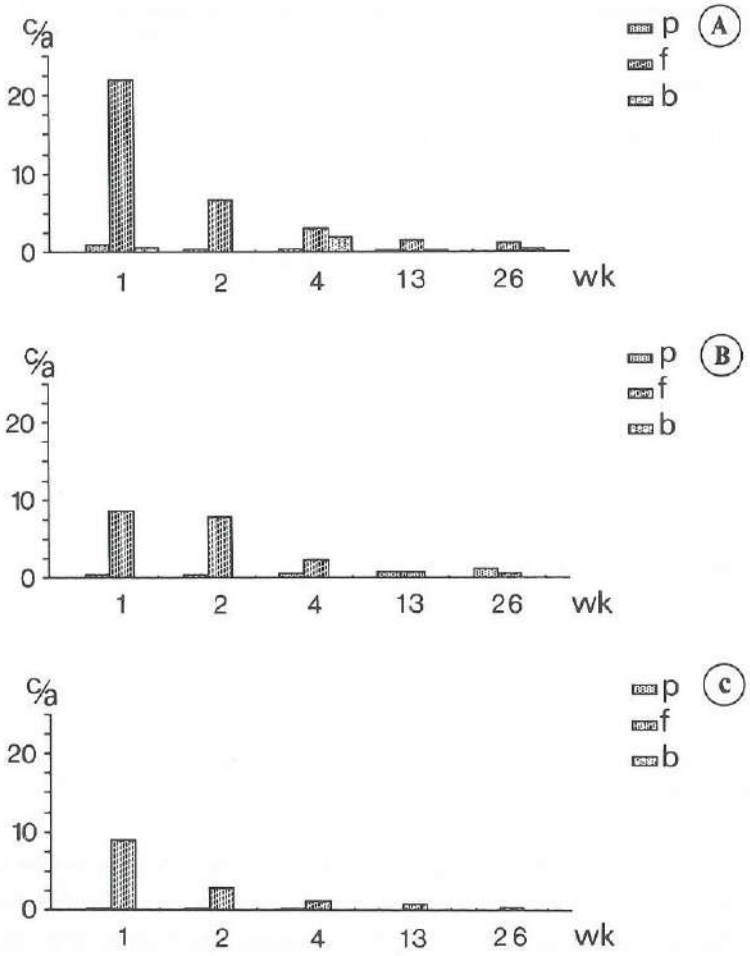


Fig. 3. Diagrams showing intensity of the average tissue proliferation calculated as number of labeled nuclei per 0.3 mm^2 tissue in cross-section (c/a) for copolymer (A), polyproplene oxide (B), and Estane^R (C). A distinction is made between exudate cells (p), fibrous tissue (f), and bone (b). Note the relatively high level of tritiated thymidine incorporation by fibroblasts and the labeling of bone cells associated with HPOE/PBT copolymer.

after one week (Table III), but the peak was much lower than that in the tympanic membrane. Thymidine incorporation by exudate cells was insignificant. The composition of the exudate-cell population was comparable to that described for tympanic membrane implants, and the same holds for the course of the changes in the exudate composition. Fibrous tissue surrounding the implants was predominantly composed of fibroblasts and macrophages but also showed small numbers of other types of exudate cell, collagen fibrils, and capillaries. Larger vessels were seen close to the bulla wall. The only significant change in the composition of the fibrous tissue was a decrease in the numbers of polymorphonuclear granulocytes, lymphocytes, and plasma cells.

Macropores and micropores of both copolymer (Fig. 6) and Estane (Fig. 7) showed ingrowth of bone, whereas bone was never seen in pores in polypropylene oxide implants. However, bone ingrowth in copolymer pores had taken place in the first week, whereas for Estane it was only evident during and after the sixth postoperative month. Partially calcified material in pores, which was characterized by fibroblast-like cells within a matrix of collagen, was only associated with the one-, two-, and four-week implantation periods for copolymer, but for Estane it occurred in all implantation periods during which bone ingrowth occurred. Mature bone in pores was characterized by the presence of osteocytes surrounded by a calcified matrix and osteoblasts, and contained an occasional central capillary. With copolymer, bone calcification was first seen at the bone/polymer interface, which was characterized by an electron-dense granular layer (Fig. 6b), whereas with Estane calcification started from the central portion of the pores. The bone/Estane interface did not show the electron-dense layer (Fig. 7b). The level of bone proliferative activity, as indicated by thymidine incorporation, was low (Table III).

Bone/muscle implants

Bone/muscle implants were located between the middle ear bulla bone and the adjacent muscle tissue. After one week all implants were completely surrounded by exudate cells (Fig. 8). The implantation area showed sparsely vascularized fibrous tissue, which contained many spindle-shaped fibroblasts showing substantial thymidine incorporation up to the first month (Table III). Multinucleated cells were present in relatively large numbers, but there were fewer polymorphonuclear granulocytes, lymphocytes, and plasma cells, than at the other implantation sites. Exudate cells showed low proliferative activity up to the sixth month (Table III). Unlike those made of Estane, some copolymer implants showed ingrowth of bone, after which an electron-dense bone/copolymer interface was invariably found.

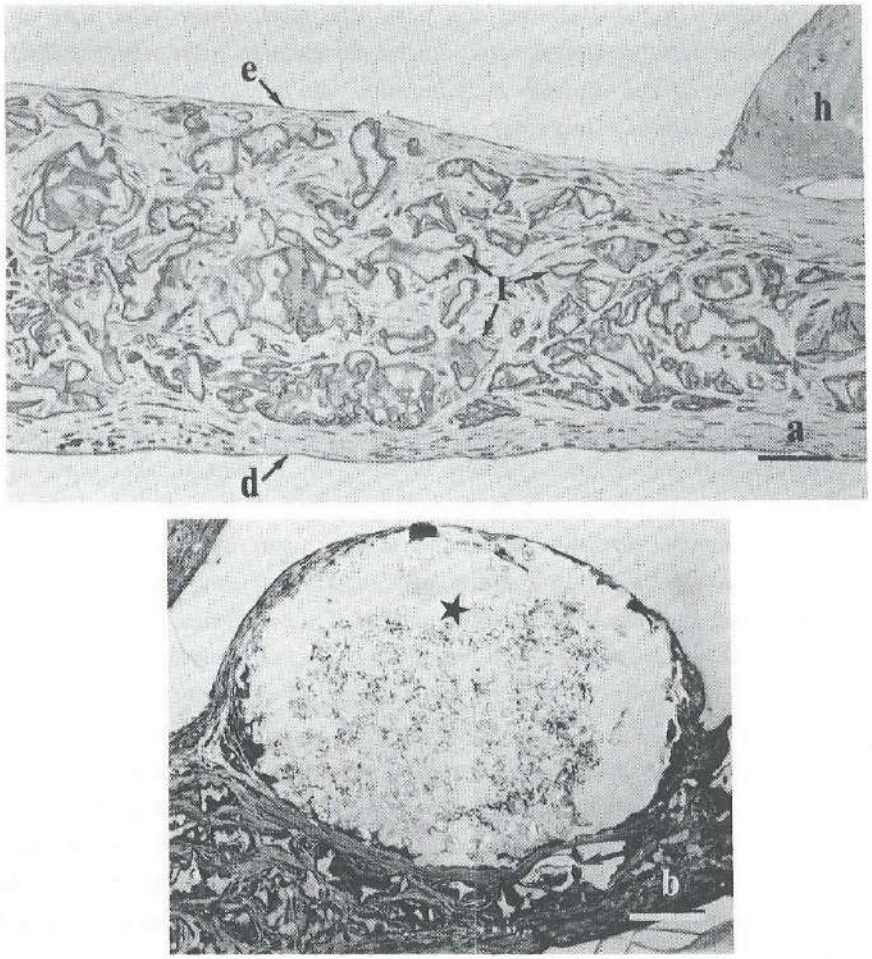


Fig. 4. Light micrographs showing Estane^{R} tympanic membrane implants three months postoperatively.
a: Estane appears as a collection of many fragments (I); the individual fragments show a relatively dark surface zone and are surrounded by phagocytes and fibrous tissue. E = epithelium; D = epidermis; H = handle of malleus. Bar = 40 µm.
b: Micrograph showing hydroxyapatite umbo (asterisk) lying close to the handle of the malleus and surrounded by fibrous tissue and covered by a thin epithelium. Note the collagen bundles and the presence of small amounts of silicone adhesive (arrow). Bar = 60 µm.

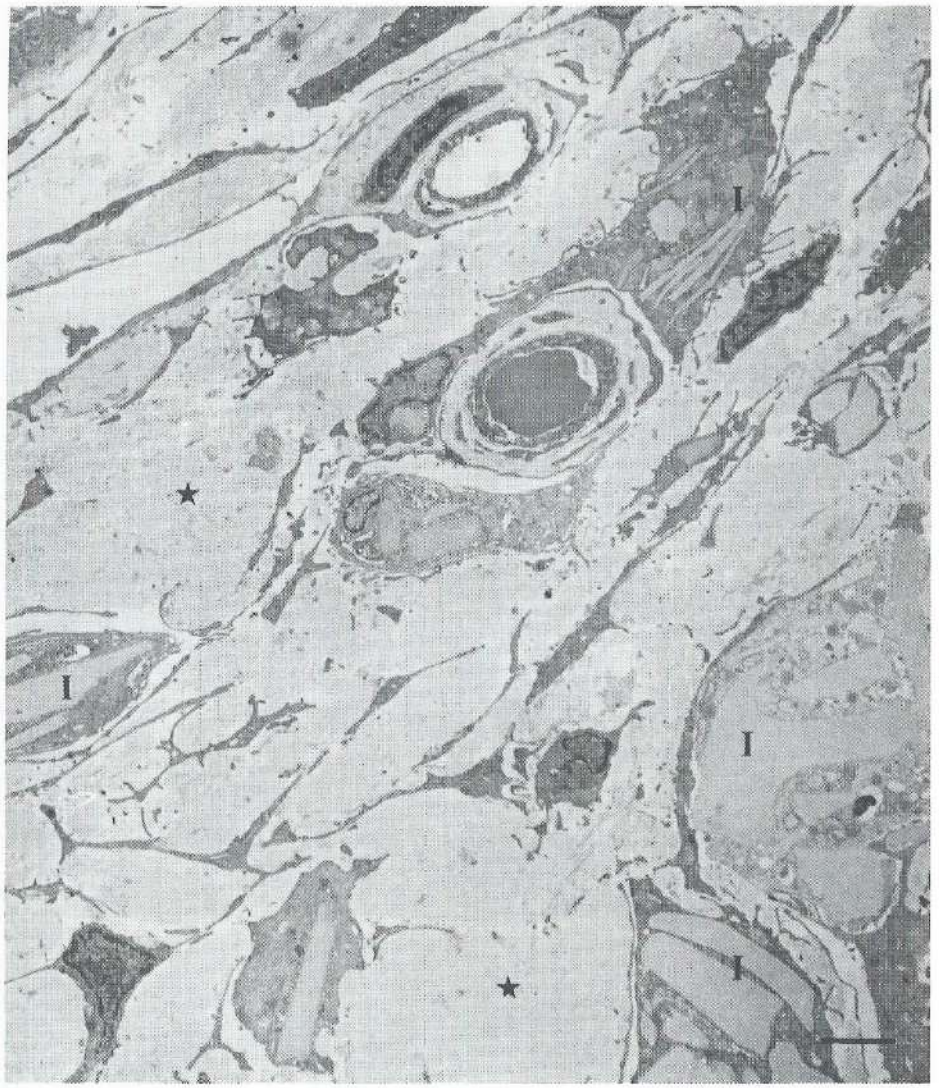
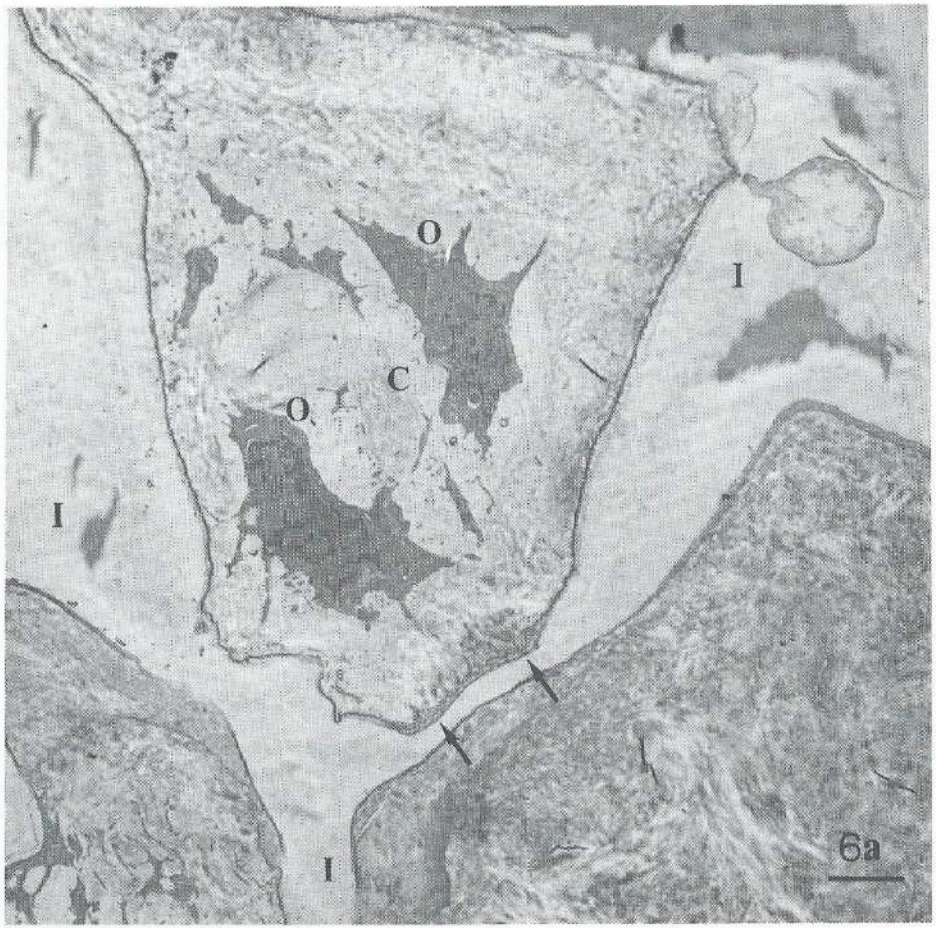
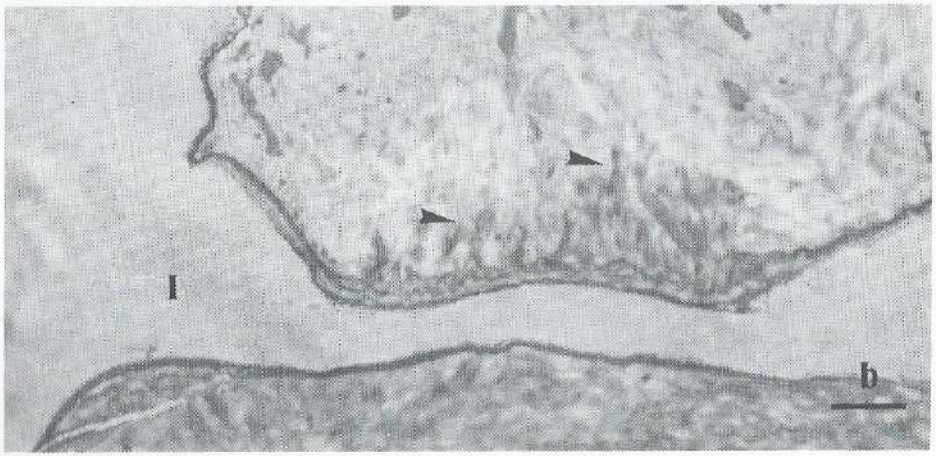


Fig. 5. Transmission electron micrograph showing a tympanic membrane with a HPOE/PBT copolymer implant after six months. Fragments of implant material (I) within the cytoplasm of macrophages are a prominent feature. Note the abundance of collagen. Bar = 7.5 µm.





Tissue composition

Light-microscopical morphometry showed that the composition of the tissue surrounding an implant varied with the observation time as a function of the implant material (Table IV; Fig. 9). With polypropylene oxide, the ratio between phagocytes (macrophages and foreign-body giant cells together) and fibrous tissue increased with time up to the sixth month, after which the amount of phagocytic tissue decreased sharply (Fig. 9a). Estane implants showed a relatively high ratio between phagocytes and fibrous tissue during the first three months (Fig. 9b). The decline of this ratio after six months was accompanied by the appearance of bone (Table IV). The composition of the tissue surrounding copolymer stayed relatively constant, the amount of phagocytes being roughly equal to the amount of fibrous tissue (Fig. 9c). After three months an increase in the amount of bone, which was earliest and most prominent for submucosal copolymer implants (Table IV), coincided with a decrease in the amount of fibrous tissue (Fig. 9c).

Implant degradation

The structure of the implant and the morphology of the polymers changed between the first and fourth postoperative week, and mononuclear and multinucleated phagocytes adhered to the implant surface. With respect to implant structure LM and TEM showed progressive loss of implant cohesion (Figs. 4, 5, 8, and 10b). With respect to the morphology of the polymers LM and TEM showed that polypropylene oxide implants were composed of many fibres up to the second postoperative week, which gave the implants a rather dense and cloudy appearance (Fig. 10a). After one month polypropylene oxide implants had become more transparent (Fig. 10b). At that time, the surface of Estane implants showed a dense layer (Figs. 4a and 11), which had not been present earlier. The thickness of the layer increased with time. Fragmentation was the most prominent feature associated with copolymer implants (Figs. 5 and 8). As time progressed the surfaces of all implants bordered by mononuclear and multinucleated phagocytes showed erosion and invasion by the cytoplasm of the adjacent cell.

Fig. 6a and b. Ingrowth of bone into submucosal HPOE/PBT copolymer implant (I) seen after three months.
a: Transmission electron micrograph of a macropore filled with bone. Two osteoblasts (O) can be seen adjacent to collagen (C) in the central part of the pore. Note the electron-dense interface between bone and implant. Bar = 3.8 µm.
b: Section of the area marked in a by arrows showing the bone/copolymer interface in more detail. The orientation of the electron-dense material (arrowheads) is suggestive of a calcification process that has started at the periphery of the pore. Bar = 0.7 µm.

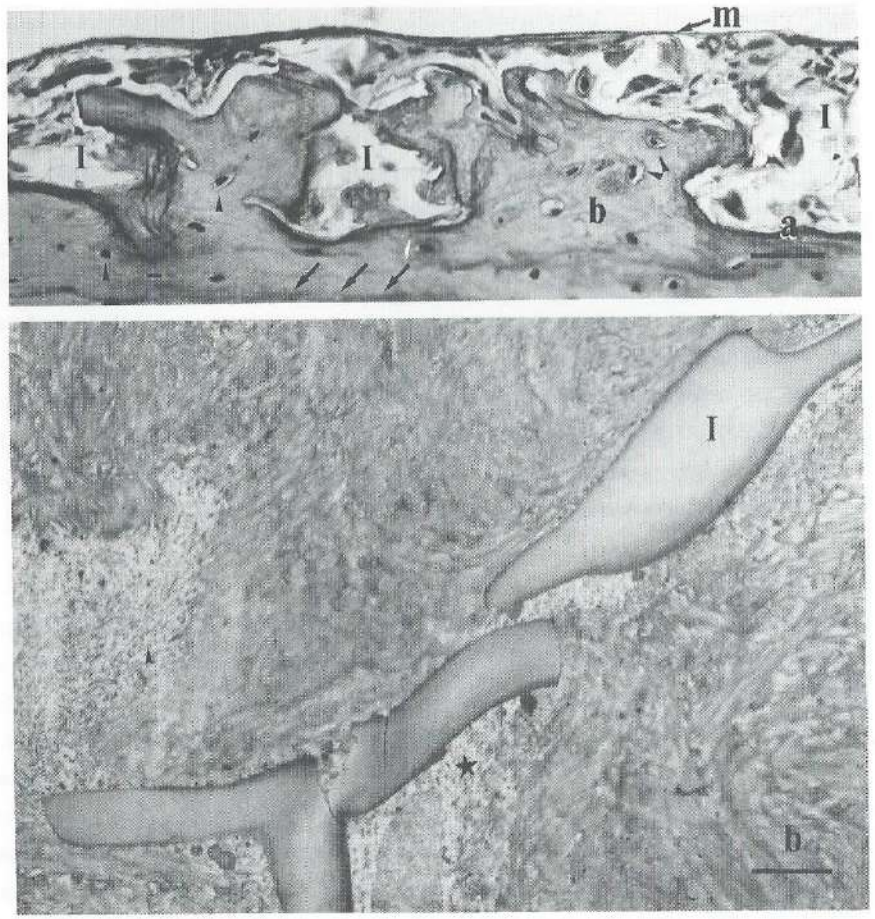


Fig. 7a and b. Ingrowth of bone in submucosal Estane^{R} implant (I) seen after six months.
a: Light micrograph showing Estane implant between the middle ear bulla bone (B) and mucosa (M). Note the osteocytes (arrow heads) and the natural lamina limitans of bone (arrows). Bar = 40 µm.
b: Transmission electron micrograph revealing the absence of an electron-dense layer between Estane and bone and the presence of uncalcified material between bone and implant (asterisk). Bar = 1.7 µm.

Table IV. Course of the area occupied by implant material and the percentage tissue occupied by exudate cells, fibrous tissue and bone*.

Polypropylene oxide					
Interval (weeks)	Area (x1000 µm²)	Exudate cells (%)	Fibrous tissue (%)	Bone (%)	n
1	14±10	42±33	58±43	0	148
2	14±11	44±40	56±34	0	211
4	12±8	53±35	47±36	0	190
13	6±6	62±48	38±24	0	256
26	2±2	69±41	31±23	0	206
52	1±1	17±15	83±61	0	123
Estane^{R}					
Interval (weeks)	Area (x1000 µm²)	Exudate cells (%)	Fibrous tissue (%)	Bone (%)	n
1	22±13	65±40	35±15	0	252
2	17±10	67±38	33±17	0	265
4	16±9	69±36	31±13	0	250
13	13±8	66±41	34±15	0	282
26	7±4	49±22	51±38	3±3	248
52	7±5	38±32	66±33	6±5	234
HPOE/PBT copolymer					
Interval (weeks)	Area (x1000 µm²)	Exudate cells (%)	Fibrous tissue (%)	Bone (%)	n
1	22±17	40±36	59±21	1±1	164
2	21±13	43±46	55±49	2±2	219
4	20±17	48±31	50±42	2±1	214
13	16±11	39±42	57±55	4±2	268
26	11±10	39±36	56±51	5±4	223
52	10±6	44±23	46±26	10±6	231

*Values are mean ± 1 standard deviation for the number of samples shown (n).

Five rats died prematurely, all of them belonging to the one-year polypropylene oxide group. Furthermore, TEM showed numerous vacuole-like artifacts in the cytoplasm of phagocytes containing polypropylene oxide (Figs. 10a and b). These cells were also characterized by mitochondria which were invariably larger and had less densely packed cristae than the mitochondria associated with Estane and copolymer (Figs. 10c and d). Tubular and plate-like cytoplasmic structures were seen in both mononuclear and multinucleated phagocytes after three months or longer (Figs. 12a-c). Mononuclear phagocytes with irregularly shaped electron-dense inclusions were also seen (Figs. 11 and 13a) after the implantation of all three biomaterials, but were most prominent in tympanic membranes and submucosa six months after the implantation of Estane. The electron-dense inclusions were occasionally associated with the tubular and plate-like structures (Fig. 12c). For all three polymers single-spot x-ray microanalysis showed the presence of silicon, titanium, aluminum, iron, or a combination of

these elements in the electron-dense inclusions (Fig. 13b and c). With copolymer, intracellular iron was also prominent in electron-dense granules occurring either dispersed or aggregated in the cytoplasm of phagocytes (Fig. 14, inset).

Light-microscopical morphometry to determine the amount of implant material as a function of time, showed degradation of Estane, polypropylene oxide, and copolymer (Table IV; Fig. 15). As can be seen from Figure 15, after one year only a small amount of polypropylene oxide was left (less than 7% averaged over all of the one-year sections compared with the first week) as against 41% for Estane and 54% for copolymer. After an implantation period of six months, copolymer degradation seemed to have decreased (Fig. 15) and Estane resorption was virtually arrested (Fig. 15).

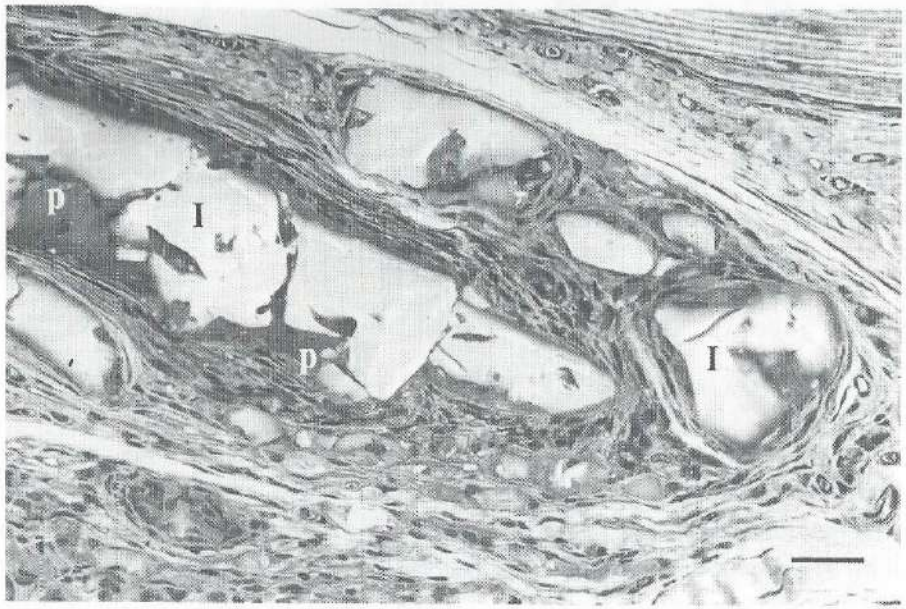


Fig. 8. Light micrograph showing copolymer implant between bulla bone and muscle tissue after one week. Note the polymer fragments (I) surrounded by phagocytes (P) and sparsely vascularized fibrous capsules containing numerous enlongated fibroblasts. Bar = 40 µm.

DISCUSSION

Alloplasts used for reconstructive middle ear surgery can be subdivided into metals, ceramics, and polymers[14,26]. Polymers are the only candidates for myringoplasty, because their elastic and vibratory properties *in vitro*[18] are comparable to those of the tympanic membrane. A number of polymers[6-11] have

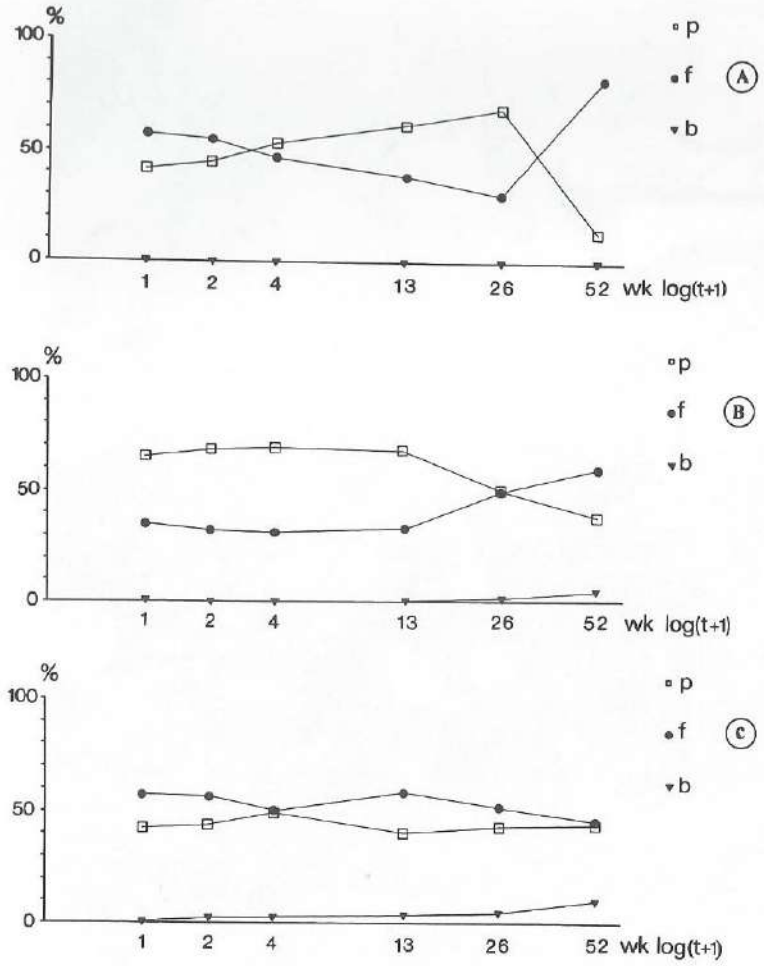
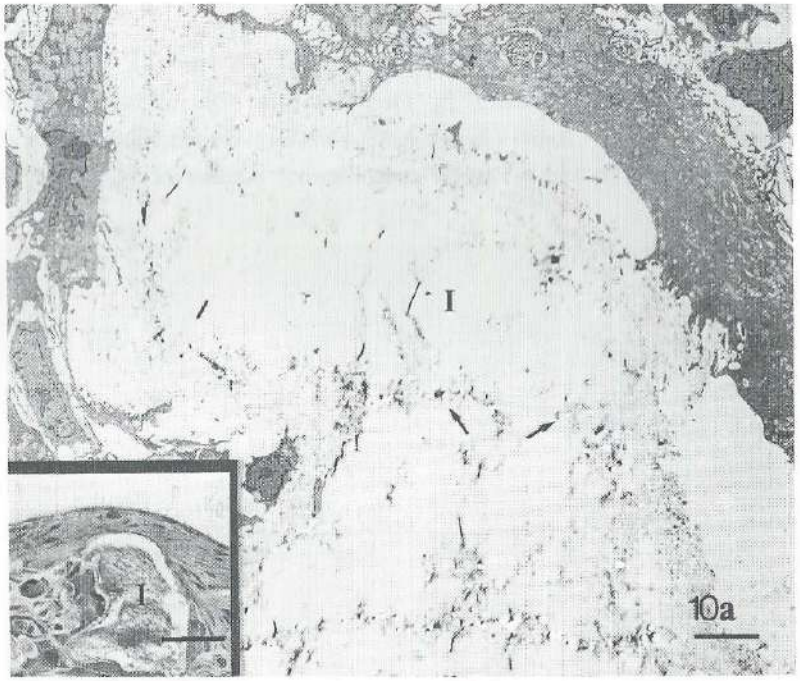
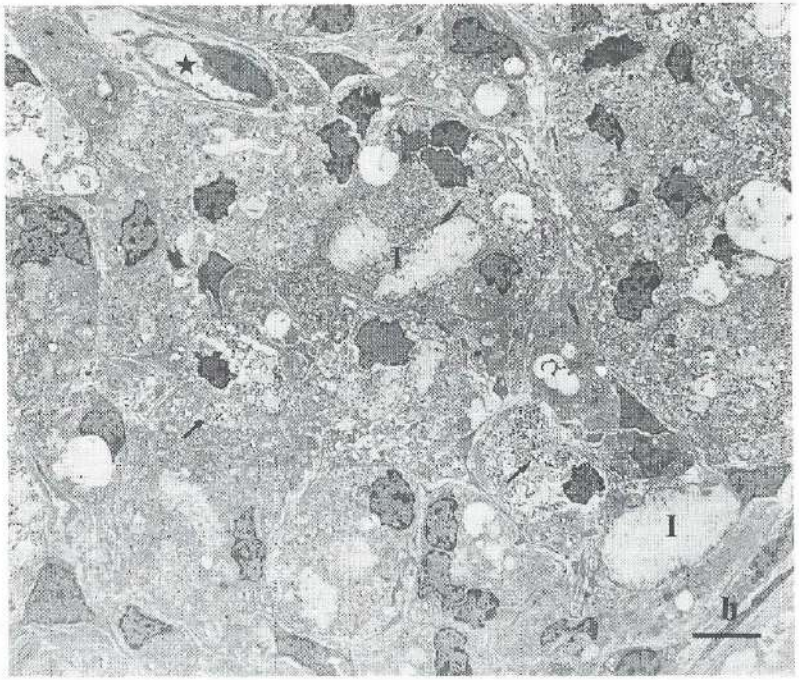


Fig. 9a-c. Course of the percentages of tissue surrounding implant and showing exudate cells (P), fibrous tissue (F), and bone (B). Values are averages of the total number of implants made of polypropylene oxide (A), Estane^{R} (B), or HPOE/PBT copolymer (C). Note that the tissue composition differs according to the implant material.





been investigated as material for closing tympanic membrane perforations but were not satisfactory. In this study the biocompatibility of macroporous elastomeric implants made of Estane[R] 5714 F1 polyether urethane, polypropylene oxide, and HPOE/PBT segmented polyether polyester copolymer was assessed to evaluate their suitability as alloplastic tympanic membrane in a total alloplastic middle ear prosthesis[2].

Implantation of these polymers led at all implantation sites to tissue responses associated successively with the wound-healing process[27] and the foreign-body reaction[28]. Eventually, all of the tympanic membranes showed an epidermis, an epithelium, and a layer of connective tissue[29]. Initially, the tympanic membranes were rather thick due to the presence of a large amount of granulation tissue, but after prolonged survival they gradually became thinner because of the disappearance of granulation tissue and the degradation of the implants. At the same time, the connective tissue of the tympanic membranes became rather acellular. The structure of the new connective-tissue layers resembled that of the normal rat tympanic membrane[29], but their arrangement differed. This course of

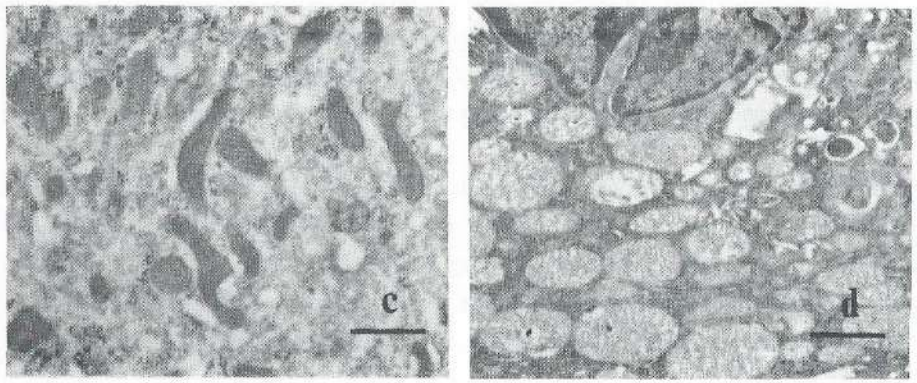


Fig. 10a-d. Tissue response to polypropylene oxide degradation in the tympanic membrane.
a: Transmission electron micrograph showing polypropylene oxide implant material after three months. Macrophages and foreign-body giant cells separate the implant material (I) from the fibrous tissue. Note artefacts (arrows) predominantly associated with the polymer. Bar = 7 µm. Inset: light micrograph showing cloudy appearance of implant (I) after two weeks. Bar = 40 µm.
b: Transmission electron micrograph made six months after implantation, showing relatively small polymer fragments (I) surrounded by phagocytes. Numerous artefacts can be seen in both the fragments and the phagocytes (arrows). Note the capillary (asterisk). Bar = 7 µm.
c and d: Transmission electron micrographs showing the mitochondrial morphology of phagocytes associated with copolymer or Estane (a) and the swollen mitochondria associated with polypropylene oxide (b). Bars = 0.7 µm.

events is in good agreement with that observed when a tympanic membrane perforation heals either spontaneously[30] or after use of a biological graft[5].

Degradation of polypropylene oxide implants was accompanied by tissue reactions in the tympanic membrane but also at the other implantation sites, the changes differing significantly from those seen during breakdown of Estane and copolymer. The amount of polypropylene oxide decreased rapidly after the first month and the phagocyte/polymer interface[31] was comparable to that characteristic of relatively rapidly degrading polymers[32]. Furthermore, between the first and six month tympanic membranes implanted with polypropylene oxide showed infiltration of polymorphonuclear granulocytes, which is characteristic of the acute inflammatory response[28], and macrophages, but also the presence of plasma cells, which together with lymphocytes suggest an immunological reaction[17]. Because the wound-healing process is limited to a postoperative period of about four weeks[27], the observed cellular response may be attributed solely to the presence of polypropylene oxide. Infiltration of exudate cells, the abnormal morphology of the mitochondria[33] of the phagocytes associated with polypropylene oxide, and the premature death of five rats all in the one-year polypropylene oxide group, strongly suggests[34] the release of toxic substances during polypropylene oxide degradation[35]. Estane and copolymer degraded less rapidly than polypropylene oxide. The increased electron density of the superficial zones of Estane implants[31] might be indicative of changes in the chemical properties[36] of this polymer during degradation.

Neither the infiltration by exudate cells that was associated with polypropylene oxide degradation nor the occurrence of phagocytes with a deviating morphology was seen during Estane and copolymer degradation, which suggests the absence of toxic degradation products or additives. This assumption is supported by the results of several tissue-culture tests[35], in which only polypropylene oxide was found to be toxic.

The electron-dense inclusions associated with the degradation and phagocytosis of polypropylene oxide, Estane, and copolymer, contained silicon, aluminum, titanium, and iron, singly and in various combinations. These elements are probably impurities deriving from e.g. the catalyst and the production of the polymers[37,38]. These inclusions are comparable to the granules described by van Blitterswijk et al.[25,39], who postulated storage of trace elements originating from tricalcium phosphate implants. Phagocyte-mediated storage of trace elements has also been reported after implantation of hydroxyapatite in the human middle ear[40] and after injection of tricalcium phosphate into the peritoneal cavity of mice[41]. Although the storage of trace elements apparently did not affect local tissue

responses, it is important to know whether any systemic effects are to be expected. Our findings underscore the importance of using implant materials of the purest grade. Hemosiderin, which is seen during periods of inflammation[42] is a probable source of the intracellular iron present in the small granules occurring in the phagocytic cytoplasm. The tubular and plate-like structures seen within phagocytes resemble the angulated lysosomes of Gaucher's cells morphologically[43]. Implant degradation followed by enhanced phagocytosis could have led to overloading and overwhelming of the lysosomal apparatus, which in turn would lead to accumulation of material in the phagocyte[43].

Whereas polypropylene oxide was almost completely degraded after one year (averaged over all implantation sites), the average degradation of copolymer slowed down after six months and that of Estane ceased after six months. This coincided with the disappearance of the majority of the phagocytes. These observations are in line with the findings made by van Blitterswijk et al.[24] for hydroxyapatite implants. Although the degradation behavior[31,34] of Estane, polypropylene oxide, and copolymer differed, the degree of this degradation and the volume of pore-occupying phagocytes were shown to depend on the implantation site[34]. The introduction of polymeric implants under the mucosa and bordering muscle tissue also led to more severe foreign-body reactions than those seen in the presence of either hydroxyapatite[23,24] or tricalcium phosphate[25] under the same conditions. Not only differences in chemical compostion and porosity but also a higher degree of polymer degradation might have been responsible for this divergence.

