CALCIUM PHOSPHATE MIDDLE-EAR IMPLANTS

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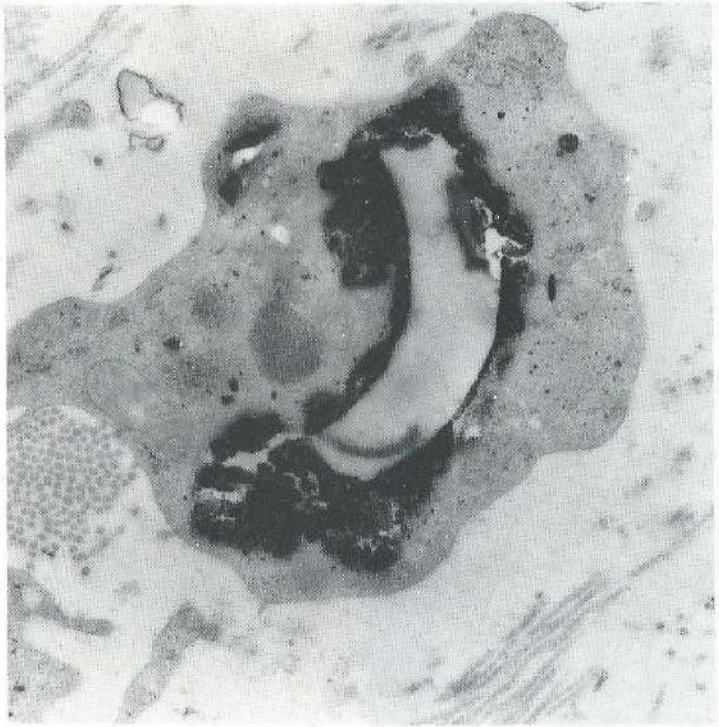

Calcium phosphate middle-ear implants

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CHAPTER I

GENERAL INTRODUCTION

Chronic middle-ear disease frequently affects vital parts of the sound transmission system, (the tympanic membrane and ossicular chain). Furthermore, for adequate treatment of this disease complete eradication of the diseased tissue is obligatory and often includes ossicles and the tympanic membrane as well as parts of the bone of the middle ear and external meatus. Restoration of the hearing capacity was already being attempted three centuries ago, when Banzer (1640) (1) tried to close a persisting perforation in the tympanic membrane with a piece of pig bladder mounted in an ivory tube. Since then a large variety of materials and techniques have been used in reconstructive middle-ear surgery. These materials can be roughly subdivided into four groups: autogenous, allogenous, xenogenous, and alloplastic, i.e. materials deriving from the same individual, the same species, another species, and an artificial source respectively.

Autogenous materials used in otologic surgery include ossicles (2-6), bone (7-10), and cartilage (10-15). Although successful application has been reported for all of them, it is not yet certain whether they are (16,17) or are not (2-15) subject to resorption, which is of crucial importance for the long-term results. The same holds for allogenous materials, which have the specific problem of requiring preservation for storage and for reduction of the antigenic activity. Several methods, including preservation in alcohol, formalin, thimerosal, and cialite (18-20), seemed to permit clinical applications (21-24), but there is circumstantial evidence that immunological problems can lead to rejection or resorption (25,26). Problems of the same nature can be expected to arise with xenogenic (27) materials. Autogenous and allogenous materials in particular have still another drawback in that only healthy donor tissue can be used. This drawback and the relative scarcity of autogenous and allogenous ossicles, tympanic membranes, and tympano-ossicular blocks for this purpose explains the interest in alloplastic biomaterials.

The first alloplastic biomaterials used for ossicular reconstruction in reconstructive middle-ear surgery were Palavit rods implanted by Wullstein in 1952 (28), followed by the application of Supramid (29). Both materials proved inadequate, but better results were obtained with polyethylene, teflon, tantalium, and steel wire (30-32). An important breakthrough in the use of alloplastic biomaterials in otologic surgery was the introduction of porous biomaterials, which allow tissue ingrowth and thus improve fixation of the prosthesis. These porous materials included Proplast, a combination of Teflon and vitreous carbon, and Plastipore, a high-density polyethylene with a sponge-like structure. Initially, most of the reports on Proplast and Plastipore were quite promising (33-46); however, failure in the form of hearing loss or extrusion occurred particularly after longer implantation periods and during infection (47-56). The more recent otologic biomaterials include bio-ceramics, a group of materials now comprising three categories, i.e., aluminum oxide, glass ceramics, and calcium phosphate ceramics.

Aluminum oxide is a bioinert biomaterial which has been succesfully applied in several fields of surgery (57-62) including reconstruction of the middle ear (61-67), where it is used

at present for canal-wall replacement and columella prostheses. So far, only dense forms of the material have been described for middle-ear surgery.

Glass ceramics derive from work done by Hench (70-73), who developed a bioactive glass (Bioglass) which has not yet been applied in clinical otology but seems promising on the basis of results obtained in animals (74,75). One of the special properties of this material is ion exchange at the interface, which is thought to lead to a firm bond with bone. Brömer et al. (76) developed another bioactive glass, called Cerativa1, which is less soluble than Bioglass due to differences in its composition (77). Results obtained with this material in animals and patients have shown its good biocompatibility (78-80). Ceravital, like Bioglass, is only available in a dense form. Besides these two glasses, another glassy material, called Macor has been tested for application in middle-ear surgery, but the available results are not unanimously encouraging (81-84).

Apart from aluminum-oxide and glass ceramics, a third group of ceramics is in use for reconstructive middle-ear surgery, i.e., calcium phosphate ceramics. The biocompatibility of two ceramics of this type has been tested, i.e., hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, and β-whitlockite $Ca_3(PO_4)_2$ also known as tricalcium phosphate (TCP). These materials have the same ions as those predominating in the bone mineral matrix, and hydroxyapatite even has the same crystal structure (85-87). Both materials can be fabricated in dense and macroporous forms, in contrast to the above-mentioned ceramics, all of which are dense.

Hydroxyapatite can be produced in various forms, i.e., microporous (micropores accounting for more than 5 volume per cent; pore diameter *ca.* 3 μm) macroporous (pore diameter *ca.* 100 μm), micro-macroporous, and dense (macropores accounting for less than 5 volume per cent). Microporosity is determined by sintering temperature and macroporosity by the addition of hydrogen peroxide to the calcium phosphate mixture before sintering (88,89). The biocompatibility of the material has been tested under several animal-experimental and clinical conditions and seems to be good (90-103). Comparable observations have been made in otology, in both experimental (104-106) and clinical studies (107-109). With respect to its mechanical properties, the compressive strength is satisfactory, but the tensile strength is limited, which makes hydroxyapatite less suitable for some orthopaedic or dental applications but not for otologic applications, where mechanical demands are light. In this connection it is also of interest that the mechanical properties of the material were reported to improve with increasing tissue ingrowth (110). The most favourable properties of the material seem to be that it permits good tissue ingrowth and firm direct bonding to bone. With respect to biodegradation, some authors have reported degradation of the material (96) whereas others did not observe it (99,100). Hydroxyapatite is used clinically to replace ossicles and the posterior meatal wall, as well as for a total alloplastic middle ear.

β-whitlockite can be produced in the same forms as hydroxyapatite (111) and seems to possess good biological properties in animals and patients (99,100, 112-115). Like hydroxyapatite it has been reported to undergo little or no degradation (112,113) as well as considerable biodegradation (99,100). Unlike hydroxyapatite, β-whitlockite is not applied clinically as an ossicular replacement prosthesis or total alloplastic middle ear.

Compared with other biomaterials, hydroxyapatite and β-whitlockite have the advantage of a composition rather similar to that of the mineral matrix of bone, which holds in particular for hydroxyapatite, and that they can be manufactured in several grades of porosity.

This situation led us to assess the biocompatibility and biofunctionality of these calcium phosphate ceramics, with special attention to hydroxyapatite.

Biocompatibility and biofunctionality

Biocompatibility and biofunctionality are useful criteria for evaluation of the performance of a biomaterial but do not necessarily go hand in hand. For instance, a prosthesis may fail abruptly to function without a corresponding avert tissue reaction (116,117) in other words, the biofunctionality is inadequate while the biocompatibility seems satisfactory. On the other hand, functional implants can be found near pathologic tissue (118), which means that poor biocompatibility is not always accompanied by reduced biofunctionality. Furthermore, implants can fail in both biofunctionality and biocompatibility, as in the case of the polyurethane polymeres used for open fixation of fractures. This material underwent hydrolysis and the products were toxic to the host tissue (119). Apparently, biocompatibility and biofunctionality are not determined solely by the characteristics of the implant or the body's reactions to the implant: the performance of biomaterials is the result of interactions between the implant material and the host tissue.

Interactions between biomaterials and tissues

The first response of the body to which an implant is exposed is the wound reaction induced by the surgery required for implantation. This reaction comprises several phenomena; in chronological order: the clotting of fibrin and platelets, the cellular response of neutrophilic granulocytes, lymphocytes, and macrophages, the local appearance of fibroblasts, capillaries, and collagen, and finally covering by epithelial cells (120,121). The presence of a biomaterial in the vicinity of the wound can affect the course of these events. For instance, it may influence oxygenation and phagocytosis, affect the orientation of cross-linked collagen, and increase the risk of infection (122,123). These and other changes lead to the characteristic features of a foreign-body reaction, such as the presence of macrophages, polykaryons, and a fibrous tissue envelope (122-124). The severity of the foreign-body reaction is determined by the interactions between the biomaterial and the host tissue.

Interface reactions: Since direct contact between the biomaterial and the host tissue occurs at the biomaterial/tissue interface (127), interactions at this site can be expected to be of primary importance for the biocompatibility and biofunctionality of a biomaterial. According to Hench et al. (128), these interactions can be subdivided into three categories: chemical, physiological and mechanical. For glass ceramics and calcium phosphate ceramics, the chemical interface reactions lead to a direct bond with bone effected by calcium and phosphate ion exchange. Because the crystal structure of hydroxyapatite ceramic is similar to that of the mineral bone matrix, this bond may be expected to be even stronger than for other bioactive ceramics due to the continuity of the crystal structure (129,130). Bioactive materials induce bonding osteogenesis, whereas bioinert material like aluminum oxide, which is extremely inert at the surface, gives rise to a kind of contact osteogenesis that does not produce a bond between the bone and the material. There are also biotolerant materials, such as metals, whose interface reactions do not permit direct bonding or contact with bony tissue;

these materials induce distance osteogenesis. The mechanical interaction at the interface is reflected by histological differences in the response to implant materials differing in shape or surface structure e.g., round or rectangular, smooth or rough (131,132). However, the cellular response has also been shown *in vitro* to alter as a function of surface characteristics of the material (smooth or rough) (133), which suggests that friction is not the only cause of this phenomenon. Surface interactions can be altered to produce firmer fixation of an implant by the use of micropores and macropores.

Porosity: Macropores are known to allow tissue ingrowth. In general, a pore diameter larger than 30 μm gives rise to ingrowth of fibrous tissue, whereas a pore diameter of more than 100 μm leads to the ingrowth of bone tissue (33, 134-137). The kind and quantity of ingrowing tissue will of course be strongly dependent on the implantation site. As already mentioned, the advantages of tissue ingrowth include firm fixation, and possibly a smaller fibrous capsule, because the foreign-body reaction is less severe (123). The use of micropores can be expected to improve fixation too, due not only to cell ingrowth but also to an increase of the surface area. The disadvantages of implant porosity include reduced compressive strength (111), and possibly a higher incidence of infection (138,139). Furthermore, macroporous implant materials seem to have fewer carcinogenic properties, as discussed in the next section.

Foreign-body carcinogenesis: Besides standard histopathological studies on implantation sites, interest has also been given to the carcinogenic properties of implanted biomaterials. Tumors have been found in both laboratory (140-142) and clinical studies (143-145), apparently directly correlated with implant presence. Experiments done in mice showed a relatively high frequency of implant-associated tumors. The only factors with an influence on the frequency of malignant malformations were the total surface area and the physicochemical surface properties. Large smooth surfaces were associated with the highest frequency of tumors, whereas perforation, macroporosity, and surface roughness were linked with a greatly reduced tumor incidence. A higher degree of degradibility also resulted in a decrease. The relatively high incidence of implant-associated tumors in mice cannot be extrapolated to the human situation because mice seem exceptionally susceptible to tumor formation induced by a foreign body. The long latent period expected for foreign-body carcinogenesis makes it difficult to predict the incidence of such tumors in man, but the data available at the moment are not disquieting (144).

Biomaterials and infection: A wound containing a foreign body is generally believed to be more likely to become infected than a wound without a foreign body. This has been confirmed for implants by a number of studies primarily concerned with the behaviour of materials destined for use in vascular surgery and as suture materials (138,139, 146-155). Some of these studies showed a correlation between the structure of a biomaterial and the occurrence of bacteremic infection, a rough surface coinciding with infection more often than a material with a smooth surface. Another significant factor seems to be the surface charge of a biomaterial, which may be a decisive factor for the adherence of bacteria to the material (156-158). Furthermore, a biomaterial may promote infection but can also reduce the efficiency of antibiotic treatment and thus lead to recurrence of infection (159). Biomaterials in use in otology have never been exposed to experimentally induced bacterial infection. This is surprising, because infection is frequently encountered in otology (160,161) and is often the reason for surgery which means a relatively high risk of implantation in a contaminated area or a similar risk of relapse or recurrent infection.

Systemic effects: In the evaluation of a biomaterial attention is generally concentrated on local tissue reactions. This approach may lead to the underestimation of systemic effects (145). An acknowledged example of the systemic effects of biomaterials is systemic hypotension attributable to the presence of polymethyl methacrylate bone cements, partially due to monomer released from these (162). Other systemic effects are the encephalopathy, osetoporosis, and anaemia caused by relatively high concentrations of aluminum in dialysis solutions (163,164). Mention may also be made here of the immune response to metals in dental alloys (165-168).

Objectives of the study

Biomaterial/host interactions are complicated and strongly dependent on the mode of application and the nature of the biomaterial in question. This means that a general testing method will not suffice, and that evaluation should be made in relation to the demands, the material must satisfy as well as the specific characteristics of the implantation site. The present study was performed to assess the biocompatibility of hydroxyapatite in three situations: as middle-ear implant material for a prosthesis to replace the incus, as posterior external canal wall prosthesis, and as total alloplastic middle ear. In addition, β-whitlockite was evaluated as an alternative material. Since these calcium phosphates are candidate materials for middle-ear implants in patients, the site chosen for implantation should have a close morphological resemblance to the human middle ear. For their studies Grote and Kuijpers chose the middle ear of the rat (39, 104), which is similar to that of man (169-175) morphologically (176,177). The epithelium of the rat middle ear is composed predominantly of flat polygonal epithelial cells alternating ciliating cells in the mucociliary tracks above and below the promontory. In the vicinity of the round window niche and the orifice of the Eustachian tube, goblet cells occur as well. A comparable middle-ear epithelium is shown by the human middle ear.