Although bone ingrowth was established for both copolymer and Estane, the progress and degree of this ingrowth, the composition of the bone, the direction of bone deposition, and the bone/implant interface differed for these two polymers. The deposition of bone and the electron-dense bone/copolymer interface[31], which morphologically resembled the natural lamina limitans of bone[44], were comparable to the bone/implant responses characterizing the bioactive ceramic hydroxyapatite[23,24,45]. The ability of bone to grow into the pores of polymers has been demonstrated e.g. for polyethylene[46], polysulfone[47], and polypropylene[46], but an electron-dense layer associated with a bone/polymer interface has to the best of our knowledge not been reported before. With respect to the attachment of the alloplastic tympanic membrane to the porous hydroxyapatite canal wall of the prosthesis, both the lamina limitans-like interface suggesting a chemical bond[48] between bone and copolymer and the degree of bone and fibrous tissue ingrowth suggest better implant fixation of copolymer than of Estane by mechanical interactions[49,50] and point to copolymer as preferable

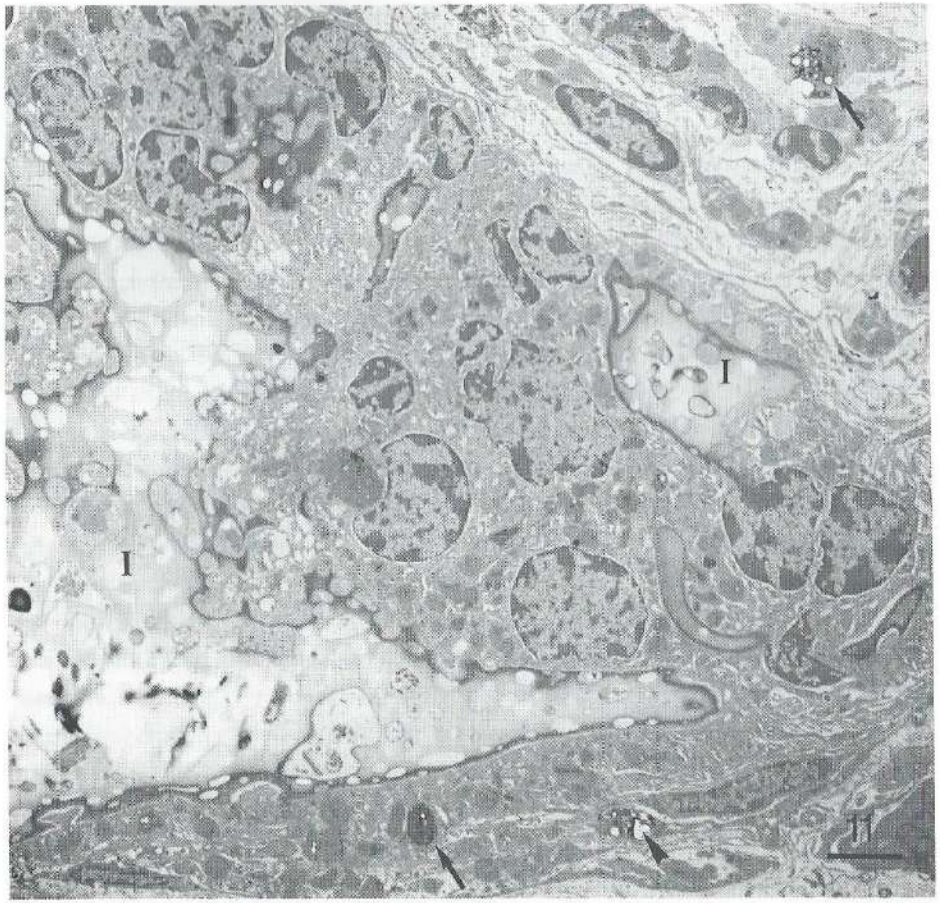
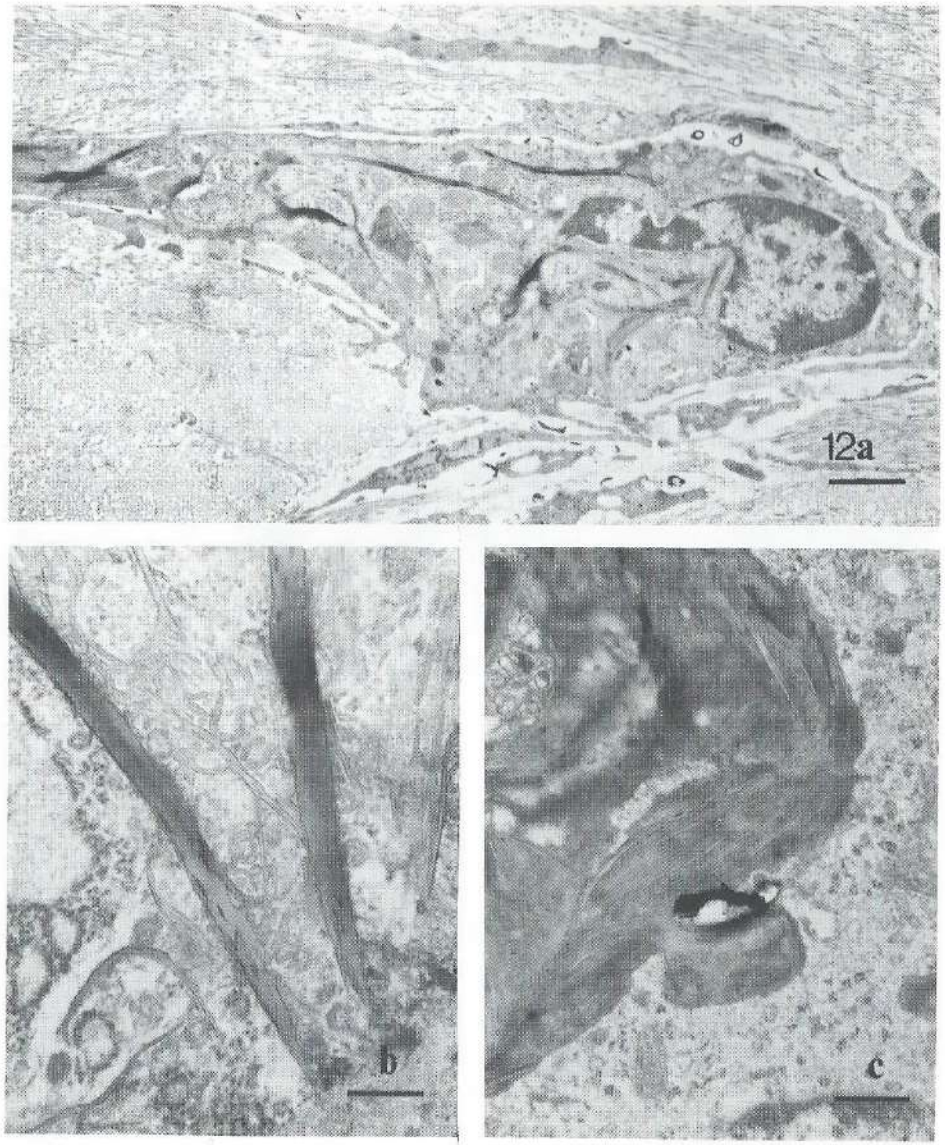


Fig. 11. Transmission electron micrograph of a fragment of Estane^{R} engulfed by a foreign-body giant cell, six months after implantation in the tympanic membrane. Note the eroded surface of a large piece of Estane and the smaller fragments (I) characterized by the presence of an electron-dense surface zone. Arrows and arrowhead point to inclusions containing trace elements. Bar = 4 µm.

Fig. 12. Transmission electron micrographs showing plate-like and tubular structures in the cytoplasm of phagocytes associated with either submucosally implanted polypropylene oxide after three months (a) or an Estane implant in the tympanic membrane after six months (b and c).
a: Cell displaying scattered tubular structures. Bar = 1.9 µm.
b and c: Cross-sections and transverse sections of tubular structures (b) and a plate-like structure (c) at higher magnifications. Note the electron-dense inclusion in c. Bars = 0.5 µm.



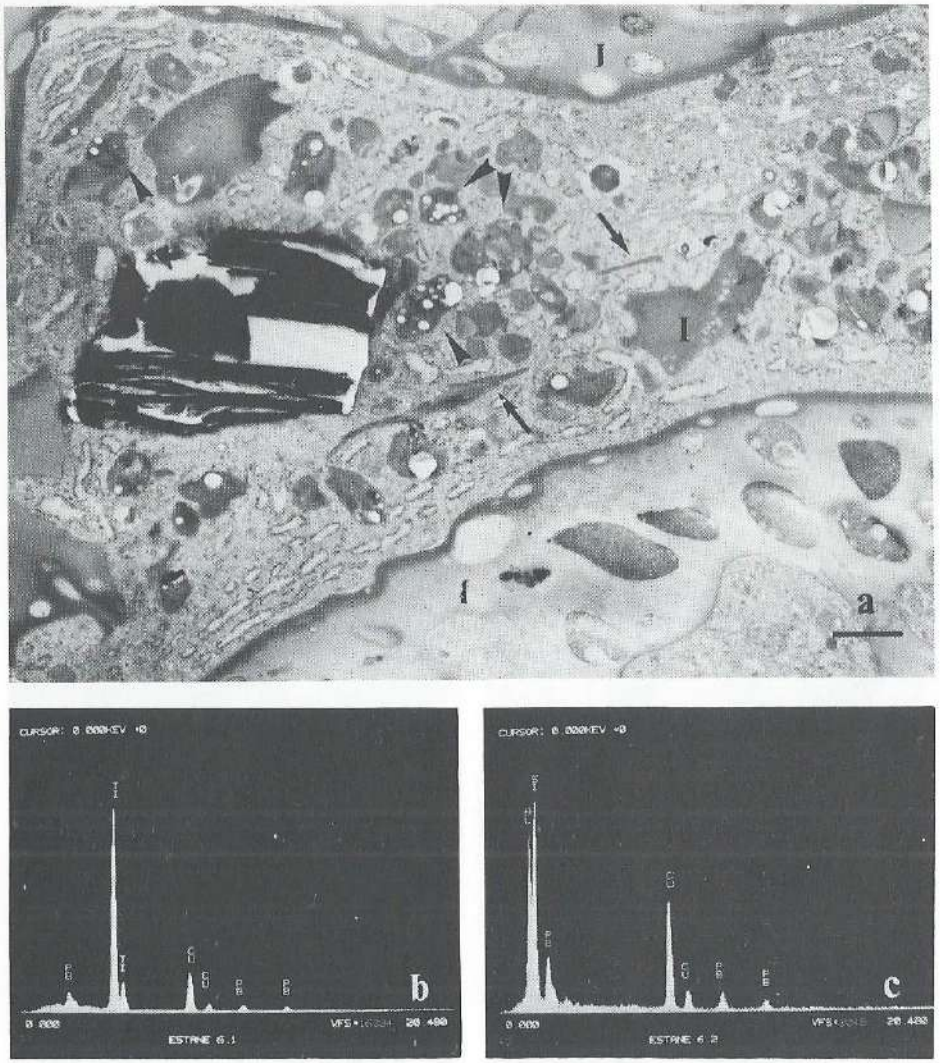
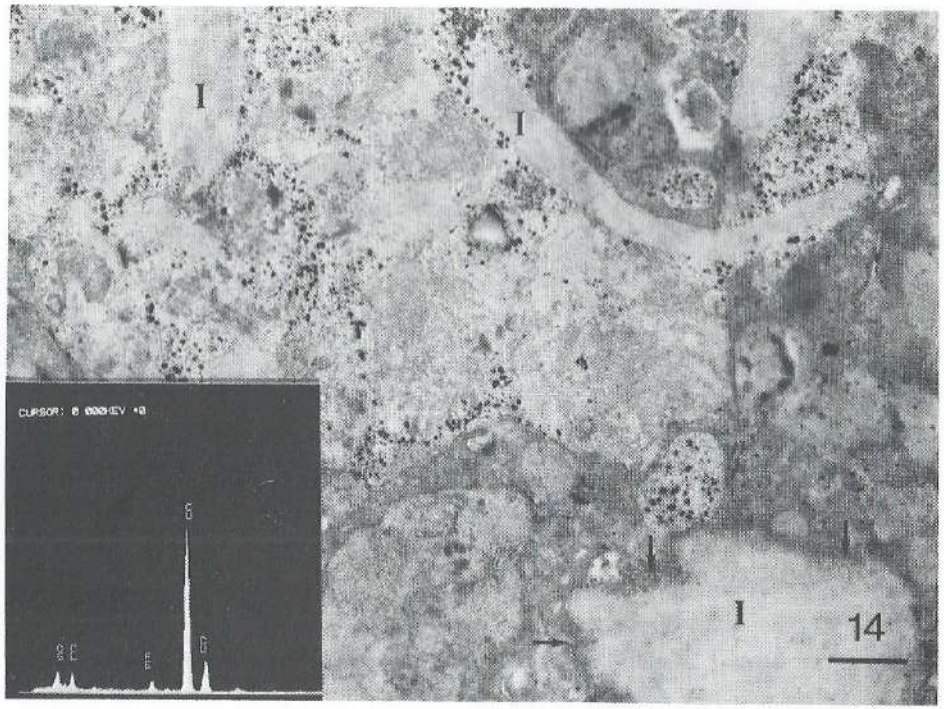


Fig. 13a-c.
a: Transmission electron micrograph of a phagocyte showing Estane (I), tubular structures (arrows), many vacuolized structures (arrow-heads) morphologically comparable to the inclusions shown in figure 11, and a large fragment of an electron-dense material. Bar = 1.9 µm.
b and c: X-ray spectra of the vacuolized structure marked in Figure 11 by an arrowhead (b) and of the large electron-dense inclusion (c) revealing the presence of titanium or aluminum, silicon, and iron, respectively. The other peaks are artefacts induced by the preparation of the sections for transmission electron microscopy.



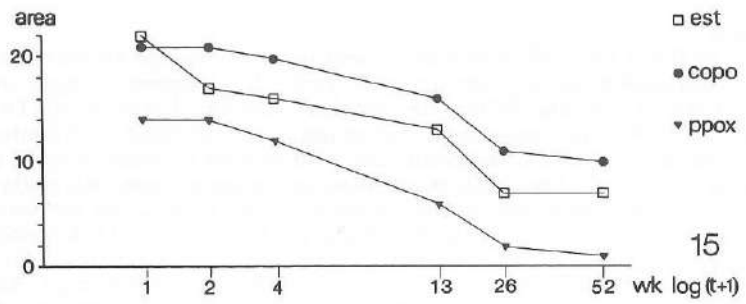


Fig. 14. Transmission electron micrograph showing phagocyte with numerous copolymer fragments (I) and iron-containing granules as determined by x-ray microanalysis (inset). Tympanic membrane implant, six months postoperatively. Note the eroded surface of HPOE/PBT copolymer (arrows). Bar = 0.6 µm.

Fig. 15. Schematic representation of the cross-sectional area (x 1000 µm²) occupied by the polymers and averaged over the total number of implants as a function of time, showing different degrees of implant degradation.

material for an alloplastic tympanic membrane. Possible explanations for the persisting empty pores in copolymer implants are insufficient interpore connections or implant structure, but this point needs further investigation.

The incorporation of the hydroxyapatite umbos without any adverse reactions is in good agreement with the observations made with hydroxyapatite implanted in rat middle ear mucosa[22-24]. These findings also show successful fixation of an alloplastic tympanic membrane together with hydroxyapatite by tympanic membrane tissue, which is of crucial importance for the integration of and sound-conduction by the TAM[14]. In sum, the results of this study suggest that a degradable macroporous alloplastic tympanic membrane as temporary scaffolding in the TAM is favorable for fixation of the hydroxyapatite ossicular chain to the canal wall of the prosthesis. Because degradation of polypropylene oxide induced tissue responses related to implant toxicity, this material is not suitable as alloplastic tympanic membrane. The degradation products of Estane and HPOE/PBT copolymer did not have toxic effects during the first postoperative year. Although Estane was more rapidly invaded by exudate cells and fibrous tissue, probably because of the more suitable pore structure, the copolymer is preferred as tympanic membrane material because its implants proved to give better fixation by tissue. Especially the possible chemical bonding with bone makes HPOE/PBT copolymer a promising implant material. Before final conclusions can be drawn, however, the quality of the epithelium covering the implants as well as the biocompatibility of these polymers during infection must be evaluated.

Acknowledgements

The authors wish to thank Prof. W.Th.Daems (Laboratory for Electron Microscopy) for his critical evaluation of the results and Prof. K.B.Wagener (Dept. of Chemistry, University of Florida, Gainsville, Florida) and Dr. J.H.Kuit and Dr. J.G.F.Bots (Dept. of Chemical Technology, Technological University of Twente, Enschede) for preparing the implant materials. The hydroxyapatite granules were a generous gift from Prof. K. de Groot (Dept. of Material Science, Free University, Amsterdam). For technical assistance we are indebted to Mr. B.M.A. van der Lans and Ms. P. van der Hoek (ENT Dept.), Mr. H.C.Choufoer (Laboratory for Anatomy and Embryology, University of Leiden), and Mr. J.J.Beentjes, Mr. L.D.C.Verschragen, and Mr. S.B.Neijman (Laboratory for Electron Microscopy). We also wish to acknowledge the support obtained from the Laboratory for Surgery of the Leiden University Hospital.

These investigations were mainly supported by the Netherlands Technology Foundation (STW); supplementary grants were received from the Heinsius Houbolt Fund.

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CHAPTER 5

THE EFFECT OF IMPLANTATION SITE ON PHAGOCYTE/POLYMER INTERACTION AND FIBROUS CAPSULE FORMATION

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SUMMARY

Implants of Silastic[R], Estane[R], polypropylene oxide, and an HPOE/PBT segmented polyether polyester copolymer were qualitatively and quantitatively evaluated, with respect to interaction with mononuclear and multinucleated phagocytes as well as fibrous capsule formation, after implantation at three sites in the rat middle ear. The volume of the phagocyte exudate surrounding the implants, the degree of implant degradation and fragmentation, and the thickness of the fibrous capsules, were found to be correlated with the implantation site. On the basis of these findings it can be concluded that it is important to assess the biocompatibility of a biomaterial at carefully chosen implantation sites.

INTRODUCTION

Degradation of a biomaterial is important in relation to biofunctionality. Relatively rapidly degrading polymers are used as adhesives and for sutures, drug-delivery devices, and resorbable implants serving as temporary scaffolding[1,2]. Metals are used frequently because of their relative stability in the body, e.g. in orthopedics. However, degradation of these materials has often led to malfunctioning of prosthetic devices[3,4]. Degradation can be due to the biological medium[5,6] as well as to cellular activity. The degradation of implants by cells is predominantly caused by interaction with mononuclear[1,7-12] and multinucleated[1,8,10-12] phagocytes, but also with granulocytes[1,13]. Implant degradation by phagocytes and encapsulation in fibrous tissue are responses which are characteristic for the foreign-body reaction[8,14,15]. The degree of these responses is affected by material factors such as chemical composition[16-18], surface texture[8,9,18-20], porosity and pore size[17,21,22], shape and size[23-25], but also by such experimental variables as animal species[26,27], implantation technique[27,28], and implantation site[10,21,24,26,27,29-31]. Hydroxyapatite, for example, is reported to degrade in the rat middle ear at a rate of 15-20 µm/year[31,32], whereas in other animals or in man the degradation of hydroxyapatite of the same composition was slower or even absent[33-35]. Both the

species of animal used and the implantation technique could have been responsible for the divergent patterns of hydroxyapatite breakdown[10,28]. The presence of multinucleated phagocytes at the hydroxyapatite interface[30,31] and the degradation of hydroxyapatite in rat middle ears[31] was shown to vary with implantation site and implantation technique[10,28,31].

If the site of implantation is taken as the only variable, the rat middle ear seems to be a good model for investigation of the effect of the implantation site on the foreign-body reaction in general and of the interaction between phagocytes and polymers in particular. In the present study, four chemically and structurally different biomaterials were implanted in the rat middle ear at three different sites. The biomaterials were selected as a possible scaffold for the restoration of tympanic membrane defects in a total alloplastic middle ear prosthesis (TAM)[36].

MATERIALS AND METHODS

Implants

The implanted biomaterials were 1) Dow Corning Silastic^R, 2) Estane^R 5714 F1 polyether urethane (B.F.Goodrich[37]), 3) polypropylene oxide[38], and 4) a poly(ethylene oxide hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer (55% HPOE and 45% PBT); Akzona Inc.[39]). Silastic implants were dense and 125 µm thick, whereas the other implants were 100 µm thick with porosities of about 50% (Fig. 1). All films were cleaned by extensive rinsing in running tapwater and distilled water. All implants of a given polymer originated from the same batch.

Animals and implantation technique

Implants made of the same material were implanted in rat middle ears at three sites: 1) in tympanic membrane tissue, 2) submucosally, and 3) between bulla bone and surrounding muscle tissue (Fig. 2). The tympanic membrane implants had a diameter of 3 mm (the diameter of the rat tympanic membrane). Submucosal implants measured 1 by 1 mm^2 and the bone/muscle implants 2 by 2 mm^2.

Male Wistar rats weighing beween 200 and 220 g were used. The middle ears were reached via a dorsal approach under Hypnorm^R anesthesia (100 µl/100 g body weight)[30]. After the bulla had been partially prepared free of muscle tissue and fascia, a small hole was drilled in the bulla bone with a diamond burr, leaving the middle ear mucosa intact (Fig. 2). Two submucosal implants were placed between the bulla bone and the middle ear mucosa. The burr defect was occluded with a bone/muscle implant placed between the bulla bone and the adjacent muscle

tissue, which was pushed back after the implantation. The tympanic membrane was also reached via a dorsal approach after perforation of the auditory canal. Part of the pars tensa was separated from the handle of the malleus and the annulus. The tympanic membrane implant was then placed over the handle of the malleus but also as an underlay below the exteriorly situated remnants of the tympanic membrane, which were still connected to the annulus (Fig. 2).

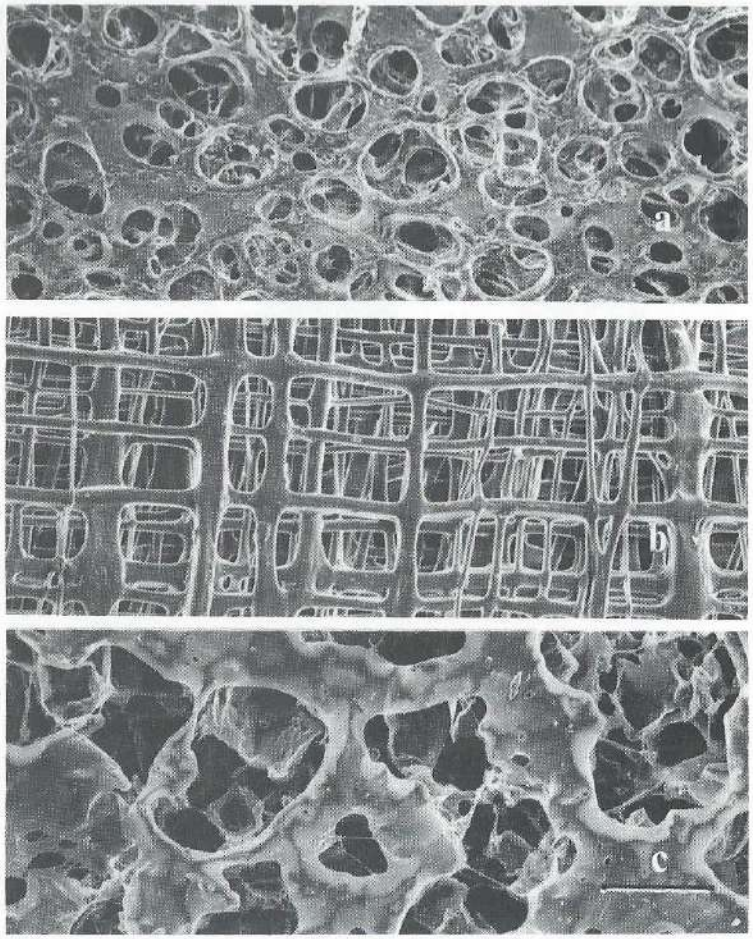


Fig. 1. Scanning electron micrograph of the surface of three biomaterials: (a) Estane^R, (b) polypropylene oxide, and (c) the HPOE/PBT copolymer. Note the relatively rough surface of Estane compared with that of polypropylene oxide and copolymer. Bar = 100 µm.

Table I. Distribution of an implant material according to time (weeks).

SUR		1	2	13	26	52*
TOT	TM#	12	12	11	11	11 (6@)
	SM	24	24	22	22	22 (12@)
	BM	12	12	11	11	11 (6@)

* Except HPOE/PBT polyether polyester copolymer.
\# Except Silastic^{R}.
@ For polypropylene oxide, due to the death of five rats.
SUR Survival time.
TOT Total number of implants.
TM, SM, and BM Tympanic membrane, submucosal or bone/muscle implant.

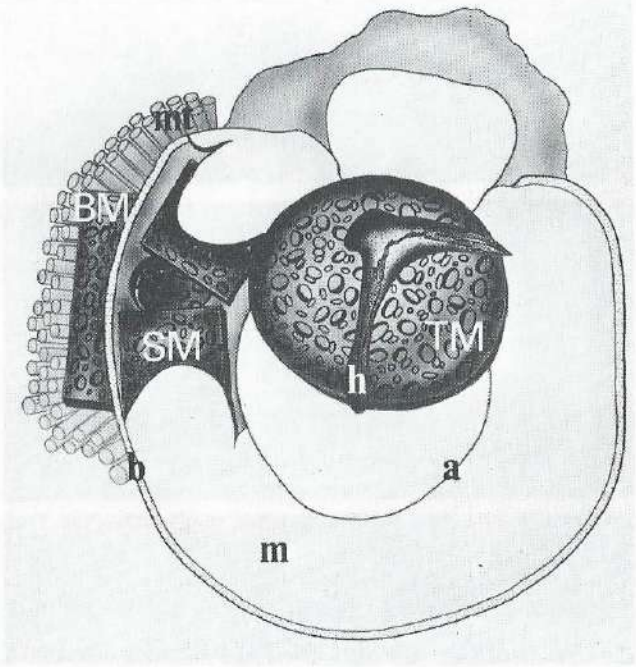


Fig. 2. Schematic representation of the implantation sites in the rat middle ear seen from the middle ear cavity. TM = tympanic membrane implant between hammer (H) and annulus (A); SM = submucosal implant between middle ear mucosa (M) and bulla bone (B); BM = bone/muscle implant surrounded by muscle tissue (MT).

Implantation periods were one, two, 13, 26, and 52 weeks, except for the copolymer, which was studied for up to 26 weeks (Table I). In all, 789 implants were investigated.

Light microscopy

Rats were killed by decapitation. After dissection, the middle ears were fixed for 2 hr in 2% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M Sörensen's buffer (pH 7.4, 4°C), and then for 14 hr in the same fixative without the glutaraldehyde. Next the ears were decalcified for 4 weeks in a 10% EDTA solution containing 2% paraformaldehyde (pH 7.4, room temperature). After being rinsed in running tap water for 24 hr, the specimens were routinely embedded in Paraplast^{R}. Sections cut 6 µm thick were stained with hematoxylin-eosin.

Morphometry

For morphometrical evaluation, 2042 Paraplast sections, which represented all of the biomaterials under study, were photographed at a standard magnification of x250, and evaluated on a computerized X-Y tablet used to measure for each implantation site: 1) the cross-sectional area seen under the light microscope occupied by phagocytes and porous biomaterial and 2) the degree of copolymer fragmentation. For the latter, the number of copolymer fragments, that derived from a single implant that covered a certain tissue area in cross-section, was counted. Additional calculation was done to obtain the number of fragments per mm^2 of tissue. For objective comparison of the three implantation sites, the measured biomaterial areas and the quantitative results on copolymer fragmentation were corrected for original implant volume, which was 0.35, 0.20, and 0.40 mm^3 for tympanic membrane, submucosal, and bone/muscle implants, respectively. The thickness of the fibrous capsules surrounding submucosal and bone/muscle implants made of Silastic in cross-section seen under the light microscope, was also measured. Data are reported as the average plus or minus the standard deviation about the mean and the number of samples is also included.

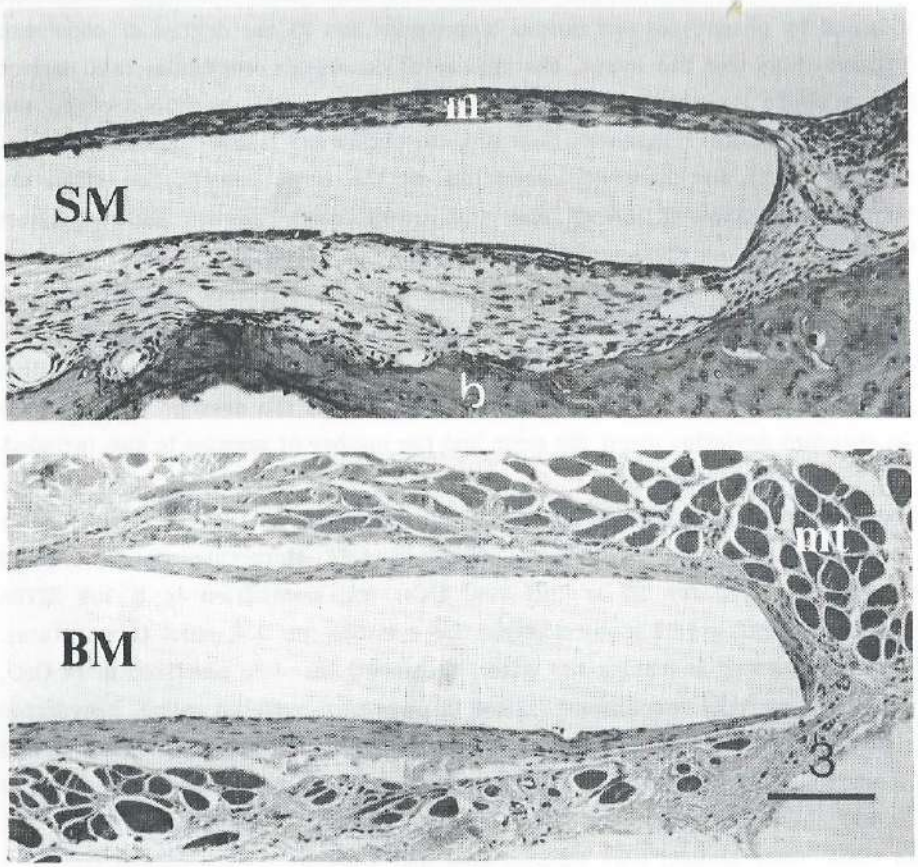
Transmission electron microscopy (TEM)

The ears destined for TEM were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer for 16 hr (pH 7.4, 4°C), and decalcified in a 10% EDTA solution containing 1.5% glutaraldehyde for 4 weeks (pH 7.4, room temperature). After being rinsed in running tap water, the specimens were postfixed in 1% OsO_4 for 30 min at room temperature, rinsed in phosphate-buffered saline, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections were cut on a LKB Ultratome, stained with uranyl acetate and lead citrate, and examined in a Philips EM 201 electron microscope at operating voltages of 60 and 80 keV. Sections, 1 µm thick, were stained with toluidin blue and studied by light microscopy.

RESULTS

The general picture was that the wound healing process could be observed for all implantations. As part of this process phagocytes were present throughout the wound site for up to two weeks. After that period most of the phagocytes were found at the surface of the implants.

Silastic^{R} implants were not incorporated into tympanic membrane tissue, whereas with Estane^{R}, polypropylene oxide, and copolymer implants the tympanic membrane healed within two to four weeks. Submucosal and bone/muscle Silastic implants were always surrounded by a capsule of non-vascularized fibrous tissue (Fig. 3). For implants between bone and muscle, the thickness of the fibrous capsules ranged from 10 to 30 µm thick (2-6 cell layers; number of samples is 388), whereas submucosally the fibrous capsules were 5 to 14 µm thick (1-3 cell



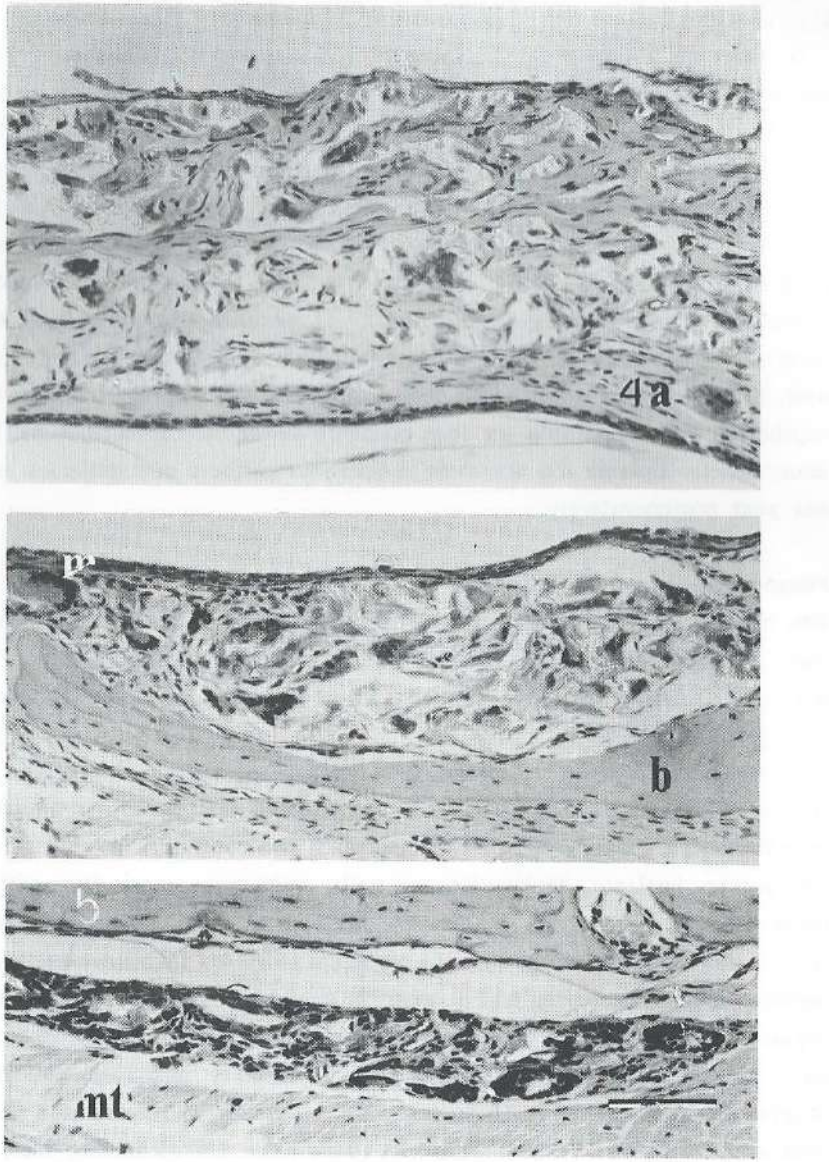


Fig. 4a-c. Light micrographs of Estane^{R} implants 13 weeks after implantation. (a) Tympanic membrane, (b) submucosal, and (c) bone/muscle implants. Note the effect of implantation site on phagocyte volume and number of multinucleated cells. M = mucosa; B = bulla bone; MT = muscle tissue. Bar = 40 µm.

Fig. 3. Light micrographs showing Silastic^{R} encapsulated in fibrous tissue after 26 weeks *in situ*. SM = submucosal and BM = bone/muscle implant, M = mucosa; B = bulla bone; MT = muscle tissue. Bar = 40 µm.

layers; 476 samples). The capsules were thinnest where Silastic bordered bone.

Implants made of Estane, polypropylene oxide, and copolymer were invariably surrounded by phagocytes and fibrous tissue, although a complete fibrous capsule surrounding the porous implants was never seen. For bone/muscle implants the organization of tissue differed from that of submucosal and tympanic membrane implants. Light microscopy showed that after 13 weeks of implantation bone/muscle implants were segregated into several foci of implant material and phagocytes. These foci were surrounded by thin, sparsely vascularized fibrous capsules (Fig. 4). This segregation of implant material was not seen at the other sites of implantation. Tympanic membrane implants were vascularized earliest and best, although at 13 weeks after implantation the infiltration of fibrous tissue by capillaries was comparable for both tympanic membrane and submucosal implants. Bone/muscle implants did not show comparable numbers of capillaries as late as one year postoperatively.

Phagocyte/polymer interactions

Few phagocytes were seen between Silastic and its fibrous capsule, and these cells were predominantly of the mononuclear type. The results of the TEM study showed that more phagocytes were attached to bone/muscle Silastic implants than to those situated submucosally.

In the first postoperative week, light microscopy showed differences between the three sites of implantation with respect to the occurrence and morphology of multinucleated phagocytes and the volume of the phagocyte population covering the porous implants. Multinucleated cells were seen most frequently where implants were surrounded by muscle tissue, and were less characteristic of submucosal and tympanic membrane implants (Fig. 4). In bone/muscle implants, multinucleated phagocytes had at most 20 nuclei as early as one week after the implantation, the number increasing to about 200 after 13 weeks. After one week both tympanic membrane and submucosal implants displayed multinucleated phagocytes with maximally 10 nuclei. After 13 weeks, multinucleated phagocytes with a maximum of 30 nuclei were associated with submucosal implants, whereas the corresponding number for tympanic membrane implants did not exceed 20. Multinucleated phagocytes usually appeared as rather flat cells, although in the case of predominantly bone/muscle implants they were less flattened and the nuclei were frequently located in the peripheral zone (Fig. 4 and 5a). TEM revealed that the cytoplasm of especially the multinucleated phagocytes was dominated by lysosomes, mitochondria, and small segments of rough endoplasmic reticulum (Fig. 5). Lamellopodial extensions were frequently seen along the

Table II. Average percentage of the tissue occupied by phagocytes in the course of time (weeks).

SUR		1	2	13	26	52*
EST	TM	43±36 (94)#	52±48 (102)	48±47 (91)	34±45 (71)	35±30 (88)
	SM	71±77 (70)	71±60 (64)	69±61 (83)	47±41 (80)	32±28 (72)
	BM	81±46 (88)	79±55 (99)	82±74 (108)	67±51 (97)	47±43 (74)
PPO	TM	24±20 (60)	20±24 (72)	56±52 (95)	62±57 (68)	11±11 (42)
	SM	48±38 (53)	51±44 (66)	63±60 (77)	74±82 (63)	11±15 (38)
	BM	54±61 (71)	62±68 (73)	66±49 (84)	70±47 (75)	28±26 (43)
COP	TM	18±21 (50)	27±44 (76)	33±33 (93)	34±23 (82)	-
	SM	42±46 (43)	42±60 (63)	37±31 (70)	38±42 (64)	-
	BM	59±39 (71)	59±65 (80)	48±62 (106)	44±38 (77)	-

* Except HPOE/PBT polyether polyester copolymer.
\# Mean ± 1 standard deviation for the number of samples shown in parentheses.
SUR Survival time.
EST Estane^{R} polyether urethane.
PPO Polypropylene oxide.
COP HPOE/PBT polyether polyester copolymer.
TM, SM, BM Tympanic membrane, submucosal or bone/muscle implant.

Table III. Mean areas (μm² x 1000) occupied by implants in the course of time (weeks).

SUR		1	2	13	26	52*
EST	TM	23±17 (94)#	23±21 (102)	19±21 (91)	12±8 (71)	14±9 (88)
	SM	25±14 (70)	20±15 (83)	15± 9 (83)	6±4 (80)	7±6 (72)
	BM	18±13 (88)	9± 6 (99)	6± 6 (108)	2±3 (97)	1±2 (74)
PPO	TM	16±11 (60)	16±11 (72)	7± 6 (95)	3±4 (68)	1±2 (42)
	SM	14± 9 (53)	13±11 (66)	6± 7 (77)	3±3 (63)	1±1 (38)
	BM	12±10 (71)	13±11 (73)	4± 4 (84)	1±2 (75)	0 (43)
COP	TM	24±15 (50)	24±21 (76)	18±13 (93)	11± 9 (82)	-
	SM	23±18 (43)	21±17 (63)	18±15 (70)	13±12 (64)	-
	BM	18±16 (71)	18±10 (80)	11± 8 (105)	9± 8 (77)	-

* Except HPOE/PBT polyether polyester copolymer.
\# Mean ± 1 standard deviation for the number of samples shown in parentheses .
SUR Survival time.
EST Estane^{R}.
PPO Polypropylene oxide.
COP HPOE/PBT polyether polyester copolymer.
TM, SM, BM Tympanic membrane, submucosal or bone/muscle implant.

unattached edge of multinucleated cells (Fig. 5a). The ultrastructural morphology of the phagocytes was comparable for all three implantation sites. With polypropylene oxide implants the phagocytes contained numerous vacuoles and showed mitochondria which were larger and had less densely packed cristae than those attached to the other biomaterials. Estane implants were most frequently associated with multinucleated phagocytes (Fig. 5b), whereas copolymer implants were predominantly associated with mononuclear phagocytes (Fig. 5c).

Light microscopy showed that the porous biomaterials contained larger volumes of phagocytic exudate after implantation between bulla bone and adjacent muscle tissue than after submucosal implantation or in the tympanic membrane (Fig. 6 and Table II). The quantitation of phagocyte volume in light-microscopical sections as a function of time showed for the three porous biomaterials that the phagocyte volumes decreased in the following site order: 1) bone/muscle tissue, 2) submucosal, and 3) tympanic membrane.

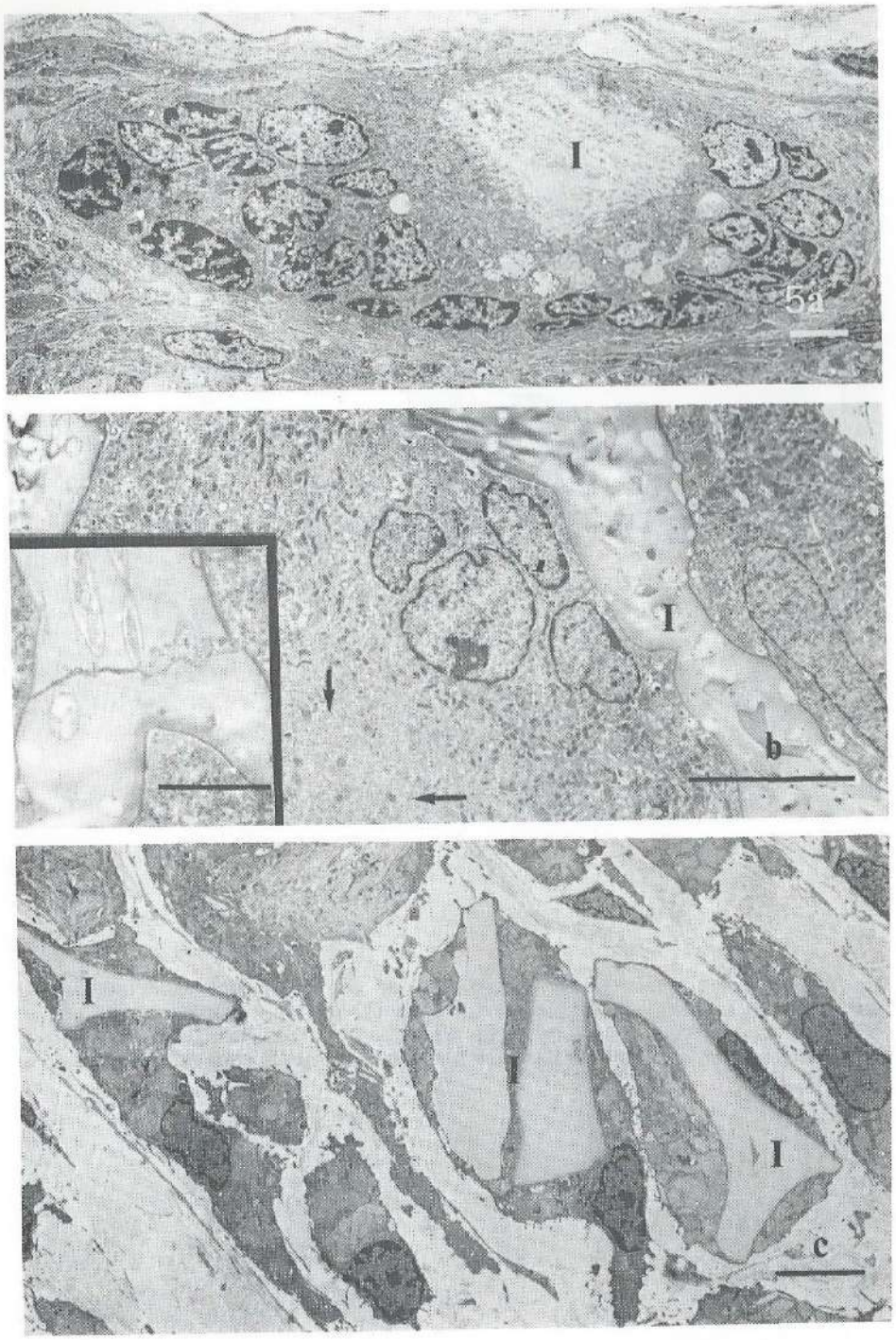
Degradation

Evaluation of the light-microscopical sections indicated, for all survival times, that the areas occupied by the porous biomaterials were smallest for the bone/muscle implants and largest for submucosal and tympanic membrane implants (Fig. 4). The size of the areas occupied by Silastic implants did not vary. Furthermore, light microscopy showed that with progression of time the integrity of bone/muscle implants was lower than that of submucosal and tympanic membrane implants.

Morphometry was performed in the area of light-microscopical sections occupied by the implants made of Estane, polypropylene oxide, and copolymer (Fig. 7 and Table III). These diagrams show a time-dependent decrease of the areas occupied by the biomaterials. Furthermore, those areas were smallest for bone/muscle implants and generally largest for implants in the tympanic membrane.

Results of the TEM study showed that phagocytosed pieces of the bone/muscle implants were smaller than those seen in submucosal and tympanic membrane sections. In the case of Estane, such pieces were also more irregular

Fig. 5a-c. Transmission electron micrographs showing submucosal (a) and tympanic membrane (b, c) implants after 13 weeks.
a: Polypropylene oxide (I) within Langhans cell. Bar = 7 µm.
b: Estane^{R} implant (I) surrounded by a multinucleated phagocyte. Note the centrioles in the cell (arrows); inset shows crack in Estane. Bars = 7 µm.
c: Macrophages containing fragments of copolymer (I). Bar = 7 µm.



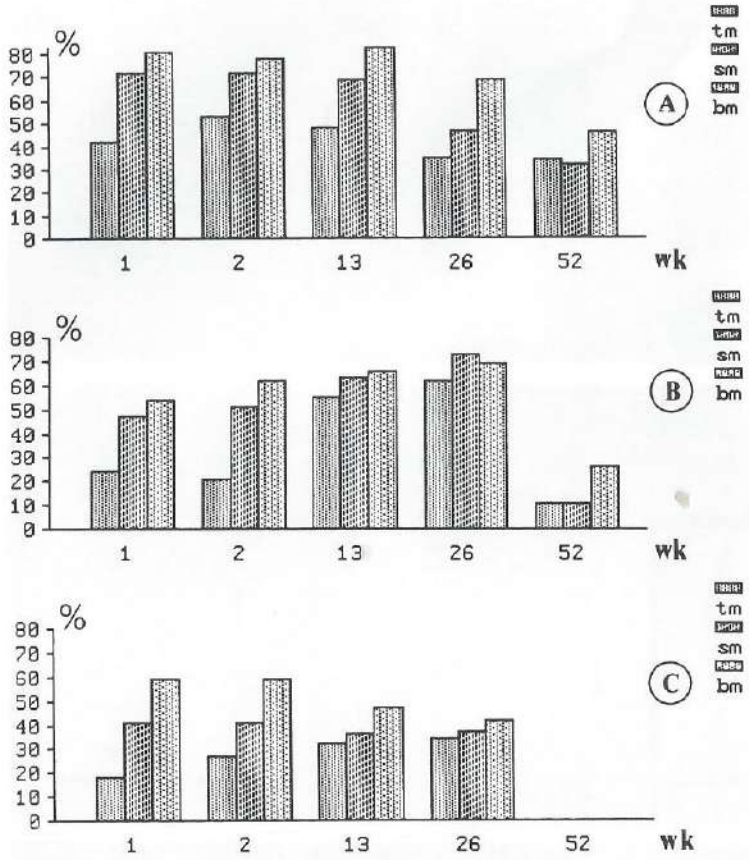


Fig. 6. Diagrams showing the percentage of the tissue area occupied by phagocytes as a function of time for (a) Estane^{R}, (b) polypropylene oxide, and (c) copolymer implants. TM = tympanic membrane, SM = submucosal, and BM = bone/muscle implant. Standard deviations and number of samples are reported in Table II.

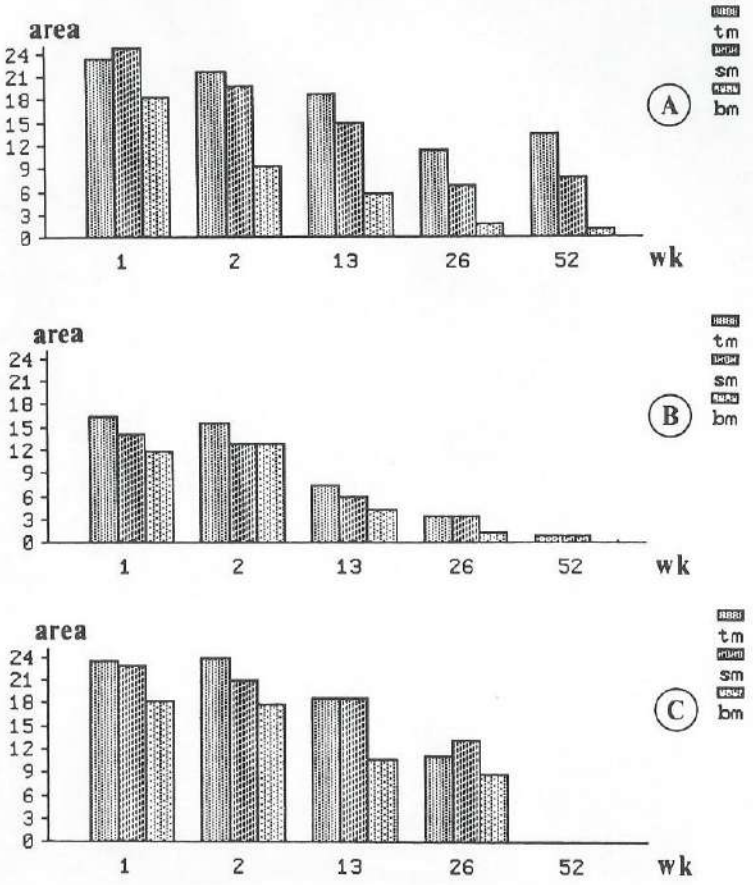


Fig. 7. Diagrams showing the mean areas (μm² x 1000) occupied by (a) Estane^{R}, (b) polypropylene oxide, and (c) copolymer implants in the course of time. TM = tympanic membrane, SM = submucosal, and BM = bone/muscle implant. Standard deviation and number of determiniations are shown in Table III.

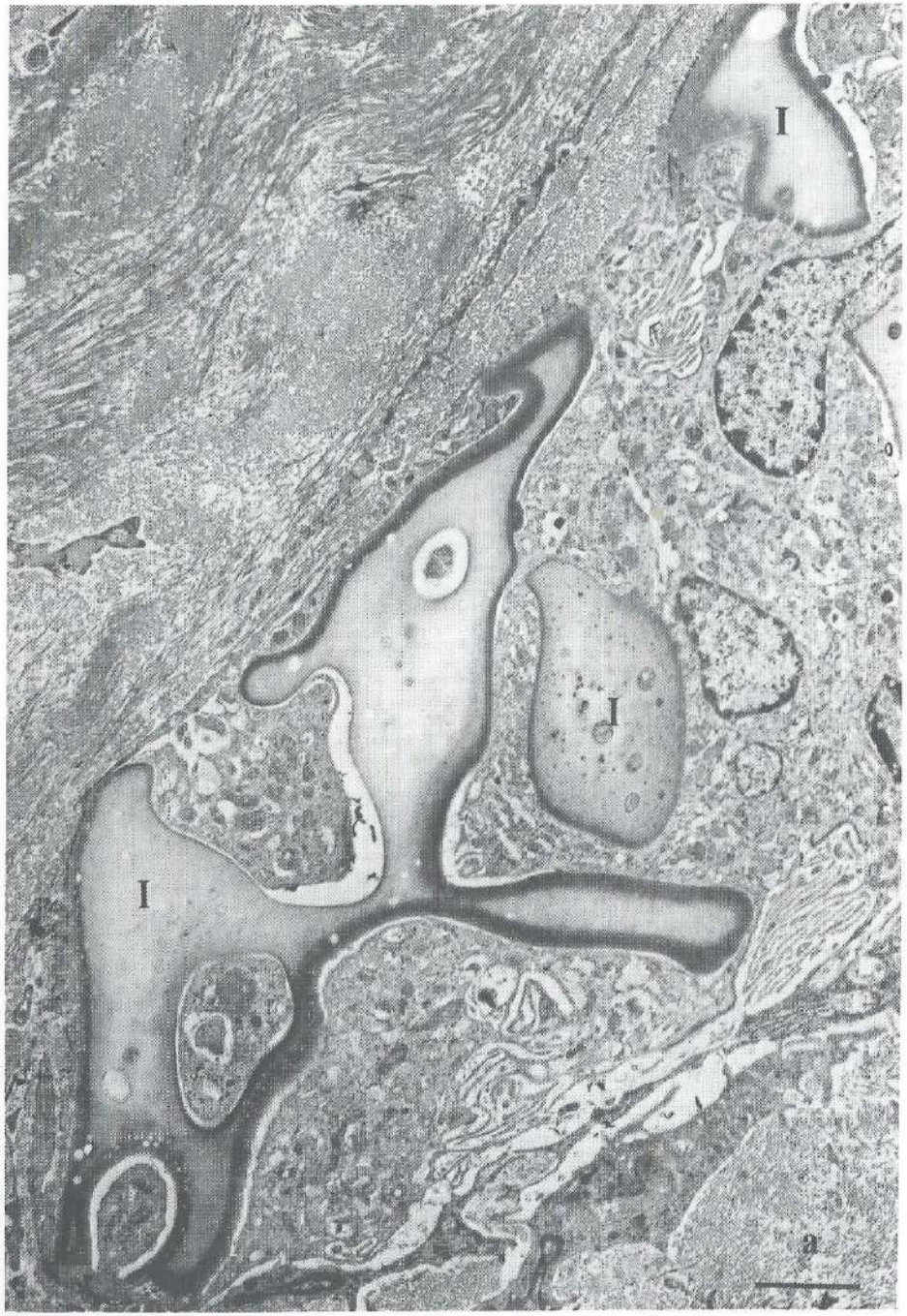
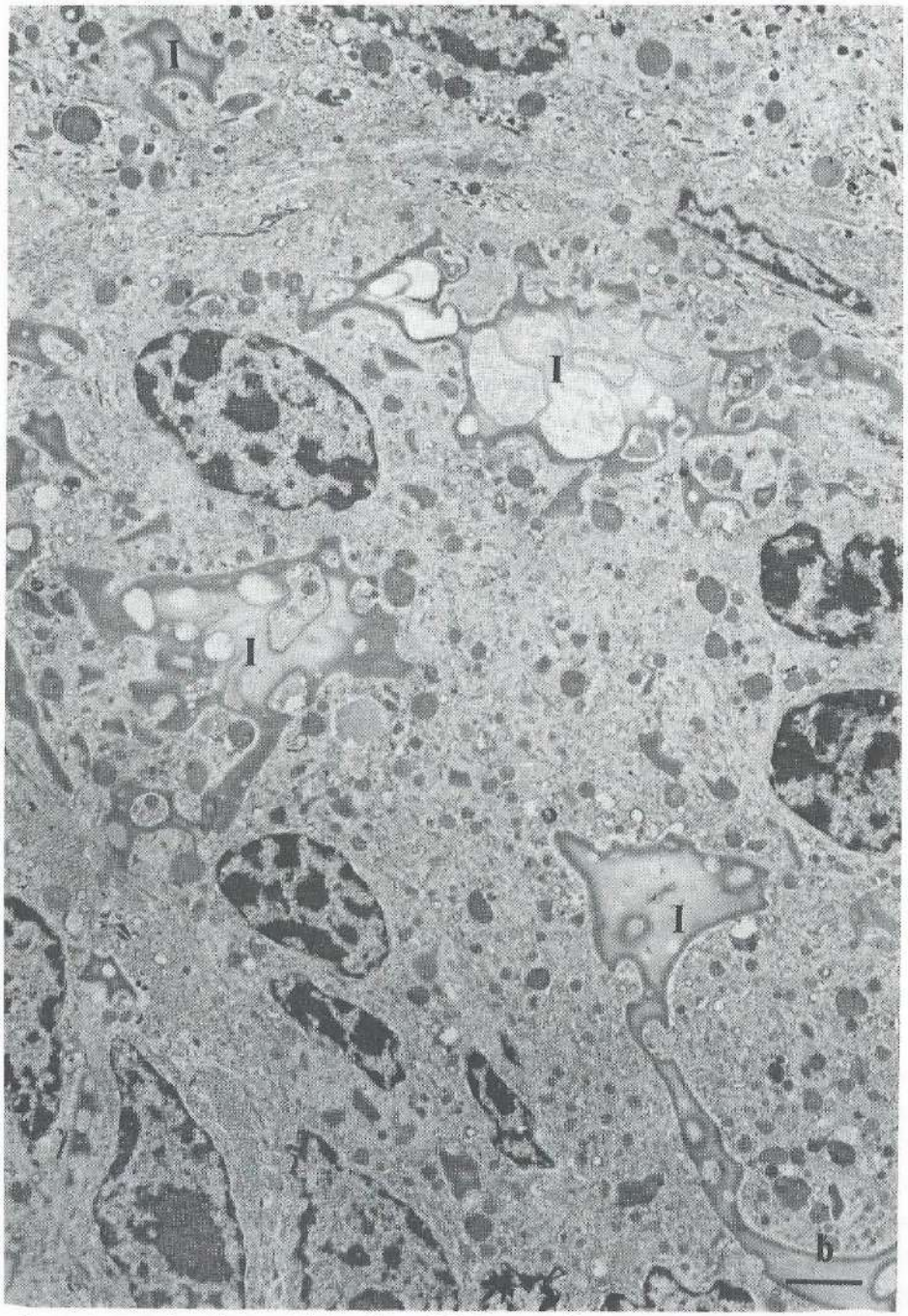


Fig. 8. Transmission electron micrographs showing Estane^{R} implants (I) at 52 weeks post-operatively: (a) in the tympanic membrane and (b) between bone and



adjacent muscle tissue. Note the different surface zones and the many irregularly shaped pores in b. Bars = 4 μm.

in shape (Fig. 8). The edges of phagocytosed polypropylene oxide showed signs of erosion and cellular invasion (Fig. 5a). The edges of copolymer implants appeared roughened, granular, and in some cases invaded by the cytoplasm of the adjacent phagocyte (Fig. 9). The morphological appearance of neither polypropylene oxide nor copolymer was affected by the implantation site.

Fragmentation

Phagocytosed Estane and copolymer displayed cracks which in the latter case frequently separated biomaterial fragments from the bulk of the implant (Figs. 5b (inset) and 9). No cracks were seen in polypropylene oxide implants. The mode of copolymer fragmentation depended on the implantation site (Fig. 10 and Table IV). Copolymer implanted between bulla bone and adjacent muscle tissue became fragmented more often and sooner than those in the submucosa and in the tympanic membranes (Fig. 11). Prominently fragmented copolymer implants showed predominantly mononuclear phagocytes which contained many copolymer fragments of different sizes and shapes (Fig. 9b).

Table IV. Quantitative results pertaining to HPOE/PBT polyether polyester copolymer fragmentation. Number of fragments* per mm² tissue as a function of time (weeks).

SUR	1	2	13	26
TM	1±2 (50)	5±3 (76)	26±13 (93)	49±30 (82)
SM	3±2 (43)	4±3 (63)	25±18 (70)	37±33 (64)
BM	6±4 (71)	15±6 (80)	63±44 (105)	85±52 (77)

* Mean ± standard deviation for the number of samples shown in parentheses.
SUR Survival time.
TM, SM, BM Tympanic membrane, submucosal or bone/muscle implant.

DISCUSSION AND CONCLUSIONS

During the early postoperative periods, the presence of the biomaterials under study resulted in tissue responses which were comparable to those characteristic of surgical trauma without an implant[8,14]. The presence of an implant is known to alter the normal sequence of events during the healing process[14,21,26]: a foreign-body reaction, which is considered to be a chronic inflammatory response, is usually the result[8,14]. At two weeks postoperatively, the implantation sites were characterized by a granulation tissue composed of fibroblasts and small capillaries in a matrix of collagen and by the presence of mononuclear and multinucleated phagocytes predominantly surrounding the

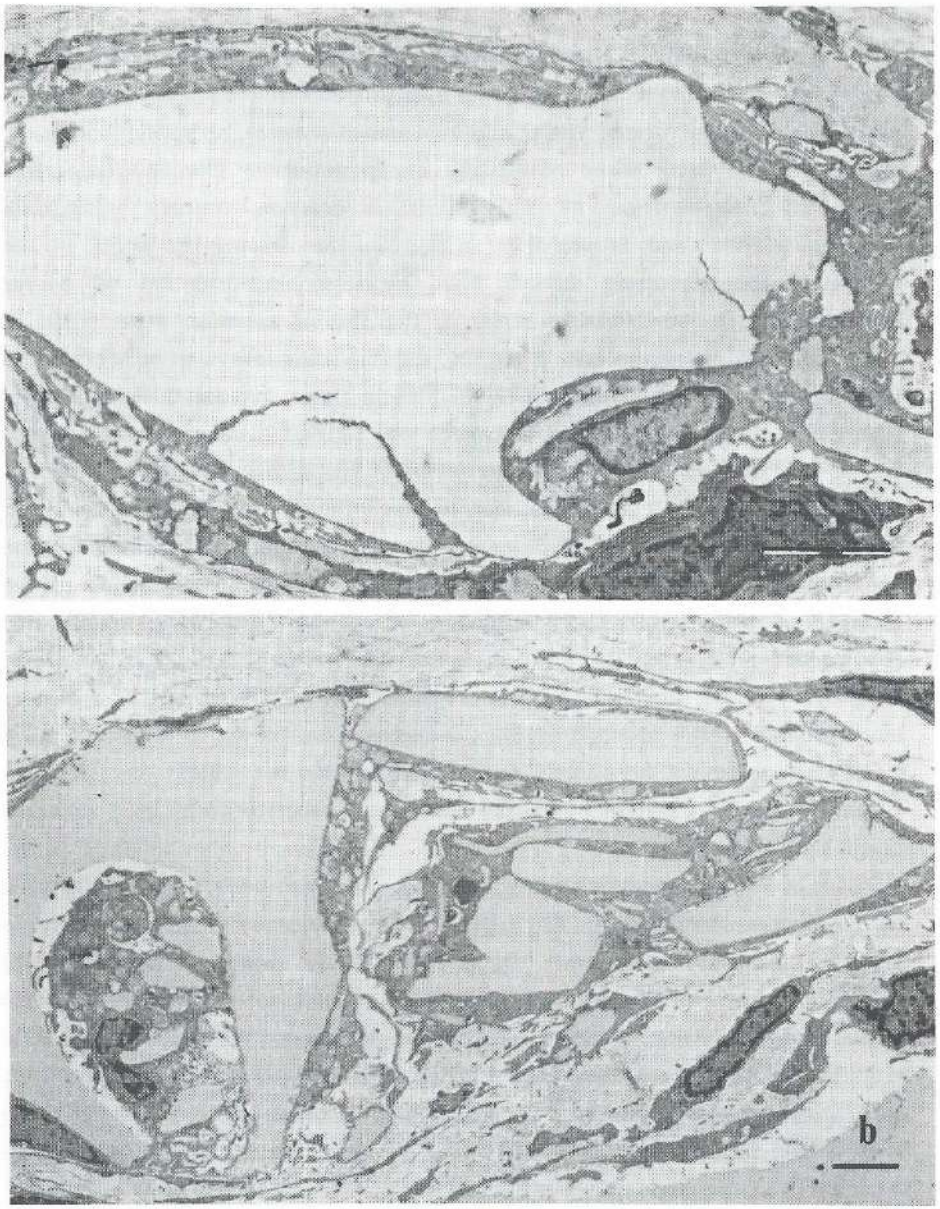


Fig. 9. Transmission electron micrographs of tympanic membrane implants showing HPOE/PBT copolymer fragmentation; (a) 13 and (b) 26 weeks post-operatively. Bars = 6 µm.

biomaterials. This picture is reported to be characteristic for the foreign-body

granuloma[14,15,26].

Silastic^R, which was the only non-porous material used, was not incorporated into tympanic membrane tissue, but was always completely encapsulated by non-vascularized fibrous tissue. The porous implants of Estane^R, polypropylene oxide, and copolymer were never completely encapsulated by fibrous tissue. The complete encapsulation of Silastic is a common feature with bulk materials[23,24,29,40,41] and is probably related to the absence of pores in the implant[21], although some authors have reported encapsulation of porous biomaterials[16]. In bone/muscle implants, the fibrous capsules were twice as thick as those on submucosal implants, and the thickness was comparable to that of intramuscular implants in rats[42]. Muscle tissue induces thicker capsules than other soft tissues do[24,27,29], although the size of a dense implant is also known to exert an influence. Larger implants (bone/muscle) generate thicker capsules[23]. Few phagocytes were seen between the Silastic implants and their fibrous capsule. The TEM findings led to the conclusion that most of the phagocytes were of the mononuclear type and were mainly attached to the interface of bone/muscle Silastic implants. A correlation between toxicity and the amount of degradation product on the one hand and the prominence of phagocytes or the fibrous capsule on the other, has been established for several biomaterials[40,41]. The absence of Silastic degradation for up to 52 weeks made it impossible to establish such correlation in the rat middle ear. Silicone rubber degradation has been reported by other investigators who used different conditions[43].

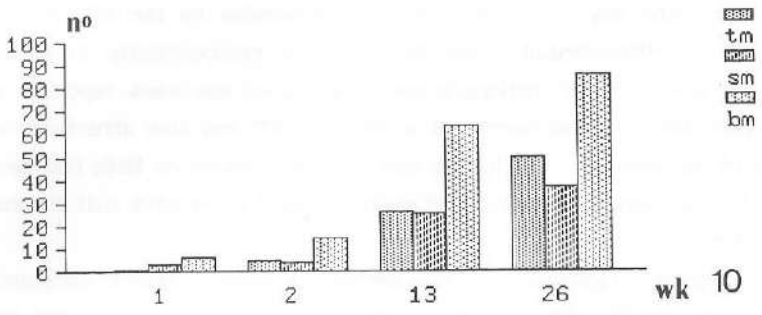
Pores in Estane, polypropylene oxide, and copolymer were always infiltrated by phagocytes, fibrous tissue, and capillaries. Light microscopically, bone/muscle implants displayed the strongest foreign-body reaction, which led to several foci of implant material surrounded by phagocytes and non-vascularized fibrous tissue. These foci were morphologically comparable to the small foreign-body granulomas often associated with relatively rapidly degrading materials[44]. At such sites vascularization was minimal, which is often explained by assuming that newly formed capillaries had been disrupted by the movement of the implants[21]. Morphological evaluation of the phagocyte volumes in the light-microscopical sections of the porous biomaterials showed that this volume was a function of the implantation site. For the three biomaterials, phagocyte volumes decreased in the following site order: 1) surrounded by or adjacent to muscle tissue, 2) submucosally, and 3) in the tympanic membrane. The light-microscopical picture showed that most of the multinucleated phagocytes were located in the pores of bone/muscle implants. Some investigators attribute the

large numbers of multinucleated phagocytes to an attempt by the body to isolate an implant from the environment[45] in a way comparable to the effect of a fibrous capsule. Multinucleated phagocytes attached predominantly to Estane implants. The frequency of multinucleated phagocytes has been reported to depend on both the chemical nature of a biomaterial[46] and fine structures on the surface of an implant[9,19,20]. Whether one of these factors or both had been responsible for the marked association of multinucleated giant cells with Estane, is not yet clear.

The area occupied in light-microscopical sections by porous implants decreased with time, most rapidly for polypropylene oxide and most slowly for the copolymer. For the three porous implants degradation decreased in the site sequence: (1) adjacent to or surrounded by muscle tissue, (2) submucosally, and (3) in the tympanic membrane. TEM showed particles of all of the implant materials in the cytoplasm of mononuclear and multinucleated phagocytes. Mononuclear and multinucleated phagocytes are known to phagocytose biomaterial[1,9,12]. In phagocytes occurring with bone/muscle implants, implant-derived particles were more strongly eroded than those in the tympanic membrane. This suggests that phagocytes show greater activity in association with bone/muscle implants.

The signs of erosion of polypropylene oxide implants as well as the large number of vacuoles occurring in phagocytes containing polypropylene oxide, indicate a relatively rapid degradation of this material[7,9,44,47]. The polypropylene oxide we used has been reported to undergo rapid hydrolysis releasing aldehydes[48]. Both the deviant morphology of most of the mitochondria in phagocytes containing polypropylene oxide and the untimely death of five rats in the polypropylene oxide group, all of which were scheduled for decapitation one year postoperatively, point to toxic effects of the aldehydes released during polypropylene oxide breakdown. These results, which confirm the negative findings made for polypropylene oxide in an *in vitro* biocompatibility study[49], underscore the importance of long-term implantation studies.

Whereas polypropylene oxide did not fragment and Estane did so only incidentally, copolymer implants underwent fragmentation in the course of time. The mode of copolymer fragmentation depended on the implantation site, and the degree decreased in the following order: 1) adjacent to or surrounded by muscle tissue and 2) both submucosally and in the tympanic membrane. Although the numerous copolymer fragments must have considerably enlarged the surface area of the implant relative to the volume of the material, an increase of copolymer fragmentation was not accompanied by a comparable increase of



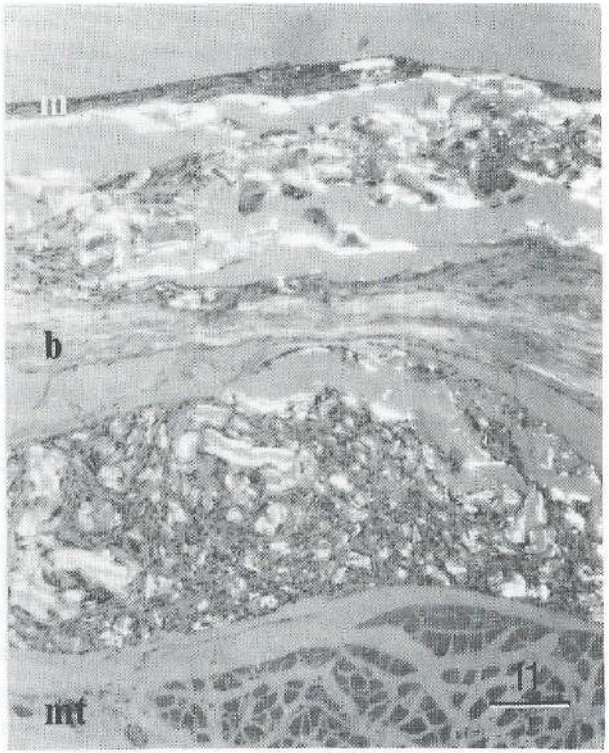


Fig. 10. Quantitative results pertaining to polyether polyester copolymer fragmentation, as number per mm^2 tissue in cross-section for tympanic membrane (TM), submucosal (SM), and bone/muscle (BM) implantation site. Standard deviation and number of samples are reported in Table IV.

Fig. 11. Polarized-light micrograph. Copolymer fragments between bulla bone (B) and mucosa (M) are larger and less numerous than those between bone and muscle tissue (MT). Bar = 40 µm.

copolymer degradation. Such coupling was to be expected, because the rate of degradation of a material is proportional to the exposed area[1,27,50]. It is possible that copolymer did not undergo degradation, but that the observed decrease of copolymer area as a function of time was the result of transportation of copolymer fragments by macrophages to e.g. lymph nodes, where they accumulated[51] and went undetected by our measurements. With respect to this postulated copolymer transportation, it is interesting that the largest numbers of copolymer fragments were seen in the cytoplasm of mononuclear phagocytes, which are more motile *in vitro* than multinucleated phagocytes are[52] and, further, that small particles are generally phagocytosed by macrophages[45,53], whereas larger particles are surrounded by multinucleated phagocytes[53]. It is also conceivable that quantitation of the copolymer area was hampered by the minuteness of some of the copolymer fragments; TEM frequently showed fragments too small to be observed at the light-microscopical level. But a group of small fragments could also have appeared as a single fragment on a light micrograph, leading to overestimation of the copolymer area. The edges of copolymer were roughened, granular, and occasionally invaded by cytoplasm, all of which are considered characteristic of polymer degradation by phagocytes[7,8,44]. On these grounds it seems likely that copolymer degradation had contributed to the decreased copolymer area we observed. For correct assessment of copolymer degradation, morphometry must be performed at the electron-microscopical level.

The encapsulation of Silastic by fibrous tissue, the phagocyte volume, and the polymer degradation - all of which were associated with the porous implants made of Estane, polypropylene oxide, and copolymer - and the fragmentation of copolymer, were shown unequivocally to depend on implantation site in the rat middle ear. Although each polymer interacted specifically with tissue, these exponents of the foreign-body reaction invariably decreased in the following site order: (1) adjacent to or surrounded by muscle tissue, (2) submucosally, and (3) in the tympanic membrane. The differences observed between bone/muscle and submucosal implantation sites are in good agreement with the findings on hydroxyapatite degradation and the incidence of phagocytes at the hydroxyapatite surface in the rat middle ear[10,28,30,31], and support the hypothesis that mismatch of the elastic moduli of implant and tissue causes stress and movement at the interface, resulting in mechanical irritation of the surrounding or adjacent tissue; the greater the irritation the stronger the foreign body reaction[10,24,27,29,30].

Mechanical irritation does not seem to be the only explanation for the

implantation-site dependency of the foreign-body reaction. First, the differences between tympanic membrane and submucosal implants found in this study are less easily explained by the hypothesis of mechanical irritation than the differences between either of these sites and the bone/muscle site; at two of the implantation sites the porous biomaterials were not in contact with muscle tissue. Second, biomaterials implanted in muscles with different activities provoked similar responses[14,29], although different responses were to be expected.

It would be interesting to find out whether mismatching of elastic moduli of implant and tissue in the absence of muscle tissue adequately explains the differences observed between tympanic membrane and submucosal implantation sites, or whether such variables as aeration and the incidence of capillaries play a role.

In sum, the results of this study made it clear that four chemically and structurally different biomaterials induced foreign-body reactions that showed similar variations as functions of implantation site. This finding underlines the importance of assessment of the biocompatibility of a biomaterial at implantation sites where the tissue response to the presence of the implant will match as closely as possible those provoked by the same material when applied clinically.

Acknowledgements

The authors are indebted to Dr. J.H.Kuit and Dr. J.G.F.Bots (Department of Chemical Technology, Twente University of Technology, Enschede) in the preparations of the biofilms. The polyether polyester copolymer was a generous gift from Prof. K.B.Wagener (Department of Chemistry, University of Florida, Gainesville, Florida). The technical assistance of Ms. P. van der Hoek and Mr. B.M.A. van der Lans (ENT Department), Mr. H.C.Choufoer (Dept. of Anatomy and Embryology, University of Leiden), and Mr. L.D.C.Verschragen, Mr. J.J.Beentjes, and Mr. S.B.Neijman (Laboratory for Electron Microscopy) is gratefully acknowledged.

These investigations were supported by the Netherlands Technology Foundation (STW).

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CHAPTER 6

TISSUE/MATERIAL INTERFACE CHARACTERISTICS OF FOUR ELASTOMERS.

A transmission electron microscopical study

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SUMMARY

The tissue/biomaterial interface reactions of four elastomers - selected as candidates for scaffolding for tympanic membrane tissue in a total alloplastic middle ear prosthesis - were studied at the electron microscopical level after implantation in the rat middle ear. Time-dependent changes in the phagocyte/polymer interface suggested degradation of porous implants made of Estane^{R}, polypropylene oxide, and a poly(ethylene oxide hydantoin) and poly(tetramethelene terephthalate) segmented polyether polyester copolymer, but not of dense Silastic^{R} implants. Silastic was always encapsulated in fibrous tissue. Contact between fibrous tissue and HPOE/PBT copolymer or Estane was established in the third month, but fibrous tissue was never seen close to polypropylene oxide. Bone made direct contact only with Estane and the copolymer implants. The bone/copolymer interface showed an electron-dense layer morphologically similar to that seen between bone and hydroxyapatite; this layer was absent at the bone/Estane interface. Estane and especially the HPOE/PBT copolymer seem to be suitable as alloplastic tympanic membrane because of their interface behavior with respect to fibrous tissue and bone.

INTRODUCTION

For the reconstruction of an empty middle ear cavity, we have been developing a totally alloplastic middle ear prosthesis (TAM) consisting of a porous hydroxyapatite canal wall segment as suspension system and a dense hydroxyapatite ossicular chain as sound-conducting system[1]. This prosthesis requires an alloplastic tympanic membrane for fixation of the ossicular chain to the suspension system[2] to prevent extrusion of the ossicular chain and for better sound-transducing properties. In this situation the porous tympanic membrane material should act as a scaffolding to support proliferating tissue.

Implant fixation by tissue, brought about by bonding across an interface, can be based on mechanical bonds, chemical bonds, or bonds created by physical forces[3]. Generally, mechanical bonds underlie fixation of porous implants[3-5], whereas chemical interface reactions lead to a direct bond between bioactive implants and bone[3,6-12]. For permanent fixation chemical bonding seems to be the most

effective, and bonds established by physical forces are not promising[3]. With polymeric biomaterials, implant fixation seems to result solely from the growth of fibrous tissue and bone into pores, because chemical bonding between bone and polymers has not been observed[13-17].

Appreciable degradation of the tympanic membrane material before it has been integrated into the middle ear by tissue ingrowth endangers the connection between the ossicular chain and the canal wall of the Total Alloplastic Middle ear. Macrophages[11,12,18-22] and foreign-body giant cells[11,12,18-22] are frequently associated with implant degradation. In a number of these studies[11,20,21] the phagocyte/biomaterial interface was found to provide important morphological information about material degradation.

Silastic^R silicone rubber, Estane^R 5714 F1 polyether urethane, polypropylene oxide, and a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer were chosen for a series of studies on candidate materials for alloplastic tympanic membrane in the TAM[23,24]. In the present study electron microscopy was used to investigate the interaction between each of the four biomaterials and phagocytes, fibrous tissue, and bone at the tissue/biomaterial interface.

MATERIALS AND METHODS

Implants and implantation technique

Dense implants made of the Dow Corning silicone rubber Silastic^R and porous implants (50% over-all porosity, pore sizes up to 160 μm) made of Estane^R 5714 F1 polyether urethane (BFGoodrich), polypropylene oxide[25], and a poly(ethylene oxide hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer (55% HPOE and 45% PBT; Akzona Inc.[26]) were used. The implants, which were 100-125 μm thick, were cleaned by extensive rinsing in running tap water and distilled water. The implants were placed at three sites in the middle ear of male Wistar rats (body weight 200-220 g): in the tympanic membrane, between the middle ear mucosa and bulla bone, and between the bulla bone and adjacent muscle tissue[24]. In all, 266 implants made of the four biomaterials under study, were evaluated after one, two, four, 13, 26, and 52 weeks *in situ*.

Transmission electron microscopy

After fixation in 1.5% glutaraldehyde in 0.14 M cacodylate buffer for 16 hr (pH 7.4, 4°C) the middle ears were decalcified by immersion for 4 weeks in 10% EDTA

and 1.5% glutaraldehyde (pH 7.4, room temperature). Postfixation was performed in 1% $0s0_4$ for 30 min at room temperature. After embedment in Epon, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Philips EM 201 electron microscope.

RESULTS

Phagocyte/polymer interface

Compared with the porous implants, relatively few phagocytes adhered to Silastic^R. The majority of the phagocytes were macrophages. Only implants bordered by muscle tissue showed appreciable numbers of multinucleated phagocytes for up to one year. Rough endoplasmic reticulum and ribosomes were the predominant structures in the cytoplasm of the phagocytes, and mitochondria and lysosomes were seen occasionally. The phagocyte/Silastic interface was smooth and did not change in the course of time. Cell organelle-poor adherence zones were not seen and the same holds for phagocytosis and penetration of Silastic by cytoplasm (Fig. 1).

The surface of polypropylene oxide implants remained smooth during the first postoperative month. Early contact between polypropylene oxide and mononuclear and multinucleated phagocytes was characterized by the presence of phagocytes with relatively large organelle-poor adherence zones containing numerous filaments

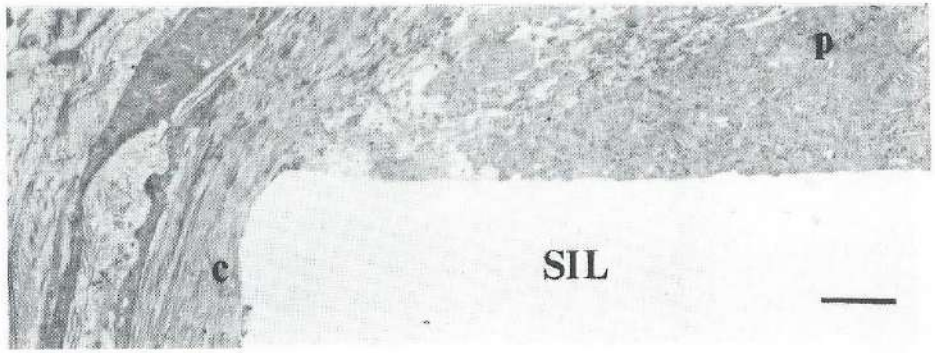

Fig. 1. Part of a fibrous capsule surrounding a Silastic^R implant (SIL). Note the presence of a phagocyte (P) and collagen (C). Bar = 1.4 μm.