Testing of biomaterials by implantation in the animal is a valuable procedure, but the complexity of the reactions and the long duration of such experiments are disadvantages. To avoid these problems use is made of *in vitro* experiments (178), which provide quantitative and qualitative data rapidly. Since a material should be tested in a situation resembling the clinical situation as closely as possible the choice of middle-ear epithelium seemed logical. However, except for papers on cholesteatomous tissue (179-181), no reports on the culture of middle-ear tissue were available. We therefore developed a culture method suitable for middle-ear epithelium and used tissue from the rat. The culture technique and tissue characteristics are described in *Chapter II.*

Since infection is a common occurrence before and after middle-ear surgery, a biomaterial destined for reconstructive middle-ear surgery should be tested in both non-infected and infected environments. Despite the many studies on middle-ear infection (182-187) there are few reports on experimentally induced middle-ear infection in animals (188-192) and none at all for the rat. This made it necessary to develop a middle-ear infection model for this species. *Staphylococcus aureus* was chosen as infectious organism because it was frequently found in contaminated wounds (155) and has been isolated from middle-ear effusions (193-195), although less frequently than *Streptococcus pneumoniae* and *Haemophilus influenzae* (193, 195-199). The experimental infection model is described in *Chapter III.*

The middle-ear epithelium plays an important role in the defence mechanism against infection of the middle ear (200), as examplified by the transport function of the mucociliary tracks. In view of this phenomenon and the importance of covering by epithelium as a stage in wound healing that is also expected to limit the occurrence of infection, evaluation of the epithelium on an implant material is crucial in this context. *Chapter IV* deals with the epithelial covering of hydroxyapatite.

As already mentioned, interfacial reactions exert an influence on the biofunctionality and biocompatibility of implant materials and this seems to hold especially for the category of bioactive materials to which the calcium phosphate ceramics belong. The bioreactions at the hydroxyapatite/tissue interface are discussed in *Chapter V.*

In this study use was made of both macroporous and dense materials. The tissue reactions in the pores can be expected to influence the functioning and biological performance of a biomaterial and might also provide information about the bioactivity of such materials. Macropore characteristics are treated in *Chapter VI.*

As mentioned above, materials destined for implantation should be assessed during infection and in particular with respect to use in reconstructive middle-ear surgery. We used the infection model described in Chapter III to obtain information about the behaviour of hydroxyapatite under the conditions prevailing during infection and applied the same time-points as those mentioned in Chapters IV-VI. In these studies infection was always induced three weeks before decapitation. The results of this experiment are given in *Chapter VII.*

Besides the long-term effects of infection, evaluation of the short-term effects was expected to provide interesting information on the behaviour of an implant material. This study is reported in *Chapter VIII.*

We investigated the behaviour of macroporous β-whitlockite for comparison with hydroxyapatite. The study was done in non-infected and infected middle ears. The same techniques were used as for the hydroxyapatite experiment except *in vitro*. The β-whitlockite results are discussed in relation to the data on hydroxyapatite in *Chapter IX.*

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CHAPTER II

Culture And Characterization Of Rat Middle-Ear Epithelium

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SUMMARY

This study was performed to design a method to culture rat middle-ear epithelium and to apply the method to investigate the characteristics of this epithelium. Culture of explants of middle-ear epithelium in the presence of the epidermal growth factor was succesful, whereas serial cultivation required 3T3 feeder cells in addition to the epidermal growth factor. Cultured middle-ear epithelium was studied by phase contrast microscopy, transmission and scanning electron microscopy, and combined light and scanning electron microscopy (LM/SEM). These techniques showed similarity between the cultured and the natural middle-ear epithelium. Explants and outgrowths showed both flat polygonal and ciliated epithelial cells. In serial cultivation, however, only the first of these cell types was observed. Frequently, a single primary cilium was found on the cell surface. Transmission electron microscopy showed cross-linked envelopes, formation of which was promoted by ionophore X537A.

Cytokeratin was demonstrated by immunoblotting, immunofluorescence, and immunoperoxidase methods using an anti-cytokeratin monoclonal antibody.

The model described here permits study of the differentiation of middle-ear epithelium *in vitro* and may be of future value for the study of chronic middle-ear diseases.

Since the introduction of the 3T3 feeder-layer technique (14) serial cultivation of epithelia has become a routine procedure allowing the cultivation of epithelia of various origins (2,5). The terminal differentiation of cultured epithelial cells has been studied (3,26) and the most prominent features seem to be the presence of cross-linked envelopes (15, 16, 27) and a relatively high cytokeratin content in the cytoplasm. In culture this differentiation pattern was not specific for cornifying epithelia, but also occurred in non-cornifying epithelia (2,5).

In spite of the fair number of epithelia cultured so far, there have been no reports concerning the cultivation of middle-ear epithelium, whether in the form of explants or in serial cultivation. The only reports available on the culture of ,,middle-ear tissue'' concern the culture of cholesteatoma matrix (11, 12, 19), a pathological tissue originating from either the middle-ear epithelium or the external meatus. Middle-ear epithelium is part of the upper airway epithelium and is generally composed of flat polygonal cells. In addition, ciliated and goblet cells are present in two mucociliary tracks (4, 7, 8). The presence of cytokeratin in middle-ear epithelium

has been predominantly demonstrated for pathological tissue (18), and no reports are available on cross-linked envelopes.

The present study aimed at establishing cultures of middle-ear epithelium of the rat. The cultured epithelium was studied by phase contrast, scanning and transmission electron microscopy. Furthermore, the presence of cytokeratin was investigated in relation to cross-linked envelope formation. The use of a combined light and scanning-electron microscope (29) allowed direct comparison of the light-microscopical and scanning electron-microscopical images.

MATERIALS AND METHODS

Culture conditions

Explants: Samples of middle-ear mucosa (*ca.* 1 mm²) were prepared from the middle-ear wall of male Wistar rats (body weight *ca.* 200 g). After incubation in physiological saline (4°C, pH 7.4) with penicillin (100 U/ml) and streptomycin (100 μg/ml) for at least 2 hr, the explants were allowed to adhere to the culture-dish surface in a small volume of culture medium for ca. 24 hr. The culture medium consisted of a mixture of Dulbecco's Vogt and Ham's F12 medium (3 : 1) supplemented with fetal calf serum (5%), hydrocortisone (0.4 μg/ml), isoproterenol (10^{-6}M), penicillin (100 U/ml), and streptomycin (100 μg/ml). After adherence, a normal amount of medium was supplied. All material was cultured in 10% CO_2 at 37° C and the medium was renewed twice a week.

In some of the experiments epidermal growth factor (10 ng/ml) (collaborative research) was added to the medium after 4 days of culture. Explants and their outgrowths were studied after 1 and 2 weeks.

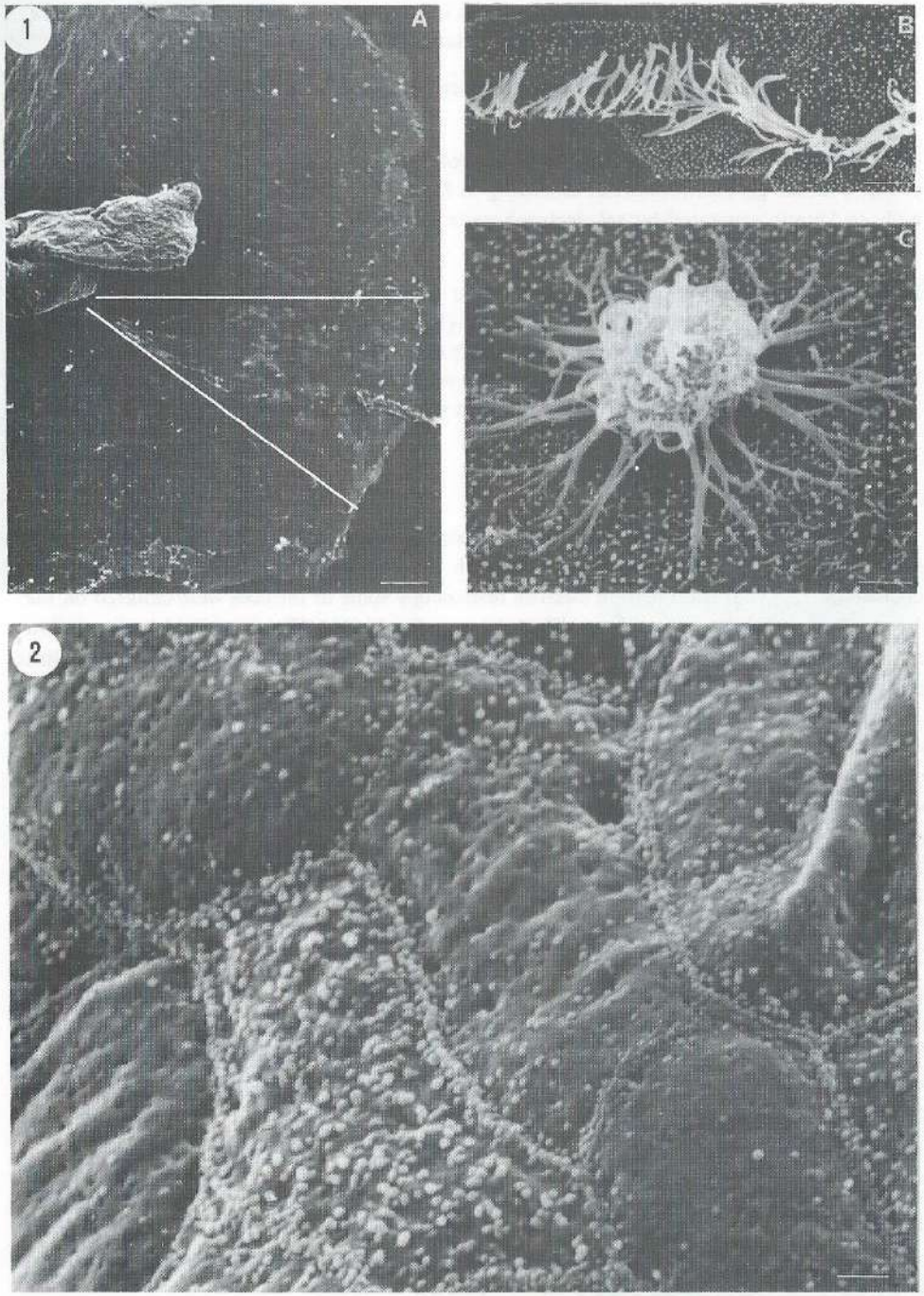
Serial cultivation: To find the most suitable culture technique, three culture methods were used. In all three, epithelial cells were harvested from the outgrowth of an explant, cultured for 2 weeks, by tripsinization and plated at a density of 2×10^4 cells/cm². For the first method, hydrocortisone, isoproterenol, penicillin, and streptomycin were added to the basic medium. For the second method, epidermal growth factor was added to the medium after four days. The third method resembled the second except that 1.2×10^4 lethally irradiated 3T3 cells per cm² were plated with the epithelial cells. The third method was used routinely for characterization of the epithelial cells.

Cytokeratin demonstration

Cells were fixed with methanol and stained with an anti-keratin monoclonal antibody, clone 80, which recognizes keratin specifically, as demonstrated in human tissue (10).

Fig. 1a-c. Scanning electron microscopial survey of an explant (2 weeks) and its outgrowth. In the indicated triangle there are ciliated cells with normal (b) and deviate morphology (c). Bar a: 300 μm, Bar b: 3 μm, Bar c: 1.25 μm.

Fig. 2. Scanning electron micrograph of explant surface with polygonal cells. Note the microvilli on the cell surface and border. Bar: 1 μm.



Immunoperoxidase staining: Rabbit-anti-mouse Ig conjugated to horse radish peroxidase was used as second antibody. As substrate, 3,3[4]- diaminobenzidine was applied.

Immunofluorescence: Direct immunofluorescence was performed with clone 80 conjugated to TrITC.

Gel electrophoresis and immunoblotting: Total cell lysates were analysed by SDS-polyacrylamide gel electrophoresis according to Maizel (9). Binding of anti-cytokeratin antibody to keratin separated by gel-electrophoresis was assayed by the immunoblot technique described by Towbin et al. (23).

Promotion of cross-linked envelope formation: Cross-linked envelope formation was promoted and scored according to Rice and Green (16), using ionophore X537A.

In this experiment envelope formation was only determined for serially cultivated cells. Control cells were incubated in medium without the ionophore. Envelopes were scored after 4, 6, 10, 14, and 28 days of culture. At least 4 dishes were used for each of the culture intervals.

Electron microscopy

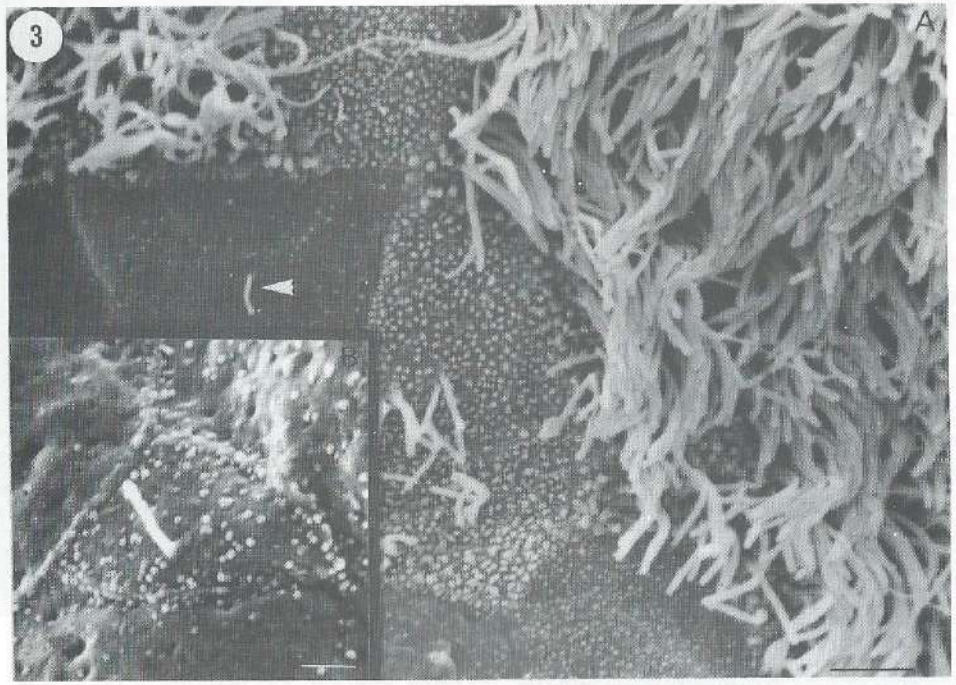
Explants, outgrowths, and surface cultures were studied by transmission electron microscopy, scanning electron microscopy, and with the combined light and scanning electron microscope (29). For transmission electron microscopy some of the cells were cultured on the dish surface and the rest on melinex. The cells were fixed in a 1.5% glutardialdehyde solution in sodium cacodylate buffer (0.14 M, pH 7.4, 4° C) for 15 min. After being washed with phosphate-buffered saline (pH 7.4), the cells were exposed to a 1 % $0s0_4$ solution at room temperature for 30 minutes. Next, the specimens were dehydrated in a graded alcohol series and embedded in Epon. After polymerization, the dishes were fractured and the cells in the Epon were re-embedded for horizontal and perpendicular sectioning. Thin sections were contrasted with uranyl acetate and lead hydroxide, and studied with a Philips EM 200 electron microscope.