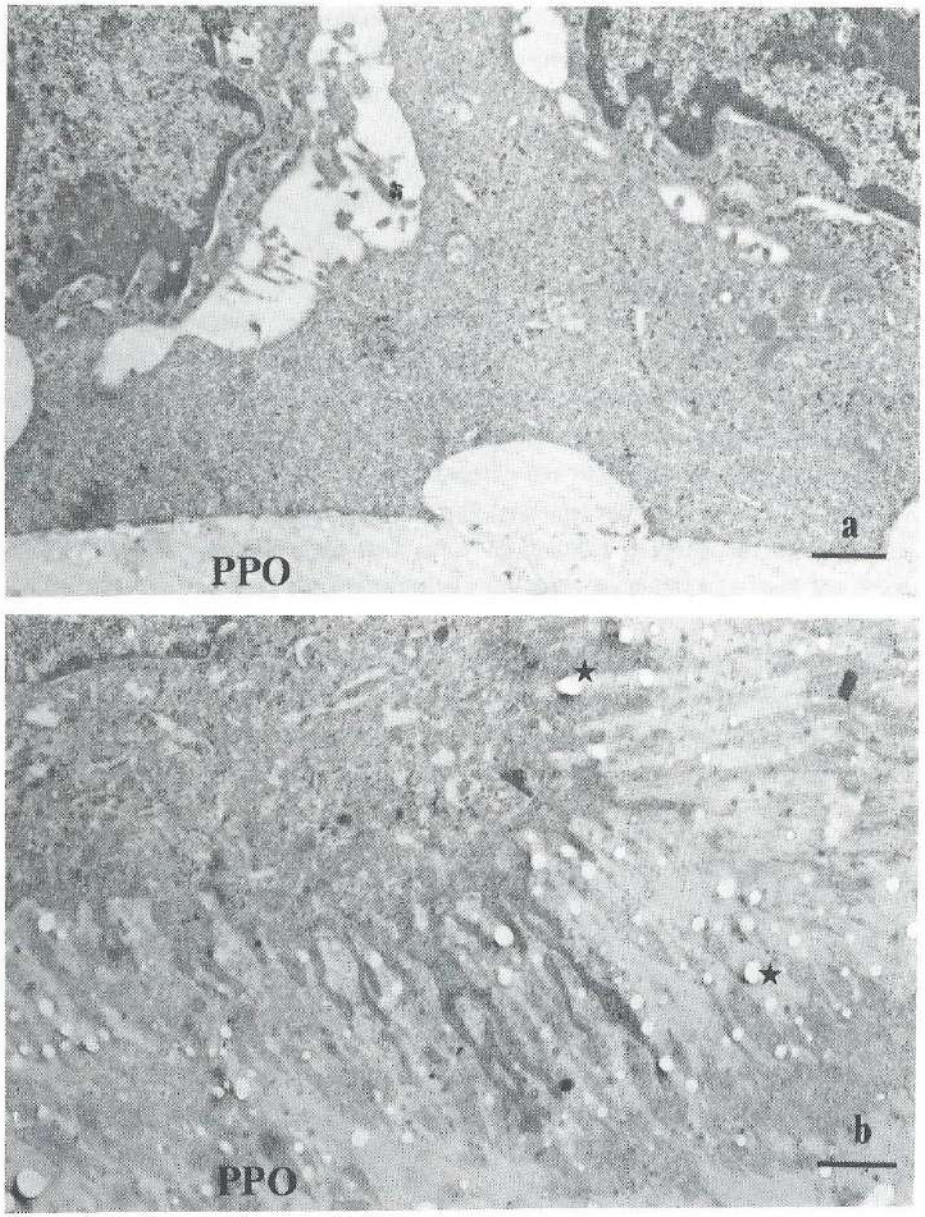

Fig. 2a and b. Phagocyte/polypropylene oxide (PPO) interface showing a smooth polymer surface 2 weeks postoperatively (a) and an interdigitated cell membrane 3 months after implantation (b). The polymer shows artefacts associated with resin infiltration (asterisk). Note in a the distance between the cell organelle-poor adherence zone and the polymer surface. Bars = 0.7 µm.

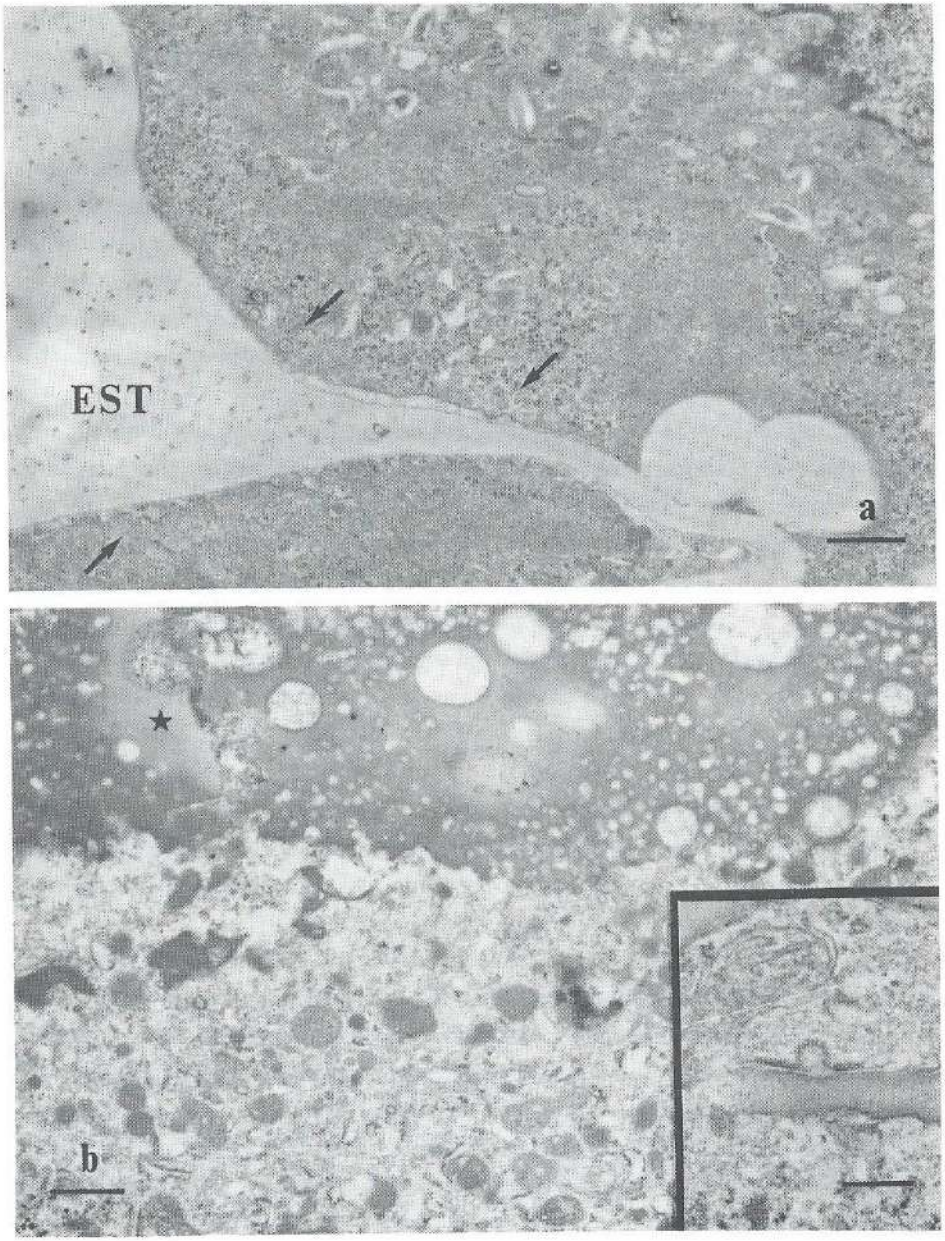

Fig. 3a and b. Phagocyte/Estane^{R} interaction 2 weeks (a) and 12 months (b) postoperatively.
a: Section showing cell organelle-poor adherence zone with Estane implant (EST). Arrows indicate coated pits. Bar = 0.7 µm.
b: Micrograph showing increased electron-density of the eroded Estane surface zone. Asterisk indicates less eroded part of the polymer. Note the abundance of cell organelles. Bar = 0.7 µm. Inset: Coated pit at Estane surface. Bar = 0.25 µm.

and some ribosomes. Adherence zones occurred close to the implant, although small segments were also observed about 1 µm from the surface (Fig. 2a). Between the third month and the end of the first half year, the area of interaction surface increased considerably (Fig. 2b). Organelle-poor adherence zones were less prominent than in the shorter periods, and showed many finger-like projects invading the implant by as much as several microns. Cross-sections of these cytoplasmic extensions, which were 0.1-0.2 µm in diameter, showed an abundance of pinocytotic vesicles, which suggests that the extensions were sites of pinocytotic activity. Mitochondria, ribosomes, and rough endoplasmic reticulum were the cell organelles seen most frequently within the phagocytes that surrounded polypropylene oxide.

Estane^{R} implants were completely electron lucent before implantation but also during the first postoperative weeks (Fig. 3a). After one month, the surface of Estane bordered by phagocytes was more opaque and the thickness of this electron-dense surface zone increased with time (compare a and b in Fig. 3). Six and twelve months after implantation, the electron-dense surface zone of Estane showed many small holes that had not originally been present (Fig. 3b). After postoperative periods of three months and longer, small fragments of biomaterial with the increased electron density were seen. Coated pits and pinocytotic vesicles (Fig. 3a and b, and inset) as well as cells with organelle-poor adherence zones occurred frequently (Fig. 3a). The cytoplasm of the cells adjacent to the biomaterial contained mitochondria, rough endoplasmic reticulum, ribosomes, multivesicular bodies, and lysosomes. After three months, the electron density of the surface zones of cracks in Estane implants was comparable to that of non-implanted Estane.

The phagocyte/copolymer interface was relatively smooth up to one month postoperatively. During this interval adherence zones with cells poor in organelles were present and were associated with early copolymer fragmentation, since they usually occurred between the larger copolymer fragments. Copolymer fragmentation was very prominent. Before implantation, copolymer was electron lucent and had a smooth surface. Transmission electron microscopy showed that the fragments increased in number and their morphology changed during the longer implantation periods. In due time, furthermore, the phagocyte/copolymer interface became granular and was frequently invaded by the cytoplasm of the phagocytes (Fig. 4a and b). Smaller fragments showed more erosion and greater electron density than the larger ones did (Fig. 4b). Mitochondria, rough endoplasmic reticulum, and lysosomes dominated in the phagocyte cytoplasm. Fusion of lysosomes with implant fragments was seen six and twelve months after implantation (Fig. 4b).

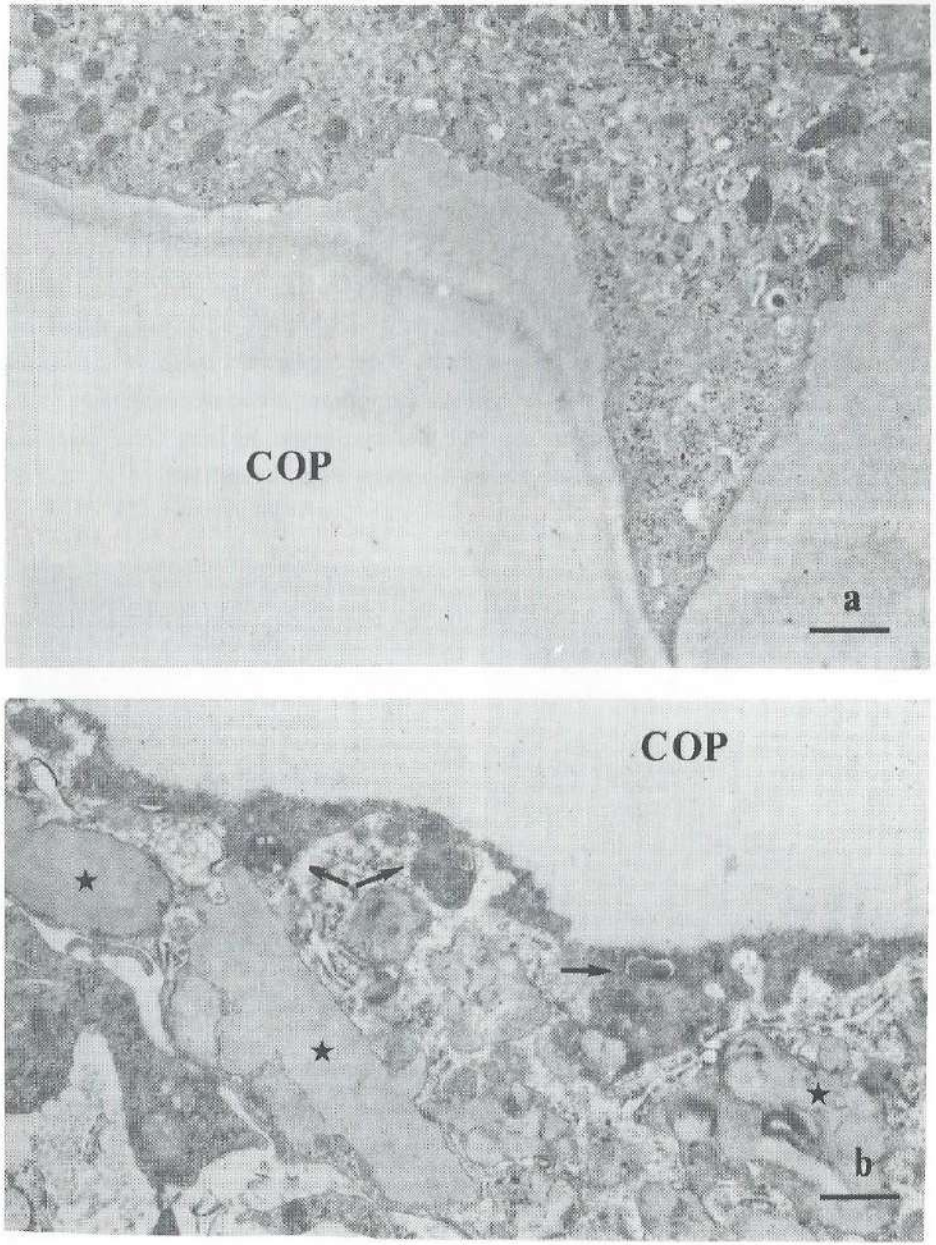

Fig. 4a and b. Interface between phagocyte and HPOE/PBT copolymer (COP) 3 months (a) and 6 months (b) after implantation.
a: Note the penetration of cytoplasm into the polymer and the abundance of mitochondria. Bar = 1 µm.
b: Numerous HPOE/PBT copolymer fragments (asterisks) which are more osmiophilic than most of the bulk copolymer. Note the multivesicular bodies (arrows). Bar = 0.7 µm.

Fibrous tissue/polymer interface

Fibrous tissue bordered Silastic, Estane, and copolymer implants but not those made of polypropylene oxide. This tissue encapsulated Silastic (which was not incorporated into tympanic membrane tissue) after the first week and was composed of fibroblasts, striated collagenous fibrils about 50 nm in diameter, and thinner filaments of about 10 nm without a banding pattern, all running parallel to the implant surface (Fig. 5a). Fibrous tissue established contact with copolymer and Estane during the third month but only in tympanic membranes (Fig. 5b). This tissue was composed of striated collagenous fibrils about 50 nm in diameter. Fibroblasts and 10-nm filaments were absent. With copolymer, some of the fibrils ran parallel to the surface of the biomaterial but others ended perpendicular to it. In this region Estane did not display the electron-dense surface zone that was seen at the phagocyte/Estane interface (compare Figs. 3 and 5b).

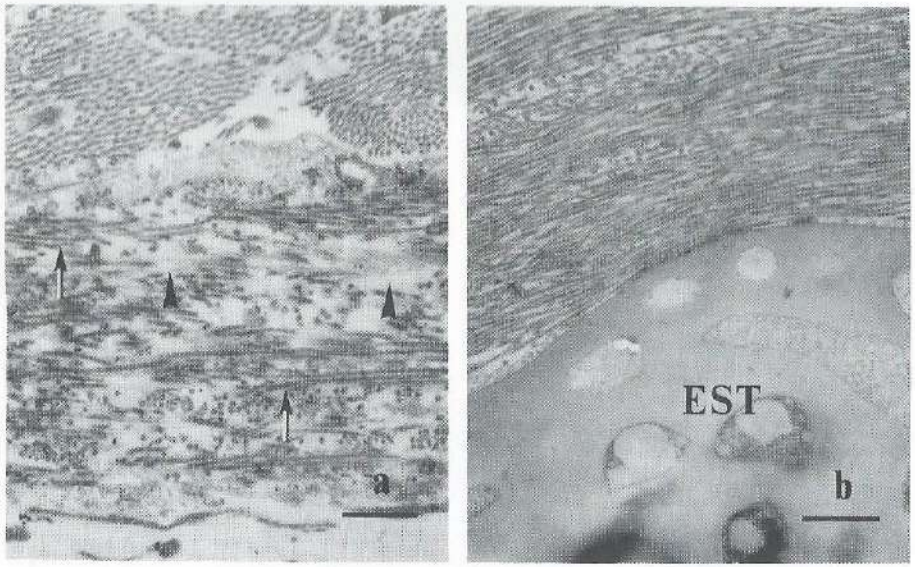

Fig. 5a and b. Electron micrographs of Silastic^{R} (a) and Estane^{R} (b) surrounding fibrous tissue.
a: Note the collagenous fibrils (arrows) and the 10-nm filaments (arrowheads) in the fibrous capsule. Bar = 0.5 µm.
b: The fibrous tissue/Estane (EST) interface is composed of collagenous fibrils running parallel to the surface of the implant. There are no 10-nm filaments and the electron-dense surface zone of Estane seen after the interaction of Estane with phagocytes is absent. Bar = 0.35 µm.

Bone/polymer interface

Bone was in direct contact with porous implants made of copolymer (Fig. 6) and Estane (Fig. 7), but not with those of polypropylene oxide. Bone was invariably separated from Silastic by an intervening layer of fibrous tissue. Bone/copolymer contact was seen as early as one week after the implantation and occurred with both submucosal and bone/muscle tissue implants. The resulting interface was characterized by an electron-dense layer on the surface of the copolymer. This layer was sometimes granular in appearance and between 100 and 700 nm thick (Fig. 6a) or multilayered with a thickness of up to 1000 nm (Fig. 6b). The electron-dense interface was frequently continuous with the lamina limitans of bone (Fig. 6b, inset). Two weeks postoperatively, the bony tissue occupying some of the pores in copolymer was composed of fibroblast-like cells and randomly orientated striated collagenous fibrils with diameters ranging from 50 to 130 nm. The axial periodicity shown by the electron microscope was about 65 nm. After the longer implantation periods the bony tissue in the copolymer pores seemed to be ossified and contained osteocytes. Ultrathin sections showed copolymer pores with diameters smaller than 5 µm and completely filled with calcified matrix, but also larger pores with only the peripheral part occupied by bone. Copolymer surrounded by bone was not fragmented.

Bone made only contact with submucosal Estane implants, but not before the end of the sixth month (Fig. 7). The bone/Estane interface (Fig. 7, inset) lacked the electron-dense layer associated with copolymer and was predominantly composed of collagenous fibrils with diameters similar to those seen for copolymer implants. In Estane pores partially filled with bone, the bone was confined to the central portion of the pores. Furthermore, where bony tissue bordered Estane it seemed less ossified than the centrally deposited tissue. Estane bordered by bone did not display the electron-dense polymer surface zone seen at the phagocyte/Estane interface but unlike copolymer it showed fragmentation.

DISCUSSION AND CONCLUSIONS

The tissue-implant interface is the region where host tissue and biomaterial interact[27,28]. Implantation of the candidate alloplastic tympanic membranes in the rat middle ear made it possible to evaluate interface reactions with phagocytes, fibrous tissue, and bone. The interaction between phagocyte and polymer is frequently associated with implant degradation[11,12,19-22], whereas the interactions with both fibrous tissue and bone promote implant integration by tissue[3-5,11,12]. Important information about the tissue/implant interaction is provided by the

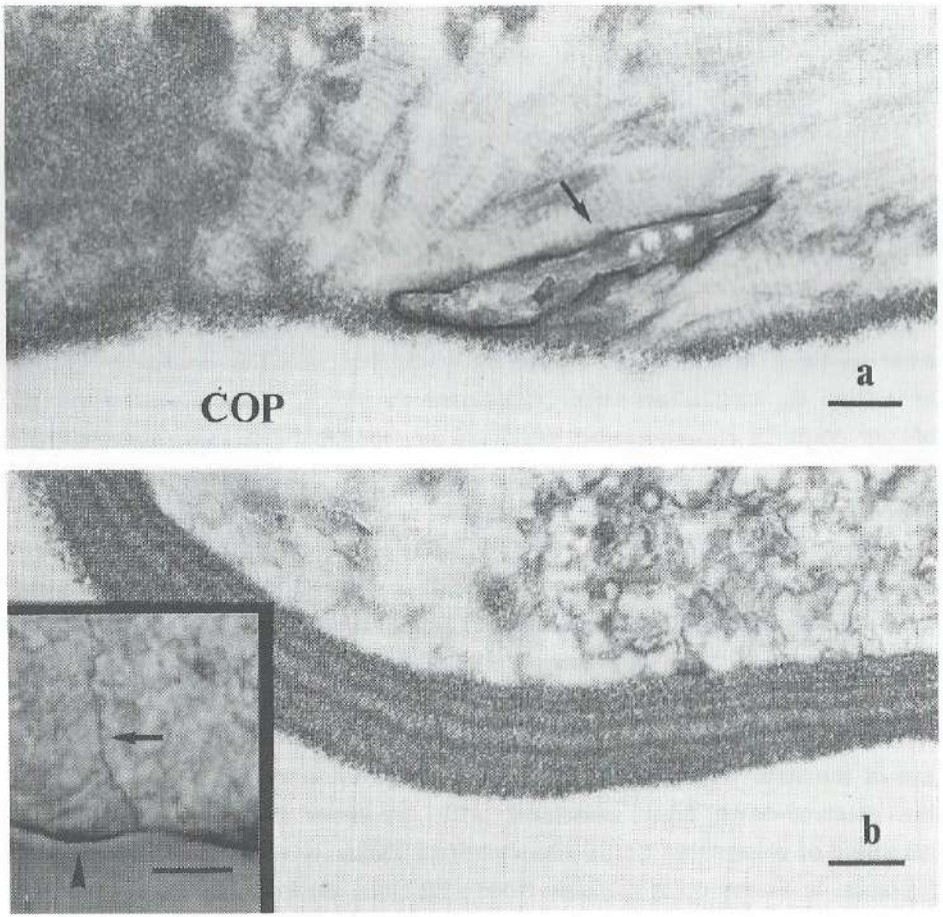

Fig. 6a and b. Bone/copolymer (COP) interface showing the monolayer (a) and the multilayered (b) electron-dense surface zone. Note in a the canaliculum (arrow). Bars = 0.2 µm. Inset shows continuity between the natural lamina limitans of bone (arrow) and that of the bone/copolymer interface (arrowhead). Bar = 0.7 µm.

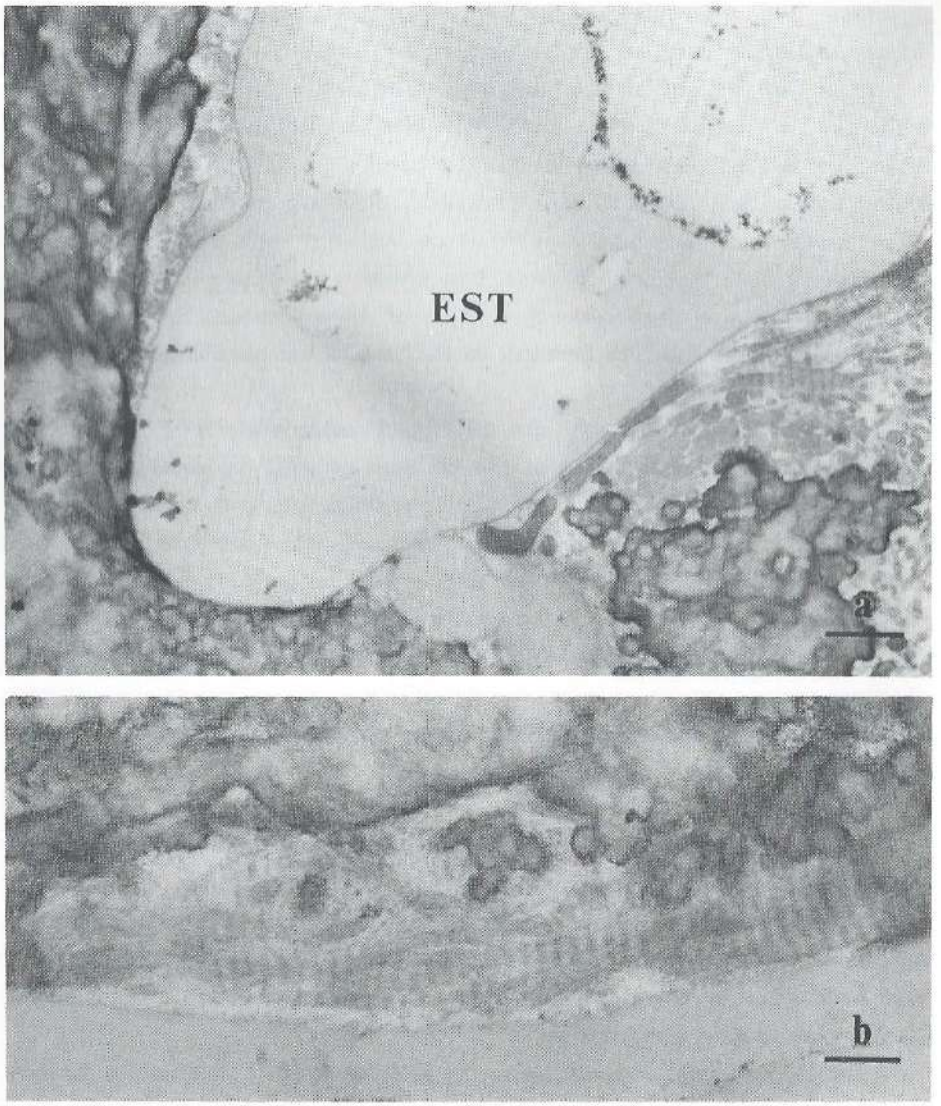

Fig. 7a and b. Interface between bone and Estane^{R} (EST).
a: Although bone touches the implant, most of the Estane is separated from it by a non-ossified collagenous material. Bar = 0.7 µm.
b: The same interface in more detail. Note the collagenous material parallel to the surface of the implant. There is no lamina limitans-like interface like that seen with copolymer or the electron-dense surface zone on Estane associated with interaction with phagocytes. Bar = 0.2 µm.

interface. The phagocyte/implant interface can indicate whether degradation occurs under the prevailing conditions and, if it does, to what extent[11,19,21]. The interface with fibrous tissue[6], and especially that with bone, may provide evidence of tissue/implant binding[3,6-9]. With respect to the bone/implant interface, a subdivision can be made according to bioactive, bioinert, and biotolerant, or better, biotolerated, materials[8]. Bioactive materials induce bonding osteogenesis, whereas bioinert materials do not bond with bone upon contact. Biotolerated materials do not come into contact with bone at all; they are invariably separated from bone by a layer of fibrous tissue. Polymers do not belong to the first category because, to the best of our knowledge, they do not bond to bone[13-17].

Degradation of a biomaterial can take place extracellularly[11,20,29] as well as intracellularly[11,19,21]. Components of the aqueous external environment affecting polymer breakdown are numerous and include enzymes, pH, temperature, salts, and lipids[30,36], whereas those of the intracellular milieu are limited to enzymes and pH[37-39]. The enzymes in the extracellular fluid[33,35] derive from such cells as phagocytes[37,38]. Macrophages and multinucleated phagocytes adhered to the surfaces of the implants under study, but only Silastic^{R} was not degraded. The chemical composition[30,32] of Silastic may have been responsible for this absence of degradation, but it is also possible that the environment of Silastic implants was less destructive because phagocytes were relatively scarce; Estane^{R} and copolymer implants surrounded by bone were not degraded either. Furthermore, because the Silastic implants were not porous, the area of interaction between the implant surface and the environment was significantly smaller than that for porous implants. The absence of Silastic degradation is consistent with findings on subcutaneously implanted silicone rubber[40], although degradation[41] and fracture[42] have been reported. These conflicting results might be explained by e.g. the use of different implantation sites[24].

On the electron-microscopical level, the interactions at the interface between phagocytes and porous implants made of polypropylene oxide, Estane, and copolymer suggest correlation between polymer degradation and phagocyte activity[11,12,19,22]. It is not clear[39], however, whether the presence of phagocytes follows the degradation of a polymer by extracellular fluid[29] or whether already present phagocytes are themselves responsible for breakdown[20]. Interface behavior led to changes in the morphology[38,43-45] of the phagocytes as well of the polymer surface. Specimens of the porous biomaterials before and one and two weeks after implantation were completely electron lucent and had smooth surfaces, as shown by transmission electron microscopy. Starting in the fourth

postoperative week, the polymer surfaces showed erosion that increased with time. In the order of decreasing degree, the following porous polymers were invaded by the cytoplasm of adjacent phagocytes: polypropylene oxide, copolymer, and Estane. The invasive behavior of phagocytes with respect to polypropylene oxide is characteristic for relatively rapidly degrading polymers[19,21,29], mainly polyesters[46]. The ester linkage is susceptible to non-enzymatic hydrolysis[19,29,33,47], which can take place readily in the extracellular environment[29,47] and can be mimicked in a synthetic extracellular fluid or in water[33,35,47]. The instability of polypropylene oxide, which is a hydrophobic polyether[25], was surprising, and is probably attributable to impurity-catalysed hydrolysis[48].

An interesting feature is the electron-dense surface zone shown by the Estane implants surrounded by phagocytes after implantation periods longer than one month. The increase of the thickness of the surface zone with increasing duration of implantation and the fact that the electron-dense zone was only observed where phagocyte/Estane interactions occurred, suggest an association between the electron-dense zone and Estane degradation. Mechanisms postulated to explain the increase of electron density, which might indicate a change in Estane structure[36], are increasing osmiophilicity of the interface during Estane degradation or absorption of osmiophilic material, e.g. lipids or the contents of lysosomes, to the Estane surface prior to or during the interaction.

The cracks in Estane did not seem to have been the result of interaction with phagocytes, because the surfaces of most of these cracks did not display the electron density characteristic of phagocyte/Estane interaction. Mechanical stress exerted by the tissue surrounding the implant could have been responsible for the formation of such cracks. Environmental stress cracking is frequently seen in polyurethane implants[49], but for copolymer a relationship between polymer fragmentation and phagocyte/polymer interaction seems more likely. Copolymer fragmentation did not occur where ingrowth of bone had taken place and phagocytes were absent. Furthermore, as shown earlier[24], both the degree of copolymer fragmentation and the number of phagocytes present here too were functions of implantation site and time, whereas this was not the case for Estane fragmentation.

Silastic implants were always incorporated into fibrous capsules which were probably non-adherent[6]. The porous Estane and copolymer implants showed not only overgrowth but also ingrowth of fibrous tissue, which seems to lead to better implant fixation[3-5,11,12]. The composition of the collagenous matrix surrounding Silastic differed from that of Estane or copolymer. The presence of

10-nm filaments and the absence of 30-nm fibrils might be characteristic for fibrous capsules, but the differences can also be explained by the differences in implantation site[50], because Estane and copolymer were bordered by fibrous tissue only in the tympanic membrane, whereas Silastic was not integrated into tympanic membrane tissue.

Implants made of Estane, copolymer, and polypropylene oxide had pores with diameters theoretically allowing bone ingrowth[5]. Bone ingrowth has been established for a number of ceramics[3,4,7,10-13,51,52], as well as several polymers[14-17], and direct contact with bone has been reported for certain ceramics[8]. With polymeric materials the ingrowing bone was invariably separated from the alloplast by a layer of a fibrous tissue[53]. Direct contact between bone and the polymers under study was seen for Estane and copolymer but not polypropylene oxide. Bone formation occurred earliest and most extensively inside pores of copolymer. Furthermore, in ultrathin sections only the bone/copolymer interface showed an electron-dense layer with a morphological resemblance to the layer observed between bone and the bioactive ceramic hydroxyapatite[10-12,54]. The fact that the layer found between bone and copolymer closely resembled and was continuous with the natural lamina limitans of bone, constitutes evidence that copolymer participates at least partially in normal bone metabolism[55]. It would be interesting to know whether the composition of the layer on copolymer is similar to that of the layer on hydroxyapatite or other bioactive ceramics[9,11,12,54]. Copolymer is the only polymer which, to the best of our knowledge, shows a lamina limitans-like interface with bone. If this interface is the product of a bioactive polymer surface, it suggests that in relation to bone copolymer behaves like a bioactive material. However, only push-out experiments can show whether there is a chemical bond between bone and copolymer and, if so, provide information about its strength[56].

The bone/hydroxyapatite interface consists of calcium phosphate incorporated into an organic matrix, which is probably responsible for firm and direct bonding with bone[8]. Both the crystal structure and the stoichiometry of hydroxyapatite seem important in this process, which is called bonding osteogenesis[8]. Copolymer does not have either of these properties of hydroxyapatite, although the present findings concerning the bone/copolymer interface suggest a bond similar to that associated with hydroxyapatite. Inorganic compounds incorporated into copolymer[57] and leached after implantation, chemically reactive sites in copolymer[58], and the absorption of organic or inorganic substances derived from the tissue fluid might have affected conditions at the bone/copolymer interface and given rise to a surface layer responsible for the bond with bone. Contact between bone and

implant can be established by bonding osteogenesis or contact osteogenesis[8]. With the former, the deposition of bone starts from the pore wall and proceeds to the center, whereas with the latter bone is first seen in the central portion of the pore and then grows out to the implant surface. A combination of osteogenic mechanisms is also possible[11]. Copolymer showed bone deposition on the pore wall first, which is in accordance with bonding osteogenesis, and incomplete bone ingrowth in Estane pores was characterized by a tissue-free space between bone and implant, which is characteristic of contact osteogenesis.

In sum, the present results show that both the interacting tissue and the properties of the individual biomaterials influenced the tissue/material interface characteristics. Non-porous implants made of Silastic were not incorporated into tympanic membranes, but at the other implantation sites they were always enveloped by a loose capsule of fibrous tissue. Silastic cannot be considered suitable for use as alloplastic tympanic membrane in a dense form, but in view of the relative absence of phagocytes at the interface it might prove suitable in a porous form. For polypropylene oxide, Estane, and copolymer, the time-dependent changes of the phagocyte/polymer interface suggested polymer degradation. The degradation of Estane was accompanied by changes of unknown origin in the osmiophilic properties of the implant surface. Since polypropylene oxide was surrounded by phagocytes only and showed rapid degradation, it too cannot be considered suitable for use as a scaffold for tissue overgrowth. Estane and copolymer showed ingrowth of and direct contact with fibrous tissue and bone suggesting implant fixation by the surrounding tissue. Although bone made contact with both Estane and copolymer, only the interface with copolymer showed an electron-dense structure similar to that described for the bioactive material hydroxyapatite. A bond with bone should greatly improve fixation of the alloplastic tympanic membrane to the hydroxyapatite canal wall of the total alloplastic middle ear prosthesis. This interface behavior makes copolymer a more promising candidate for use as alloplastic tympanic membrane than Estane, although final conclusions must await evaluation of polymer degradation and fragmentation as well as implant behavior during infection.

Acknowledgements

The authors gratefully acknowledge the cooperation of Prof. K.B.Wagener (Department of Chemistry, University of Florida, Gainsville, Florida, USA), and of Dr. J.H.Kuit and Dr. J.G.F.Bots (Department of Chemical Technology, Twente University of Technology, Enschede), who supplied the porous biomaterials. They are also indebted to Ms. P. van der Hoek and Mr. B.M.A. van der Lans (ENT Department) and Mr. J.J.Beentjes and Mr. L.D.C.Verschragen (Laboratory for Electron Microscopy) for technical assistance, and wish to express their gratitude to the co-workers of the Laboratory for Surgery of the University of Leiden for their help.

These investigations were supported by the Netherlands Technology Foundation (STW).

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CHAPTER 7

BIOCOMPATIBILITY OF FOUR ELASTOMERS DURING A *STAPHYLOCOCCUS AUREUS*-INDUCED MIDDLE EAR INFECTION

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SUMMARY

The biocompatibility of dense Silastic[R] implants and porous implants made of Estane[R] 5714 F1 polyether urethane, polypropylene oxide, and an HPOE/PBT segmented polyether polyester copolymer was evaluated during an induced *Staphylococcus aureus* middle ear infection.

The middle ear response to infection seemed not to be affected by the presence of implants made of either of the polymers. Light microscopical morphometry and transmission electron microscopy showed degradation of the porous implants under study, but not of Silastic implants, which were invariably surrounded by a fibrous capsule. This finding, combined with the degree of porous implant degradation, the composition of the tissues surrounding the implants, and the tissue/biomaterial interface reactions are consistent with the results obtained in the non-infected middle ear. Round-cell infiltrates however, were predominantly associated with implants made of polypropylene oxide and HPOE/PBT copolymer; while the presence of (phagocytosed) microbial debris was associated with copolymer.

The present findings indicate that with respect to implant behavior in infected surroundings Estane is the best porous material, whereas the behavior of Silastic implants did not deviate from that in non-infected ears.

INTRODUCTION

In the development of a total middle ear prosthesis[1,2] an alloplastic tympanic membrane is necessary[3]. The biocompatibility of a number of polymers that might be suitable to serve as alloplastic tympanic membrane was assessed *in vitro*[4] and in the non-infected rat middle ear[5,6].

Because infection occurs frequently in otology[7,8] and its sequelae can be an important reason for reconstructive middle ear surgery[9,10] its effects are of interest for the evaluation of the biocompatibility of a biomaterial used in that region. If an infection develops the incidence and course of infection and the response to this process can be affected by the presence of an implant[11-14]. Infection can also interfere with the tissue/implant interface and implant fixation

by fibrous tissue and bone[15,16] and implant degradation[17-20]. In addition, it has been reported that the incidence of an implant site infection is related to the biocompatibility of implant e.g. the amount of tissue within pores[12,21-24], the performance of the implant covering epithelium[15], and the degree of tissue reaction to the implant[25].

This report concerns the biocompatibility of four candidates for alloplastic tympanic membrane in the rat middle ear during a *Staphylococcus aureus* infection[26].

MATERIALS AND METHODS

Implant materials

The following biomaterials were evaluated: 1) Dow Corning Silastic^{R} silicone rubber, 2) Estane^{R} 5714 F1 polyether urethane[27], 3) polypropylene oxide[28], and 4) poly(ethylene oxide hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer (HPOE/PBT = 55/45; Akzona Inc.[29]). Silastic implants were non-porous and 125 µm thick. Implants made of the other polymers were about 100 µm thick and porous, with overall porosities of about 50%. The materials were cleaned by extensive rinsing first in running tap water and then in distilled water.

Animals and implantation technique

The middle ears of 160 male Wistar rats (body weight 200-220 g) were reached via a dorsal approach under Hypnorm^{R} anesthesia (100 µl/100 g body weight)[30]. Both middle ears were given four implants made of the same material[5]. A hole of about 2 mm^2 was drilled in the middle ear bulla and two submucosal implants were then placed between the middle ear mucosa and the bulla bone. The burr defect was closed by placing a single bone/muscle implant between the bulla bone and the adjacent muscle tissue. After opening of the dorsal part of the auditory canal, giving full view of the tympanic membrane, part of the tympanic membrane was separated from the handle of the malleus and the annulus, and a tympanic membrane implant was placed in the resulting defect.

Implantation periods were one, three, six, and 12 months except for the copolymer, for which the longest period was six months. All Silastic, Estane, and copolymer implants originated from the same batch, whereas those made of polypropylene oxide and used for the one month survival period were from a different batch than those used for the other intervals. In all, 596 implants were studied in 160 infected left middle ears, and 596 implants in 160 non-infected

middle ears served as controls. Both infected and non-infected implants were distributed among the evaluation techniques as shown in Table I.

Table I. Distribution of implant materials according to duration of implantation time (month) and evaluation technique.

		1	3	6	12*
SUR					
LM	TM#	4	4	4	4(2ß)
	SM	8	8	8	8(4ß)
	BM	4	4	4	4(2ß)
AU	TM#	1	1	1	1
	SM	2	2	2	2
	BM	1	1	1	1
TEM	TM#	3	3	3	3(2ß)
	SM	6	6	6	6(4ß)
	BM	3	3	3	3(2ß)
SEM	TM#	3	3	3	3(1ß)
	SM	6	6	6	6(2ß)
	BM	3	3	3	3(1ß)

* Excluding the polyether polyester copolymer.
\# Except Silastic^{R}.
ß For polypropylene oxide.
SUR Survival time in months.
LM Light microscopy and light-microscopical morphometry.
AU Light-microscopical autoradiography.
TEM Transmission electron microscopy.
SEM Scanning electron microscopy.
TM, SM, BM Tympanic membrane, submucosal or bone/muscle implant.

Infection

Three weeks before decapitation, infection of the left middle ear was induced by injecting of 0.1 ml of saline containing approximately 1.4 x 10^7 *Staphylococcus aureus*[26], administered through the tympanic membrane into the middle ear cavity. Right middle ears were not infected.

Light microscopy and light-microscopical autoradiography and morphometry The rats were decapitated and the middle ears dissected. For fixation, the middle ears were immersed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Sörensen's buffer (pH 7.4, 4°C) for 2 hr and then for 14 hr in the same fixative but without glutaraldehyde. For decalcification, the specimens were held for 4 weeks in 10% EDTA in distilled water containing 2% paraformaldehyde (pH 7.4, room temperature). After being rinsed for 24 hr in running tapwater, the middle ears destined for routine light microscopy and light-microscopical morphometry

(Table I) were dehydrated in graded alcohols and embedded in Paraplast^R. Sections 6 µm thick were stained with a hematoxylin-eosin.

A total number of 983 light-microscopical sections was photographed at a standard magnification of 250 times and evaluated on a computerized X-Y tablet (Videoplan^R). The composition of the tissue surrounding the biomaterials under study was quantitated by measuring the areas in cross-sections occupied by phagocytes, fibrous tissue, and bone and relating them to implantation time. This was also done for the amount of implant material. For determination of the thickness of the Silastic surrounding fibrous capsules, 286 sections were evaluated by measuring the capsule thickness on a photographic print enlarged 250 times.

For autoradiography, one rat for each biomaterial and each implantation time of one, three, and six months, was injected intraperitoneally with (6-^{3}H)thymidine (specific activity 25 mCi/mmol; Radiochemical Centre Amersham, England) at a dose of 0.1 mCi/100 g one hr before decapitation (Table I). Fixation and decalcification of the middle ears were as for light microscopy, and specimens were embedded in glycol methacrylate (GMA, JB-4 embedding kit, Polyscience). Sections (2-µm) were coated with K5 nuclear emulsion and exposed for 4 weeks at 4°C. After development and fixation of the emulsion, sections were stained with methyl-green pyronin and analysed.

Transmission electron microscopy (TEM)

The ears destined for TEM were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer for 16 hr (pH 7.4, 4°C), and decalcified for 4 weeks (10% EDTA, 1.5% glutaraldehyde, pH 7.4). After a rinse in running tap water (24 hr) and two rinses in phosphate-buffered saline (5 min. each), pieces of the middle ears containing implant materials measuring about 1 mm^3 were postfixated in 1% $0s0_4$ (30 min, room temperature) and then rinsed in phosphate-buffered saline. Finally, after being dehydrated in graded alcohols, the specimens were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Philips 201 electron microscope. Semithin sections were stained with toluidine blue and studied by light microscopy.

Scanning electron microscopy (SEM)

The middle ears reserved for SEM were rinsed in phosphate-buffered saline to remove the mucus from the cavity prior to fixation for 2 hr in 1.5% glutaraldehyde in cacodylate buffer (pH 7.4, 4°C). The ears were then washed three times in phosphate-buffered saline, dehydrated in graded alcohols, critical point dried in carbon dioxide, and sputter coated with gold. A Cambridge 180 S stereo scanning electron microscope was used to study the ears at operating voltages of 15 and 20 keV.

RESULTS

Normal rat middle ear morphology

The rat middle ear cavity is of the bullar type (Fig. 1). The greater part of the lateral wall of the cavity is formed by the tympanic membrane. The central part of the medial wall, the promontory, is formed by the inner ear wall. The thickness of the tympanic membrane gaines between 6 and 15 µm and consists of an epidermis that is continuous with the epidermis of the external ear canal, a connective tissue layer, and an epithelium that is continuous with the epithelial lining of the middle ear mucosa. The epithelium of the tympanic membrane is composed of flat polygonal cells, whereas that of the mucosa is predominantly composed of flat polygonal cells but also of the ciliated and secretory epithelium of the mucociliary tracts, which are located inferior and superior to the promontory.

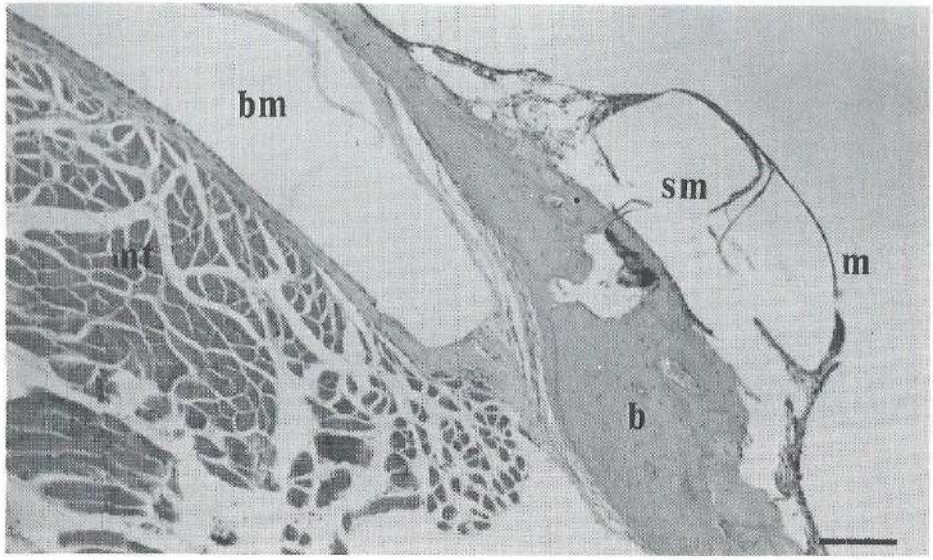


Fig. 1. Light micrograph of a non-infected rat middle ear showing submucosal (SM) and bone/muscle (BM) implants made of Silastic^R. M = mucosa; B = bulla bone; MT = muscle tissue. Bar = 40 µm.

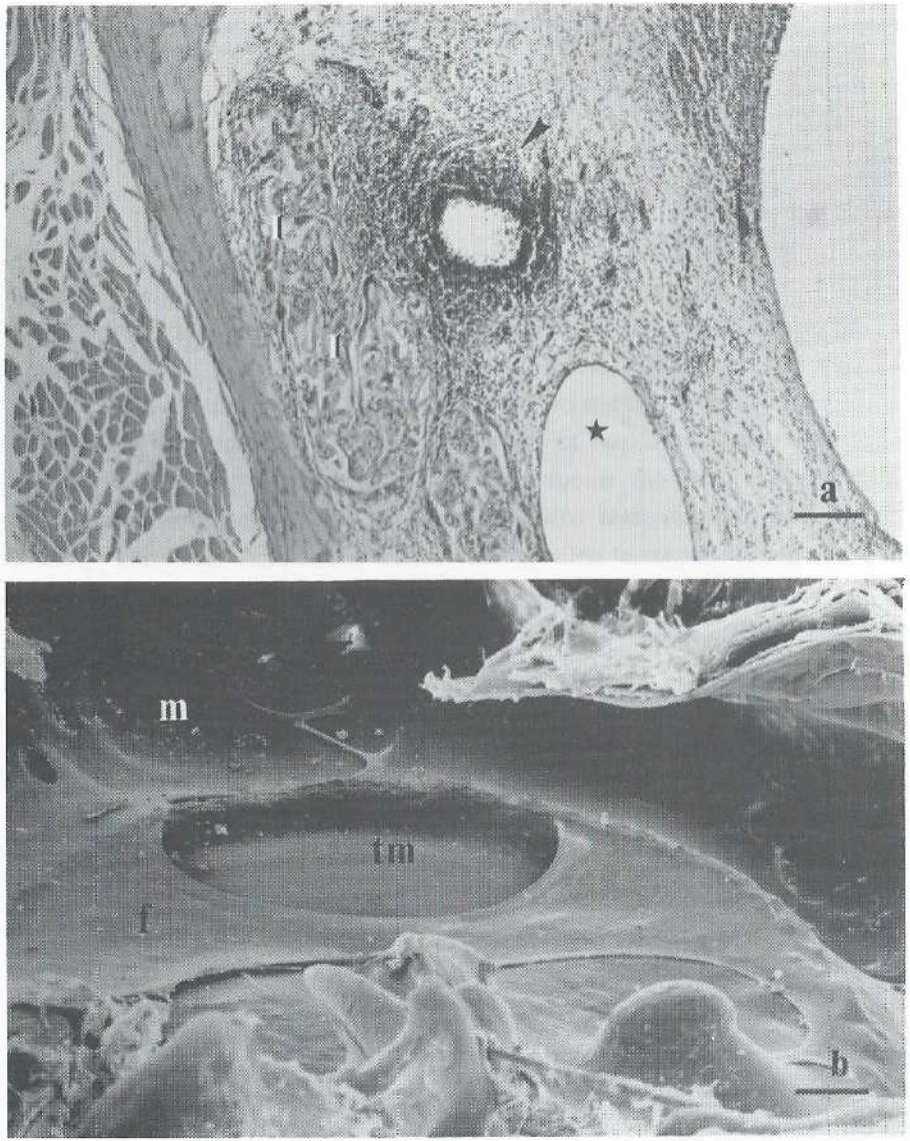


Fig. 2a. Light micrograph of a section showing *Staphylococcus aureus* infected rat middle ear showing Estane^{R} submucosal implant (I). Fibroblastic ingrowths with pockets (asterisk) and inflammatory cells (arrowhead) are evident. Bar = 40 µm.
Fig. 2b. Scanning electron micrograph showing fibroblastic ingrowth (F) originating from the mucosa (M) and partially covering the tympanic membrane (TM). Bar = 150 µm.

Table II. Percentages of ears that have developed an infection after instillation of *S.aureus*.

Implant material	percentage
Silastic^{R}	89
Polypropylene oxide	89
Estane^{R}	86
Copolymer	94

Infected rat middle ears

The first part of the following section describes the rat middle ear responses with implants three weeks after the instillation of *Staphylococcus areus*. The percentage of ears in the various experimental groups that had become infected is shown in Table II. The second part of this section deals with the biocompatibility of the materials in the infected ear.

Response of the infected ear containing implants

After infection, the general picture was that of a partially obstructed middle ear cavity (Fig. 2a and b) caused by the swelling of the lamina propria of both the mucosa and the tympanic membrane and by fibroblastic ingrowths originating from the middle ear mucosa. The severity of swelling varied from site to site. Scattered throughout the swollen lamina propria there were numerous dilated capillaries with monocytes, lymphocytes, plasma cells, and polymorphonuclear granulocytes in the lumen. Fibroblasts and macrophages predominated in the connective tissue and plasma cells and mast cells were regularly seen close to capillaries.

The severely swollen submucosa displayed numerous pockets filled with exudate. These pockets were lined predominantly by apparently secretory active cells but also by ciliated epithelium. The shape of the majority of the cells forming the epithelial lining of the tympanic cavity were flat polygonal or cuboid polygonal. The numbers of active ciliated and secretory cells were higher than in the non-infected middle ear, but these types of cell were still confined predominantly to the two mucociliary tracts. Both the middle ear bulla bone bordering the mucosa and the bone of the handle of the malleus showed signs of osteoresorption and osteogenesis. The formation of new bone dominated over bone resorption. A layer of new bone was separated from the original bone by a distinct lamina limitans.

Biocompatibility during infection

In the following, the biocompatibility of the four polymers during infection is described with special attention to (1) the composition of the implant surrounding tissue, (2) the tissue/implant interface, and (3) polymer degradation.

Tissue composition

Submucosal and bone/muscle implants made of non-porous Silastic^R were encapsulated in fibrous tissue at one week, whereas all tympanic membrane implants made of the silicone rubber were rejected from the middle ear before the second week. Only a few phagocytes were seen between the Silastic and the fibrous capsule. Quantitation showed that the capsules associated with the bone/muscle implants made of Silastic were twice as thick as those surrounding Silastic submucosal implants and that their thickness was not affected by the infection (Fig. 3). Nor was the proliferative activity of the fibroblasts forming the fibrous capsules as indicated by the incorporation of tritiated thymidine shown in figure 4a.

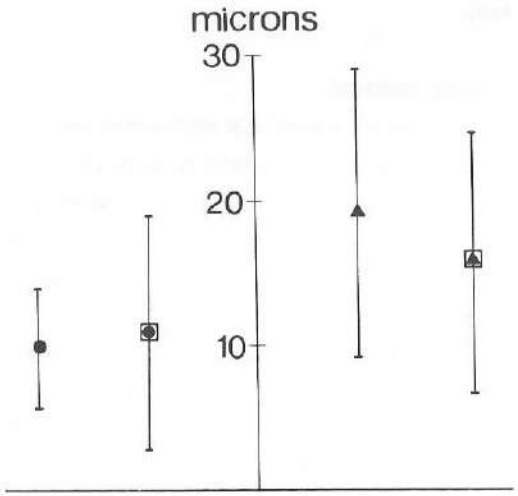


Fig. 3. Diagram showing the effect of infection on the thickness of the wall of fibrous capsules surrounding Silastic^R. • Submucosal implant (n = 72 samples). ◉ Submucosal implant from infected ear (n = 68). ▲ Bone/muscle implant (n = 81). ◬ Bone/muscle implant, infected (n = 65).

The porous implants made of polypropylene oxide, Estane, and copolymer, were surrounded by mononuclear and multinucleated phagocytes and fibrous tissue. Estane submucosal implants and copolymer submucosal and bone/muscle implants were surrounded by bone too. Most of the phagocytes were present on the surface of the implant, and fibrous tissue usually occupied the central portion of the pores. When ingrowth of bone occurred, the implants still showed the original pore structure. Otherwise, the implantation sites showed fragments of the biomaterial, most of them completely surrounded by or ingested by phagocytes.

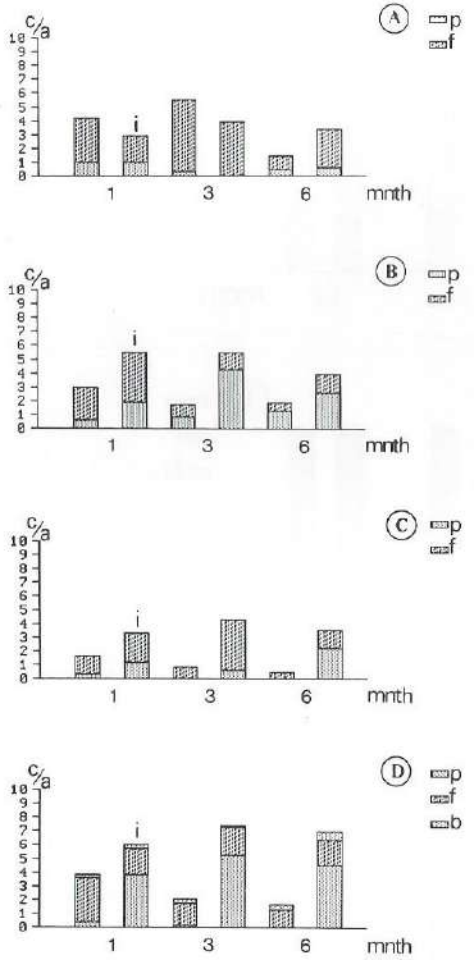


Fig. 4. (a-d). Diagrams showing influence of infection on (^{3}H)-thymidine incorporation in phagocytes (P), fibrous tissue (F), and bone (B). Left bars refer to data of non-infected ears, right bars (i) the infected situation. C/a = counts/0.1 mm^2 tissue averaged over total numbers of implants made of Silastic^R (a), polypropylene oxide (b), Estane^R (c), and HPOE/PBT copolymer (d).

The morphometric findings in the light-microscopical sections of middle ears given porous implants averaged over all implantation sites showed changes in the composition of the tissue in the course of time. Figure 5 shows these effects for both infected and non-infected ears. Polypropylene oxide was associated with a decrease of the area occupied by phagocytes between six months and one year. Between one and six months the average size of the phagocyte population remained approximately the same. Comparable changes in the numbers of

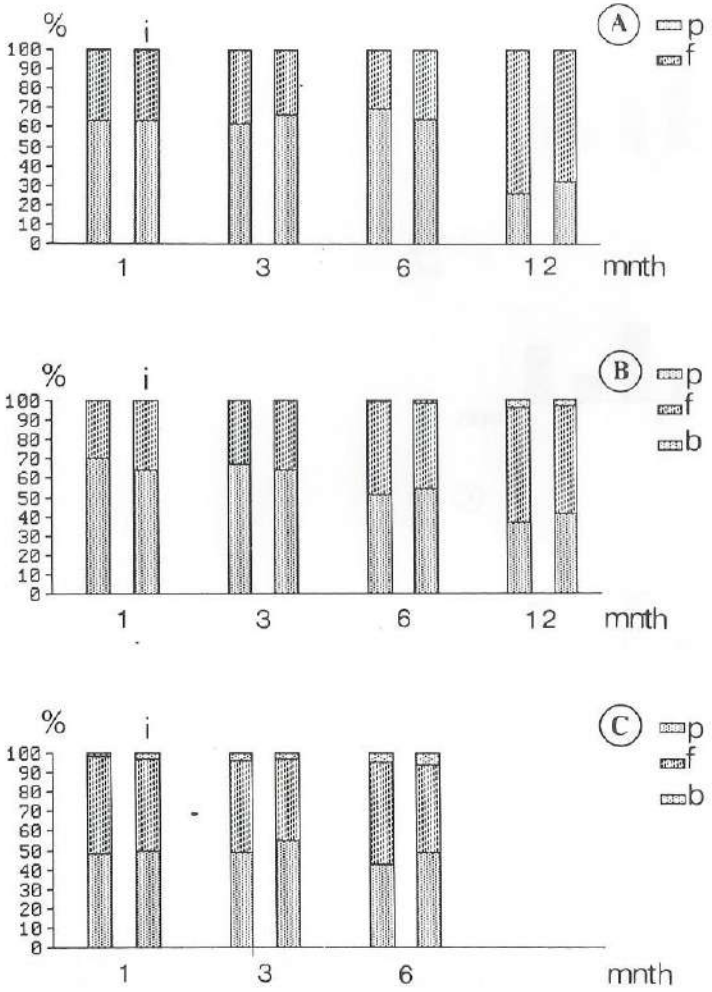


Fig. 5 (a-c). Course of the percentages of implant-surrounding tissue occupied by phagocytes (P), fibrous tissue (F), and bone (B) without infection (right bars) and with infection (left bars; i). Values are means of the total number of samples of Estane^R (a), polypropylene oxide (b), and copolymer (c).

phagocytes were seen in the non-infected ear (Fig. 5a). As Figure 5b shows for both infected and non-infected middle ears, the percentage of phagocytes surrounding Estane decreased between one and twelve months, whereas the amount of fibrous tissue and bone (the latter present since the sixth postoperative month) increased during that period. The percentage of phagocytes

surrounding fibrous tissue and copolymer fluctuated around 50% during all evaluation periods in both infected and non-infected ears (Fig. 5c). With copolymer, bone appeared before the first month and the amount of bone was greater after one year than after one month.

For the detection of possible effects of the implantation site on the interaction between infection and tissue composition, the percentage of a porous biomaterial

Table III. Distribution of the percentage of tissue occupied by phagocytes (a), fibrous tissue (b), or bone (c) as function of implantation site in the course of time (months).

a)	SUR	1	3	6	12*
EST	TM	53#/49@	48/51	35/27	34/42
	SM	70/63	69/60	47/62	32/38
	BM	85/81	83/80	69/73	46/44
PPO	TM	29/33	55/60	62/58	11/30
	SM	56/61	63/66	73/70	11/18
	BM	75/66	69/73	72/65	55/49
COP	TM	35/39	34/52	36/56	---
	SM	43/48	36/43	37/40	---
	BM	65/63	72/70	56/50	---

b)	SUR	1	3	6	12*
EST	TM	47#/51@	52/49	65/73	66/58
	SM	30/37	31/40	50/33	58/54
	BM	15/19	17/20	31/27	52/55
PPO	TM	71/67	45/40	38/42	89/70
	SM	44/38	37/34	26/30	89/82
	BM	25/34	31/27	28/35	45/51
COP	TM	65/61	66/48	64/44	---
	SM	52/45	57/47	50/43	---
	BM	35/34	24/30	41/48	---

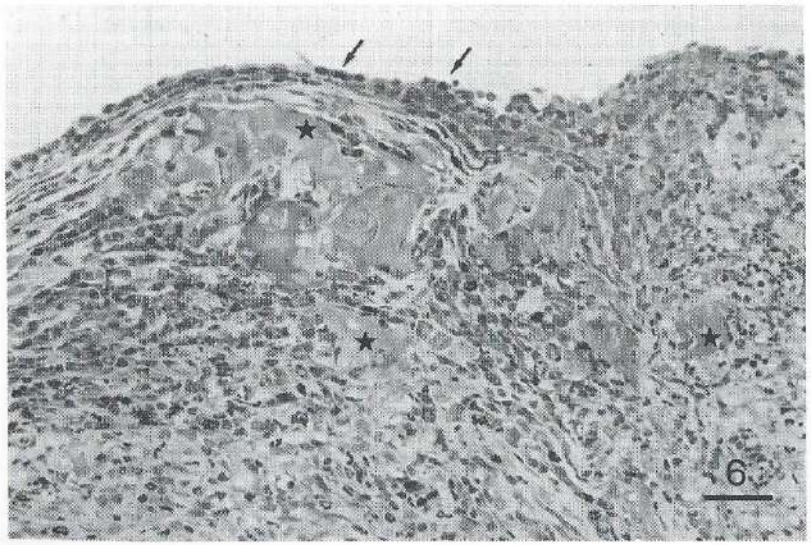
c)	SUR	1	3	6	12*
EST	SM	0#/0@	0/0	0/0	9/8
	BM	0/0	0/0	0/0	0/0
COP	SM	5/7	7/10	13/17	---
	BM	2/3	4/0	3/2	---

* Except the polyether polyester copolymer.
\# Non-infected middle ears.
@ Infected middle ears.
SUR Survival time.
EST Estane^R.
PPO Polypropylene oxide.
COP HPOE/PBT polyether polyester copolymer.
TM, SM, BM Tympanic membrane, submucosal or bone/muscle implant.

occupied by phagocytes, fibrous tissue, and bone was averaged for each implantation site as a function of time (Table III). The most prominent changes attributable to infection were associated with tympanic membrane implants made of copolymer and concerned an increase of the percentage of tissue occupied by phagocytes and a decrease of the amount of fibrous tissue.

Infection generally led to an increase in the incorporation of tritiated thymidine by the tissue surrounding the porous implants (Fig. 4b-d). For copolymer and polypropylene oxide, the increase was associated with a relatively high proliferative activity of phagocytes (Figs. 4d and 6). Tympanic membranes with polypropylene oxide or copolymer implants showed relatively large numbers of macrophages and polymorphonuclear granulocytes that had infiltrated the fibrous tissue surrounding the polymers (Fig. 6), but smaller numbers of mast cells and plasma cells. The latter type of cells was seen less frequently in association with submucosal and bone/muscle implants made of polypropylene oxide, copolymer, and Silastic, and all of the implants made of Estane.

Mononuclear and multinucleated phagocytes containing microbial debris were associated with all of the biomaterials under study, but most prominently with the copolymer (Fig. 7a). Furthermore, most of the ears given polypropylene oxide and copolymer, as well as some of those given Silastic or Estane, showed a relatively high incidence of calcification islands which, in the case of copolymer, were seen not only in the tympanic membrane and the middle ear mucosa, but also in the middle ear bulla bone (Fig. 8).



Interface reactions

With increasing survival time, the smooth surfaces of the porous implants became rough. Polypropylene oxide and copolymer implants were penetrated by cytoplasmic protrusions of phagocytes (Fig. 7a and inset; Fig. 7b). The phagocyte/Estane interaction was associated with an increase in the electron-density of the surface of the polyurethane prior to polymer erosion (Fig. 7c). The phagocyte/Silastic interface remained smooth. The phagocytes surrounding the porous implants contained numerous ribosomes, rough endoplasmic reticulum, mitochondria and lysosomes, whereas the phagocytes associated with Silastic had considerably fewer mitochondria and lysosomes. Cell organelle-poor adherence zones were predominantly associated with polypropylene oxide and Estane, and to a lesser extent with copolymer. They were not associated with Silastic.

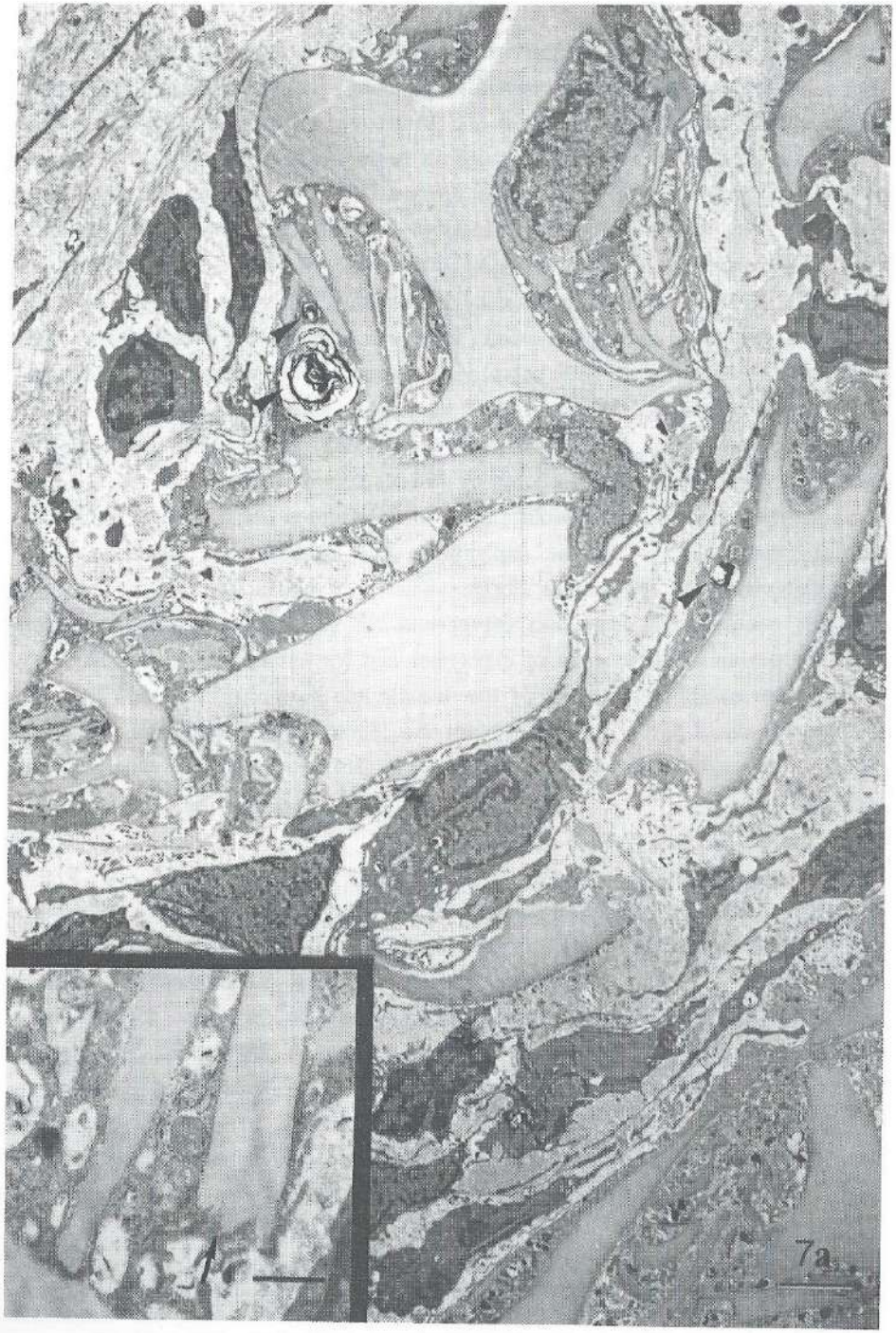
Silastic was surrounded by fibrous tissue one month after implantation, copolymer and Estane were bordered by fibrous tissue three and six months after implantation, respectively. Fibrous tissue never bordered polypropylene oxide. The fibrous tissue surrounding the implants in the infected rat middle ear was composed mainly of collagenous fibrils.

Direct contact with bone was established for submucosal as well as muscle tissue bordering implants made of copolymer and for Estane submucosal implants. It was never established for any of the Silastic and polypropylene oxide implants. An electron-dense layer continuous with the lamina limitans of bone was found regularly at the bone/copolymer interface (Fig. 8c) but was absent where Estane bordered bone.

Polymer degradation

The effects of the introduction of *S.aureus* into the middle ear cavity on polymer degradation was assessed by morphometric analysis of the average cross-sectional area seen in the light microscope of the polymers under study after each postoperative interval. Figure 9 shows the degradation (averaged for the three implantation sites) of the porous implants in both non-infected and infected surroundings. The area occupied by polypropylene oxide decreased by 90% between the fourth week and one year in both non-infected and infected ears. Implants made of Estane and copolymer were also degraded in the course of time, and the degree of degradation was not influenced by the presence of the pathogen.

Fig. 6. Light micrograph showing (^{3}H)-thymidine incorporation indicating cell proliferative activity. Most of the labeled cells are fibroblasts, but there are also some labeled macrophages and epithelial cells. Note the copolymer fragments (asterisks). Arrows point to tympanic membrane epithelium. Bar = 44 µm.



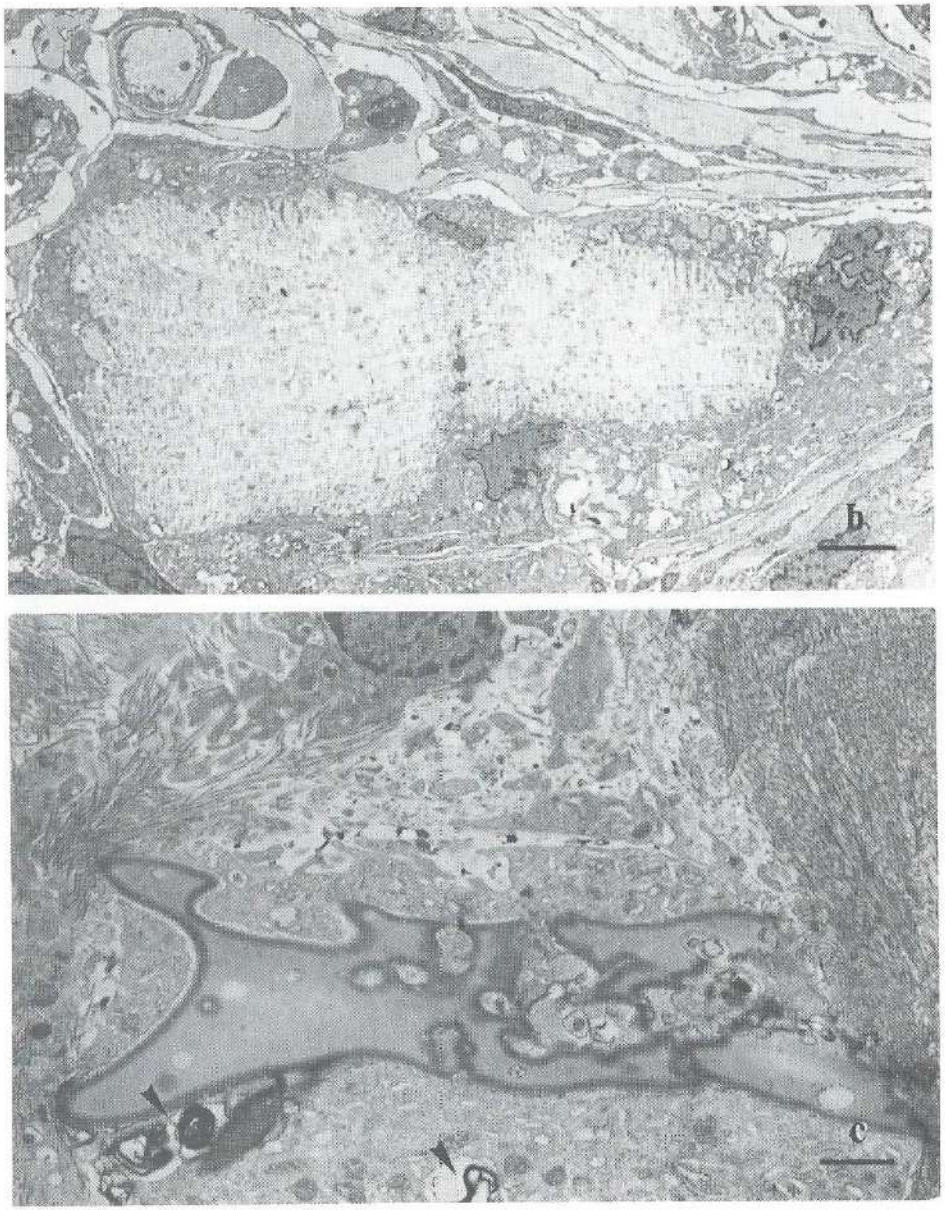
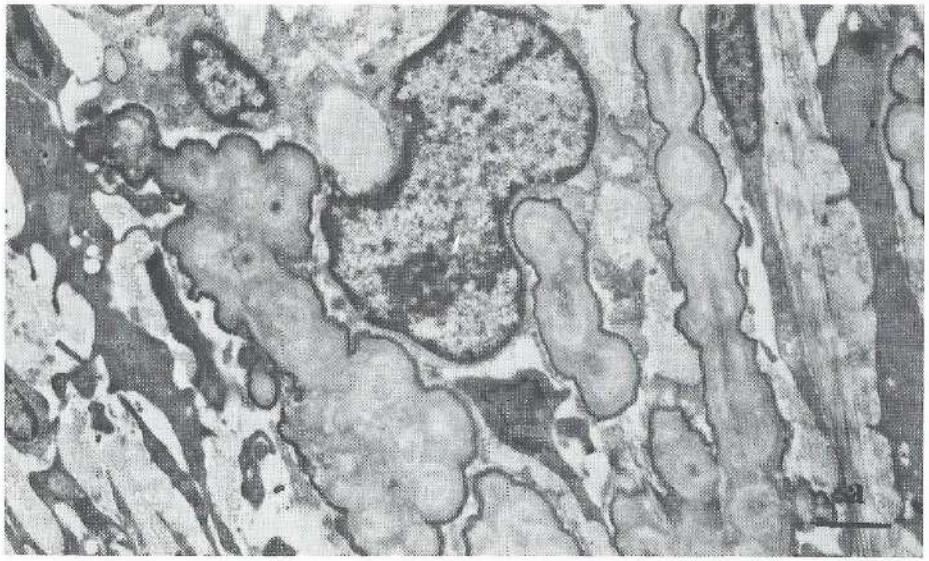
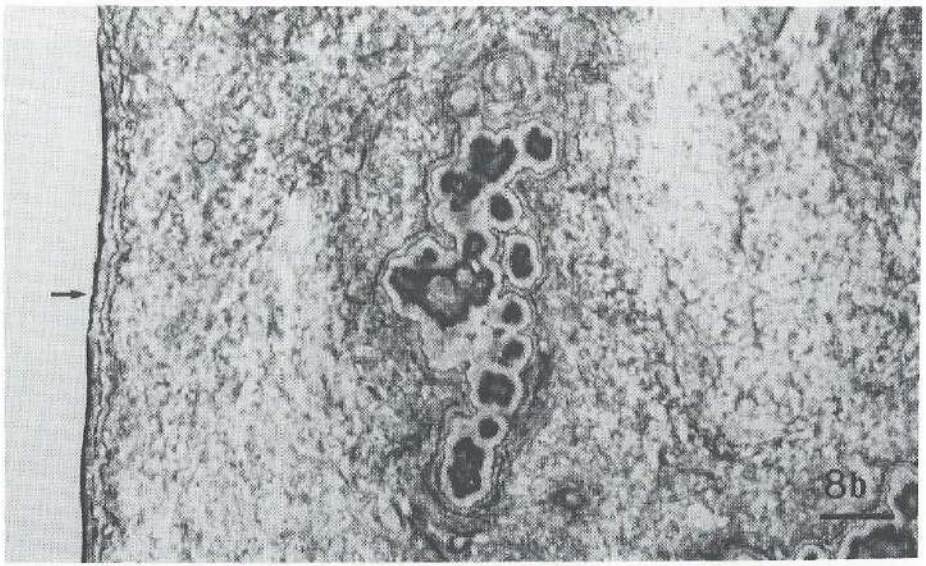


Fig. 7 a-c. Transmission electron micrographs suggestive of polymer degradation.
a: Macrophages containing HPOE/PBT copolymer and microbial debris (arrowheads). Bar = 3.5 µm. Inset: phagocyte/copolymer interface. Note the infiltration of polymer by cytoplasm (arrow). Bar = 0.7 µm.
b: Polypropylene oxide implant displaying eroded surface surrounded by phagocytes. Bar = 5.2 µm.
c: Electron-dense zone associated with Estane^{R} degradation. Note the microbial debris (arrowheads). Bar = 1.5 µm.





DISCUSSION AND CONCLUSIONS

Staphylococcus aureus is the most common micro-organism causing wound infection in hospitals[20], and can be regularly demonstrated in middle ear effusions[31]. This pathogen, which has been used in a number of studies on the effect of infection on implant materials and sutures[13,16,20,21,23,24,32], has proven its value in research on middle ear infection in the presence[15,33,34] and absence of an implant[26,35,36].

Infiltration of the lamina propria of both the tympanic membrane and the middle ear mucosa by polymorphonuclear granulocytes, macrophages, and lymphocytes, which are known to phagocytose microbial and cellular debris[26,35,37,38], is indicative of a cellular response in the infected middle ear[26,35,39]. The occurrence of numerous round-cell infiltrates in tympanic membranes implanted with polypropylene oxide or copolymer seems to support the

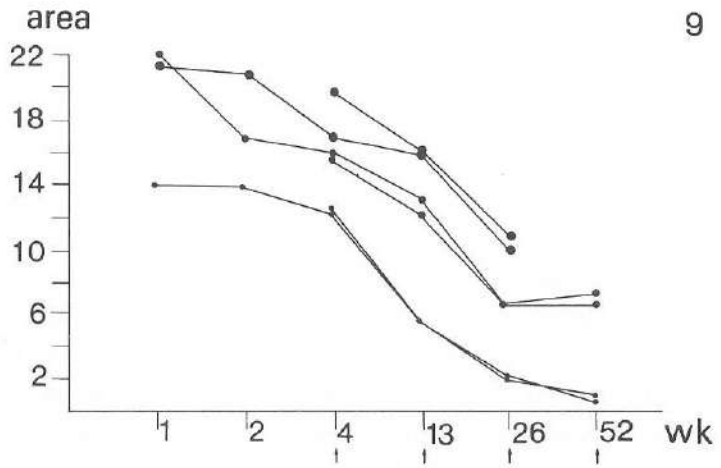


Fig. 9. Diagram showing the effect of infection on polymer degradation. Amount of HPOE/PBT copolymer (●), Estane^R (●), and polypropylene oxide (•), quantitated as average cross-sectional area seen under the light microscope (in μm^2 x 1000). Intervals in which infection was carried out are indicated by arrows. The one and two weeks periods, which are part of a different study, are included to give the proper picture.