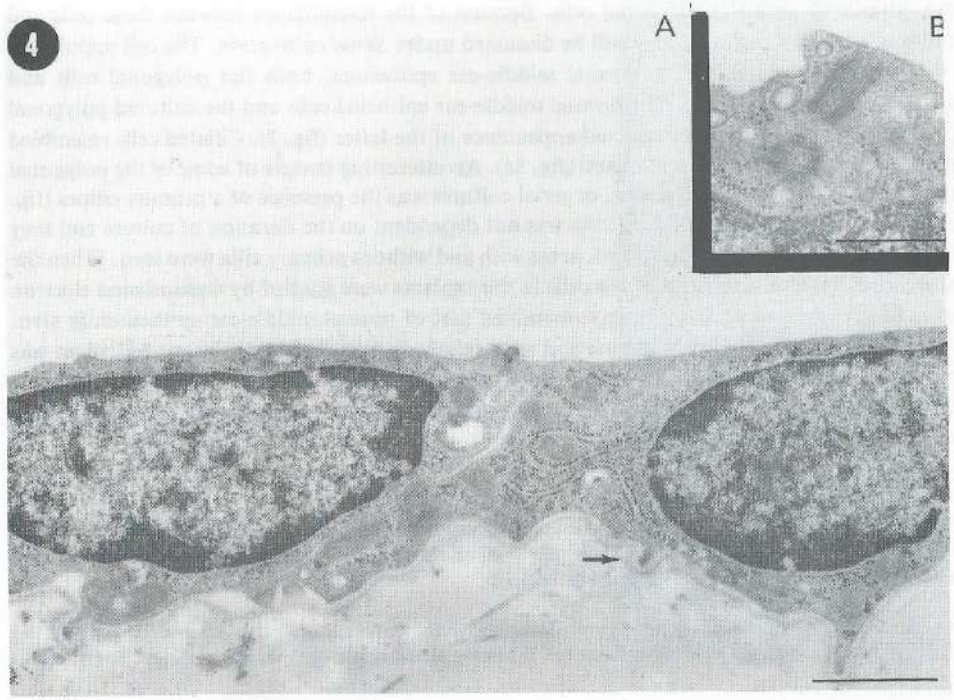
The material destined for scanning electron microscopy was cultured either on the dish surface or on a cover glass. After fixation in glutardialdehyde, the adhering cells on the dish surface or cover glasses were dehydrated, critical point dried, gold coated, and studied with a Cambridge S 180 scanning electron microscope.

Combined light and scanning electron microscopy: Use was made of surface cultures on cover glasses. Specimens were treated as described for immunoperoxidase staining and then dehydrated, critical point dried, and gold coated. The preparations were studied with a Leitz LM/SEM microscope consisting of a Leitz 1200 B SEM with a special stage and LM optics connected to a Leitz LM (29).

Fig. 3a and b. Part of an explant with ciliated epithelium. A primary cilium can be seen (arrow); a similar structure is shown in the inset (b). Bar a: 3 μm, Bar b: 1 μm.

Fig. 4a and b. Transmission electron micrograph of an explant (a). A basal lamina is still present (arrow) and centrioles occurred occasionally (b). Bar a: 1 μm, Bar b: 0.5 μm.





RESULTS

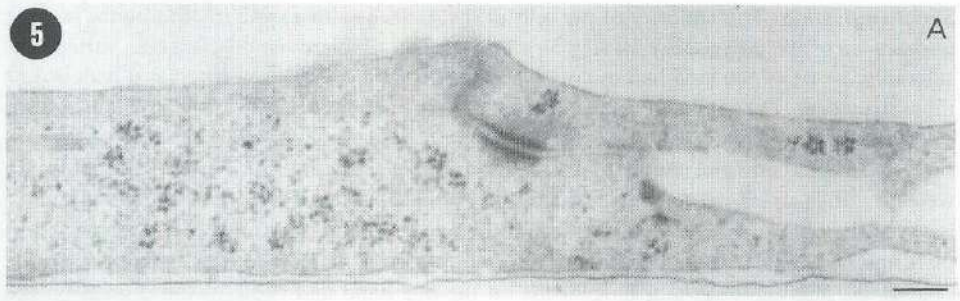
Explants cultured in the absence of epidermal growth factor generally showed outgrowth of a single layer of cells after one week. After that, however, no further increase occurred and explants and outgrowth started to float. The addition of epidermal growth factor to the medium 4 days after culture was started resulted in significant improvement. Outgrowth became more prominent, although still usually as a single layer, and floating only started after 2 weeks at the earliest. Serial cultivation was started after trypsinization of the explants. In medium without epidermal growth factor, proliferation of epithelial cells was slow. The addition of epidermal growth factor gave better results, but the best proliferation was obtained with a combination of epidermal growth factor and 3T3 feeder cells. Surface cultures were composed, like the outgrowths, of a single layer of cells and after trypsinization could be replated easily for at least 10 passages. Since phase contrast light microscopy did not add any significant findings to those obtained by scanning and transmission electron microscopy, attention was focussed mainly on the electron-microscopical results. Only the explants cultured with growth factor and by serial cultivation with 3T3 cells and growth factor were investigated extensively.

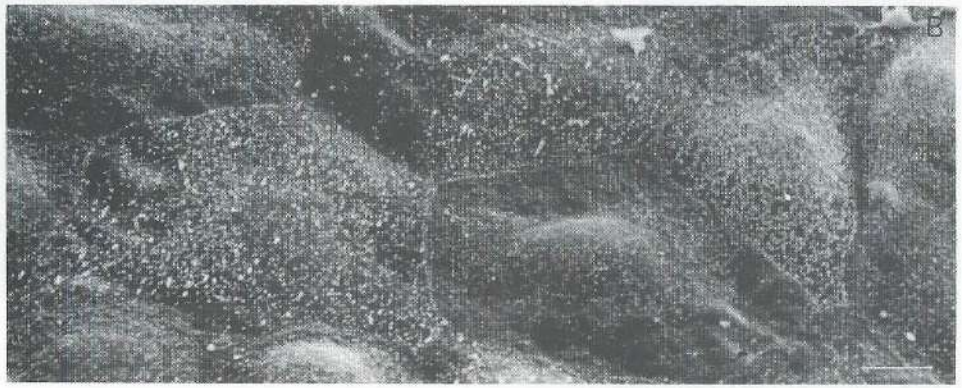
Since no clear differences in cell morphology were found between the time-points, only the over-all results will be discussed.

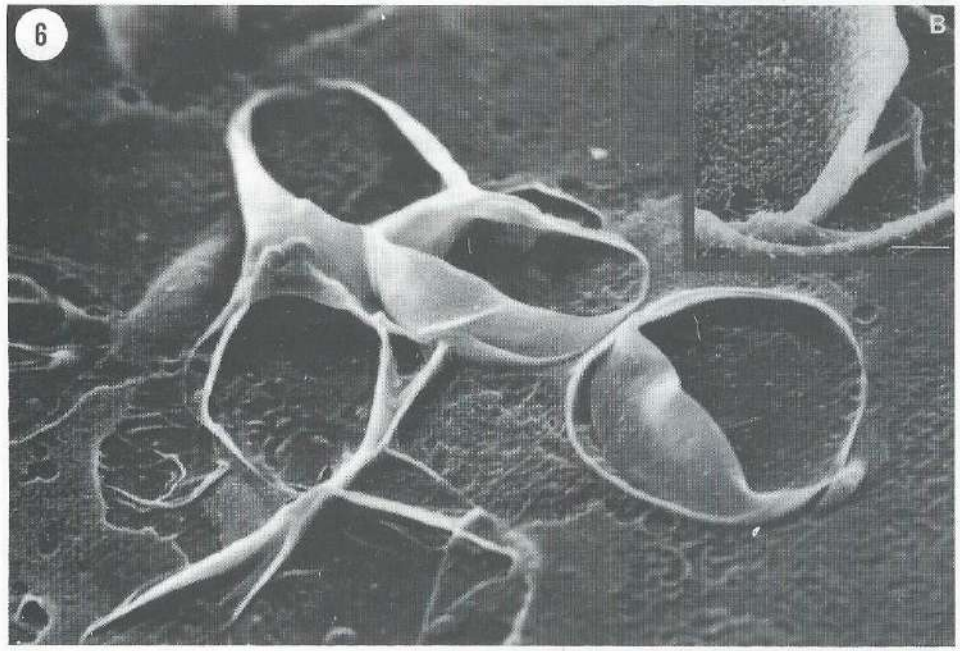
Explants: Figure 1a shows an electron micrograph of an explant. The outgrowth of cells is prominent and there is a triangular configuration containing ciliated cells. The ultrastructural features of the cilia sometimes differed considerably, cilia with a normal appearance (fig. 1b) and cilia with a deviating ultrastructure (fig. 1c) being observed. The majority of the cells in the outgrowth were flat polygonal cells. Because of the resemblance between these cells and those in the serial cultures, they will be discussed under *Serial cultivation*. The cell population of the explant resembled the normal middle-ear epithelium, both flat polygonal cells and ciliated cells, being present. The normal middle-ear epithelial cells and the cultured polygonal cells differed by the more contracted appearance of the latter (fig. 2). Ciliated cells resembled normal middle-ear ciliated epithelium (fig. 3a). An interesting feature of some of the polygonal cells in the explant, the outgrowth, or serial cultures was the presence of a primary cilium (fig. 3, a and b). The presence of such cilia was not dependent on the duration of culture and they occurred mainly in cell clusters, thus, areas with and without primary cilia were seen. When the ultrastructural characteristics of the cells in the explants were studied by transmission electron microscopy, the morphology closely resembled that of normal middle-ear epithelium *in vivo*. The cells still lay on the basal lamina and were relatively thin. The endoplasmic reticulum was well developed, and many ribosomes were present in the cytoplasm. Mitochondria had a normal appearance and microvilli were also present (fig. 4a). Occasionally, a centriole was seen near the cell surface (fig. 4b).

Fig. 5a and b. Transmission (a) and scanning (b) electron micrographs of flat polygonal epithelium in a serial culture. The cells are thin and show microvilli on the surface and desmosomes are present at contact sites between cells. Bar a: 0.2 μm, Bar b: 5 μm.

Fig. 6a and b. Envelope-like structure protruding above the basal culture plane after serial cultivation. The inset shows the highly irregular surface (b). Scanning electron micrograph. Bar a: 10 μm, Bar b: 5 μm.







Serial cultivation: The majority of the cells in the surface cultures and in outgrowths had the appearance of flat polygonal cells (fig. 5b). The number of microvilli on these cells varied considerably: some of the cells had many on cell surface and cell border, whereas others lacked these structures. The cells were large and even thinner than those of the explant. Transmission electron microscopy brought out this phenomenon and also showed that outgrowths and surface cultures consisted mainly of a single layer of cells (fig. 5a). Furthermore, desmosomes were found in areas of contact between the epithelial cells. The most striking difference between the cells in the serial cultures and those in the explants and outgrowths was the complete absence of ciliated epithelium in the former. Another difference was found between the cells in the serial cultures and outgrowths on the one hand and those in the explant on the other. Both of the former showed structures projecting above the basal plane of culture, had more electron luminescence, a rough surface, and resembled rather empty envelopes. They occurred singly or in clusters (fig. 6, a and b). The rough surface of these structures was similar to that on cells in transmission electron microscopy, i.e., an electron-dense structure associated with the outer cell membrane (fig. 7). This structure resembles the cross-linked envelope. This envelope was seen most clearly in sections cut parallel to the culture surface; its thickness varied considerably and numerous microvilli-like structures at the surface were a characteristic feature. Besides mitochrondria, endoplasmic reticulum, and other cell organelles, filamentous structures could be distinguished (fig. 8a). These filaments were either in contact with or lay in the vicinity of the cell membrane (fig. 8, a and b). Frequently, filamentous structures were associated with desmosomes (fig. 8c). Electron-dense inclusion bodies with widely differing morphology (fig. 8d) were also present.

Demonstration and localization of cytokeratin

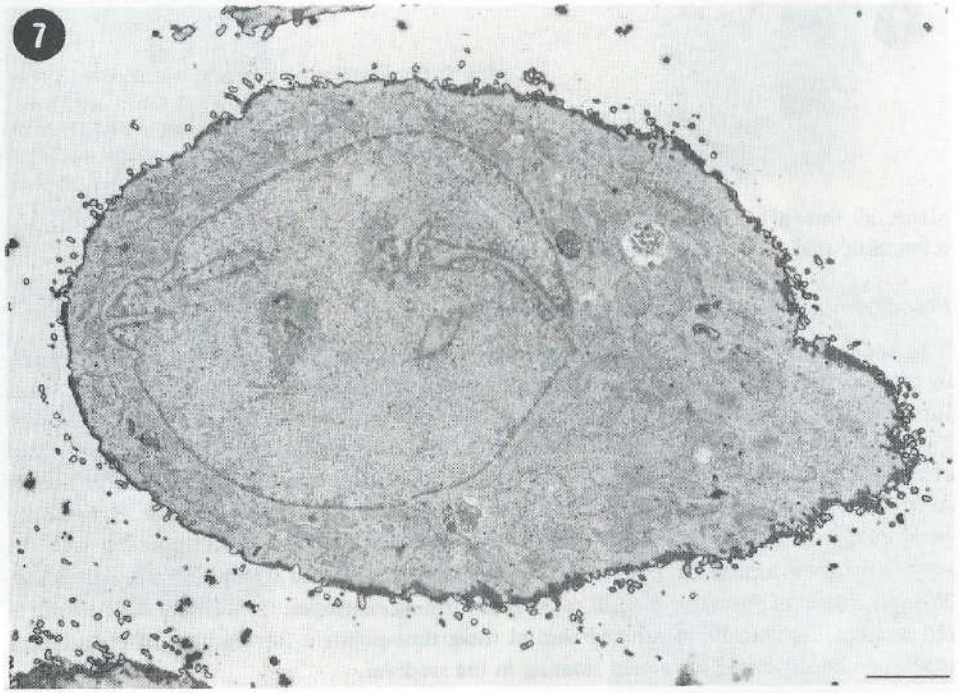
Immunofluorescence obtained with an anti-cytokeratin monoclonal antibody showed cytokeratin in middle-ear epithelium immediately after removal from the rat middle ear. However, the number of cells obtained was too small to allow immunoblotting. Since serial cultivation provided larger quantities of epithelial cells, this culture technique was chosen for the demonstration of cytokeratin by immunoblotting and immunostaining.

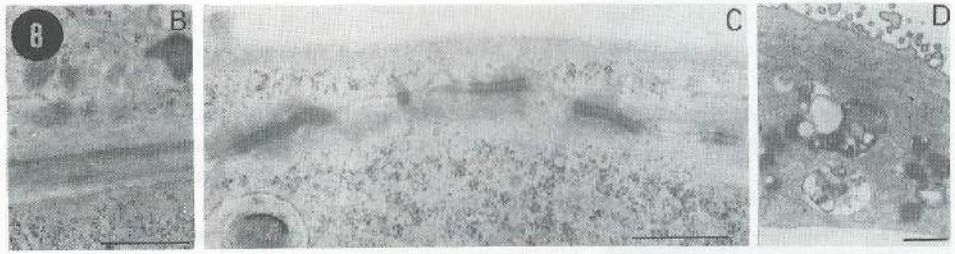
Immunoblots stained with anti-cytokeratin clone 80 showed bands at 50 KD. The cytokeratin pattern was the same whether the epithelium was cultured for 1, 2, or 4 weeks (fig. 9). In addition to immunoblotting, serially cultured cells were stained for cytokeratin by direct immunofluorescence with clone 80 (fig. 10a). Almost all of the cells were cytokeratin-positive.