Fig. 8. a and b. Transmission electron micrographs of calcification islands.
a: Tympanic membrane three months after implantation of polypropylene oxide. Note the electron-dense surface zone of the aggregates. Bar = 1.3 µm.
b: Completely encapsulated calcification islands in middle ear bulla bone (HPOE/PBT copolymer implant, six months postoperatively). Arrow point to electron-dense layer between bone and the copolymer (C). Bar = 3.8 µm.

hypothesis that the presence of an implant increases the risk of infection[40]. However, the Estane[R] implants and the submucosal and bone/muscle implants made of Silastic[R], polypropylene oxide, and the copolymer were less frequently associated with round-cell infiltrates. This suggests that other factors besides the presence of an implant are associated with implant-site infectivity. The route used for infection via the tympanic membrane could have been responsible for the observed differences between polypropylene oxide and copolymer tympanic membrane implants and submucosal and bone/muscle implants made of the same polymers. Different structural[16,21,32,41] and physical[42] properties of tympanic membrane implants made of copolymer and polypropylene oxide may have been responsible for the divergence from the behavior shown by tympanic membrane implants made of Estane.

Studies on the effect of bacteria on material degradation have yielded varying evidence: increased[19], decreased[18], and unchanged[15] degradation of biomaterials have been reported. The transmission electron microscopical findings on the phagocyte/polymer interface reported in this paper are similar to those seen in non-infected ears. The changes in the surface of porous implants used in this study - erosion and increased osmiophilicity - and the global characteristics of the phagocytes associated with these polymers - e.g. organelle-poor adherence zones and the many mitochondria and lysosomes - reflect the degradation of polypropylene oxide, Estane, and copolymer[6]. The phagocyte/Silastic[R] interface showed none of the features associated with implant degradation, which suggests that the silicone rubber is not subject to degradation within a one-year implantation period. Furthermore, the quantitative results on the degradation of porous implants correspond well with the findings on polymer breakdown in the non-infected rat middle ear[5]. It was found that the degree of degradation decreased in the following order: polypropylene oxide, Estane, and copolymer. From these observations it can be concluded that no noticable bacterial degradation[20] or enhanced polymer degradation associated with the inflammatory process[15] has occurred.

The mucociliary response and the fibrous tissue and bone response patterns are very similar to those observed in studies on the infected rat middle ear, both in the absence[26] of a biomaterial and in the presence of implants made of hydroxyapatite[15,33], which is the bulk material of the total alloplastic middle ear prosthesis[2,3], and tricalcium phosphate[35].

Non-porous Silastic implants invariably became surrounded by a capsule of fibrous tissue, that was comparable for both infected and non-infected ears as to composition and thickness. The tissue surrounding Silastic implants seemed not to

be more inflamed than that surrounding the porous implants. This deviates from observations made by Merrit et al.[21]. The results on the average composition of the tissue surrounding the porous implants three weeks after the onset of infection were a declining percentage accounted for by fibrous tissue and a comparable increase of the percentage taken by the phagocytes. However, it can not be expected that these small changes would seriously interfere with the implant fixation by fibrous tissue and bone[15,16].

The origin of the calcification islands is obscure. Similar structures have been reported as part of tympanosclerotic plaques[44], frequently associated with a chronic middle ear disease. Since these islands were not observed by van Blitterswijk et al. in comparable experiments with calcium phosphate implants[15,33,34], it might be suggested that the used biomaterials contribute to their formation. Ingrowth of bone, which occurred more frequently in HPOE/PBT copolymer pores than in Estane pores, the lamina limitans-like interface, which was associated only with HPOE/PBT copolymer and suggests a bond with bone[15,16], and the absence of direct contact between bone and implants made of Silastic and polypropylene oxide, correspond well with the response of bone of these biomaterials in the non-infected rat middle ear[6].

In sum, Silastic, polypropylene oxide, Estane, and copolymer implants did not seem to significantly influence the reaction pattern associated with middle ear infection. Although the infection did not significantly affect the biocompatibility of any of these biomaterials as to (1) the degree of implant degradation, (2) the composition of the tissue surrounding the implant, and (3) the tissue/implant interface reactions, inflammatory cells were predominantly associated with HPOE/PBT copolymer and polypropylene oxide, while the presence of (phagocytosed) microbial debris was most frequently seen in relation to copolymer. Regarding the porous implants, Estane showed the best behavior during infection, while the results on Silastic did not deviate from those in non-infected ears. Further investigation seems advisable if the divergent behavior between the porous implants should prove to be due to differences in e.g. porosity or chemical composition.

Acknowledgements

The authors wish to express their gratitude to Dr. A.B.M.Klaassen (Radboud Hospital Nijmegen), Ms. P. van der Hoek and Mr. B.M.A. van der Lans (ENT Dept.), and Mr. J.J.Beentjes, Mr. L.D.C.Verschragen and Mr. S.B.Neijman (Laboratory for Electron Microscopy) for their technical assistance. They also gratefully acknowledge the cooperation obtained from the Laboratory for Surgery and the Laboratory for Bacteriology and Parasitology of the University of Leiden. The porous implants were generous gifts from Prof. K.B.Wagener (Dept. of Chemistry, University of Florida, Gainesville, Florida, USA), and Dr. J.H.Kuit and Dr. J.G.F.Bots (Dept. of Chemical Technology, Twente University of Technology, Enschede).

These investigations were mainly supported by the Netherlands Technology Foundation (STW). Supplementary grants were received from the Heinsius Houbolt Fund.

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CHAPTER 8

EPITHELIAL REACTIONS TO FOUR TYMPANIC-MEMBRANE ALLOPLASTS.

A morphological study in non-infected and infected rat middle ears

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SUMMARY

Epithelial reactions to Silastic[R], Estane[R] polyether urethane, polypropylene oxide, and a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer, were investigated after implantation in tympanic membranes and submucosa of non-infected and *Staphylococcus aureus*-infected rat middle ears.

Porous implants made of Estane and polypropylene oxide were completely covered by tympanic-membrane epidermis and epithelium in two weeks and those made of copolymer in between two and four weeks postoperatively. Silastic implants, which were dense, were not enveloped by tympanic-membrane tissue. Starting in the sixth postoperative month the proliferative activity and structure of the tympanic membrane epithelium became normal except for the presence of iron-containing cells near polypropylene oxide. After initial swelling caused by the surgical trauma, neither the proliferative activity nor the composition of the epithelium covering submucosal implants was affected by the presence of any of the biomaterials. Infection of middle ears bearing implants induced epithelial reactions similar to those associated with infected middle ears without an implant.

None of the polymers under study hampered differentiation of epithelial cells into ciliated and secretory cells or affected the normal middle ear response to infection, but Estane proved to be the best material for alloplastic tympanic membranes with respect to epithelial overgrowth and morphology.

INTRODUCTION

Implant failure due to infection has been attributed to incomplete covering of the alloplast by epithelium[1,2], endothelium[3,4], and epidermis[5], but also to unstable implant-skin junctions[6,7]. Furthermore, insufficient epithelial overgrowth can lead to implant extrusion[8] and inadequate endothelialization can lead to thrombus formation[9,10].

Although epithelium is invariably separated from the implants by other tissues[1,2,6,7,9], it is often the ultimate and complete covering by epithelial, epidermal, or endothelial cells that makes implants successful when used in e.g. otorhinolaryngology[1,2] and vascular surgery[3,4,9,10]. This success has been ascribed to special properties of the epithelial tissue, e.g. the non-thrombogenic surface of

endothelium[11], the relative impermeability of stratum corneum to pathogens[12], or the mucociliary activity of ciliated and mucous-producing cells[2,13,14].

In the present study the epithelial reactions to Silastic^R, Estane^R, polypropylene oxide, and a polyether polyester copolymer, which are candiate materials for an alloplastic tympanic membrane in a total alloplastic middle ear prosthesis for the restoration of the sound-conducting system (TAM[15]), were assessed in non-infected as well as in *Staphylococcus aureus* infected rat middle ears[16]. Together with the results of an earlier study[17], the epithelial reactions described here give a complete picture of the biocompatibility and biofunctionality of the four candidate materials in the infected rat middle ear. Results on the behavior of hydroxyapatite - the bulk material of the TAM[15] - in infected surroundings have been published elsewhere[13,14].

MATERIALS AND METHODS

Implants, implantation technique, and infection

Implants were made of four materials: Dow Corning Silastic^R, Estane^R 5714 F1 polyether urethane (BFGoodrich[18]), polypropylene oxide[19], and a poly(ethylene oxide hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer (55% HPOE and 45% PBT; Akzona Inc.[20]). Silastic implants were non-porous and 125 µm thick, whereas the other implants were porous (pore diameters up to 160 µm; over-all porosities of about 50%) and about 100 µm thick. Implants were cleaned in running tapwater, rinsed in distilled water, and air-dried before implantation.

Implantation was performed in the middle ears of male Wistar rats (body weight 200-220 g). The method has been described in detail elsewhere[21]. Briefly, under Hypnorm^R anesthesia a small hole was drilled in the middle ear bulla bone, leaving the middle ear mucosa intact. Two submucosal implants, each measuring about 1 x 1 mm^2, were placed between the bone and the mucosa. After opening of the auditory canal, part of the tympanic membrane was separated from the handle of the malleus and the annulus for the insertion of a tympanic-membrane implant with a diameter of 3 mm. In all, 733 implants from non-infected and 469 implants from ears infected by the injection of *Staphylococcus aureus*.

Middle ears were infected three weeks before decapitation by intratympanic injection of approximately 1.4 x 10^7 *S.aureus* suspended in sterile physiological saline[16]. Only left middle ears were infected; the right middle ears served as controls. The treatment modalities and observation periods are summarized in Table I.

Table I. Numbers of ears used for study of the implant materials in rat middle ears according to method and observation period (weeks).

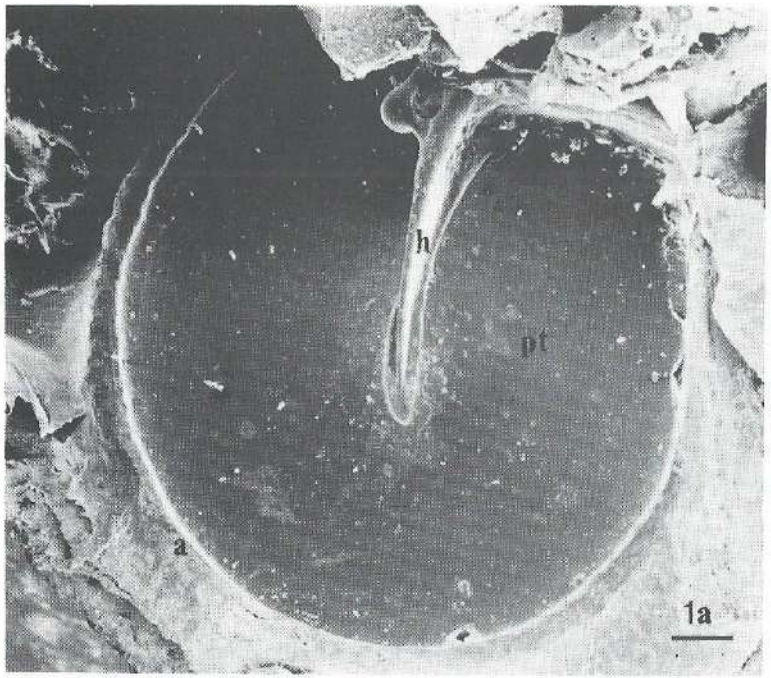
	SUR	1	2	4		13		26		52		TOT	
		N	N	N	I	N	I	N	I	N	I	N	I
LM	TM*	6	6	5	5	5	5	5	5	5(3)	5(3#)	32(30)	20(18)
	SM	12	12	10	10	10	10	10	10	10(6)	10(6)	64(60)	40(36)
TEM	TM	3	3	3	3	3	3	3	3	3(2)	3(2)	18(17)	12(11)
	SM	6	6	6	6	6	6	6	6	6(4)	6(4)	36(34)	24(22)
SEM	TM	3	3	3	3	3	3	3	3	3(1)	3(1)	18(16)	12(10)
	SM	6	6	6	6	6	6	6	6	6(2)	6(2)	36(32)	24(20)

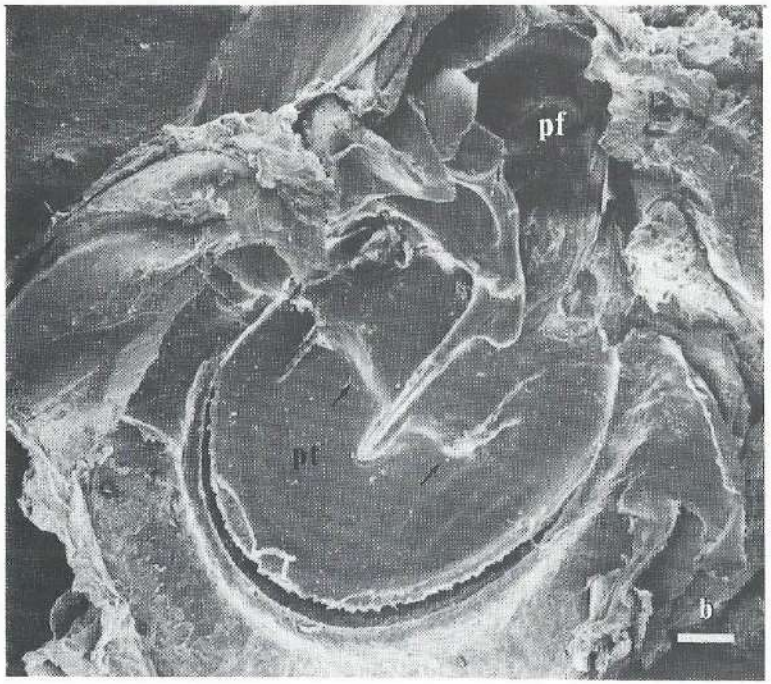
* Silastic^R was not studied in tympanic membranes.
\# Fewer polypropylene oxide implants were studied (number between parentheses).
N Not infected.
I Infected.
LM Light microscopy (one ear was used for light-microscopical autoradiography).
TEM Transmission electron microscopy.
TM, SM Tympanic membrane or submucosal implant.

Light microscopy and light-microscopical autoradiography

Middle ears destined for light microscopy (Table I) were fixed for 2 hr in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Sörensen's buffer (pH 7.4, 4°C) and then for 14 hr in the same fixative but without the glutaraldehyde. After decalcification for 4 weeks (10% EDTA and 2% paraformaldehyde in distilled water, pH 7.4, room temperature) and overnight rinsing in running tap-water, the ears were dehydrated in graded alcohols and embedded in Paraplast^R. Sections were cut 6 µm thick and stained with hematoxylin-eosin.

Autoradiography was performed in one rat for each observation period up to the twenty-sixth week (Table I). These rats were injected intraperitoneally with tritiated thymidine (S.A.: 25 mCi/mmol; Radiochemical Centre, Amersham, England) at a dose of 100 µCi/100 g body weight, one hr before decapitation. The methods used for fixation, decalcification, and dehydration were the same as those used for light microscopy. Embedding was done in glycol methacrylate (GMA, JB-4 embedding kit, Polyscience). Sections cut 2 µm thick were coated with K5 nuclear emulsion and exposed for 4 weeks at 4°C. These sections were stained with methyl-green pyronin. Epithelial proliferative activity was quantitated by counting the labeled nuclei in all cross-sections of tympanic membranes (with and without implants) and sections with submucosal implants.





Electron microscopy and x-ray microanalysis

For transmission electron microscopy (TEM; Table I) sections were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 4°C, 16 hr). Decalcification was performed by submersion for 4 weeks in 10% EDTA and 1.5% glutaraldehyde in distilled water (pH 7.4, room temperature). After a 24-hr rinse in running tap-

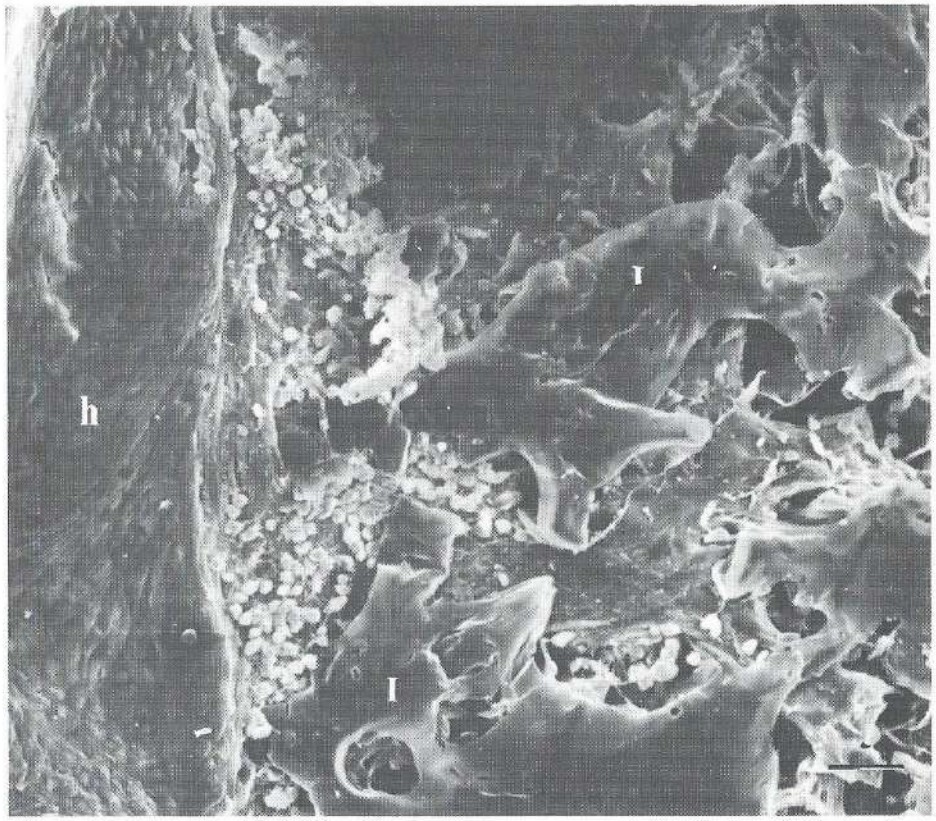


Fig. 1a-c. Series of three scanning electron micrographs showing tympanic membrane epithelium. Important structures in a and b are the pars tensa (PT), pars flaccida (PF), annulus (A), and the handle of the malleus (H). a: Normal rat tympanic membrane. Bar = 275 µm.
b: Tympanic membrane with Estane^{R} implant (arrows) two weeks postoperatively. Bar = 410 µm.
c: Copolymer tympanic membrane implant two weeks after implantation, showing bare copolymer (I) adjacent to cobblestone-like epithelium of the mallear handle (H). Note the presence of exudate. Bar = 43 µm.

water, tympanic-membrane and submucosal implants were cut into small pieces, postfixed in 1% OsO_4 (phosphate-buffered, room temperature, 30 min), dehydrated in alcohol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 201 transmission electron microscope.

For scanning electron microscopy (SEM; Table I), ears were fixed for 2 hr in the same fixative as for TEM, rinsed in phosphate-buffered saline, dehydrated in graded alcohols, critical point-dried in carbon dioxide, gold sputtercoated, and studied with a Cambridge S 180 stereo scanning electron microscope.

For single-spot x-ray microanalysis, use was made of some of the material prepared for TEM and a Philips EM connected to a Tracor Northern (TN) 2000 X-ray microanalyser.

RESULTS

Tympanic membrane epithelium

Light microscopy showed that porous tympanic membrane implants were first covered by a layer of exudate cells and then became surrounded by fibrous tissue. Scanning electron microscopy and light microscopy showed complete epithelial covering of Estane^R and polypropylene oxide tympanic-membrane implants after two weeks (Fig. 1b), whereas complete covering of tympanic membrane implants made of copolymer took between two weeks and one month (Fig. 1c). Tympanic membranes of middle ears which invariably rejected dense Silastic^R implants healed in between two and four weeks. Porous implants were never rejected. Throughout the experiment, the epithelium of implant-bearing tympanic membranes as well as that of tympanic membranes initially given Silastic, was predominantly composed of flat polygonal and pseudo-stratified cuboidal cells bearing variable numbers of microvilli (Fig. 2). After three months, cuboidal and pseudo-stratified epithelium were only seen occasionally, i.e., in the annulus and the handle of the malleus, and the numbers of microvilli had decreased. Both the flat polygonal and cuboidal epithelial cells contained a nucleus, small quantities of rough endoplasmic reticulum, some ribosomes, a Golgi apparatus, and a few mitochondria (Fig. 3a). Epithelial cells were connected to each other by desmosomes and to the lamina basale by hemidesmosomes. Besides the flat polygonal epithelial cells, there were ciliated as well as secretory cells in the pars flaccida and in the part of the pars tensa extending from the annulus halfway to the handle of the malleus (Fig. 3b). Secretory cells contained relatively electron-lucent and electron-dense granules. In the presence of polypropylene oxide, some of the secretory cells on the pars

tensa had electron-dense granules (Fig. 3b) containing iron, as established by x-ray microanalysis (Fig. 3c).

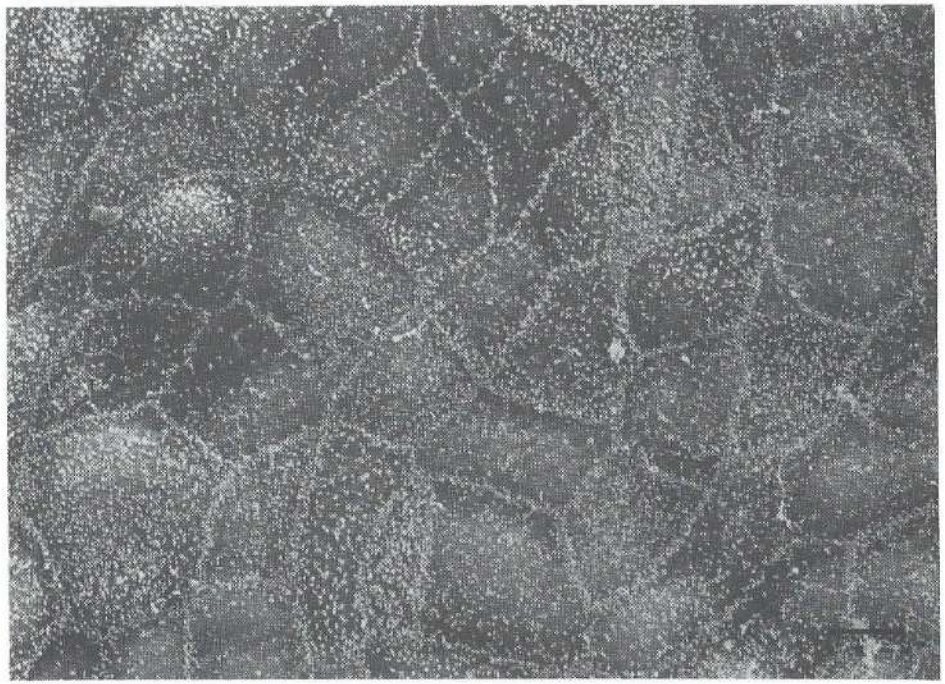


Fig. 2. Scanning electron micrograph showing microvilli-bearing flat polygonal epithelium on the medial side of a tympanic membrane bearing a polypropylene oxide implant, two weeks after implantation. Bar = 3.5 µm.

Epidermis

The lateral surface of the healing tympanic membrane was completely covered by epidermis after two (Estane and polypropylene oxide) to four (copolymer and Silastic) weeks. After the first week the epidermis was thickened and consisted of four layers of keratinocytes in various stages of differentiation, i.e., the cell layers of the strata basale, spinosum, and granulosum, and the cornified cells of the stratum corneum. The keratinocytes of the thickened epidermis were more cuboidal, especially those of the stratum spinosum, and there was more intercellular substance (Fig. 4b and inset). After an observation period of six months, the thickness of the epidermis of a tympanic membrane with an implant was comparable to that of the untreated tympanic membrane, as established by light microscopy and transmission electron microscopy. With Silastic implants the epidermis was normal after three months. Epidermis cells penetrating the tympanic

connective tissue and basal epidermal cells isolated within the lamina propria of the tympanic membrane were seen occasionally.

Quantified incorporation of tritiated thymidine by the epithelial and the epidermal cells of tympanic membranes with a biomaterial implant is shown in Figure 5a. Proliferative activity associated with Silastic and copolymer tympanic-membrane implants was relatively high compared with the data for Estane and polypropylene oxide (Fig. 5a). Basal cells displayed relatively strong incorporation of ^{3}H-thymidine until the third postoperative month (Fig. 5c).

Middle ear mucosa

Submcosal implants, whether porous or dense, were surrounded by exudate cells and encapsulated in fibrous tissue underneath an intact middle ear mucosa composed predominantly of flat polygonal cells (Fig. 6a). Ciliated and secretory epithelium were also seen, especially on implants bordering the mucociliary tracts of the middle ear. Coblestone-like epithelium was observed up to the second week. Autoradiography showed a low proliferative activity of the flat polygonal epithelium bordering implants made of the biomaterials under study, which was relatively constant with progression of time, although this activity was initially slightly higher with Silastic (Fig. 5b). Labeled ciliated and secretory cells were not seen. The number of microvilli on the flat polygonal cells varied considerably. Some of the cells had many microvilli distributed over the entire cell surface, whereas others had only few which lay close to the cell border. During the one- and two-week observation periods there were some cells with long filopodia extending over the surface of the flat polygonal epithelium covering all of the biomaterials under study (Fig. 6b). These cells were also seen on the surface of tympanic membrane epithelium, although less frequently.

The TEM picture of the flat polygonal cells covering implants was the same for all of the biomaterials tested and was comparable to that of the normal epithelium lining (Fig. 6c). These cells, which were situated on a basal lamina and connected by desmosomes, showed a flat nucleus, rough endoplasmic reticulum, ribosomes,

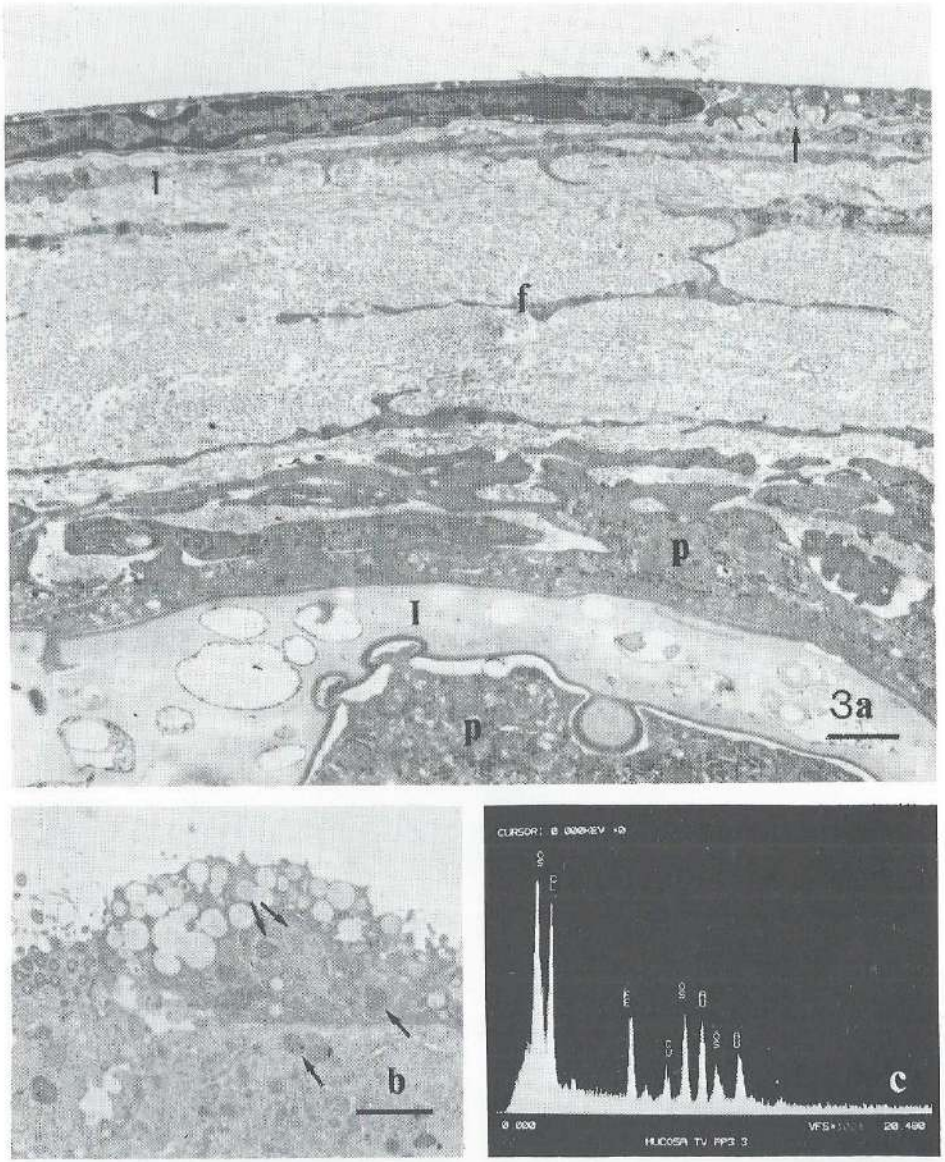
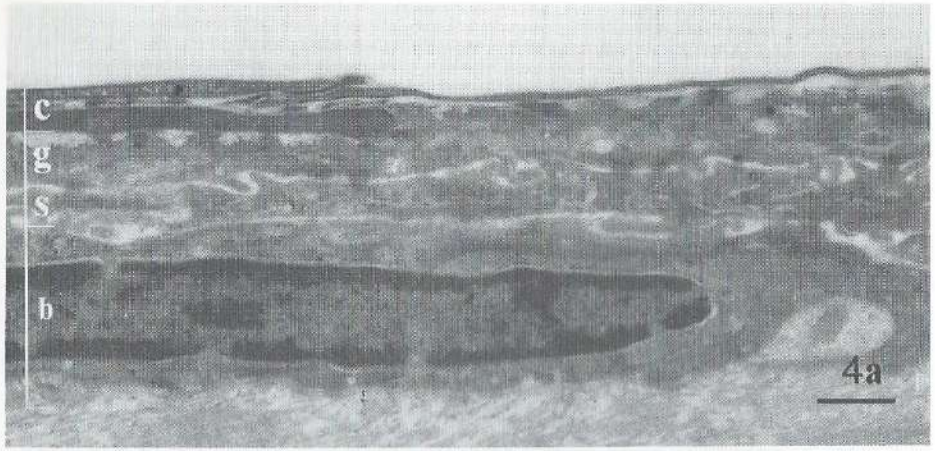
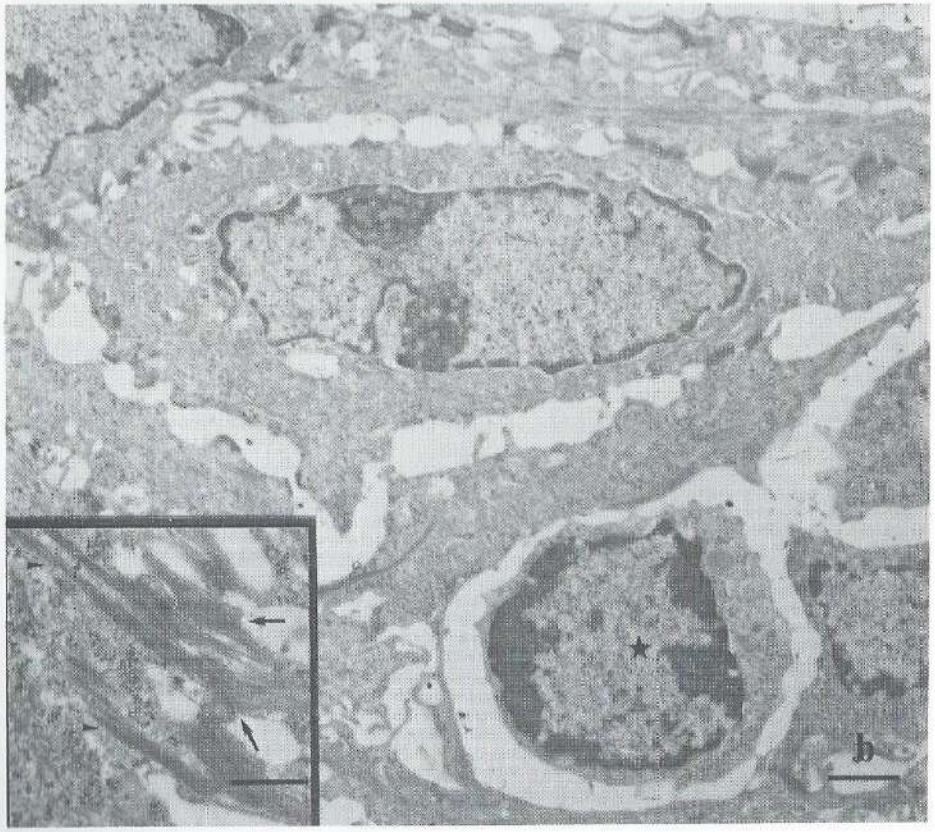


Fig. 3a and b. Transmission electron micrographs of tympanic-membrane epithelium, three months postoperatively.
a: Part of an Estane^{R} implant (I) surrounded by phagocytes (P) and fibrous tissue (F) and covered by flat polygonal epithelium. Arrows indicate the basal lamina of the epithelium. Bar = 1.1 µm.
b: Ciliated and secretory epithelium on tympanic membrane containing fragments of polypropylene oxide. Note the electron-dense inclusions within some of the granules (arrows). Bar = 1.6 µm.
c: X-ray spectrum of the inclusions shown in b, revealing the presence of iron (Fe peak).





mitochondria, and a Golgi apparatus (Fig. 6c). The morphology of the ciliated and secretory epithelium covering submucosal implants was also similar to that in the normal middle ear. SEM showed that the apical side of ciliated cells was completely covered by cilia with a uniform length and that secretory cells bulged into the middle ear lumen (Fig. 7a). Ciliated cells contained a nucleus, numerous ribosomes, and small mitochondria, but no granules (Fig. 7b). Secretory cells were characterized by numerous granules and abundant rough endoplasmic reticulum (Fig. 7b). Occasionally, a basal cell was seen (Fig. 7b).

Infection experiment

Three weeks after the injection of *Staphylococcus aureus*, both the thickness of the epidermis and the proliferative activity (Fig. 8a) of the epidermal basal cells were increased. Furthermore, lymphocytes had frequently infiltrated the epidermis, and polymorphonuclear granulocytes were also seen.

After infection, the numbers of ciliated and secretory cells covering tympanic membrane implants were higher for all implant materials and observation periods. The structure and composition of the epithelial lining of the middle ear cavity were significantly affected by the infection, whereas at that time the proliferative activity was not influenced (Fig. 8b). The middle ear epithelium was pseudostratified and showed increased numbers of microvilli-bearing cells. As Figure 9a shows, mucosa-derived fibrous tissue partially obliterated the middle ear cavity. These fibrous strands, some of them fused with the tympanic membrane, were covered mainly by flat polygonal cells (Fig. 9b) but also by smaller numbers of ciliated and secretory epithelial cells. The area covered by the mucociliary tracts was increased, as shown by scanning electron microscopy. Furthermore, areas filled with ciliated and secretory cells and continuous with the mucociliary tracts, were frequently seen on both of the upper quadrants of the pars tensa. The lamina propria was considerably thickened and infiltrated by inflammatory cells. Numerous pockets covered by ciliated epithelium and islands of epithelial cells suggesting secretory activity, were observed within the submucosal connective tissue.

Fig. 4. Transmission electron micrographs showing morphology of the epidermis.
a: Epidermis of a normal rat tympanic membrane displaying the single-cell layers of the strata basale (B), spinosum (S), granulosum (G), and corneum (C). Bar = 0.5 µm.
b: Part of the basal cell layer one month after insertion of a copolymer implant. Note the cuboidal keratinocytes and the increased amount of intercellular substance. Asterisk indicates a lymphocyte. Inset: Keratinocytes displaying desmosomes (arrows) and keratin fibers (arrowheads). Bars = 1.3 µm.

DISCUSSION AND CONCLUSIONS

Epithelial reactions to an implant are important for the adequate functioning of implants used in a number of surgical disciplines[1-9]. Middle ear implants should neither increase the risk of infection[22] nor interfere with the defence mechanisms of the middle ear[16,23,24]. With respect to the risk of infection, rapid and complete covering of an otologic implant by middle ear epithelium is crucial[13,14]. Because ciliary and secretory epithelium are known to play a role in middle ear defence[25], the composition of the epithelium covering the implant is important too. Furthermore, epithelial reactions play a role in the extrusion of implants[8].

Except for Silastic^{R}, which was rapidly rejected after implantation in the tympanic membrane, all of the tested implants were covered by epithelium. Epithelial reactions to implants in the tympanic membrane comprised changes in proliferative activity, migratory activity, the cell population, and the cell morphology of both the epidermis and epithelium. Because submucosal implants were placed underneath an intact middle ear mucosa and the procedure was not very traumatic, the reactions of the middle ear mucosa were limited to the cell morphology.

It was found that epidermis and epithelium took more time to completely cover copolymer tympanic-membrane implants than to cover polypropylene oxide and Estane^{R}, and that epidermal proliferative activity was more prominent and lasted longer in the presence of copolymer than of the other porous materials. *In vitro* studies showed that neither the proliferative activity nor the morphology of rat middle ear epithelium cultured on dense copolymer films differed significantly from material cultured on Estane; epithelium could not be cultured on polypropylene oxide[27]. It seems more likely that copolymer behaved as a temporary barrier for migrating tympanic-membrane tissue[27] (due to unfavorable structural properties[28]) than that the chemical composition[29] of copolymer influenced cell adhesion and cell growth.