The combined light and scanning electron microscope permitted comparison between the light-microscopical findings for keratin after immunoperoxidase staining and the ultrastructural features of the cells in which the cytokeratin was present (figs. 10b, 11). Immunoperoxidase labeling was more intense in cells lying partially or totally above the basal culture

Fig. 7. Cell from a serial culture in a section cut parallel to the culture surface. Note the electron-dense layer associated with the cell membrane. Bar: 1 μm.

Fig. 8a-d. Transmission electron microscopical view of the cytoplasm of a cell from a serial culture (a). Filament bundles are present. Such bundles occurred near the cell membrane (b) or near desmosomes (c). Granules showed marked morphological variation (d). Bar a: 1 μm, Bar b: 1 μm, Bar c: 0.5 μm, Bar d: 1 μm.





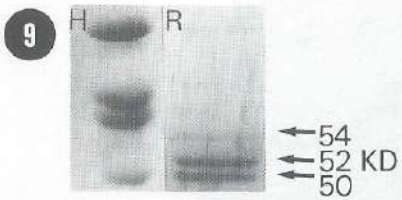


Fig. 9. Immunoblot of serially cultured rat middle-ear epithelium *(R)*. Clone 80 has labelled two clear bands, at 50 and 52 KD, and a fainter band at 54 KD. At the left as control experiment an immunoblot of human epidermis *(H)*.

plane, as shown by scanning electron microscopy. Some of these cells too had a surface resembling that of the envelope-like structures.

Promotion of cross-linked envelope formation

In addition to the cross-linked envelope-like structures shown by scanning and more clearly by transmission electron microscopy, the potentiality of cultured middle-ear epithelial cells to form cross-linked envelopes was investigated. For this purpose the cells were incubated with ionophore X537A. The number of envelopes formed during this incubation was determined after 4, 6, 10, 14, and 28 days of culture. The results of this experiment are given in fig. 12. After 4 days of culture about 50% of the epithelial cells were able to form cross-linked envelopes, whereas 100% showed this characteristic after 10 days, corresponding with the peak in the growth curve. In spite of the cell-number decrease seen after the longer periods (14, 28 days), 100% of the cells were still capable of forming envelopes. Concerning the decrease in cell number it shoud be mentioned that at these time-points a fair number of cross-linked envelope-like structures were seen floating in the medium.

DISCUSSION

There are a number of reports in the literature describing the culture of a variety of epithelia from various anatomical sites and from different species. These epithelia were cultured under various conditions. The method introduced by Rheinwald (14), who used 3T3 feeder cells, allowed serial cultivation of human and other epithelia.

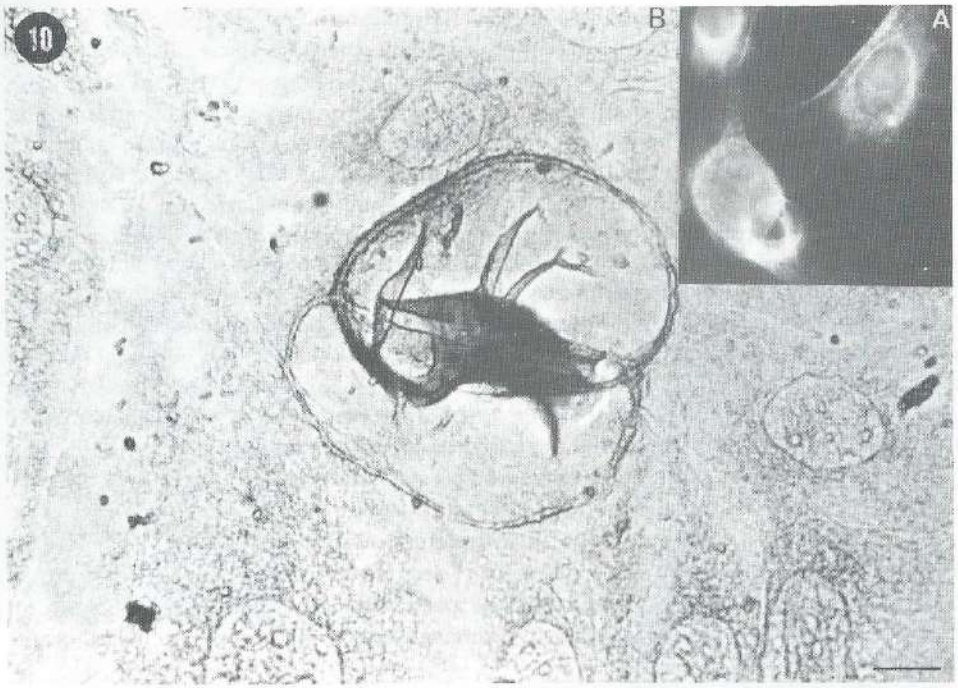
The present results show that the use of lethally irradiated 3T3 cells in combination with epidermal growth factor gives good results for the serial culture of rat middle-ear epithelium. Explants of this tissue did not need the presence of the feeder layer for good outgrowth, but the addition of epidermal growth factor to the medium had a positive effect on outgrowth.

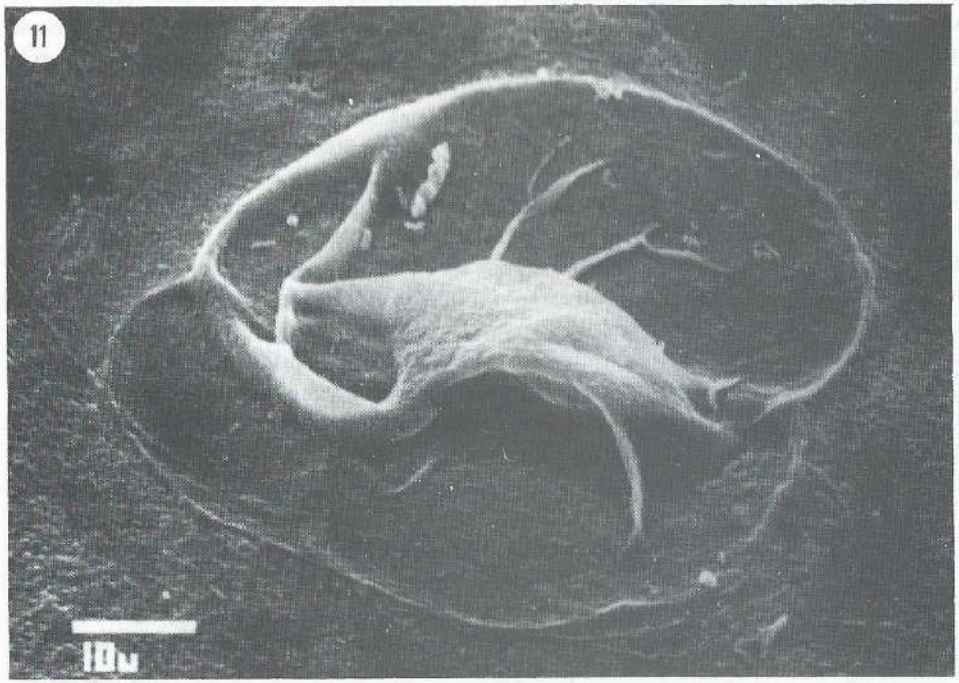
Comparison of the morphological characteristics of rat middle-ear epithelium in culture with those of the same epithelium *in vivo* (4, 7, 8) showed a close resemblance.

In the explants flat polygonal cells and ciliated cells were seen, types of cell which are present in the middle-ear cavity. Just as in the middle ear, the flat polygonal cells were characterized by varying numbers of microvilli on the cell surface and border.

Fig. 10a-b. Keratin demonstrated in cultivated cells by labeling with clone 80, as shown by immunofluorescence (a) and immunoperoxidase (b). Bar b: 10 μm.

Fig. 11. Scanning electron microscopical appearance of the same structure as shown in fig. 10a. Bar: 10 μm.





The outgrowth of the explants also showed flat polygonal epithelial cells together with ciliated cells. The outgrowth of ciliated cells started from explant sites where ciliated epithelium was present. The triangular configuration of the zones in the outgrowth (fig. 1a) where these cells occurred suggested that proliferative activity must have taken place. In contrast to the findings in the explant, the morphology of the cilia was not always the same in the outgrowth and the normal middle ear. Culture seems to have a negative influence on the differentiation and/or proliferation of these cells. None of the serial cultures showed any ciliated cells, which suggests that the trypsin flotation procedure applied before plating inhibits the adherence of proliferation of this cell type. It is also possible, however, that both were weaker than in the flat polygonal epithelium, even before trypsinization. Generally, however, the cells in serial cultures were similar to those in the outgrowth. A prominent finding made with all of the culturing techniques was the presence of primary cilia on the cell surface. The occurrence of primary cilia has been frequently reported (6, 13, 17, 20, 22, 28), although until now not for middle-ear epithelium. The presence of this structure has been thought to be associated with the centriole (1, 24, 25), which would explain its disappearance during mitosis.

Another characteristic feature seen in outgrowths and serial cultures but not in the explants was the envelope-like structures protruding above the culture plane, as observed by scanning electron microscopy. The surface of these structures was highly irregular and showed microvilli-like projections. Since transmission electron microscopy showed association between the presence of a cross-linked envelope and an irregular surface, it may be assumed that the envelope-like structures found by scanning electron microscopy are indeed cross-linked envelopes.

Cross-linked envelopes are composed of cross-linked proteins (15, 21), and formation of these structures can be promoted by an ionophore, for example X537A (16). In the present study ionophore addition promoted cross-linked envelope formation in rat middle-ear epithelium *in vitro*. When the peak in the growth curve was reached, 100% of the suspended cells showed the ability to form an envelope, and even though the total cell number decreased after the peak, all cells remained capable of forming an envelope. Since the medium showed floating envelope-like structures during these longer culture periods, this decrease in cell number is probably attributable to terminal differentiation and detachment of some of the cultured cells.

Until now, the presence of cytokeratin in middle-ear epithelium has mainly been reported for pathological tissue (18). In the present study we applied a monoclonal antibody (clone 80) directed against cytokeratin, as shown earlier for human epithelial tissue. With total lysates of cultured rat middle-ear epithelium, immunoblotting showed distinct bands at 50 KD and 52 KD, indicating that clone 80 also recognizes rat cytokeratin. In addition to these two distinct bands, some very weak bands appeared. It is not yet clear whether only a limited amount of these cytokeratins was present or clone 80 does not recognize these cytokeratins as efficiently as it does the 50 KD and 52 KD bands. Cytokeratin was also visualized by immunoperoxidase staining and by immunofluorescence. With these techniques cultured cells clearly showed the presence of keratin.

The use of combined light and scanning-electron-microscopy (29) showed comparison of the light-microscopical findings provided by immunoperoxidase staining and the ultrastructural findings. The results showed that especially cells protruding above the culture plane contained relatively large amounts of the immunoperoxidase stain. Moreover, these cells often resembled the envelope-like structures seen in the outgrowths and surface cultures. With respect to

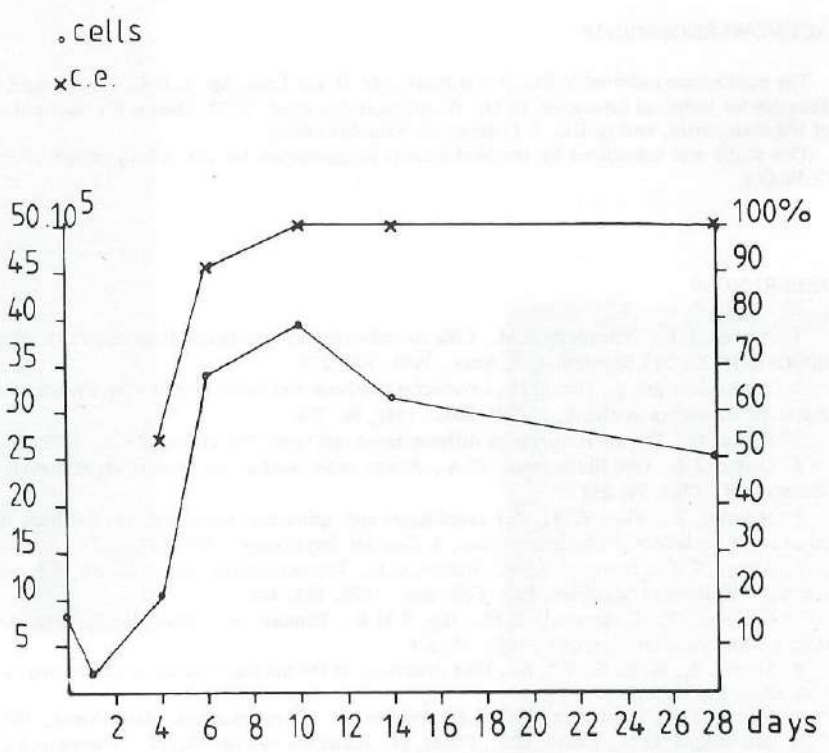


Fig. 12. Growth curve of serially cultured middle-ear epithelium, and the percentage of cross-linked envelopes found after incubation with ionophore X537A.

keratin in the cytoplasm, a question arose. Although immunoblotting, immunoperoxidase staining, and immunofluorescence showed the presence of keratin in these cells, it was not clear where the keratin was located ultrastructurally. The electron microscope showed filament bundles connected with desmosomes in the cytoplasm, but from our observations it could not be concluded whether these structures represented microfilaments or intermediate filaments, because the prominent cytokeratin filaments seen in most keratinocytes were not found in this study.

In sum, it can be concluded that rat middle-ear epithelium can be cultured both as explants and serially. Cultured rat middle-ear epithelium closely resembled the normal *in vivo* middle-ear mucosa. Terminal differentiation occurred, and resembled the process described for other epithelia. Some questions concerning the localization of keratin in the cytoplasm remain unanswered, but these problems can be solved by application of electron-microscopical immunocytochemical techniques.

The experimental model described here can be expected to prove useful to study the differentiation of middle-ear epithelium and thus contribute to the understanding of chronic middle-ear disease.

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CHAPTER III

Acute Otitis Media

An Animal Experimental Study

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SUMMARY

This paper describes an investigation performed to study the middle ear events ensuing from *Staphylococcus aureus* induced infection in the middle-ear cavity of the rat. To obtain an evaluation at both the cell and the tissue level, scanning electron microscopy, transmission electron microscopy and histology were used. *Staphylococcus aureus* infection appears to be characterized by five major events: (i) cellular response, (ii) humoral response, (iii) mucociliary response, (iv) fibroblastic response, (v) bony response. Since these occurrences correspond to the events witnessed in the human middle ear affected by acute otitis media, *S. aureus* achieved infection might prove a useful tool for the further study of this disease, by means of animal experiments.

The number of clinical, epidemiological and bacteriological studies dedicated to acute otitis media appears impressive (2, 15, 26, 27). Animal experimental studies on this disease, however, are relatively sparse (7, 8, 11, 17). This is a surprising phenomenon, since animal experimental studies seem to offer an ideal instrument for a systematic approach to the pathogenesis of acute otitis media.

Though *S. aureus,* the organism used throughout this study, is not the most common organism in otitis media, it can regularly be demonstrated in middle ear effusions (4, 21, 22), and offers a suitable way of evoking a mild reaction in the middle ear cavity of the rat.

The purpose of this study was to obtain a more profound knowledge of the pathogenesis of acute otitis media, by a reproducible method of inducing acute infection in the middle ear of the rat. The use of histology, scanning and transmission electron microscopy provided a way to evaluate the evoked events at both the tissue and the cellular level.