Because of the conflicting results on epithelial reactions to polypropylene oxide and the finding that degradation of this compound in the body leads to the

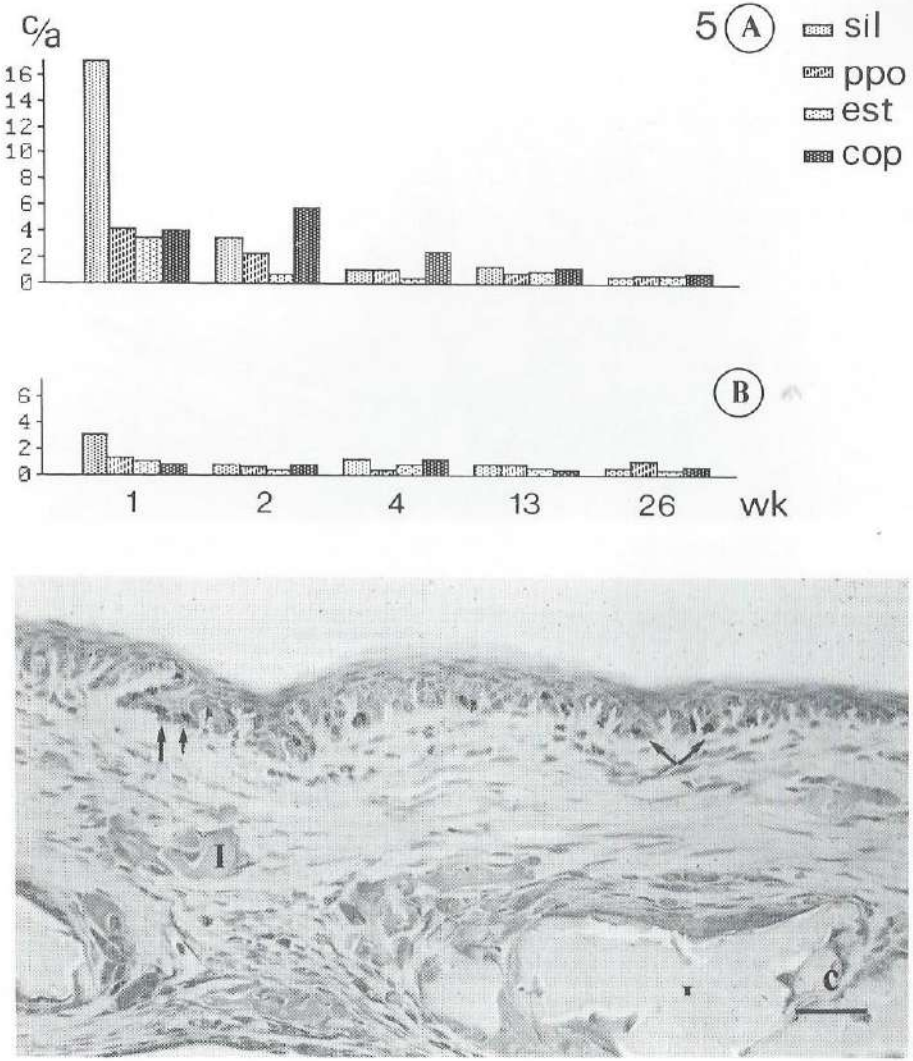
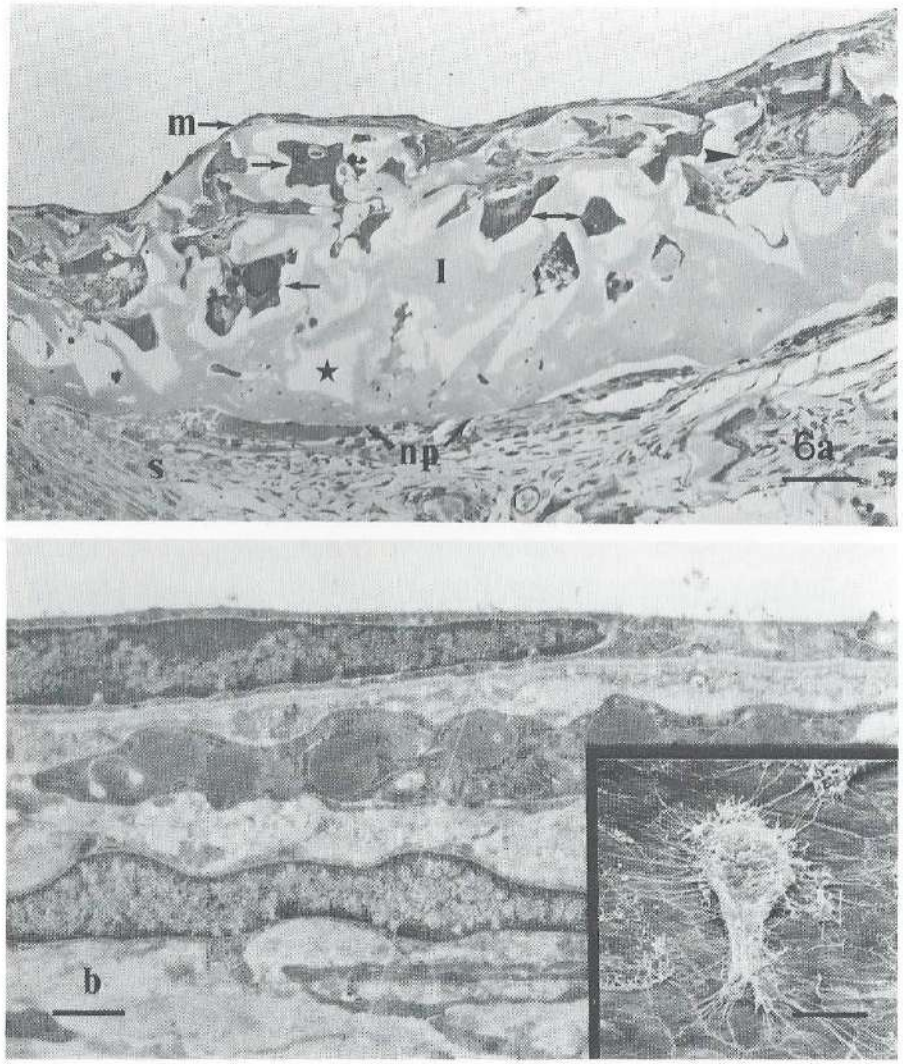


Fig. 5a-c. Autoradiographic results.
a and b: Schematic representation of the results of the quantification of the autoradiographic findings for the four biomaterials. Average number of counts per section (c/a) of a tympanic membrane or submucosal implant expressed as function of time for tympanic membrane epidermis and epithelium (a) and middle ear epithelium (b). Sil = Silastic^{R}; Ppo = polypropylene oxide; Est = Estane^{R}; Cop = copolymer implants.
c. Autoradiograph of copolymer three months after implantation, showing thickened epidermis with labeled basal cells (arrows). I = Implant. Bar = 40 µm.



release of toxic substances[21], epithelial reactions to polymers would seem to be less sensitive guages of biocompatibility and biofunctionality *in vivo* than *in vitro*[30], or even that it must be concluded that the *in vivo* epithelium response cannot be considered to offer a reliable criterion for the biocompatibility of implants at all[31].

After implantation, the numbers of ciliated and secretory cells on the tympanic membrane increased. Cells of both types were probably originated by transformation or from the mucociliary tracts bordering the upper quadrants of the pars tensa[32]. The effects of iron uptake by secretory epithelium covering polypropylene oxide tympanic membranes on the biocompatibility of an implant material is not known and should be investigated. The cobblestone-like appearance, the increased numbers of microvilli, and the pseudo-stratification of part of the flat polygonal epithelium covering the tympanic membrane and the middle ear cavity were of relatively short duration and are probably attributable to the surgical procedure associated with the implantation. Comparable reactions of the epithelium have been observed after the implantation of hydroxyapatite[1,13,14] and tricalcium phosphate[33] in the rat middle ear. The origin of the filopodia-rich cells, also observed by van Blitterswijk et al.[1], could have been either epithelial or exudative. The absence of desmosomes between these cells and the underlying epithelium points to an exudative origin. After longer intervals the morphology of the epithelium of both the tympanic membrane[34] and the mucosa[1,32,35] was similar to that in normal ears.

Infection affected the structure and proliferative activity of the tympanic membrane epidermis as well as the composition and morphology of the tympanic membrane epithelium and of the middle ear mucosa. The reactions of the tympanic membrane epithelium and the middle ear mucosa exposed to Silastic, Estane, polypropylene oxide, and copolymer to infection were comparable to the epithelial reactions in rat middle ears without implants[16,23-25,34], which means that the presence of the biomaterials under study was not a complicating factor.

Fig. 6. Morphology of the mucosa of the rat middle ear after implantation of an alloplast.
a: Light micrograph showing early reactions to copolymer submucosal implant, two weeks postoperatively. Note the presence of exudate (arrows) and fibrous tissue (arrow head) in the copolymer (I) pores and also empty pores (asterisk) and the non-porous side of the implant (NP). M = mucosa; S = submucosa. Bar = 40 µm.
b: Transmission electron micrograph showing part of the submucosa covering a Silastic^{R} implant three months after implantation. Bar = 1 µm. Inset: Scanning electron micrograph of a cell adhering with many filopodia to middle ear epithelium covering Estane^{R} implant, one week postoperatively. Bar = 2.7 µm.

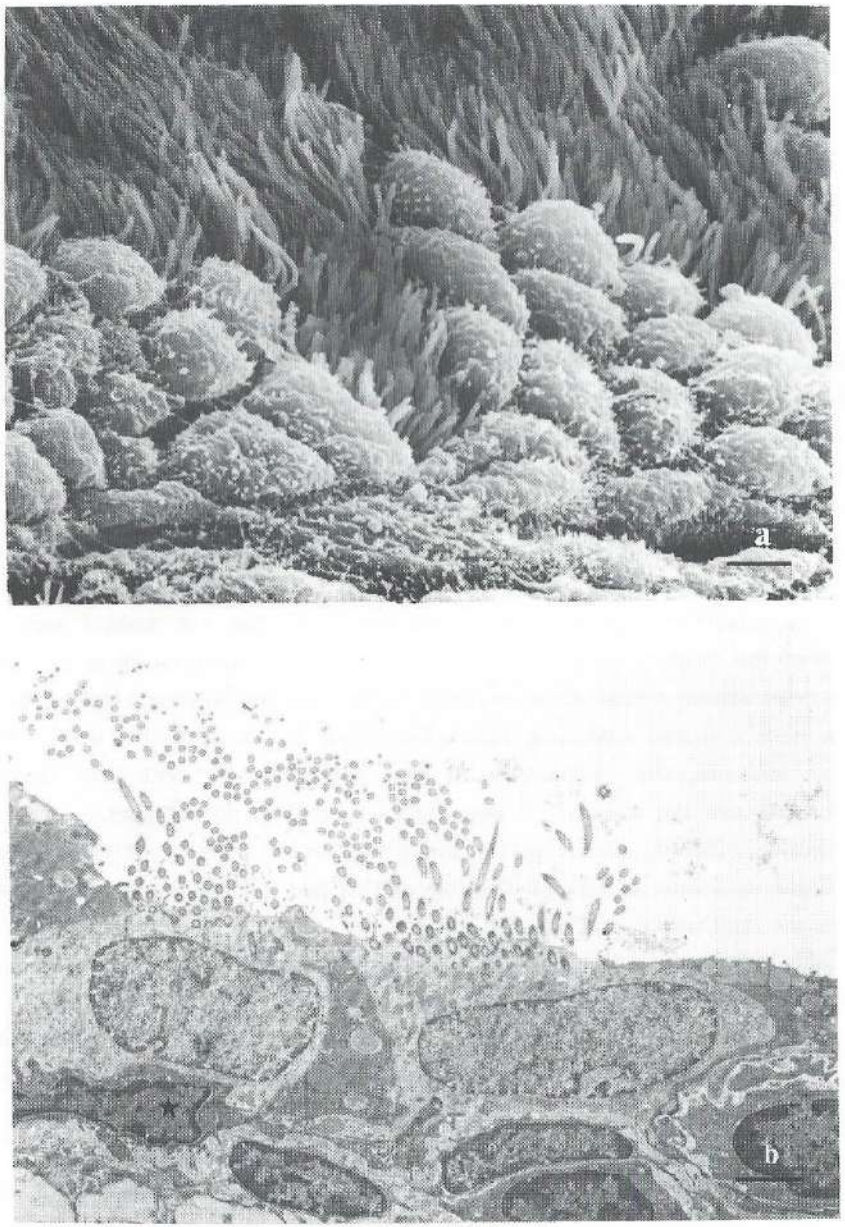


Fig. 7. Scanning (a) and transmission (b) electron micrographs showing ciliated and secretory epithelium covering submucosal implants.
a: Ciliated and bulging secretory cells of a mucociliary tract covering Estane^{R} one week after implantation. Bar = 2.1 µm.
b: Part of a mucociliary tract covering copolymer implants one week postoperatively. A basal cell (asterisk) is also visible. Bar = 1.8 µm.

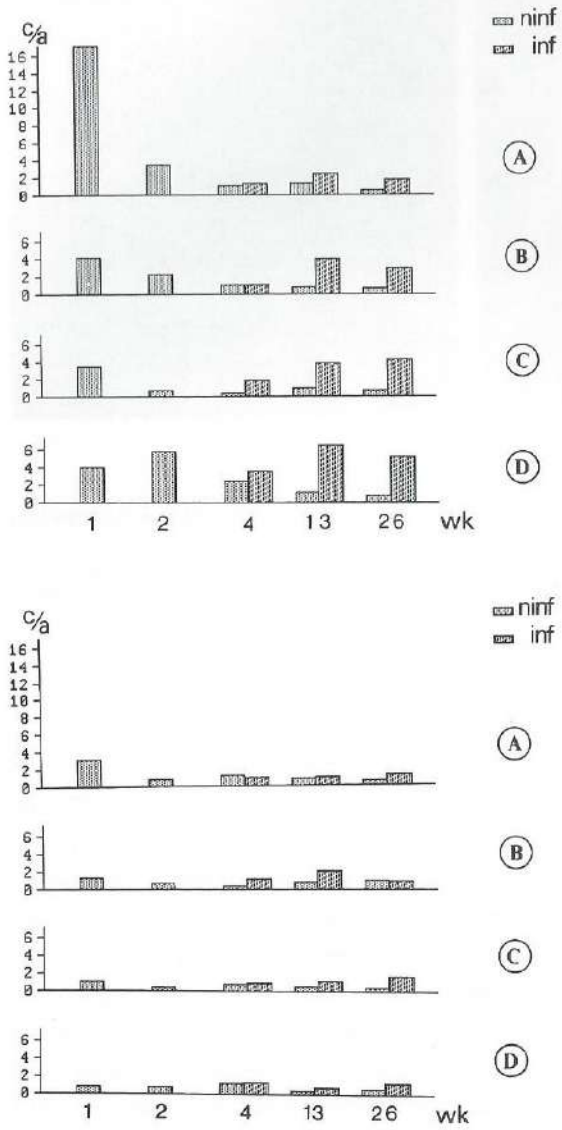


Fig. 8. Schematic representation of the effect of infection on the incorporation of tritiated thymidine by epithelium. Average number of counts per section (c/a) of a given tympanic membrane or submucosal implant as a function of time for tympanic membrane epithelium and epidermis (top) and middle ear epithelium (bottom). Note the relatively large increase of the proliferative activity associated with tympanic membrane implants relative to that of submucosal implants. A = Silastic^{R}, B = polypropylene oxide; C = Estane^{R} polyether urethane; D = HPOE/PBT copolymer.

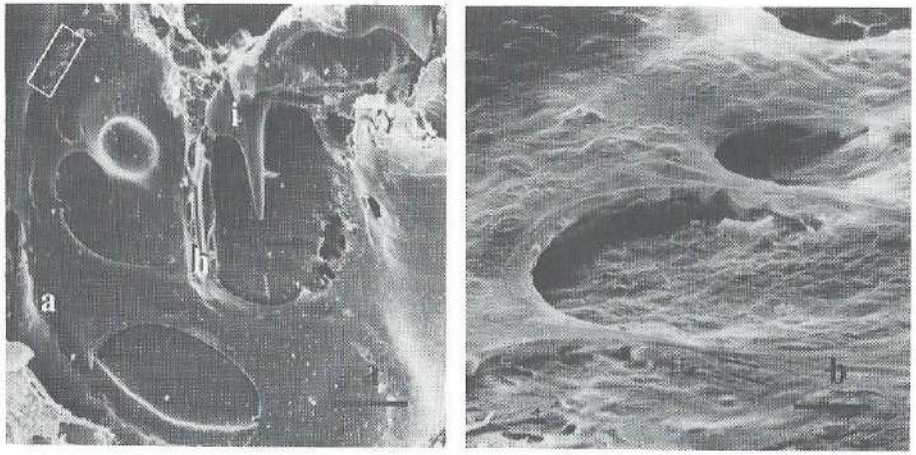


Fig. 9. Effects of infection on epithelium of the tympanic membrane and middle ear.
a: Scanning electron micrograph showing fibrous ingrowths spread over and occasionally fusing with the tympanic membrane. Estane^{R} three months after implantation. Arrow: possibly the opening made to introduce *Staphylococcus aureus*. A = annulus; H = handle of the malleus; I = incus. Bar = 160 µm.
b: Detail of left upper quadrant in a (see box), showing flat polygonal epithelium covering the fibrous strands originating from the mucosa. Bar = 20 µm.

From the present data it may be concluded that when implants made of one of the four polymers under study are covered by epithelial cells, the differentiation of epithelium into ciliated and secretory epithelium is normal. Ultimately, the preoperative situation is restored. Furthermore, the presence of these biomaterials did not affect the normal epithelial responses to middle ear infection. However, the finding that Silastic is not incorporated into tympanic membranes, as well as earlier results[21,26] suggesting that polypropylene oxide yields toxic degradation products, indicate that these materials are not suitable for an alloplastic tympanic membrane. Nevertheless, the use of Silastic in middle ear surgery at other sites seems acceptable, because submucosal implants of Silastic are tolerated well. This has also been found in other studies[17,21,36]. Estane, which is covered more rapidly by tympanic-membrane epithelial cells than copolymer is, seems to be the most appropriate material for an alloplastic tympanic membrane. Before final conclusions can be drawn, further experimental studies on animals are needed to find out whether the structure of the HPOE/PBT copolymer implants was responsible for the relatively slow covering of the material by epithelium.

Acknowledgements
The authors gratefully acknowledge the critical discussion of the results by Dr. W.Kuijpers (Radboud Hospital, Nijmegen) and are indebted to Mr. H.K.Koerten (Laboratory for Electron Microscopy, University of Leiden) for carrying out the x-ray microanalysis.

They also wish to express their gratitude for the technical assistance provided by Dr. A.B.M.Klaassens (Radboud Hospital, Nijmegen), Mr. B.M.A. van der Lans and Ms. P. van der Hoek (ENT Department, University Hospital, Leiden), and Mr. L.D.C.Verschragen, Mr. J.J.Beentjes, and Mr. S.B.Neijman (Laboratory for Electron Microscopy, University of Leiden). The cooperation obtained from the Laboratory for Bacteriology and Parasitology and the Laboratory of Surgery of the University of Leiden is also gratefully acknowledged.

Films of Estane^{R} 5714 F1 polyether urethane, polypropylene oxide, and HPOE/PBT polyether polyester copolymer were kindly supplied by Dr. J.H.Kuit and Dr. J.G.F.Bots (Technological University of Twente, Enschede) and Prof. K.B.Wagener (University of Florida, Gainesville, Fla, USA).

These investigations were supported by the Netherlands Technology Foundation (STW) and the Heinsius Houbold Fund.

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CHAPTER 9

CHEMICAL STRUCTURE AND TOXICITY OF IMPLANT MATERIAL

SUMMARY

A review of the literature on the chemical composition and toxicity of Silastic^R silicone rubber, Estane^R 5714 F1 polyether urethane, the polyether polypropylene oxide, and a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer was performed. The findings were analysed in relation to the results of our tissue-culture experiments and implantation studies in rats done to assess these polymers as candidate materials for an alloplastic tympanic membrane.

Polypropylene oxide was found to be toxic for both cultured cells and the tissue surrounding the implant. This toxicity was attributed to non-polymerized substances and degradation-released aldehydes, respectively. Cells exposed to Silastic degradation products obtained in an artificial aging experiment *in vitro* showed a morphology differing from that of normal cells by their less-flattened appearance, the presence of silicon-containing inclusions, and structures resembling dilated endoplasmic reticulum. However, since neither Silastic degradation nor silicon accumulation occurred *in vivo*, Silastic is considered to be harmless to the body. Biocompatibility studies on Estane and copolymer showed that neither these polymers nor their degradation products were toxic.

INTRODUCTION

For assessment of the behavior of a permanent implant within the body, the effect of the local milieu on the properties of the implant and the cytological effect of the implant on adjacent and distant tissue must both be examined[1]. The manifestation of toxicity varies according to the nature of the substance, the route by which it is introduced into the body, and the duration of the exposure[2,3]. The composition of such materials must be known before tissue reactions and systemic responses to the implant material can be studied[4]. This chapter gives a review of the literature on the chemical composition of four candidate materials for an alloplastic tympanic membrane and the implications for implant toxicity. The polymers studied were Silastic^R, polypropylene oxide, Estane^R 5714 F1 polyether urethane, and a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer (55/45 HPOE/PBT). For 55/45 HPOE/PBT copolymer - the most promising polymer in terms of the results of the biocompatibility implantation study discussed in the preceding chapters - the published information was supplemented with results of additional implantation studies in the rat undertaken to obtain more information about possible toxic effects of this polymer.

SILASTIC[R]

Polymer chemistry and toxicity

Dow Corning Silastic[5,6] is a medical-grade silicone rubber that is widely used and commercially available[5] in the form of non-porous (dense) films. Silastic is a heat-vulcanizing silicone rubber, the vulcanization occurring by reaction with water absorbed from the air[8,9]. The structural formula of polydimethyl siloxane, i.e., silicone rubber, is shown in Fig. 1.

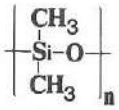

Fig. 1. Chemical formula of polydimethyl siloxane.

Silicone rubbers, which are hydrophobic polymers, were found to be relatively inert[9,10] and stable[9] during three years of implantation subcutaneously, intramuscularly, intraperitoneally, and in the cerebellum of animals. However, there are reports of mechanical changes related to lipid absorption of silicones during implantation in patients for periods of up to eight years as part of heart valves[11], subcutaneously in dogs[12] for two years, and as human joint prostheses for five years[13]. Autian mentioned degradation of silicone rubbers implanted in the body[14]. Systemic toxicity of medical-grade silicone rubbers has not been reported[9], but studies in animals on the use of silicone fluid are less encouraging[15] because of the deposition of silicone droplets in e.g. the spleen, liver, adrenals, and kidneys. Silicone elastomers formulated for biomedical applications do not contain organic additives[9] such as antioxidants, dyes, and plasticizers that would be major contributors to the acute toxicity of plastics[12]. Although Silastic is tolerated well by the body[9,10], solid-state carcinogenesis cannot be completely excluded because of findings[12,16] in animal experiments, predominantly in rats; it has been reported for dense implants made of a variety of materials[15], but has never been firmly established in man[16].

Discussion

The morphology of the samples of rat middle ear epithelium serially cultured in medium prepared with the artificially aged silicone rubbers Silastic and Dow Corning[R] Elastomer (Chapter II) differed from that of normal cells of the same type. Treated cells of both groups were less flat and showed cytoplasmic

structures resembling dilated endoplasmic reticulum. With Silastic, part of these divergent cells also showed electron-dense inclusions containing silicon. Although the aberrant cell morphology suggests a toxic response to degradation products of Silastic and Elastomer, cell toxicity apparently does not always lead to the formation of silicon bodies, since such bodies were not seen in the cells exposed to Elastomer. Although it seems likely that the silicon in these bodies originated from artificially aged Silastic, we found no evidence indicating that they are also formed during Silastic degradation *in vivo* (Chapters IV-VIII), because in our animal experiments Silastic did not undergo visible degradation during implantation periods of up to one year. Foreign-body carcinogenesis was not observed for Silastic implanted in the rat middle ear.

POLYPROPYLENE OXIDE

Polymer chemistry and toxicity

The polyether glycol polypropylene oxide (PPOX) has received attention as a segment in segmented block copolymers for use in contact with blood[17-20] or as biodegradable implant system[21,22]. The chemical and physical nature of the segments of a block copolymer affect surface properties[20,23] and blood response[17,24] as well as the mechanical properties and stability in the biologicial environment[24]. Polypropylene oxide is more hydrophobic than the readily soluble polyether glycol polyethylene oxide (PEO)[25]. Blends of PPOX (as the more hydrophobic and mechanically stronger component) and PEO (as the component with greater blood compatibility) have been used for small-diameter blood vessel prostheses[18,19] with promising results as to mechanical properties and blood compatibility[19].

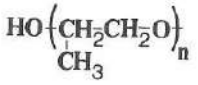

Fig. 2. Chemical formula of polypropylene oxide.

The PPOX we studied (Fig. 2) had a molecular weight of about 200 kDalton and was synthesized[26] from propylene oxide with a catalyst prepared from diethylzinc and triphenyltin hydroxide. Fibers were spun from a PPOX solution in dichloromethane[18,19]. Winding of the fibers on a rotating axle produced a porous tubing with a woven structure[18,19]. Dicumylperoxide (DCP) was added (5% w/w)

to crosslink the tubing after fabrication. This 'spinning from solution method' makes it possible to vary the pore size of a prosthesis. Films were washed with acetone to remove non-crosslinked PPOX, DCP, and such decomposition products as acetophenone and 2-phenylisopropanol[18,19].

Discussion

Results of the three *in vitro* studies (Chapter II) and the implantation study (Chapters IV-VIII) constitute evidence that the presence of the polypropylene oxide studied led to cell responses indicative of implant toxicity. Both the absence of cell attachment and the divergent cell morphology could have been due to incompletely eliminated toxic substances initially present in the polymer[18,19]. The cell death in the artificial aging experiment, the altered morphology of the mitochondria of the phagocytes adhering to the implant after one month or longer in the rat middle ear, and the death of five rats in the one-year group, suggest that toxic substances were released during polypropylene oxide degradation[19].

For assessment as biomaterial the concentration of the toxic DCP and catalyst had to be as low as possible and exposure to UV irradiation avoided as much as possible to prevent degradation of the polymer and incorporation of peroxy radicals into the polymer network[18]. Furthermore, besides being toxic, traces of catalyst do also promote PPOX degradation leading to the release of other toxic substances such as aldehydes[19], which offers a likely explanation for the rather unexpected rapid degradation of PPOX and the toxic responses observed *in vivo*.

ESTANE^{R} 5714 F1 POLYETHER URETHANE

Polymer chemistry

The third polymer under study was Estane 5714 F1 segmented polyether urethane[27-32]. Polyurethanes are a family of diverse polymers with a wide range of properties determined by the molecular composition[33]. Many polyurethanes have been investigated for use in biomedical devices, Biomer^{R} probably the most extensively[33-38]. Moreover, different polyurethanes are produced in a variety of physical forms, which complicates the discussion of the individual properties[37].

For our studies, Estane was synthesized according to the prepolymer method[38]. Initially, methylene bis(p-phenylisocyanate) (MDI) was reacted with polytetramethylene glycol (PTMG) to form an intermediate oligomer. This prepolymer was converted into the polyurethane (Fig. 3) by further reaction with 1,4-butanediol (1,4-BD). n,n-Dimethylacetamide (DMA) has a solvent effect on Estane. In Estane, chains of the aromatic hard segment MDI are connected by flexible polyether soft segments composed of PTMG. The length of the hard

segment can be increased by use of the chain extender 1,4-BD[38]. For the synthesis of Biomer, use is made of diamines or water as chain extender[34].

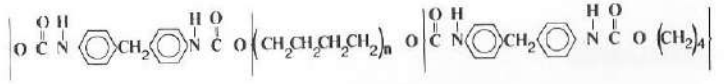

Fig. 3. Possible chemical formula of Estane^{R}.

Polymer toxicity

Although polyester urethanes can be degraded relatively rapidly *in vitro* by a hydrolytic process[39], polyether urethanes are rather resistant to degradation[40]. However, it has been shown both *in vitro*[35] and *in vivo*[33,39,41-43] that the urea linkages of the polyether urethane Biomer is susceptible to hydrolysis. According to Smith et al.[35], if it may be accepted that degradation takes place hydrolitically at urea linkages of polymers with a structural resemblance to Biomer, the most likely end product is the chain extender[34]. Extrapolation of these authors' findings to Estane 5714 F1 polyether urethane suggests that hydrolysis leads to the release of butanediol[36] (for details, see under 55/45 HPOE/PBT copolymer). MDI, which is used as the hard segment, is a highly toxic substance[3], but MDI systems are generally approved by the Food and Drug Association (FDA) for the packaging and processing of dry foods[33]. There is some concern as to whether by-products of MDI, e.g. 4,4-diamino diphenylmethane (MDA), can induce mutagenic effects of implants[44]. MDA, the presence of which has been attributed to polymer degradation[42], was detected in polyether urethane after processing and steam sterilization, albeit in very low concentrations[46].

Acute toxicity was investigated by Szeicher[47] for polyurethanes, and no adverse reactions were found. Pellethane^{R}[48], Texin^{R}[49], and Estane[27] have passed class VI tests of the US Pharmacopeia[50] test for acceptable pyrogen levels and tissue-culture tests to detect cytopathic effects and Biomer has been approved by the FDA. However, the tests used by the US Pharmacopeia are very insensitive with respect to biocompatibility and have little relevance for implant materials for long-term use[4]. For example, *in vitro* cell cultures have shown growth inhibition by several polyurethanes[51,52], and toxic responses attributed to degradation byproducts of polyurethanes *in vivo* have also been reported[1,53,54].

Discussion

The results concerning Estane biocompatibility *in vitro* (Chapter II) and *in vivo* (Chapters IV-VIII) imply that this polymer and its breakdown products are not

toxic. The rather strong foreign-body reaction to Estane was probably elicited by the relatively rough surface of the implants[87] rather than by possible irritating degradation products[34-36].

55/45 HPOE/PBT COPOLYMER

Polymer chemistry

The fourth polymer we studied is a poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer[55,56] (Fig. 4) belonging to a class of biocompatible copolymers especially developed for biomedical use[55]. The hard segments are composed of tetramethylene terephthalate (number-average molecular weight of about 1400 D), which confers the requisite properties, and the soft segments, composed of ethylene oxide hydantoin, provide the desired biological properties[56]. The hard segments make up 45 percent by weight of the polymer, the soft segments contributing 55 weight percent. The number-average molecular weight of ethylene oxide hydantoin is 1,006. 1,4-Butanediol and dimethyl terephthalate were used to prepare the hard segments and 5,5-dimethylhydantoin and ethylene oxide were used to synthesize the hydantoin polyester prepolymer[57]. The synthesis of 55/45 HPOE/PBT copolymer is discussed in detail in US Patent No. 4,262,114[57]. 55/45 HPOE/PBT copolymer dissolves in choroform.

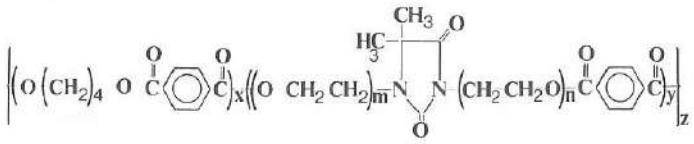

Fig. 4. Chemical formula of HPOE/PBT segmented copolymer.

Incorporated into the soft segment is a thermally stable heterocyclic unit, hydantoin, that prevents crystallization of polyethylene oxide (PEO), a substance which by nature is highly crystalline[25]. The elastomer state of the copolymer, which persists at room temperature, is obtained by the presence of hydantoin. PEO also forms highly crystalline complexes with a wide variety of small-molecule substances such as calcium chloride, bromine, and iodine[25]. At present, comparable copolymers with sufficient elastomeric properties can be synthesized without the use of hydantoin (PEO/PBT copolymers). Hydantoin also provides a chemically reactive site for the incorporation of other moieties to enhance material properties[56].

Toxicity - Review of the literature

With respect to possible toxic effects of this polymer, a distinction should be made between the original ingredients and substances released during copolymer degradation. Reed and coworkers[58-60] showed that chemical hydrolysis is the mechanism underlying the degradation of poly(ethylene oxide) / poly(ethylene terephthalate) copolymers (PEO/PET) *in vitro*[60]. Furthermore, the ester bond of the PET segment linking the ester and the hydrophilic ether segments, was the primary cleavage point[58-60]. In view of the similarity between PEO/PET and 55/45 HPOE/PBT copolymers, the HPOE/PBT copolymer is expected to hydrolyse in a similar way, resulting in the release of poly(ethylene oxide hydantoin) and poly(tetramethylene terephthtalate). The HPOE might degrade further into hydantoin derivatives and polyethylene oxide segments. PBT itself will be affected the least by hydrolysis, because of its crystallinity in polymeric form[55]: the more crystalline the polymer, the less likely it is to swell because of water uptake and the less susceptible it is to hydrolysis[61]. In the long run, however, it might dissociate into butanediol and terephthalate derivatives.

1,4-Butanediol given orally or intragastically is known to be toxic to animals and man (for references, see Poldrugo et al.[62]). The liver and kidneys appear to be particularly susceptible[62]. However, these findings are based on experiments in which animals were fed 1 g diol per day per kg body weight, which is significantly more than the amount of diol that might be released in the human middle ear, for example, during HPOE/PBT copolymer tympanic membrane degradation.

Diphenylhydantoin (phenytoin) is used as an anticonvulsant drug and has been reported to induce in the unborn child teratogenic effects[63,64] that can become manifest as anomalies of e.g. the middle ear and inner ear[65]. Diphenylhydantoin intoxication can manifest itself as nystagmus, dizziness, and, occasionally, tinnitus[66]. The prescribed therapeutic blood level is 10 to 20 mg/ml, which is significantly higher than the concentration resulting from degradation of a HPOE/PBT copolymer tympanic membrane.

Terephthalic acid (the para-form of benzenedicarboxilic acid) and its esters are important as starting chemicals for certain types of polymer. They are also used in animal feed to enhance the physical properties of the feed and the bioavailability of added compounds such as antibiotics[67]. Compound esters of terephthalic acid with a lower molecular weight (C1 up to C5) showed cytotoxic and toxic effects, but the C6 compounds did not[68]. Implantation studies on polyethylene terephthalate[69-72] with the proprietary names Dacron^{R}, Mylar^{R}, Terylene^{R}, and Melinex$^{R;37,73-77}$, and on polytetramethylene terephthalate[78] did

not reveal toxicological effects. Furthermore, in 1985 the FDA removed limitations on the use of polytetramethylene terephthalate for material that would be in contact with food[79].

Polyethylene oxide, whose shorter chain segments are commonly referred to as polyethylene glycol[25], has shown good blood compatibility[17]. Studies on PEO or PEO-based copolymers suggest that neither orally taken PEO[80] nor any of its breakdown products is toxic[58,81].

Fillers that might be used[57] to modify polymer properties of the 55/45 HPOE/PBT copolymer include titanium dioxide, chopped fiberglass, carbon black, silica gel, alumina, and clays. With respect to toxicity, no information is available for the first two of these fillers and the others have been reported to be non-toxic[3,37], although Blais[4] does not recommend the use of carbon black because it has substantial amounts of highly mutagenic polyaromatic hydrocarbons. The copolymer also contains Ionox 330 (Irganox 1330) antioxidant (1.0 weight %)[57], which has been approved in many countries for use in food-packaging materials[82].

Toxicity - Experiments

The 55/45 HPOE/PBT copolymer has been tested with respect to blood compatibility[56]. For these experiments, a bag was formed from a film made of 55/45 HPOE/PBT copolymer by heat-sealing along three of the edges, leaving the top open. After 1.5 ml heparinized rabbit blood had been brought into such a bag, the bag and its contents were placed in a refrigerator for testing over a 72 hr period. The sedimentation rate of the blood was normal and no signs of hemolysis were seen. The clotting time of unheparinized rabbit blood in another bag made of 55/45 HPOE/PBT copolymer was normal too. The tissue culture-agar overlay test[83] was used to assess the cytotoxicity of 30/70, 50/50, and 70/30 HPOE/PBT copolymers, but not that of the 55/45 copolymer[56]. The 50/50 and 30/70 samples were not cytotoxic, but the 70/30 was found to be cytotoxic. This means that it is advisable to purify the copolymer before implantation in order to remove low molecular weight polyether compounds, which were considered responsible[56] for the cytotoxic behavior of the 70/30 specimens (Fig. 5a). Purification can be done by dissolving the copolymer in chloroform and inducing precipitation, or simply by washing or postcondensation of the low molecular weight compounds (Fig. 5b)[56]. In general, because of leaching, the lower the molecular weight of a polymer or plastic used for an implant, the more toxic it may be to tissues[84]. This may also hold for monomers that remain in the implant.

In further studies, extracts[56] of each of the three samples (70/30, 50/50, and 30/70) in saline, polyethylene glycol (molecular weight of 400 D), and cottonseed

oil were tested with respect to toxicity according to Guess et al.[83]. Extracts of all 30/70 samples and specimens of 50/50 and 70/30 extracted in cotton seed oil only were shown to be cytotoxic in the agar overlay test. Extracts of 50/50 and 70/30 samples in saline and PEG-400 were not cytotoxic. The same extracts were used to study cell growth inhibition assessed by a specific assay[85]. The 30/70 sample did not show any significantly increased inhibition at any of the weights of the samples added to the extraction media. The same holds for the 50/50 sample at sample weights up to 400 mg, but 100% cell growth inhibition occurred at 4000 mg. The 70/30 sample showed a significant increase of cell growth inhibition at sample weights higher than 400 mg.

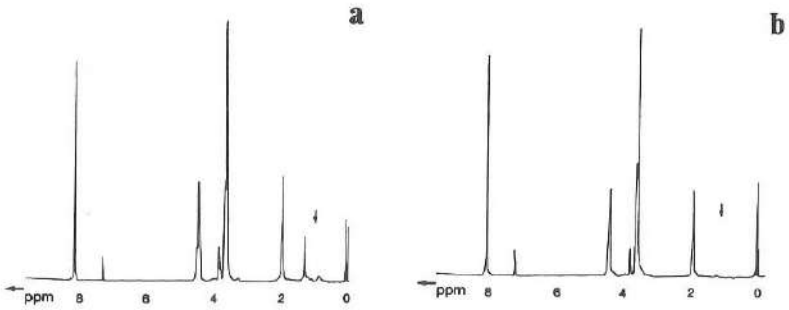

Fig. 5a and b. NMR-spectra of 55/45 HPOE/PBT copolymer.
a: Spectrum of un-purified copolymer. Note the two peaks between 0.8 and 1.3 ppm (arrows) suggestive of low molecular weight impurities.
b: Spectrum after purification. Peaks as in a (arrows) are absent.

For analysis of the chronic 55/45 HPOE/PBT copolymer toxicity in rats, nine of the animals given the copolymer implants and belonging to the one-year observation group were examined for signs of chronic toxicity (see Chapters IV-VIII). The morphology of the liver, testes, lungs, and brain was similar to that of normal one-year-old rats. In the studies done with transmission electron microscopy, the morphology of two perfusion-fixed livers was found not to deviate from the normal picture. Comparison of the average weights of the testes, liver, and lungs of rats belonging to the copolymer one-year group with those of the corresponding organs of normal one-year-old rats showed that the organ weights of the two sets of rats did not differ significantly (Table I).

Possible acute toxic effects of the copolymer or its degradation products in the rat were investigated in a comparative study done in normal rats into whose back a porous copolymer implant 100 µm thick and measuring 2x4 cm^2, was inserted intramuscularly. The morphology and the average weight of the liver,

spleen, gonads, and lungs were determined after implantation periods of three, six, and nine weeks (Table II). Neither the light-microscopical picture of these organs and brain tissue nor the organ weights showed significant differences between the treated and untreated groups.

Table I. Average organ and animal weight (g) of normal rats and rats given an HPOE/PBT copolymer implant one year earlier.

	untreated*	treated#
testes	1.6±0.6@	1.9±0.3
lungs	1.7±0.4	1.8±0.3
liver	17±4.4	18±2.4
animal	525±35	535±25

* number of samples: 4.
\# number of samples: 9.
@ mean ± standard deviation about the mean.

Table II. Effect of dorsally implanted HPOE/PBT copolymer on organ weight (in g per 500 g animal weight) as function of implantation time (weeks).

time	3		6		9	
	untreated*	treated#	untreated	treated	untreated	treated
liver	18±1.9@	19±1.0	24±5	18±1.8	2.1±1.6	20±2.1
spleen	1.0±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.7±0.1
testes	1.7±0.1	1.6±0.2	2.0±0.2	2.2±0.2	1.9±0.3	1.9±0.2
lungs	2.0±0.3	1.8±0.1	1.5±0.3	1.7±0.3	1.4±0.5	1.5±0.1
animal	465±45	460±15	480±21	490±45	500±78	493±12

* number of samples: 2.
\# number of samples: 5.
@ mean ± standard deviation about the mean.

Discussion

Both the tissue culture experiments (Chapter II) and the implantation studies (Chapters IV-IX) showed the absence of notable toxic responses to 55/45 HPOE/PBT copolymer. The foreign-body reaction provoked by 55/45 HPOE/PBT copolymer implants was rather mild. Storage of the polymer-derived[4] impurities (iron, silicon, titanium, and aluminum) in the cytoplasm of phagocytes following degradation of copolymer, but also of Estane and polypropylene oxide, does not completely exclude the possibility of systemic effects. With respect to the impurities in the 55/45 HPOE/PBT copolymer, titanium, silicon, and aluminum might have been added as fillers[57] (in the form of titanium dioxide; chopped fiberglass or clay or silica gel; and alumina, respectively) to modify its properties, but they could also have derived from the glassware and the reaction vessels used to synthesize the polymer. Although copolymer fragmentation was very prominent,

systemic accumulation[84] of biomaterial fragments, for example in liver or lung tissue, was not seen.

CONCLUSIONS

Toxic responses were only associated with implanted polypropylene oxide, which is in good agreement with the outcome of the *in vitro* tests on this polymer. The storage of Silastic^{R}-derived silicon *in vitro* could not be confirmed after Silastic implantation, which is attributed to the absence of Silastic degradation *in vivo*. Toxic responses to Silastic are therefore not to be expected. Neither the tissue culture experiments nor the implantation study showed signs pointing to toxic responses to Estane or 55/45 HPOE/PBT copolymer implants. On the basis of all these findings, the polypropylene oxide under study is considered to be unsuitable as a biomaterial. The frequent use of Silastic as implant material seems justified in view of the results of our studies. It is also concluded that Estane and 55/45 HPOE/PBT copolymer should be examined further in small-scale clinical investigations.

Acknowledgements
The authors are indebted to Mr. B.M.A. van der Lans and Ms. P. van der Hoek (ENT Dept.) for their technical assistance and to Mr. M. van 'tZelfde (Gorlaeus Laboratoria, Leiden University) for carrying out the NMR. The cooperation obtained from Mrs. K.H. van der Nat-van der Meij, Ms. H.Holtslag and Ms. J.Heijlen (Department of Surgery of Leiden University) and from J.Vermeulen (Laboratory for Electron Microscopy, Leiden University) is also gratefully acknowledged. Estane^{R} 5714 F1 polyether urethane, polypropylene oxide, and 55/45 HPOE/PBT polyether polyester copolymer were generously provided by Dr. J.H.Kuit and Dr. J.G.F.Bots (Twente University of Technology, Enschede), J.G.C.Wolke (Dept. of Biomaterials, Free University, Amsterdam), and Prof. K.B.Wagener (University of Florida, Gainesville, Florida, USA).

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other elastomers from the pattern obtained on TCPS. The third approach used in the *in vitro* study was the most sensitive. The polymers were aged artificially according to Homsy[11,12] in physiological saline solution which was then used in the preparation of the various polymer-specific tissue culture media. The epithelium was serially cultured on TCPS in normal medium for three days before being given the experimental media. Normal cell morphology and behavior were seen after culture in the two polyurethane and the copolymer media, whereas the polypropylene oxide medium invariably led to cell death and culture in the Elastomer and Silastic media resulted in a decrease of cell density and an altered morphology. After exposure to Silastic, which is frequently used in otology[13-15], some of the epithelial cells showed inclusion bodies containing silicon probably derived from the polymer.

In vivo **biocompatibility**

The rat middle ear was chosen as implantation site to assess the biocompatibility of Silastic, Estane, polypropylene oxide, and HPOE/PBT copolymer, not only because it has proven suitable for otosurgical experiments[16-19], but also because it offered a model for investigation of the behavior of implants in infected surroundings[20-22]. The latter was of interest because of the frequency with which infection is encountered in otology[23,24]. The materials under study were implanted in the rat middle ear according to Kuijpers and Grote[25] at three sites: in the tympanic membrane as underlay, between the middle ear mucosa and the middle ear bone, and between the bone and the adjacent muscle tissue. The method applied for infection of the ears was according to Grote and van Blitterswijk[26], who used *Staphylococcus aureus* to induce an acute middle ear infection for their study on the effects of such an infection on the mucosa of the middle ear[26]. The use of the rat tympanic membrane as implantation site in the present study enabled us to investigate the response of the tympanic membrane to this infection as well. The results of this control study, which are presented in Chapter III, are summarized in the next section.

Tympanic membrane structure

Although the rat tympanic membrane has been the subject of a number of studies[16,27,28], the structure of the tympanic membrane after an acute middle ear infection has not been dealt with. The normal rat tympanic membrane is composed of a pars tensa and a pars flaccida, as in man[29,30] and several animals[16,31-33]. In the rat, too, both of these parts are composed of an epidermis on the meatal side, a lamina propria, and a single layer of epithelium on the side facing the

middle ear cavity[16,31-33]. In response to a *Staphylococcus aureus*-induced middle ear infection, the connective tissue layers of the tympanic membrane showed infiltration of polymorphonuclear granulocytes, lymphocytes, and macrophages, but also increased numbers of ciliary and secretory epithelial cells. Both reactions, which were comparable to the cellular[26,34-36] and mucociliary[26,36] responses of the middle ear mucosa during infection, respectively, were restricted to the pars flaccida and to predominantly the annular and manubrial regions of the pars tensa, which means that the largest part of the pars tensa had hardly been affected by or responded to this infection.

Tissue behavior

The introduction of an alloplast disturbs the continuity of the epithelial layer and underlying tissues. Epithelium and damaged tissue are repaired by a wound-healing process[37] involving exudation of polymorphonuclear granulocytes, lymphocytes, and monocytes as well as the phagocytosis of debris by macrophages. The acute response is followed by the formation, underneath the recovering epithelium, of new connective tissue which is composed of fibroblasts and capillaries in a matrix of collagen and is called granulation tissue[37,38].

Foreign-body reaction

The presence of an implant is known to alter the normal sequence of events during the healing process[37]: a foreign-body reaction occurs[37,39], and gives rise to a foreign-body granuloma containing macrophages and foreign-body giant cells together with connective tissue[37,38,40]. In our experiments, the implantation of Silastic, Estane, polypropylene oxide, and the HPOE/PBT copolymer in the rat middle ear indeed led to the formation of foreign-body granulomas (Capters IV and V). The severity of the foreign-body reaction serves as a measure of biocompatibility[41-43]. Because the foreign-body reaction is influenced not only by the chemical composition of the implant[44,45], but also by its porosity, the pore size[46], the surface texture[39,45-47], and its shape and size[48,49], the evaluation of the biocompatibility of the four elastomers required a subdivision between non-porous Silastic and the other implants, all of which were porous.

Silastic implants were invariably surrounded by a capsule of non-vascularized fibrous tissue and small volumes of exudate (composed of predominantly macrophages but also foreign-body giant cells) occurring between the capsule and the implant, whereas the pores in the other implant materials were always infiltrated by exudate, fibrous tissue, and occasionally bone. The volume occupied by the exudate surrounding the porous implants was considerably larger than for

non-porous Silastic. The complete encapsulation of Silastic is the common feature of a foreign-body granuloma associated with non-porous materials[42,43,49-51] and is probably related to the absence of pores in the implant[52]. The biocompatibility of Silastic was quantitated *in vivo* on the basis of the thickness of the fibrous capsules. The average capsule thickness was between 5 and 14 µm for submucosal implants and for implants surrounded by muscle tissue values between 10 and 30 µm were obtained, comparable to those usually seen for intramuscular implants in rats[53].

To quantitate the biocompatibility of the porous implant materials we determined the total volume occupied by macrophages and foreign-body giant cells in and around the porous implants. The smallest volumes of these cells were found for copolymer implants. Estane and polypropylene oxide implants were associated with larger volumes. The results show unequivocally that both the total volume of exudate associated with all types of porous implant and the thickness of the capsules enveloping Silastic depended on the implantation site (Chapter V). These exponents of the foreign-body reaction invariably decreased in the following order of implant sites: within muscle tissue, submucosal, and in the tympanic membrane. Mismatch of elastic moduli of implant and tissue, causing stress and movement at the tissue/implant interface and leading to mechanical irritation, probably explains this implantation-site dependency[49,50,54,55].

Implant degradation

Macrophages and foreign-body giant cells can release enzymes into the extracellular fluid[40,47,56], and these enzymes, together with other fluid components[56-58], can contribute to extracellular implant degradation[54,59,60] manifested as, e.g., changes of the cell/implant interface[39,47,54,59-61]. Macrophages and the foreign-body giant cells may also degrade implant materials intracellularly[39,47,54,59-61]. Although macrophages and foreign-body giant cells adhered to the surfaces of all of the materials investigated in the present study (Chapter VI), interface changes were not associated with their interaction with Silastic. This suggests that Silastic was not susceptible to degradation, possibly because of its hydrophobic character[62]. The phagocyte/implant interface produced by interactions with polypropylene oxide resembled the severely eroded surface of polymers showing relatively fast degradation[59,60]. The increased electron-density of the Estane implant surface, which suggested that degradation took place in the surface regions of this polymer[63], might have been the result of chemical changes in the polymer structure[64]. With progressive degradation the phagocyte/copolymer interface became rougher and the material became more fragmented.

With respect to the functioning[18] of the alloplastic tympanic membrane complete degradation of the biomaterial is preferable to incomplete degradation or no degradation at all. However, degradation should not lead to responses of a toxic, mutagenic, or carcinogenic nature[65]. For the quantitation of implant degradation, morphometry was performed (Chapters IV and V). Polypropylene oxide showed the most rapid degradation: averaged over all implantation sites, at most 7% of the original material was present after one year. After six months, Estane degradation was arrested at about 40%, whereas after one year copolymer degradation had slowed down and 54% of the polymer was still present. The incomplete degradation of Estane and HPOE/PBT copolymer was probably due to the relative absence of phagocytes during the later observation periods. The slower rate of copolymer degradation compared with that of Estane was surprising, because copolymer underwent progressive fragmentation (Chapters IV-VI) that must have considerably enlarged the area of exposed polymer, which is often reported to be proportional to the rate of degradation[52,59]. By far the most copolymer fragments were seen in the cytoplasm of macrophages[66,67] and although macrophages show greater motility than multinucleated phagocytes[68] *in vitro* and might migrate to e.g. lymph nodes and the liver where undigested particles would be stored[52], accumulation of copolymer fragments in the liver could not be established in our experiments (Chapter IX). Not only degradation of polypropylene oxide, Estane, and copolymer but also the degree of HPOE/PBT copolymer fragmentation were found to be functions of the implantation site in the same order of importance as seen for the capsule thickness and the total volume occupied by macrophages and foreign-body giant cells.

The ultrastructural morphology of mononuclear and multinucleated phagocytes differed between the various polymers. Cells with polypropylene oxide in or near the cytoplasm had mitochondria with an altered morphology suggesting a toxic response[69]. This finding together with the premature death of five rats in the one-year polypropylene oxide group and the culture failure with polypropylene oxide strongly suggested the release of toxic substances during polypropylene oxide degradation[70], as discussed in Chapter IX. After three months, phagocytes associated with polypropylene oxide, Estane, or copolymer displayed tubular and plate-like structures resembling the angulated lysosomes of Gaucher cells[71] (Chapter IV). Because these structures were not associated with non-degradable Silastic, polymer breakdown and the accumulation of breakdown products must have contributed to their genesis. Inclusions containing silicon, titanium, aluminum, and iron, or combinations of these elements were also associated with the degradation of porous implants. Their presence can be accounted for by

polymer impurities originating from the equipment used to synthesize the polymers or from organometallic initiators or the catalyst[72]. Although trace-element impurities in the body usually originated from orthopedic metallic implants[73], impurities comparable to those mentioned above have been found in phagocytes after implantation of tricalcium phosphate[22] and hydroxyapatite[74] in the rat and man, respectively, as well as after the injection of tricalcium phosphate into the peritoneal cavity of mice[75], but also in cultured macrophages[76]. The storage of trace elements seems to be commonly associated with biomaterial degradation, which underscores the importance of using implant materials of the purest grade (Chapter IV).

Implant fixation

Mechanical bonds underlie fixation of porous implants[77-79], chemical interface reactions produce direct bonding between bioactive implants and bone[17,20,54,61,78,80,81]. Because chemical bonds between tissue and polymers have never been observed[82-85], implant fixation of porous polymers is thought to depend solely on growth of fibrous tissue and bone into pores. However, chemical bonding is often more desirable than mechanical bonding for the permanent fixation of an implant[78].

Ingrowth of fibrous tissue and bone leads to mechanical implant fixation with a strength depending on the amount and kind of pore-occupying tissue[77,86]. Because the pore size influences these tissue variables[86,87], the majority of the pores in the candidate materials chosen for the study had diameters of at least 30 µm, allowing good ingrowth of fibrous tissue and bone (Chapter IV). Pores in polypropylene oxide implants showed about 50% ingrowth of fibrous tissue, which suggested mechanical implant fixation, but after the first postoperative month the fibrous tissue was partially replaced by macrophages and foreign-body giant cells, which reduced implant fixation. Bone could not contribute to fixation either, since it did not occur in polypropylene oxide pores. During the first three months the pores in Estane implants were occupied by fibrous tissue having about half the volume of the phagocyte population. After six months the volume of fibrous tissue surpassed that of the phagocyte population, and bone was present in Estane pores. This suggests that mechanical fixation of Estane implants increases with time. The tissue in and surrounding HPOE/PBT copolymer implants showed since the first week not only roughly equal volumes of fibrous tissue and exudate, but also bone in steadily increasing amounts. Bone was seen twice as often in HPOE/PBT copolymer as in Estane. The presence of fibrous tissue and especially of bone in its pores makes HPOE/PBT copolymer the most promising of the alloplastic

tympanic membrane materials under study with respect to mechanical implant fixation.

The tissue/implant interface, which is the region were host tissue and biomaterial interact[52,88], supplies important information on tissue/implant bonding[54,61,77-79]. We therefore studied the interactions of the candidate alloplastic tympanic membrane materials with fibrous tissue and bone at the interface (Chapter VI).

Fibrous tissue bordered Silastic, copolymer, and Estane implants but not those made of polypropylene oxide. Silastic was invariably surrounded by a fibrous capsule; although interaction with fibrous tissue can lead to tissue/implant binding[45,89], this does not seem to be the case for fibrous capsules similar to those surrounding Silastic[45]. In contrast with the pattern seen for Estane some of the collagenous fibrils in the fibrous tissue surrounding copolymer ended perpendicular to the implants, which suggests tissue/copolymer binding[45].

Although bone grows into the pores of a number of ceramics[54,78-80,82] and polymers[82-85], contact between bone and implant has been reported convincingly for ceramics[81,90] but only rarely and not convincingly for polymers[92]. Polymers were usually separated from bone by a layer of other tissue[40,90]. Implants made of Estane, HPOE/PBT copolymer, and polypropylene oxide had pores with diameters theoretically allowing ingrowth of bone[77], but ingrowth did not occur with polypropylene oxide. Bone formation started earlier and was most prominent inside pores of copolymer; it began at the wall of the pore and proceeded toward the center. This is consistent with the pattern of bone growth leading to bonding osteogenesis[81]. In ultrathin sections the bone/copolymer interface showed an electron-dense layer morphologically resembling and sometimes continuous with the natural lamina limitans of bone. The morphology of this electron-dense layer is characteristic[54,61] for the bond between bone and the bioactive calcium phosphate hydroxyapatite, where it consists of calcium phosphate incorporated into an organic matrix[81]. If for HPOE/PBT copolymer the interface with bone can be attributed to bioactivity of the polymer, it must be concluded that in relation to bone HPOE/PBT copolymer behaves like a bioactive material. Bioactive properties, which to the best of our knowledge have not been reported for a polymer, make HPOE/PBT copolymer and possibly also polymers chemically comparable to it, exceptional. Inorganic compounds incorporated into this copolymer[92] and leached after implantation, chemically reactive sites in the copolymer[93], and the absorption[94] of inorganic and organic substances derived from the tissue fluid, could have affected conditions at the bone/copolymer interface and given rise to a surface layer responsible for the bond with bone. Bone ingrowth into Estane

pores was usually associated with a tissue-free space between bone and implant, which is characteristic of contact osteogenesis[81], or an intervening layer of tissue, which is characteristic of distance osteogenesis[81]. In the relatively rare cases of contact between bone and Estane, a lamina limitans-like structure was not observed.

Epithelial reactions

Epithelial reactions (Chapter VIII) are important for the success of an implant, because implant failure due to infection[10,95,96] or extrusion[97] is often preceded by slow or incomplete covering by epithelium. Furthermore, because the activity of ciliary and secretory epithelium is important for middle ear defence[98], the composition of the epithelium covering an implant has a significant influence on the course of events in the infected middle ear. Implantation in the tympanic membrane was accompanied by substantial surgical trauma leading to rather prominent migratory and proliferative activity of epithelium and epidermis, whereas the epithelium lining the middle ear mucosa was hardly affected in this way, thus indicating that the trauma due to submucosal implantation was small. Except for the Silastic tympanic membrane implants, which were invariably rejected within two weeks after implantation, all of the implants studied were ultimately covered by flat polygonal, ciliated, and secretory epithelial cells, i.e., the normal constituents of the epithelial lining of the rat middle ear[19,99,100] and the inner site of the tympanic membrane[16]. It took the epithelium and the epidermis more time to cover copolymer tympanic membrane implants than similar implants made of polypropylene oxide or Estane. This difference is attributed to the divergent structure of the copolymer implants, which had one non-porous site and one site with irregularly-shaped and sharp-edged pores.

Epithelial reactions to submucosal implants made of the candidate materials were comparable to the reactions to implants made of either hydroxyapatite[10,20,21] or tricalcium phosphate[22] placed in the rat middle ear. Because tympanic membrane and middle ear epithelium behaved normally in the presence of polypropylene oxide *in vivo* and epithelial cells could not be cultured on polypropylene oxide implants at all, showing that the *in vivo* behavior of rat middle ear epithelium (Chapter VIII) differs from that *in vitro* (Chapter II), we conclude that epithelial reactions *in vivo* are less sensitive for the assessment of polymer biocompatibility than those occurring *in vitro* or may not even be suitable for such assessment[55]. On the basis of the slower covering of copolymer tympanic membrane implants, the rejection of Silastic, and the *in vitro* results on polypropylene oxide, Estane must be considered the best elastomer with respect to the epithelial response.

Infection

The presence of implants made of one of the polymers under study (Chapters VII and VIII) did not affect the course of events in either the middle ear mucosa[26] or the tympanic membrane (Chapter III) as usually seen three weeks after a *Staphylococcus aureus*-induced middle ear infection. The exceptions to this were the round-cell infiltrates, which were predominantly associated with polypropylene oxide and HPOE/PBT copolymer, and the presence of microbial debris associated with the copolymer. The structure of copolymer implants might have been responsible for the divergent behavior of this material in the infected tympanic membrane. This supposition is based on the finding that HPOE/PBT copolymer tympanic membrane implants were the slowest of the group of porous implants to be covered by epithelium. Slower covering by epithelium, e.g. after the instillation of *S.aureus* through the tympanic membrane, is frequently associated with an increased risk of implant infection[95,96,101,102].

The absence of degradation of Silastic or the degree of degradation of porous implants, the composition of the tissue surrounding the porous implants, and the tissue/biomaterial interface reactions are consistent with the results obtained in the non-infected middle ear, which makes it likely that a local *S.aureus* infection does not influence the behavior of the polymers under study.

CONCLUSIONS

Silastic^{R} was found to be an adequate substrate for tissue *in vitro*, but the absence of pores in Silastic implants, which were not incorporated into the rat tympanic membrane, led to envelopment of the implants by a non-adherent capsule of fibrous tissue. Silastic was well tolerated by both the non-infected and the infected rat middle ear, did not deter the development of differentiated epithelium, and did not evoke toxic responses. Although Silastic is not suitable for tympanic membrane prostheses because it is not fixed adequately by tissue and does not degrade, this material can be considered to deserve a place in middle ear implantology for cases where both implant fixation and implant degradation are not essential.

It may be concluded from the results of both the tissue-culture experiments and the implantations in rat middle ears that the polypropylene oxide studied is not suitable as a biomaterial because of its toxic effects.

Films made of Estane^{R} 5714 F1 polyether urethane allowed normal behavior of epithelial cells *in vitro*. *In vivo*, Estane implants were associated with the ingrowth of fibrous tissue and small amounts of bone leading to mechanical fixation as well as to the presence of differentiated epithelium, which is

important for the middle ear defence against infection. The degree of foreign-body reaction accompanying Estane degradation was, however, rather strong which makes Estane less suitable as alloplastic tympanic membrane than HPOE/PBT copolymer.

Poly(ethylene oxide hydantoin) / poly(tetramethylene terephthalate) segmented polyether polyester copolymer implants showed good biocompatibility *in vitro*. The quality of HPOE/PBT copolymer implant fixation seemed unexpectedly good for a polymer, not only because of the strong ingrowth of connective tissue, but also because of the chemical bond that may exist between the copolymer and bone. The degradable HPOE/PBT copolymer implants provoked a relatively mild foreign-body reaction. The drawbacks of HPOE/PBT copolymer, which were probably attributable to the unfavorable structure of copolymer implants, were the rather slow covering by tympanic membrane epithelium and epidermis, and the infiltration of round cells and the presence of microbial debris after an induced infection. It is therefore concluded that an implant made of HPOE/PBT polyether polyester copolymer with a proper pore structure is suitable as an alloplastic tympanic membrane. Furthermore, a bonding with bone makes HPOE/PBT copolymer unique among polymers with respect to the interaction with bone and suggests that HPOE/PBT copolymer is bioactive. We therefore consider HPOE/PBT copolymer to be a potential candidate for application in all surgical disciplines where there is a need for an elastomeric biomaterial that allows chemical bonding with bone.

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SUMMARY

Chronic otitis media can affect the entire ossicular chain and its removal may be necessary for treatment of this disease. Ossicular chain prostheses in current use are susceptible to such problems as loosening and extrusion, both of which lead to unsatisfactory postoperative hearing gains. For these patients we developed a total alloplastic middle ear prosthesis consisting of a porous hydroxyapatite canal-wall segment as suspension system to which an hydroxyapatite ossicular chain is connected. To connect the ossicular chain, we required an alloplastic tympanic membrane made of an elastomeric polymer, but which can be used for the repair of tympanic membrane defects as well. The results reported in this thesis concern the biocompatibility (the reaction of the body to the implant material (biomaterial) and *vice versa*) of several elastomers. Six biomaterials were investigated using tissue culture techniques and four biomaterials after implantation in both non-infected and infected rat middle ears to assess their suitability as substrate for tissue, the degree to which they are fixed by tissue, and their degradation behavior. In the first instance the research was concentrated on Silastic^{R}, Estane^{R} 5714 F1 polyether urethane, polypropylene oxide, 55/45 poly(ethylene oxide hydantoin) and poly(tetramethylene terephthalate) segmented polyether polyester copolymer (55/45 HPOE/PBT), Dow Corning^{R} MDX-4-4210 Clean Grade Elastomer, and Pellethane^{R} 80-A polyester urethane. The studies in animals were only done with implants made of the first four polymers.

Quantitative, morphological and 'artificial aging' tissue-culture experiments done with non-porous films made of the various polymers (Chapter II) showed that polypropylene oxide allowed neither adhesion nor growth of rat middle ear epithelial cells, possibly due to released toxic substances. Elastomer too was less suitable as a substrate for cell growth, but cell behavior on Silastic, Estane, Pellethane, and HPOE/PBT copolymer was normal. However, part of the cells exposed to artificially aged Silastic showed accumulated silicon, which, if reproducible *in vivo*, is a potential hazard in the use of Silastic.

The implantation in the rat middle ear of any the four polymers selected for this purpose gave rise to wound healing and the foreign-body reaction at all three implantation sites (in the tympanic membrane, under the submucosa, or adjacent to muscle tissue). The degree of the foreign-body reaction, which was characterized by the presence of phagocytes (e.g. macrophages and foreign-body giant cells) and the promotion of a capsule of fibrous tissue, was shown to depend on the site of implantation (Chapter V), being the strongest for implants that contacted muscle. Tissue covered the porous tympanic membrane implants made of Estane or polypropylene oxide more rapidly than was the case for implants made of HPOE/PBT copolymer, which had one porous and one dense side (Chapters IV and VIII). The non-porous Silastic implants were not incorporated into tympanic membranes. These findings combined with the *in vitro* results for these polymers suggest that the incorporation of a polymer implant is determined less by its chemical properties than by its structure.

After six months, tympanic membranes with an implant were covered by normal-looking epidermis composed of keratinocytes and epithelium consisting of flat polygonal and ciliated cells (Chapter VIII). Submucosal implants made of each of the four polymers - including polypropylene oxide - were covered by a mucosa-like layer, the surface of which was composed of flat polygonal and ciliated epithelial cells and goblet cells. In view of the normal composition and morphology of the epithelium it may be concluded that unlike those *in vitro* (Chapter II), epithelial reactions *in vivo* (Chapter VIII) are not suitable indicators of biocompatibility.

None of the polymers influenced the epithelial response to a *Staphylococcus aureus*-induced middle ear infection as can be concluded from the composition and morphology of the epithelium and its reaction to the infection. However, after infection with *S.aureus* (Chapters VII and VIII), tympanic membranes given HPOE/PBT copolymer displayed a more severe reaction, possibly as a consequence of the slower covering of the copolymer by tissue. The normal tympanic membrane structure during an *S.aureus*-infection is presented in Chapter III.

Both mechanical and chemical fixation of implants occurred (Chapters IV and VI). The fibrous capsules that invariably surrounded Silastic implants did not provide good fixation of the silicone rubber. The composition of the tissue in porous polypropylene oxide implants suggest inferior fixation of implants made of this polymer as well. The presence of fibrous tissue in tympanic membrane implants as well as the ingrowth of bone in submucosal implants point to mechanical fixation of Estane implants by these tissues. Implants made of HPOE/PBT copolymer showed largest amounts of fibrous tissue and earliest and most prominent ingrowth of bone. With HPOE/PBT copolymer, bone deposition was started on the surface of the pores (Chapters IV and VI). Furthermore, the site of bone/copolymer contact was characterized by an electron-dense interface (Chapters IV and VI). These findings suggest that with respect to bone, HPOE/PBT copolymer behaves like a bioactive implant material, resulting in a chemical bond with bone. This makes HPOE/PBT copolymer the most firmly fixed by tissue, both mechanically and chemically, of all the polymers studied. Implant fixation was not disturbed by the induced infection (Chapter VII).

Degradability of the alloplastic tympanic membrane is a preferred feature, since

it may lead to complete absence of the foreign material and long-term foreign body-associated complications. The degree of implant degradation, which was independent of the infection, differed between the polymers (Chapters IV, VI, and VII). Morphometry showed that implants made of HPOE/PBT copolymer and Estane had a moderate degree of breakdown (average values of breakdown of 46 and 60%, respectively, at the end of one year), and that an average 93% of polypropylene oxide disappeared within one year. Differences in breakdown behaviors were also evident from the electron-microscopical picture of the phagocyte/polymer interactions, which indicated absence of Silastic degradation (Chapter VI). Because the total volume of macrophages and foreign-body giant cells, which is considered to be a measure of the foreign-body reaction caused by porous implants, was largest for Estane and polypropylene oxide implants and smallest for implants made of HPOE/PBT copolymer, this last material seems to be the least foreign to the organism (Chapters IV and VI). Since complete covering and incorporation by tissue always preceded implant degradation, it may be concluded that neither the scaffolding of tissue by an implant nor the fixation of an implant by tissue will be endangered by implant degradation. Only degradation of polypropylene oxide led to the release of toxic substances (Chapters IV and V). Breaking down of the three degradable polymers led to the presence of iron, silicon, aluminum, and titanium in the phagocyte cytoplasm after six months, but there were no signs of any toxic or systemic reactions associated with the accumulation of these polymer-derived trace elements (Chapter IV).

It can be concluded from the present findings that due to its toxic behavior the polypropylene oxide under study is not a suitable implant material and that both the absence of degradation and the lack of firm fixation make the dense Silastic implants unsuitable as an alloplastic tympanic membrane. Although both Estane and HPOE/PBT copolymer not only allowed the adhesion and proliferation of epithelial cells *in vitro*, but also degraded without notable toxic responses and were fixed by tissue *in vivo*, only HPOE/PBT copolymer seems promising as alloplastic tympanic membrane. The milder foreign-body reaction provoked by this material, and especially the probable binding with bone make HPOE/PBT copolymer suitable for clinical application. Chemical bonding with bone, which is exceptional for a polymer, insures not only optimal fixation of an alloplastic tympanic membrane but also makes HPOE/PBT copolymer applicable in biomedical situations where a bone-binding elastomer is needed.

SAMENVATTING

Chronische infecties van het middenoor kunnen leiden tot een aantasting van de gehele gehoorbeenketen die omwille van het genezingsproces eventueel moet worden verwijderd. Als gevolg van problemen zoals het loslaten of het afstoten is de gehoorsverbetering door middel van protheses die de gehele gehoorbeenketen vervangen, onbevredigend. Daarom is voor patiënten zonder gehoorbeenketen een totaal kunstmiddenoor ontwikkeld. Hierin is de hydroxyapatiet gehoorbeenketen door middel van een kunsttrommelvlies, dat gemaakt is van een elastisch polymeer, bevestigd aan een poreus hydroxyapatiet ophangsysteem. Dit elastomeer kan echter ook worden gebruikt voor het sluiten van trommelvliesperforaties. In het onderhavige onderzoek staat de biocompatibiliteit (hoe reageert het lichaam op de aanwezigheid van het implantaatmateriaal - biomateriaal - en omgekeerd) van diverse kandidaatmaterialen voor kunsttrommelvlies centraal. Van zes biomaterialen is met celkweektechnieken (*in vitro*) en van vier biomaterialen is ook met implantatiestudies (in zowel een niet-geinfecteerde alsook in een geinfecteerde omgeving) onderzocht of zij geschikt zijn als substraat voor weefsel. Voorts is onderzocht of deze materialen worden gefixeerd door weefsel en hoe hun degradatiegedrag is. De bestudeerde biomaterialen zijn Silastic^{R}, Estane^{R} 5714 F1 polyetherurethaan, polypropyleenoxide, poly(ethyleenoxide hydantoine) en poly(tetramethyleen tereftalaat) gesegmenteerd polyether polyester copolymeer (HPOE/PBT-copolymeer), Dow Corning^{R} MDX-4-4210-Clean Grade Elastomer en Pellethane^{R} 80-A polyesterurethaan. Proefdierstudies (*in vivo*) zijn alleen uitgevoerd met implantaten gemaakt van de eerste vier materialen.

Verschillende celkweekexperimenten (gebaseerd op celgroei, celmorfologie en kunstmatige veroudering van de polymeren) uitgevoerd met niet-poreuze films gemaakt van de diverse polymeren, toonden aan dat polypropyleenoxide de hechting en groei van ratte middenoor epitheelcellen niet toestond, mogelijkerwijs als gevolg van het vrijkomen van toxische stoffen (Hoofdstuk II). Elastomer bleek ook een minder goed substraat, terwijl het gedrag van epitheel gekweekt op Silastic, Estane, Pellethane, en HPOE/PBT-copolymeer normaal was. Cellen gekweekt in het bijzijn van kunstmatig-verouderd Silastic vertoonden insluitsels met silicium. Eventuele intracellulaire ophoping van silicium in het organisme na afbraak van Silastic maakt dit materiaal ongeschikt voor biomedische toepassing.

Alle implantaties van de polymeren die zijn bestudeerd in het middenoor van de rat resulteerden in een wond- en een vreemdlichaamreactie, onafhankelijk van de implantatieplaats: in het trommelvlies, onder het slijmvlies of tegen het spierweefsel aan dat het middenoor omgeeft. De heftigheid van de vreemdlichaamreactie, die gekenmerkt werd door de aanwezigheid van fagocyten-

zoals macrofagen en vreemdlichaamreuscellen - en een kapsel van bindweefsel, bleek afhankelijk van de implantatieplaats (Hoofdstuk V). Na implantatie in het trommelvlies werden poreuze implantaten van Estane en polypropyleenoxide sneller bedekt door weefsel dan implantaten van HPOE/PBT-copolymeer, die maar aan één zijde poreus waren. Niet-poreuze Silastic implantaten werden in het geheel niet geincorporeerd in het trommelvlies. Gezien de reacties van epitheelcellen in kweek op deze materialen mag worden geconcludeerd dat na implantatie de overgroei door weefsel in belangrijke mate bepaald wordt door de structuur van het implantaat en in mindere mate door de chemische eigenschappen van het polymeer, waaruit het implantaat bestaat.

Trommelvliezen met poreuze implantaten waren na zes maanden bedekt met een normale epidermis van verhoornend epitheel en met een normale epitheellaag bestaande uit vlak polygonaal- en trilhaarepitheel (Hoofdstuk VIII). Alle implantaten geplaatst onder het middenoorslijmvlies waren bedekt met een slijmvlies, dat aan de bovenzijde vlak polygonaalepitheel, trilhaarepitheel en slijmbekercellen bevatte. Gezien de normale morfologie en samenstelling van het epitheel dat alle materialen bedekte, dus ook polypropyleenoxide, mag worden gesteld dat, in tegenstelling tot het gedrag van epitheel *in vitro*, epitheelreacties *in vivo* onvoldoende informatie geven omtrent de geschiktheid van een polymeer als biomateriaal. De epitheelreacties tijdens de *Staphylococcus aureus*-geinduceerde infectie waren vergelijkbaar met de reacties in het normale ratte middenoor gedurende deze infectie (Hoofdstuk III). Geconcludeerd mag worden dat de normale epitheelrespons op de *S.aureus*-infectie niet werd beinvloed door de aanwezigheid van de geteste materialen. Na infectie echter, bevatten de trommelvliezen met HPOE/PBT-copolymeer relatief veel ontstekingscellen en bacterieresten (Hoofdstukken VII en VIII). Dit duidt op een heftiger reactie, hetgeen een complicatie lijkt te zijn van de tragere overgroei van HPOE/PBT-copolymeer implantaten door weefsel.

Afhankelijk van het materiaal werd mechanische en chemische implantaatfixatie waargenomen (Hoofdstukken IV en VI). Voor wat betreft de mechanische fixatie kan worden geconcludeerd dat het altijd aanwezige kapsel van bindweefsel rond de Silastic implantaten resulteerde in een slechte integratie in het organisme. De samenstelling van het weefsel in de poriën van polypropyleenoxide - een toenemende hoeveelheid fagocyten - duidde op ook een inferieure mechanische fixatie van dit polymeer. De aanwezigheid van fibreusweefsel in met name de trommelvliesimplantaten en de ingroei van bot (tussen drie en zes maanden) in de implantaten onder het middenoorslijmvlies wezen op een betere mechanische fixatie van Estane. HPOE/PBT-copolymeer implantaten bevatten het meeste

bindweefsel en bot. Bot, waarvan de ingroei al na een week werd waargenomen, werd allereerst afgezet aan het HPOE/PBT-copolymeer implantaatoppervlak en groeide vervolgens naar het centrum van de poriën toe (Hoofdstuk IV). Verder was de contactzone van bot met HPOE/PBT-copolymeer gekarakteriseerd door een elektronendichte laag (Hoofdstukken IV en VI). De bij het HPOE/PBT-copolymeer waargenomen groeirichting van bot en de elektronendichte grenslaag suggereren een bioactiviteit van HPOE/PBT-copolymeer en een chemische binding tussen bot en het copolymeer implantaat. Geconcludeerd kan worden dat implantaten van HPOE/PBT-copolymeer het beste werden gefixeerd: zowel mechanisch als chemisch. De opgewekte infectie had geen invloed op de implantaatfixatie door weefsel (Hoofdstuk VIII).

Het kunsttrommelvlies moet bij voorkeur degraderen omdat dit leidt tot de afwezigheid van het lichaamsvreemde materiaal zodat eventuele lange-termijn complicaties samenhangend met het biomateriaal niet mogelijk zijn. Door gebruik te maken van morfometrie kon worden aangetoond dat de mate van degradatie karakteristiek was voor ieder polymeer (Hoofdstukken IV en V) en niet werd beinvloed door de infectie (Hoofdstuk VII). HPOE/PBT-copolymeer en Estane gedroegen zich als matig-snel afbreekbare polymeren (respectievelijk 46 en 60% afbraak gemiddeld in een jaar) terwijl polypropyleenoxide relatief snel afbreekbaar bleek (93%/jaar). Verschillen in degradatiegedrag bleken ook uit de interacties van deze polymeren met mono- en multinucleaire fagocyten zoals werd waargenomen met de elektronenmicroscoop (Hoofdstuk VI). Hiermee is ook vastgesteld dat Silastic niet zichtbaar degradeerde. Het volume ingenomen door de populatie fagocyten rond een poreus implantaat wordt beschouwd als een maat voor de sterkte van de vreemdlichaamreactie. Dit volume bleek gemiddeld het grootst bij Estane en polypropyleenoxide en het kleinst bij copolymeer implantaten, zodat mag worden geconcludeerd dat het organisme de implantaten van HPOE/PBT-copolymeer als de minst lichaamsvreemde beschouwde (Hoofdstuk IV). Omdat volledige overgroei en incorporatie van de poreuze implantaten door weefsel vooraf gingen aan de polymeerafbraak lijkt degradatie de substraatfunctie en de fixatie van deze materialen niet in gevaar te brengen.

Alleen de afbraak van polypropyleenoxide leidde tot het vrijkomen van toxische stoffen (Hoofdstukken IV en V). Degradatie van alle poreuze implantaten resulteerde vanaf zes maanden in een ophoping van ijzer, silicium, aluminium en titanium in fagocyten, zoals bleek uit waarnemingen uitgevoerd met röntgenmicroanalyse. Er zijn geen aanwijzingen dat de opslag van dergelijke verontreinigingen - vermoedelijk afkomstig uit de polymeren - zal resulteren in effecten van toxische of systemische aard (Hoofdstuk IV).

Op grond van de resultaten van het onderzoek zoals beschreven in dit proefschrift kan worden geconcludeerd dat gezien de toxische eigenschappen het geteste polypropyleenoxide niet geschikt is als biomateriaal. Voorts blijkt niet-poreus Silastic ongeschikt als kunsttrommelvlies omdat het niet voldoende wordt gefixeerd in het lichaam en omdat het niet degradeert. Ofschoon zowel Estane als HPOE/PBT-copolymeer celhechting en celgroei toestonden, degradeerden zonder een zichtbare toxische respons te induceren en bovendien werden gefixeerd door weefsel, is alleen HPOE/PBT-copolymeer veelbelovend als kunsttrommelvlies. Behalve de mildere vreemdlichaamreactie betekent vooral de mogelijke binding met bot een motief om implantaten van dit polymeer klinisch experimenteel te gaan testen. Een chemische binding met bot, hetgeen uniek zou zijn voor een polymeer, biedt niet alleen de mogelijkheid om een kunsttrommelvlies te maken dat optimaal kan worden gefixeerd in het middenoor, maar het maakt HPOE/PBT-copolymeer ook geschikt voor biomedische toepassingen in het geval een elastomeer nodig is dat met bot bindt.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 9 december 1957 geboren te Ede. De middelbare schoolopleiding atheneum B werd in 1970 aangevangen aan het Christelijk Lyceum te Almelo en in 1977 afgerond aan het Marnix College te Ede. Na een studiejaar geofysica werd in 1978 begonnen met de studie biologie aan de Rijksuniversiteit van Utrecht. Het kandidaatsexamen B1 werd in 1981 afgelegd. Voltooiing van de opleiding vond plaats in 1984 in de richtingen Biologische Procestechnologie, Elektronenmicroscopische Structuuranalyse en Celbiologie. Het in dit proefschrift beschreven onderzoek is op 16 juli 1984 aangevangen bij de Afdeling Keel-, Neus-, en Oorheelkunde van het Academisch Ziekenhuis Leiden (onder leiding van Prof. dr. J.J. Grote en Dr. C.A. van Blitterswijk) en bij het Laboratorium voor Elektronenmicroscopie van de Rijksuniversiteit Leiden (onder leiding van Prof. dr. W.Th. Daems). Vanaf 1 augustus 1988 is de auteur werkzaam bij Holland Composite Implants b.v. en de Rijksuniversiteit Leiden.

NAWOORD

Naast de personen die genoemd zijn in de acknowledgements behorende bij de diverse hoofdstukken wil ik ook graag noemen Prof. dr. A. Bantjes en Prof. dr. J. Feijen (Technische Universiteit Twente), Dr. C.M.F. Vrouenraets (Enka bv, Arnhem), Dr. J.M. Schakenraad (Rijksuniversiteit Groningen), Dhr. H. Choufoer, Prof. dr. G.J. Mulder en Dhr. J.M. van't Noordende (Rijksuniversiteit Leiden), Dhr. H. Dudart, Dhr. V. Gerritsma, Mw. J. Kempenaar, Dr. M. Ponec en Mw. W.M. van Veen (Academisch Ziekenhuis Leiden), Dhr. R. Kamerling (Kamerling Onnes Laboratorium), Mw. D. Versleijen (Radboudziekenhuis Nijmegen) en Mw. I. Seeger-Wolf.

Stellingen behorende bij het proefschrift Alloplastic Tympanic Membrane

1. Voorzien van een goede structuur zijn implantaten gemaakt van 55% poly(ethyleenoxide hydantoine) / 45% poly(tetramethyleentereftalaat) gesegmenteerd polyether polyester copolymeer geschikt als kunsttrommelvlies.
Dit proefschrift

2. Poreuze implantaten van 55% poly(ethyleenoxide hydantoine) / 45% poly(tetramethyleentereftalaat) gesegmenteerd polyether polyester copolymeer worden na implantatie mechanisch gefixeerd door ingroei van bindweefsel en chemisch gefixeerd door een band met bot.
Dit proefschrift

3. Voor de bepaling van de biocompatibiliteit op basis van de mate van vreemdlichaamreactie moet rekening worden gehouden met de invloed van de implantatieplaats hierop.
Dit proefschrift

4. Epitheelreacties *in vivo* geven onvoldoende informatie over de implantaat compatibiliteit.
Dit proefschrift

5. Intracellulaire ophoping van spore-elementen heeft plaats bij degradatie van keramische, metalen en polymere implantaten.
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Dit proefschrift

6. De door M. De Cock et al. gehanteerde techniek van scanning elektronenmicroscopie voor de bestudering van oppervlakte structuren van diverse grafts is onvoldoende voor de herkenning van de lamina basale.
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9. Het duale karakter van hydroxyapatiet/polymeer composieten maakt het mogelijk te anticiperen op de weefselreacties die plaats hebben na de vervanging van kraakbeen of bot.

10. Gezien het inkomen van de jongste generatie promovendi behoren de kosten van het proefschrift deel uit te maken van het totale onderzoeksbudget.

11. Het is onjuist om als bezuinigingsmaatregel de wetgeving aan te passen als de controle op de naleving van de wet te duur is.

12. Het huidige straatvoetbal wordt gespeeld zonder bal en met de politie als tegenstander.

Leiden, 22 september 1988
D. Bakker