MATERIALS AND METHODS

The animals used for this study were male Wistar rats (200 g). The rats were anaesthetized by an intramuscular injection of phenobarbital (Hypnorm), after which an infection was induced by intratympanical administration of a *S. aureus* suspension in physiological saline, containing approximately 1.4×10^6 bacteria. A total of 42 middle ears were infected. The rats were decapitated after survival periods varying from one day to 4 weeks (Table I). As a control experiment, non-infected middle ears were studied and earlier studies could be used (16, 23).

Survival time (days)	Number of ears			
	Histology	T.E.M.	S.E.M.	Total
1	3	2	2	7
3	3	2	2	7
7	3	2	2	7
14	3	2	2	7
21	3	2	2	7
28	3	2	2	7
Total	18	12	12	42

Table I. A survey of the distribution of the infected middle ears among the three different techniques.

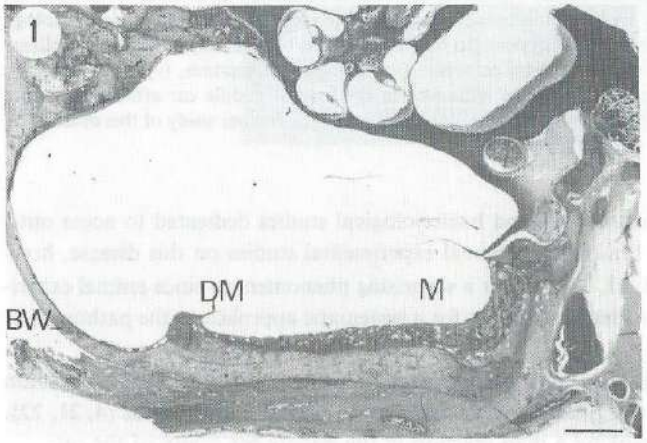


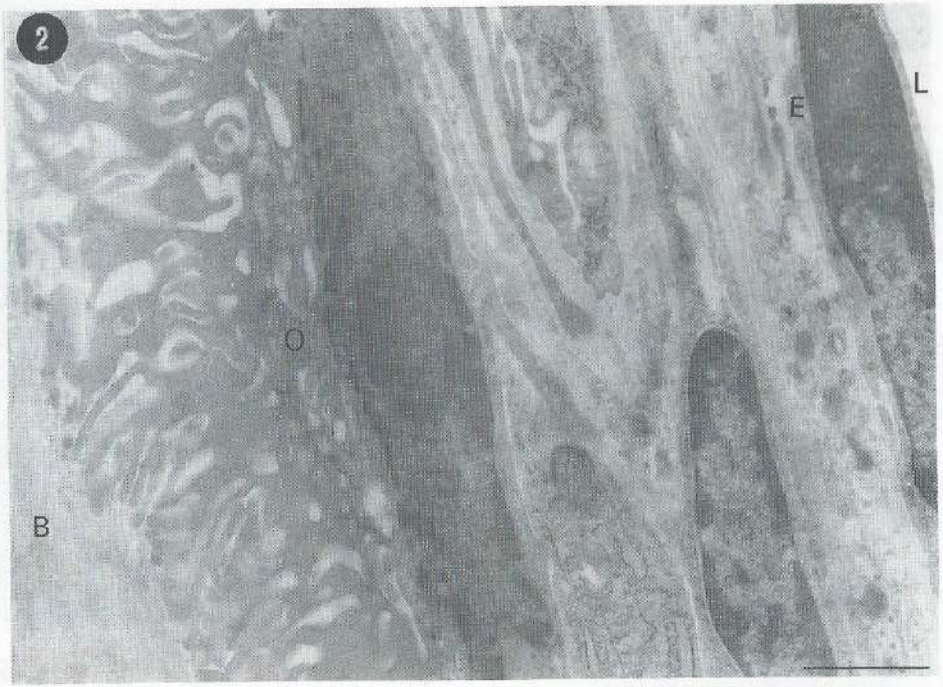
Fig. 1. Light microscopic view of part of the rat middle ear. The bulla wall *(BW)*, the drum membrane *(DM)* and the meatus *(M)* are among the structures that can be distinguished. Bar represents 250 μm.

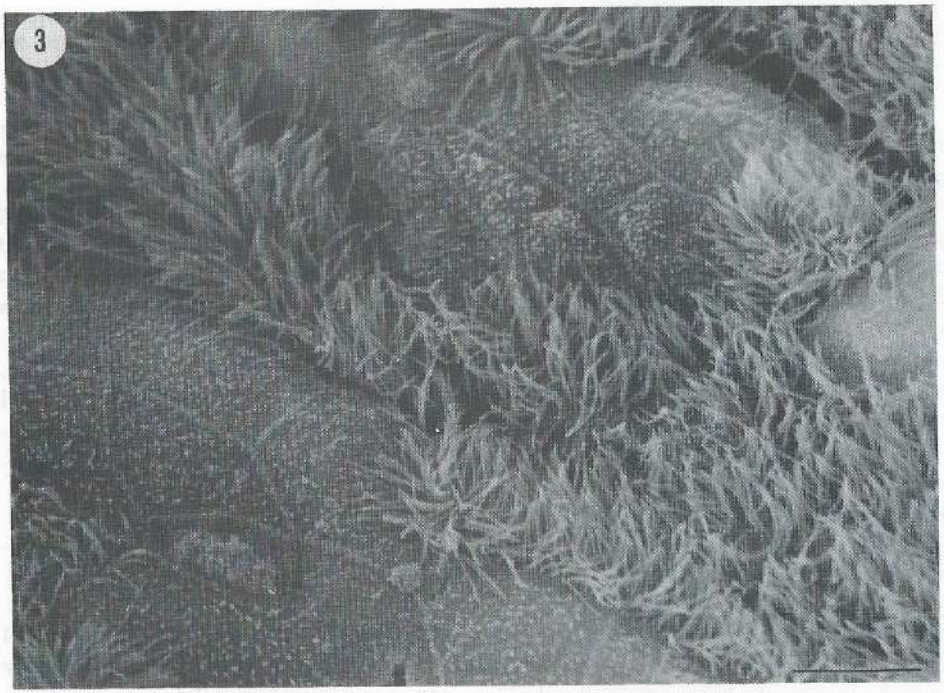
The ears destined for scanning electron microscopy were rinsed with physiological saline in order to clear the middle ear of mucus. Those middle ears prepared for histology and transmission electron microscopy were exposed to a solution of 1.5% glutaraldehyde in cacodylate buffer (0.14 M, pH 7.4, 0°C) even before dissection was completed. All material was kept in this solution at 4°C.

Histology: After a 16-hour fixation the middle ears were decalcified by means of a 4-week stay in a EDTA 10% solution (pH 7.4) containing 1.5% glutaraldehyde, embedded for routine histology and stained with hematoxylin-eosin.

Fig. 2. Transmission electron micrograph of the non-infected middle-ear mucosa. Between the bone *(B)* of the bulla and the lumen *(L)* an osteoclast *(O)* and an epithelial cell *(E)* can be witnessed. Bar represents 1 μm.

Fig. 3. Scanning electron micrograph of the non-infected middle-ear mucosa, showing part of the mucociliary tracks. Bar represents 5 μm.





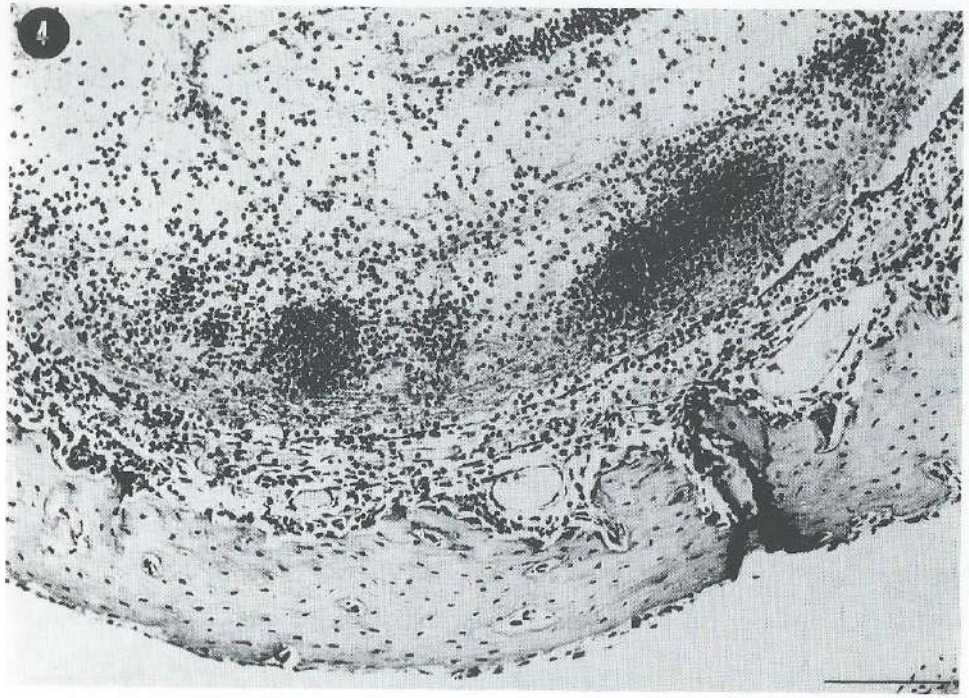


Fig. 4. Histological section of the middle-ear cavity one day after the onset of infection. Note the exudative fluid and the oedema, also lacunae due to bone resorption *(R)* can be demonstrated. Bar represents 70 μm.

Transmission electron microscopy: The mucosa was gently removed from the bulla after a 2-hour fixation, washed three times in phosphate-buffered physiological saline and post-fixed in phosphate-buffered osmium-tetroxide (1%, pH 7.4) at room temperature for 30 min.

Part of the material was obtained from middle ears destined for histology. Here, small pieces of middle ear bulla were washed and fixed in a similar way. All material was dehydrated in graded alcohols and embedded for routine electron microscopy (5). 1 μm sections were cut for light microscopy and stained with toluidine blue. Thin sections were studied by means of a Philips 200 S electron microscope.

Scanning electron microscopy: The middle ears were fixed in glutaraldehyde for 2 hours, washed three times, dehydrated in graded alcohols, critical point dried in carbon dioxide and coated with gold. The ears thus obtained were studied with a Cambridge 180 S microscope.

RESULTS

Normal middle ear morphology

The middle-ear mucosa of the rat (Fig. 1) is known to consist principally of flat polygonal epithelium, separated from the bony wall by a thin layer of fibrous tissue. Only sporadically

active osteoclasts can be perceived in the vicinity of the middle-ear bone (Fig. 2). Inferior and superior to the promontory, tracks of mucociliary epithelium can be observed. Part of this epithelium appears to alternate with polygonal cells (Fig. 3). In the neighbourhood of the round window niche and the tubal orifice, a number of these flat polygonal cells seem to be replaced by cells characterized by evident secretory activity.

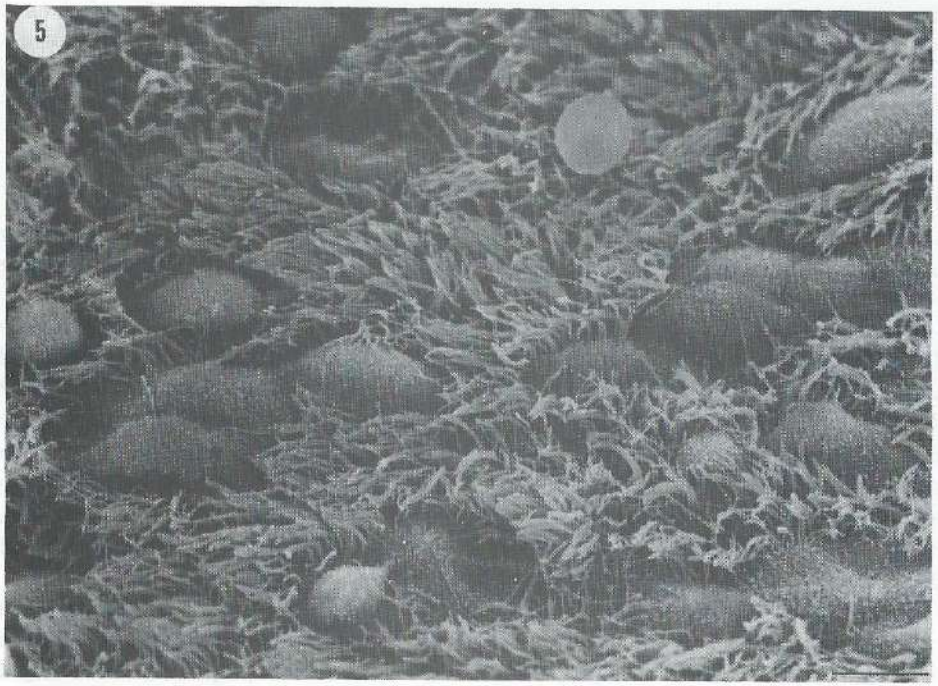
Infected middle ear morphology

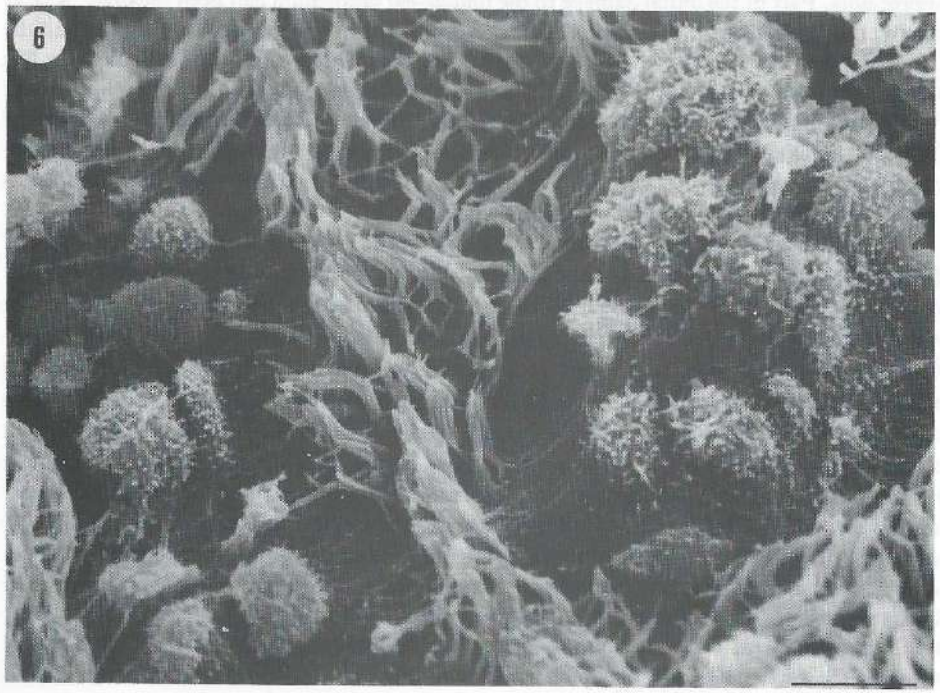
Originating with the introduction of *S. aureus* in the tympanum of the rat, a series of major events involving the lumen, the mucosa and the surrounding bone of the middle ear can be witnessed.

One day following upon intratympanical injection the middle-ear cavity becomes completely filled with mucopurulent fluid, containing a great number of neutrophilic granulocytes and considerably fewer lymphocytes. The same cells, including a number of macrophages, can be demonstrated in the lamina propria, affected by oedema and vasodilatation (Fig. 4). Though the middle-ear lumen suffers considerable damage from the *S. aureus* infection, parts of the middle-ear epithelium appear to be intact. It should be emphasized that these parts have also been subject to changes, such as swelling of the polygonal cells of the mucociliary tracks (Fig. 5). Osteoresorption, occurring on the border of the bulla and the middle-ear lumen, is one of the most striking phenomena.

Three days after infection was induced, part of the middle-ear lumen still appeared to be obliterated by mucopurulent fluid. In contrast to the onset of infection, macrophages could be demonstrated in the lumen. The proportion of cells in the lamina propria had not changed appreciably. Part of the initial damage to the middle-ear epithelium had been repaired. A large part of the originally flat epithelium had differentiated into secretory epithelium, as had the flat polygonal cells of the mucociliary tracks (Fig. 6). These cells had a cobblestone-like appearance and their activity could be demonstrated by the presence of large quantities of rough endoplasmic reticulum, mitochondria and vacuoles, as revealed by electron microscopy (Fig. 7). Though at the one-day stage some osteoblasts could be witnessed, these cells actually became prominent after 3 days, forming a layer separating the bony wall from the fibrous tissue. The number of osteoclasts decreased considerably.

One week after the onset of infection the situation in the middle ear seemed fairly stable. The amount of fluid had decreased to a small quantity, only partially covering the mucosa. Whereas the cells in this fluid were dominated by the presence of macrophages. In comparison with the earlier stages, the number of neutrophilic granulocytes had diminished in the lumen. A similar phenomenon could be seen in the lamina propria, where lymphocytes formed the majority of the cell population, although the number of macrophages almost equalled these cells. The main occurrence characterizing this stage of infection was the presence of a mucociliary track approximately twice as wide as in the non-infected situation. Scanning electron microscopy revealed almost the whole area covered by ciliated cells (Fig. 8). Transmission electron microscopy and histology, however, showed the presence of a vast number of secreting cells of goblet-cell-like appearance (Fig. 9). In some places the lamina propria had returned to its normal proportions and part of the bulla was covered by flat polygonal cells. Since the 3-day stage a large amount of bone was deposited by osteoblasts, still present in great numbers. The event, which became prominent during this stage and increased in the later stages, is the appearance of a fibroblastic ingrowth into the middle-ear cavity. This ingrowth





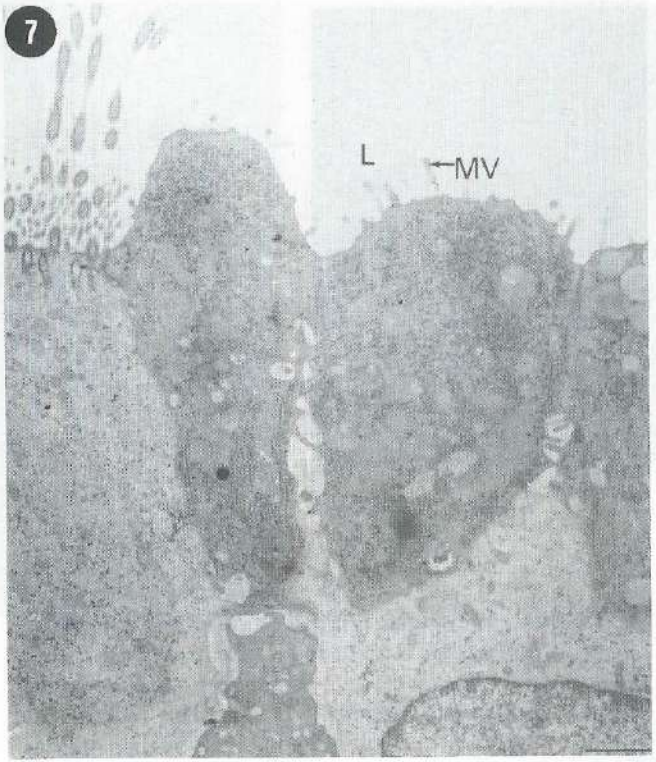


Fig. 7. Large quantities of endoplasmic reticulum, mitochondria and vacuoles, as shown by electron microscopy, suggesting secretory activity. Also microvilli *(MV)* penetrate into the lumen *(L)*. Bar represents 2 μm.

was covered by a cuboidal epithelium, part of which may be ciliated. Gland-like structures could be seen. This event may have originated in the growth of fibroblasts through the damaged epithelium into the middle-ear lumen, filled with mucopurulent fluid at the one-day stage. The ingrowth was clearly recognizable after 3 days and probably represents the predecessor of the structure present in the middle-ear cavity at one week after the onset of infection.

The next 3 weeks did not show any gross difference in comparison with the situation described one week after *S. aureus* infection. Mucopurulent fluid could only be encountered in cavities and despite the normal appearance of parts of the middle-ear epithelium the number of secreting cells was still greater than in the normal non-infected middle ear. Bone deposition was still a prominent feature, as was the fibroblastic ingrowth (Fig. 10).

Four weeks after the introduction of *S. aureus* into the middle-ear cavity, the effects originating with the onset of this infection had not yet ceased.

Fig. 5. Scanning electron microscopy reveals the swelling of originally flat epithelial cells, one day after the induction of infection. Bar represents 6 μm.

Fig. 6. Scanning electron micrograph of part of the mucociliary tracks, 3 days following the induction of infection. Note the prominent swelling of the originally flat polygonal cells. Bar represents 3 μm.

Fig. 8. A large part of the middle ear is covered by the mucociliary tracks one week after infection was induced. Bar represents 30 μm.

DISCUSSION

The experimental animal used throughout this study is characterized by a middle-ear epithelium (16, 23) very similar to that of the human ear in its histological and ultrastructural qualities (1, 12, 18, 19). Because of the relative resistance of the rat (6) to *S. aureus* initiated infection, the reaction caused by this micro-organism in the middle-ear cavity is mild. This characteristic facilitates the study and interpretation of the effects encountered in the middle-ear tympanum. Though *S. aureus* evoked infection produces a great number of effects, the authors wish to pay special attention to those reactions which in their opinion comprise the middle-ear defence system.

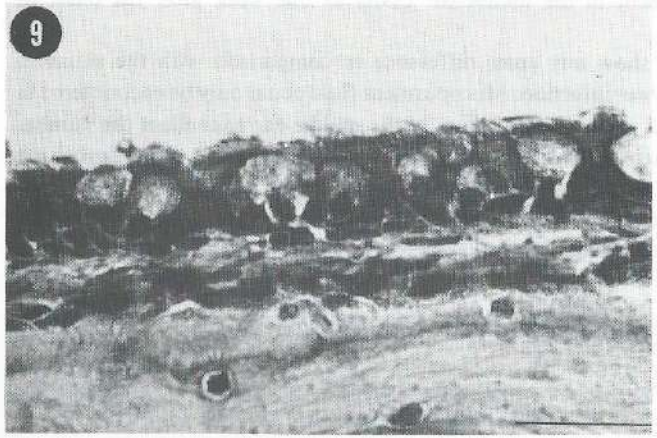


Fig. 9. The mucociliary tracks one week after the onset of infection. Note the presence of goblet cells. Bar represents 10 μm.

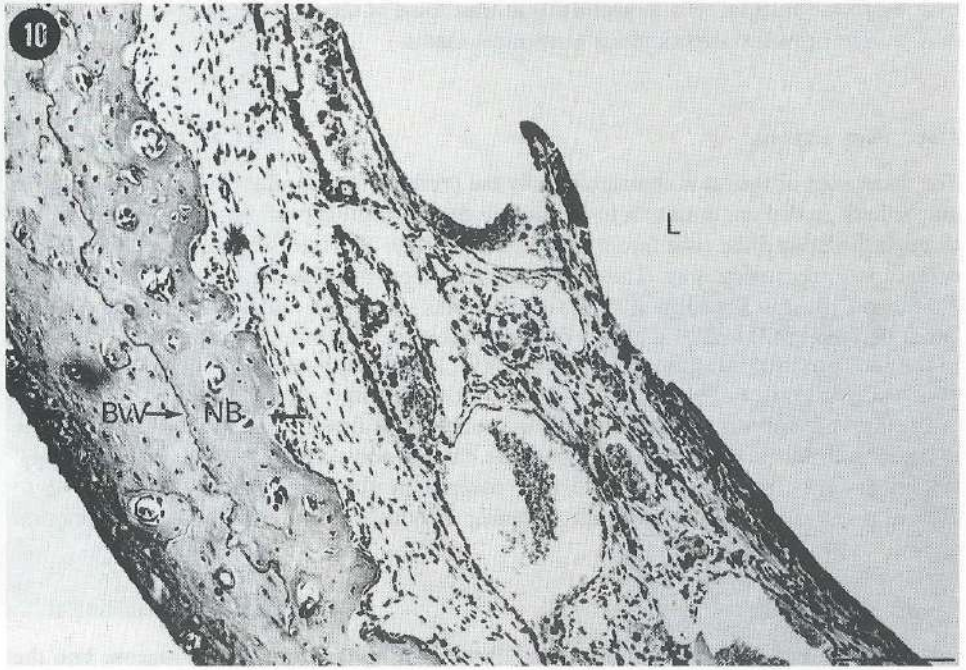


Fig. 10. Three weeks after infection was initiated, fibroblastic ingrowths into the lumen *(L)* are a prominent feature. The amount of newly deposited bone *(NB)* is impressive *(arrows)*. *BW* = original bulla wall. Bar represents 70 μm.

Cellular response

Cellular response (10) could be demonstrated as the presence of phagocytic macrophages and neutrophilic granulocytes (9) was evident. Neutrophilic granulocytes appeared to be the most prominent cells following the onset of infection, followed by lymphocytes and macrophages. As the duration of the infection was prolonged, the proportion of neutrophilic granulocytes decreased, whereas the percentage of macrophages and lymphocytes increased.

Macrophage phagocytic activity is not limited to bacteria but also affects mucoid elements and cell debris. Similar events were observed by Lim & Klainer (1971) (17).

Humoral response

Earlier studies (13, 24, 30) demonstrated an elevated level of IgA, IgG and IgD in middle-ear effusions. The presence of IgE is still a subject of controversy. Except for a rise in the immunoglobulin level, complement activation is suggested (25) and a change in lactate dehydrogenase and lysozyme level could be observed in middle-ear effusions originating from experimental otitis media (14). Lysozyme was also observed in mucosal cells (19). This study did not use techniques to demonstrate any of the previously mentioned substances. However, the presence of lymphocytes and neutrophilic granulocytes, together with a distinct secretory activity

by some of the mucosal cells, suggests that at least some of these substances must be produced in *S. aureus* provoked experimental acute otitis media.

Mucociliary response

The middle ear of the rat is characterized by the presence of mucociliary tracks (23, 31). This mucociliary epithelium is known to be capable of transporting particles from the middle ear through the Eustachian tube into the nasopharynx (28). The tracks contribute to middle-ear defence in a mechanical way. The results obtained from this study are consistent with those published by Lim & Klainer (1971) (17) as the tracks react upon middle-ear infection with an initial decrease, followed by a clearly distinguishable increase after approximately one week. In the early phase of infection the tracks may also lose some of their activity due to the effect of proteolytic enzymes on the composition of the mucus layer (3).

To sum up-it appears as if the mucociliary tracks are at first inhibited in their function due to a decrease in their area, due at least partly to damage and probably by proteolytic enzymes. Within a week's time, however, the area covered by the mucociliary tracks increase to approximately twice their original size. Whether or not the mucus is of normal composition remains to be investigated.

Fibroblastic response

One week after the induction of infection, fibroblastic ingrowths from the mucosa into the middle-ear lumen became prominent. Precursors of the ingrowths seemed to be present already 3 days after the onset of infection. They may have originated in the epithelial damage caused by the initial events of the infection, thus showing a similarity to the formation of mucosal cysts as proposed by Arnold (1977) (1). Structures similar to those demonstrated in our study were also observed by other authors in studies on experimental otitis media (16, 17). The functional aspects of these ingrowths with respect to middle-ear defence may reside in an isolatory effect or in an effect on the oxygen tension, thus creating less favorable conditions for the infectious organism (29).

Bony response

The middle-ear bone appears to be subject to two different mechanisms during acute experimental infecton. In the first stage, osteoresorption is prominent, followed by osteogenesis in the later stages of infection. The cause of the initial osteoresorption may be found in a pH shift due to the exudative reaction following upon the induction of infection, but might also be caused by osteoclastic activity. The presence of a pH shift and its effects on the middle-ear bone were not investigated, but might be present. An osteoclastic reaction could be observed, however.

Three days after infection, osteoblastic activity was evident, resulting in impressive osteogenesis after one week of infection. Similar results were witnessed by Lim & Klainer (1971) (17) and Kuypers et al. (1979) (16). The function of the new bone formation may be reconstructive and/or isolatory.

To sum up, it may be stated that *S. aureus* induced infection in the tympanic cavity of the rat is very similar to the events witnessed in the human middle ear affected by acute otitis media or even by otitis media with effusion. This way of achieving infection and the techniques used to evaluate the effects of such an infection seem to offer a suitable technique by which to study these diseases in a reproducible experimental manner.

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CHAPTER IV

Epithelial Reactions To Hydroxyapatite

An In Vivo And In Vitro Study

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SUMMARY

This study aimed to investigate the epithelial reactions to hydroxyapatite ceramic *in vivo* and *in vitro*. Shortly after implantation in the rat middle ear, hydroxyapatite was covered by a mucosal layer. In the early post-operative period the implant was almost completely covered by epithelial cells, which were found to proliferate and also showed migratory activity. After longer intervals the implant was completely covered by epithelium, which was predominantly composed of flat polygonal cells and a relatively small number of ciliated epithelium and goblet cells. All cells showed normal morphology.

In vitro experiments showed preservation of the morphology of rat middle-ear mucosa explants with good outgrowth of epithelial cells. In these outgrowths, the majority of the cells were flat polygonal, but ciliated epithelium was also seen. No difference was found between the absence and presence of hydroxyapatite. Serially cultured cells showed normal polygonal morphology, but no ciliated cells were found. Ciliated cells were also absent in control experiments without hydroxyapatite. Growth curves obtained in the absence and presence of hydroxyapatite did not differ significantly from each other.

One of the biomaterials recently in use in reconstructive middle-ear surgery is hydroxyapatite, a calcium phosphate ceramic whose stoichiometry and crystal structure resemble those of the bone mineral matrix (1). A number of reports indicate that hydroxyapatite seems to be a promising biomaterial for otologic surgery (2-5). None of these reports, however, are especially concerned with the epithelial covering of the implants.

The middle-ear epithelium can be considered to be part of the upper-airway epithelium, and is characterized by the presence of ciliated and goblet cells, although the greater part of the middle-ear cavity is covered by flat polygonal epithelial cells (6-8). One of the important functions of the middle-ear epithelial cells is their role in the host's defence against infection (9-11). An example of this defence activity is the mucociliary transport system (12).

Since infection is frequently encountered in middle-ear surgery, it seems of special interest to assess biomaterials used in middle-ear surgery in relation to their epithelial covering. Therefore, in the study reported here attention was paid to the light-microscopical and scanning and

transmission electron-microscopical characteristics of the epithelial covering of hydroxyapatite middle-ear implants. The epithelium was studied in two experimental set ups, in one of which hydroxyapatite was implanted in the rat middle ear and in the other the epithelium/hydroxyapatite interaction was studied *in vitro*. In spite of the fair number of *in vitro* tests on biomaterial performed so far (13), we felt that such materials are best tested in cultures of the tissue in which it would be implanted and by which it would eventually be covered. Therefore, we developed a new technique to assess the combined behaviour of the material and rat middle-ear epithelium in tissue cultures.

MATERIALS AND METHODS

In vivo studies

Experimental approach: Hydroxyapatite was implanted in the rat middle ear (male Wistar rats, mean body weight 200 g) at two sites: some of the implants were used to obliterate part of the middle-ear cavity, and a second group served to close the resulting defect in the bony middle-ear bulla. The post-operative periods chosen for the study ranged between one week to one year.

Implant material characteristics and distribution of the implants: Both macroporous and dense hydroxyapatite were used. The former is characterized by > 5% microporosity (pore ∅ *ca.* 3 μm) and 26% macroporosity (pore ∅ *ca.* 100 μm), and the latter has > 5% micropores and no macropores. Of the 236 implants studied, 120 were macroporous and 116 were dense implants. The exact distribution of the implant material has been published elsewhere (14), and the same holds for the sintering technique (15).

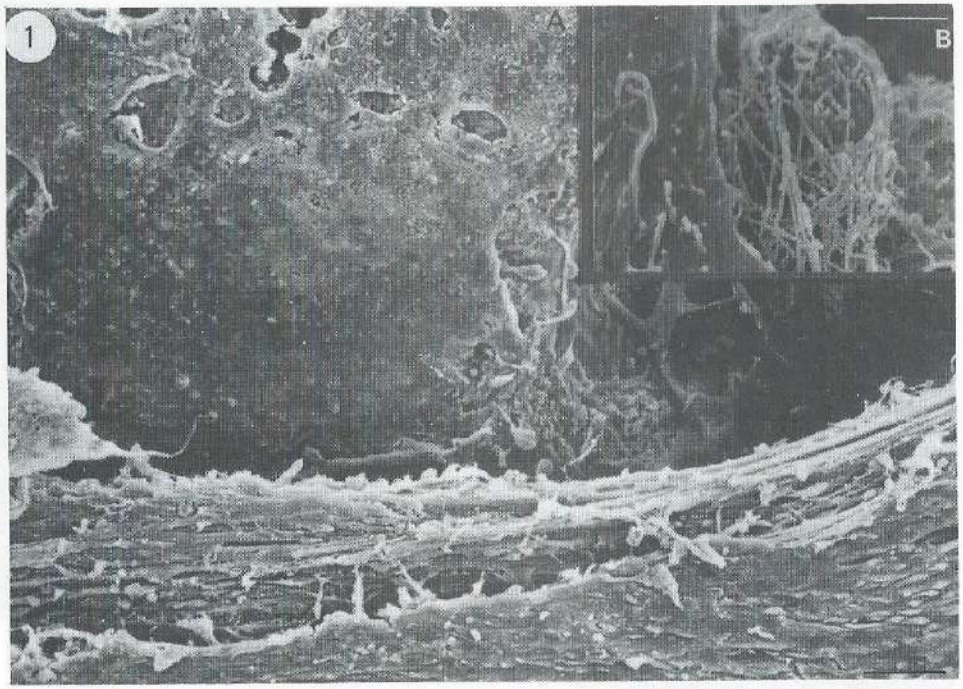
Light and electron microscopy: After dissection, the middle ears were fixed in a 1.5% glutardialdehyde solution in sodium cacodylate buffer (pH 7.4). The specimens destined for routine light microscopy were decalcified (EDTA 10%, 1.5% glutardialdehyde, ph 7.4), rinsed, dehydrated, embedded in Paraplast, and cut into 7 μm-thick sections. The material to be used for transmission electron microscopy was decalcified, post-fixed in osmium tetroxide (1%) at room temperature, dehydrated, and embedded. Ultrathin sections were examined in a Philips 200. The specimens destined for scanning electron microscopy were not decalcified but dehydrated immediately after the glutardialdehyde fixation and then critical point dried and gold coated. A Cambridge S 180 scanning electron microscope was used.

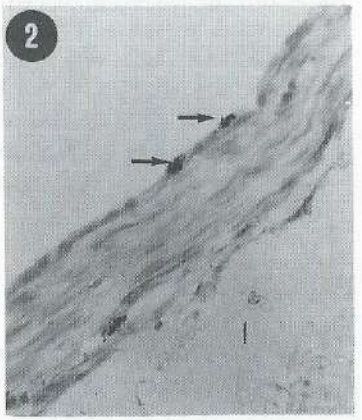
Fig. 1a. Low-power scanning electron micrograph of the hydroxyapatite/tissue interface 2 weeks after implantation. Epithelial tissue, fibrous tissue, and phagocytes covering the implant surface. Bar: 30 μm.

Fig. 1b. Detail of an epithelial cell on the fibres of the fibrous tissue. Bar: 3 μm.

Fig. 2. Light-microscopical autoradiograph of the mucosal covering of an implant *(I)* after short post-operative time. Note the incorporation of tritiated thymidine (arrows).

Fig. 3. Graph showing the results of the quantification of the autoradiographic findings. The average number of counts per histological section of an implant is plotted against time for epithelium, fibrous tissue, and bone.





Autoradiography: Some of the rats were injected with a solution of tritiated thymidine (0.1 μ Ci/g body weight) one hour before they were killed. The middle ears were treated as for light microscopy and the 7 μm sections were coated with K 5 emulsion, developed, and analysed.

In vitro studies

Explants: Small pieces (*ca.* 1 mm^2) of rat middle-ear mucosa were dissected from the bulla and placed on dense hydroxyapatite (*ca.* 3.4 mm^3). They were allowed to attach for 24 hr in a small drop of Dulbecco's modified Eagles medium and F 12 (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 after 4 days of culture epidermal growth factor (10 ng/ml) was added to the medium.

Explants and any epithelial outgrowths were studied after 1 and 2 weeks of culture. Phase contrast microscopy and scanning and transmission electron microscopy (SEM and TEM) were used. The methods applied for SEM and TEM were almost the same as those used for the *vivo* material. For the control experiments explants were cultured on the surface of the dish. At least 6 pieces of hydroxyapatite were used for each experimental technique and culture time.

Serial cultivation: Explants and 2-week-old outgrowths were treated by trypsin flotation, and the resulting cell suspension was plated at a density of 2×10^4 cells/cm^2. In the same dish, 1.2×10^4/cm^2 lethally irradiated 3T3 feeder cells were plated at the same time. The medium was identical to that used for the explants. After trypsinization the cells could be serially cultured and all studies with hydroxyapatite were done within the first 7 passages.

Cell morphology was studied by phase contrast microscopy and scanning and electron microscopy after 1 and 2 weeks of culture. Growth curves of serial cultures in the absense of hydroxyapatite and in the presence of 4 pieces of hydroxyapatite are based on cell counts in 60 mm dishes after 1, 4, 6, 10, and 14 days.

RESULTS

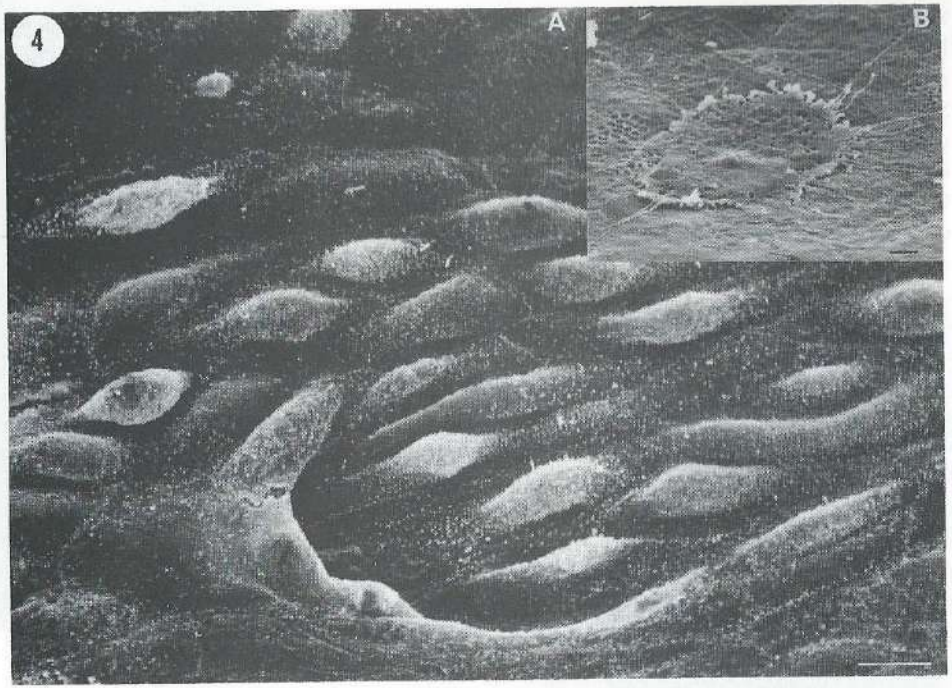
In this study two approaches were used to investigate the epithelial reactions to the presence of hydroxyapatite: one concerned the *in vivo* behaviour of epithelium in response to hydro-

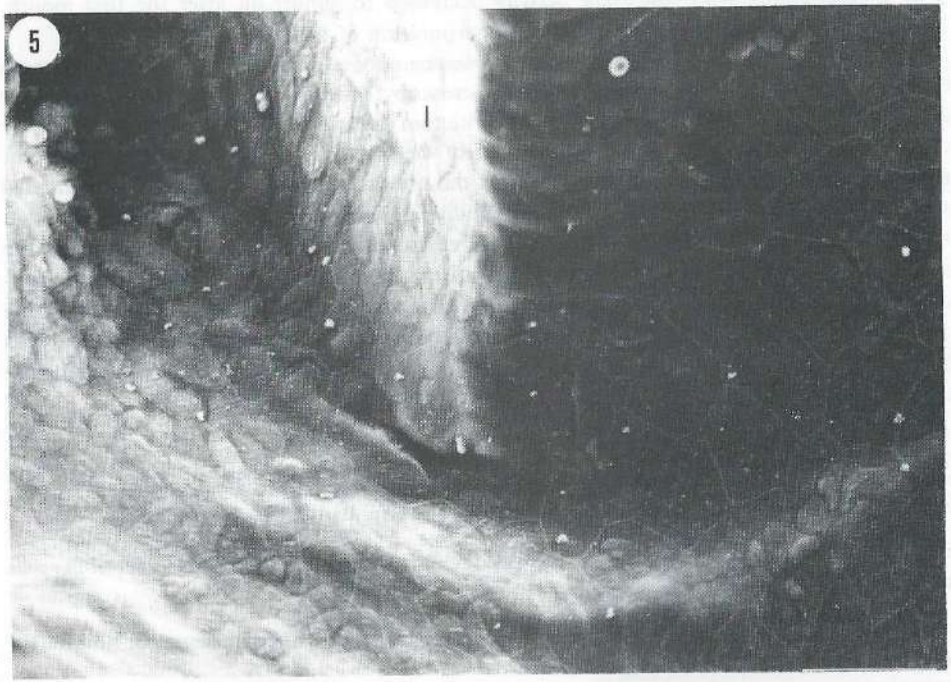
Fig. 4a and b. Scanning electron microscopical pictures of the epithelium on the hydroxyapatite implant, 2 weeks after implantation.

Fig. 4a. The cells have a cobble-stone-like appearance and some primary cilia are visible.

Fig. 4b. Flat adherent cells are present on the implant surface. Bar (a): 5 μm, Bar (b): 3 μm.

Fig. 5. Scanning electron micrograph showing the similarity between the middle-ear tissue and the tissue on the implant *(I)* 4 weeks after implantation. Bar: 30 μm.





xyapatite implanted in the rat middle ear and the other an evaluation of the *in vitro* characteristics of rat middle-ear epithelium cultured on hydroxyapatite. In the *in vivo* work both dense and macroporous forms of hydroxyapatite were used as obliterative and occlusive implants. Since there was no significant difference in the epithelial reactions to the macroporous and the dense material, they will be discussed together. During the *in vivo* studies the obliterative and occlusive implants differed initially, mainly in that events generally occurred somewhat later in the obliterative group. However, since this divergence disappeared in subsequent intervals, only the over-all epithelial reactions will be discussed.

In vivo results

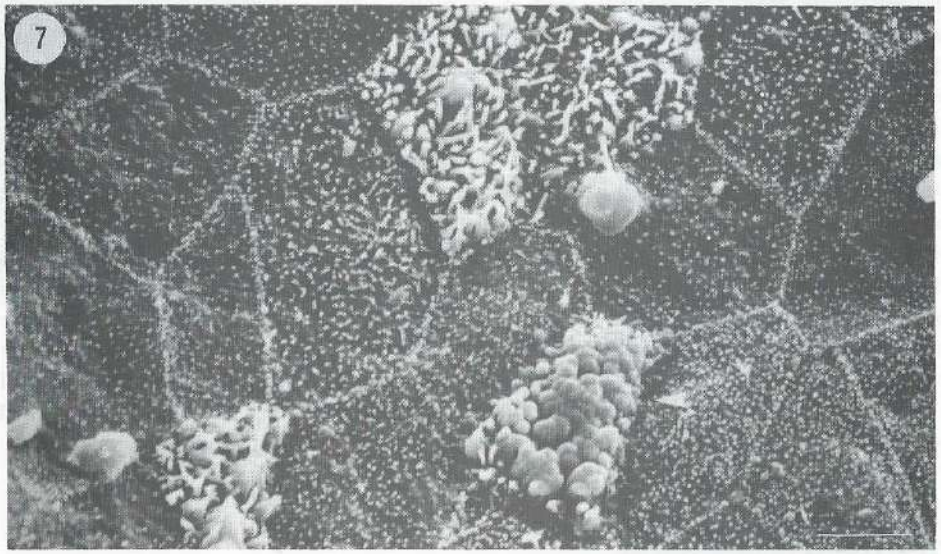
With respect to the tissue present on the implants in the early post-operative intervals, a distinction was made between three structures: 1) a layer of exudate cells, mainly macrophages and multinucleated cells as shown by the ultrastructural morphology, at the hydroxyapatite/tissue interface; 2) a surrounding layer of fibrous tissue containing capillaries, fibroblasts, and collagen fibres, which was thickiest after a week and then became thinner; 3) an epithelial layer covering the fibrous tissue. Because the whole implant was not covered everywhere by all three layers, SEM showed areas where the surface of one of them was exposed and could be studied (fig. 1a). This situation suggests that as the implant became covered, epithelial cells migrated over the filament bundles of the fibrous tissue (fig. 1b). The autoradiographic results (figs. 2 and 3) revealed a peak in the proliferative activity of the epithelial cells during the first week after implantation, after which the activity decreased to almost nil after the first month. Fibrous tissue showed a similar pattern of incorporation of tritiated thymidine.

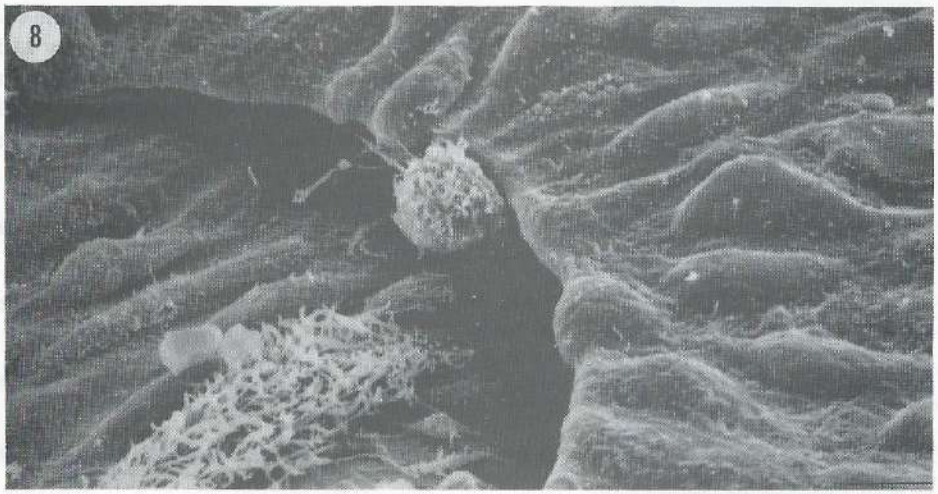
The epithelium covering the hydroxyapatite was composed predominantly of flat polygonal cells, as demonstrated by scanning electron microscopy. Initially, however, cobble-stone-like epithelium was also present (fig. 4a) together with thin cells with long filopodia extending over the epithelial surface. It could not be determined whether the latter cells were of exudative or epithelial origin. A less dominant feature was the presence of primary cilia on the epithelial surface. With increasing time the cobble-stone-like epithelium disappeared and the implant was predominantly covered by flat polygonal epithelium very similar to that seen on the middle-ear wall (fig. 5). This similarity was confirmed not only by the transmission electron-microscopical observations (fig. 6), which showed a thin epithelium with microvilli on the surface that was also clearly visible in the SEM material (fig. 7). Besides the flat polygonal cells there were goblet cells (fig. 7) and ciliated cells (fig. 8), especially on implants bordering the mucociliary tracks of the middle ear.

Fig. 6. Part of the epithelial covering on hydroxyapatite as seen by transmission electron microscopy. Note the microvilli and the collagen fibres in the fibrous tissue. Bar: 0.5 μm.

Fig. 7. Epithelium covering the implant after 13 weeks. Cells with abundant microvilli and goblet-like cells are present (arrow). Bar: 5 μm.

Fig. 8. A solitary ciliated epithelial cell on the implant surface 2 weeks after implantation. Note the exudate cell. Scanning electron micrograph. Bar: 5 μm.





Culture results

Two methods were used to assess the *in vitro* biocompatibility of hydroxyapatite. For the first, small pieces of rat middle-ear mucosa (*ca.* 1 mm²) were cultured on hydroxyapatite. The second concerned assessment of the effect of hydroxyapatite on serially cultivated middle-ear epithelium.

The use of explant cultures showed good attachment to the hydroxyapatite and all cell types characteristic for the middle ear were observed. In SEM and TEM the morphology of the cells resembled that of the original middle-ear epithelium, although some of the cells appeared slightly contracted. An interesting feature of these cultures was the peripheral outgrowth of epithelial cells from the explants. Such outgrowths, which sometimes reached impressive proportions, consisted mainly of flat polygonal cells with varying numbers of microvilli. In these outgrowths the presence of relatively large areas completely occupied by ciliated epithelium was a striking occurrence. SEM generally revealed the normal features of this epithelium (fig. 9), although ciliated cells with clustered or fused cilia were sometimes found. The ultrastructure of the cells in the outgrowth was studied by TEM (fig. 10), which showed thin cells with abundant ribosomes, a well-developed endoplasmic reticulum, and microvilli on the cell surface. Occasionally an electron-dense structure was also found at the hydroxyapatite/tissue interface (fig. 11). The morphology of this structure suggested that hydroxyapatite had been present there. In the vicinity of this structure only collagen was present. The morphology of these cells in serial cultures of rat middle-ear epithelium resembled that of the cells in the outgrowth of the explants. However, no ciliated cells were encountered in either the absence or presence of hydroxyapatite. An advantage offered by serial cultivation is the possibility of establishing growth curves. Comparison of the cell number between culture dishes with and without hydroxyapatite for several culture periods (fig. 12), showed no differences.

DISCUSSION

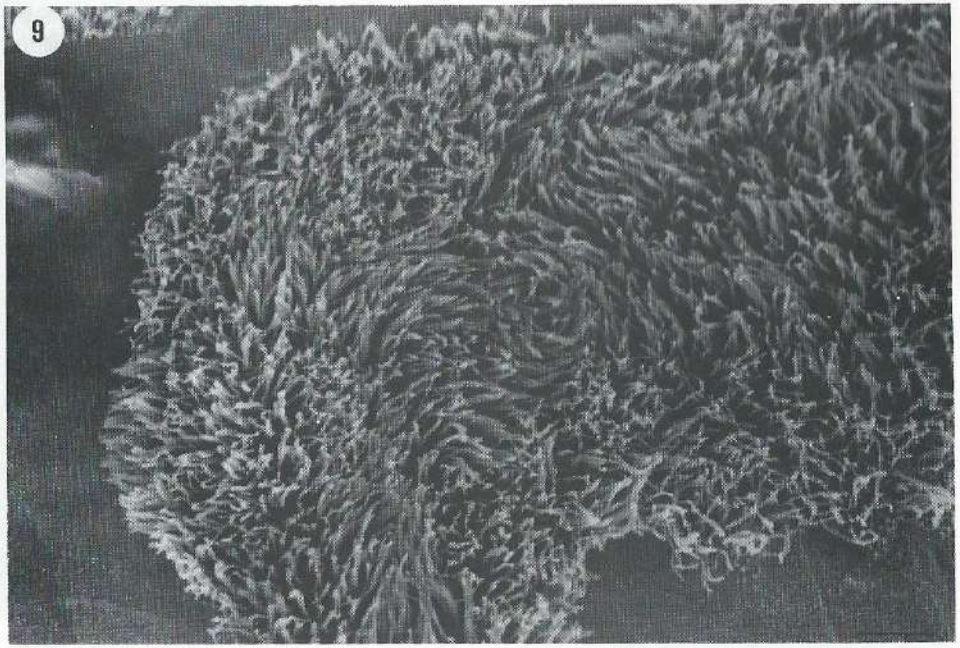
At present, a fair number of biomaterials have been used in reconstructive middle-ear surgery (2, 16-21) with varying success, which explains the continuing search for new and better materials. Recently, ceramics have attracted interest. Among these, hydroxyapatite has the closest resemblance to the mineral matrix of human bone (1) as to both stoichiometry and crystal structure. Because of this resemblance it seemed promising for use as a bone substitute

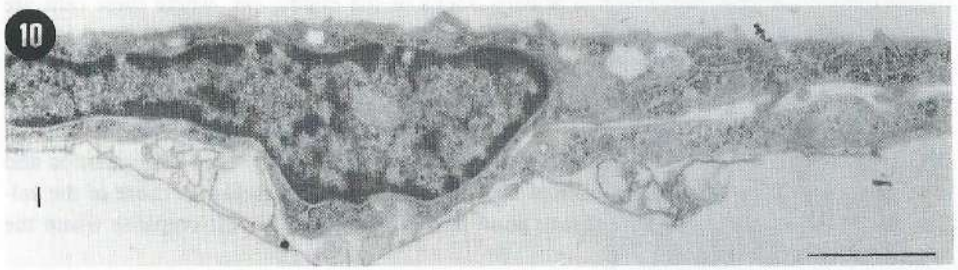
Fig. 9. Scanning electron-microscopical view of the outgrowth from an explant of rat middle-ear mucosa cultured on hydroxyapatite. Large areas of ciliated epithelium can be seen. Bar: 5 μm.

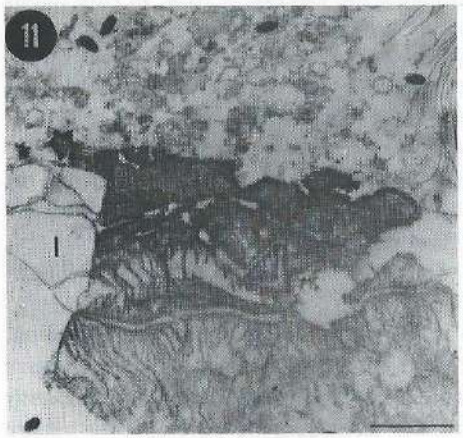
Fig. 10. Transmission electron micrograph of an epithelial cell in the outgrowth from an explant. The cell has a thin appearance. Implant *(I)*. Bar: 1 μm.

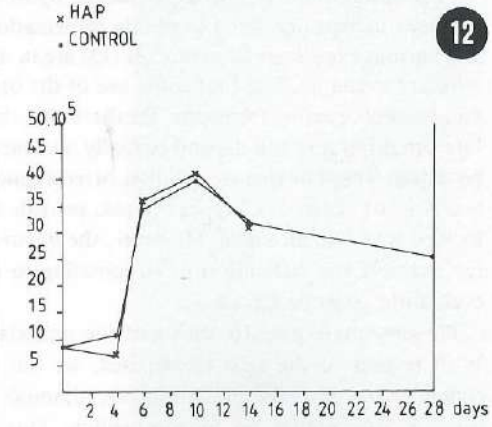
Fig. 11. Electron-dense structure at the hydroxyapatite/explant interface. The ultrastructural features suggest that calcium phosphate had been present before decalcification. *I* = implant. Bar: 1 μm.

Fig. 12. Growth curves of serial cultures of middle-ear epithelial cells in the absence and presence of hydroxyapatite.









in otologic surgery, and a number of studies were performed in which this material was implanted in animals and subsequently evaluated (2-5, 14, 22). These studies confirmed the promising features of the material. On the basis of the experimental findings, some clinical trials were started (23-25). These studies too confirmed the good biocompatibility of the material. However, in spite of the relatively large number of studies devoted to hydroxyapatite as otologic biomaterial, none of them dealt extensively with the epithelial covering of the material. Nevertheless, the epithelial covering may be considered to be of significance for the success of an implant in middle-ear surgery, since epithelium is considered to play an important role in middle-ear defence. The lack of experimental results concerning the covering of hydroxyapatite by middle-ear epithelium led us to perform the present study in which the behaviour of the epithelium under these conditions were investigated *in vivo* and *in vitro*.

The results show that in the middle ear of the rat, hydroxyapatite becomes covered by an epithelial layer which, depending on the localization of the implant in the middle-ear cavity, can already cover large parts of the implant one week post-operatively. The epithelial cells eventually covered the whole implant and the surrounding lamina propria. The covering was effected by migration and proliferative activity of the epithelial cells, as demonstrated by scanning electron microscopy and autoradiography, respectively. In the early intervals studied, cobble-stone-like epithelial cells were frequently observed but after that their number decreased, and the implant became covered predominantly by flat polygonal cells which did not differ from those of the squamous epithelium of the normal rat middle ear (7, 8, 10). In addition to the flat polygonal cells, ciliated and goblet cells were found in the epithelial covering of the implant, mainly near the mucociliary tracks of the middle ear, where these cells are normally present.

The presence of goblet cells and ciliated cells on the implant surface showed that hydroxyapatite tolerates the presence of well-differentiated cell types. This observation is of significance with respect to the role of the epithelium in the middle-ear defence mechanism (10, 26), since the *in vivo* results demonstrate that both the composition of the cell population and the morphology of the cells on implanted hydroxyapatite are very similar to those of the epithelium of the middle ear. This suggests good biocompatibility of hydroxyapatite where the epithelium is concerned.

In addition to the *in vivo* experiments, hydroxyapatite was assessed *in vitro*. *In vitro* testing has been increasingly used to obtain information about the biocompatibility of biomaterials, and various experimental protocols (13) are in use at present. Two ways to test biomaterials *in vitro* are available. The first is the use of the organ cultures and/or explants, the second uses monolayers or cell suspensions. Furthermore, the way the implant material is added to the culture can differ and will depend partially on characteristics of the material and the properties to be tested. These *in vitro* techniques offer a quick and relatively sensitive method to study the reactions of isolated cell types but also provide the only way in which experiments can be performed with human tissue. However, the *in vitro* techniques have some disadvantages as well, for example the difficulty of extrapolating to the *in vivo* situation and the impossibility of evaluating systemic effects.

We gave preference to testing of the material in the same form as used for implantation. With respect to the cells to be used, we felt that a material should be tested with tissue characteristic of the implantation site, although cultures of, for instance, fibroblasts certainly provide information on biocompability. One of the techniques we developed to culture

middle-ear epithelium of the rat concerned explants of middle-ear mucosa and the other the use of serial cultivation of rat middle-ear epithelium. The former seemed especially suitable for the evaluation of the effects on cell morphology, whereas serial cultivation permitted quantitative analysis of the effects on cell number. Culturing of the explants on hydroxyapatite showed not only that the morphology of all middle-ear cell types underwent no changes but also that outgrowth of epithelial cells occurred. In these areas ciliated epithelium was observed, indicating the presence of well-differentiated epithelial cells.

An interesting feature in the explant experiments was the occasional presence of an electron-dense structure at the hydroxyapatite/explant interface. The morphology of this structure suggested that hydroxyapatite had been present before decalcification was performed. Apparently, calcium phosphate is deposited on the hydroxyapatite surface under *in vitro* conditions, as had been established for hydroxyapatite *in vivo* even more clearly (14).

The second *in vitro* technique, i.e., the use of serially cultured cells, allowed quantitative analysis of the biocompatibility of hydroxyapatite. The results show hardly any divergence from the growth curves of the controls. This finding, together with the earlier *in vitro* observations, established the good biocompatibility of hydroxyapatite with respect to rat middle-ear epithelium.

In sum, it may be concluded that hydroxyapatite is rapidly covered by epithelial cells and permits differentiation into goblet and ciliated cells, which is of special importance for the middle-ear defence system. These features can be added to the other promising qualities of hydroxyapatite for use in reconstructive middle-ear surgery.

ACKNOWLEDGEMENTS

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CHAPTER V

Biointeractions At The Tissue/Hydroxyapatite Interface.

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SUMMARY

The events at the hydroxyapatite implant material/tissue interface in the rat middle ear were studied by light microscopy, autoradiography, morphometry, transmission electron microscopy, scanning electron microscopy, and X-ray microanalysis. Deposition of calcium, partially in the form of calcium phosphate, was found at the interface. Resorption of the implant material occurred as the result of mono- and multinuclear phagocyte activity. Resorption decreased six months after the operation, possibly due to the decreasing number of phagocytes at the interface and the increasing amount of bone in the macropores.

In middle-ear surgery it may be necessary to remove vital parts of the sound-conducting system. The resulting hearing loss can be restored by reconstructive middle-ear surgery. The use of biomaterials in such cases may improve the reconstruction. The material discussed in this paper, sintered hydroxyapatite, closely resembles the mineral matrix of human bone (1) and therefore seems promising for use in reconstructive tympanoplasty. Its suitability was confirmed by histological investigation in the middle ear of the rat (2). However, more information is needed. The present study was performed to add to the available information about hydroxyapatite ceramics by investigating the interactions between implant material and host tissue at the interface after implantation of the material in the rat middle ear.

The events at the interface (3) between an implant material and the adjacent tissue are for one the direct result of the cellular, chemical, physiological, and mechanical reactions evoked by the presence of the biomaterial (4), and for the other the result of the surgical procedure, the wound reactions (5), and the response of the tissue in the vicinity of the implant. Although not all phenomena at the implant/tissue interface are caused by the implant itself, the biomaterial is of considerable significance for the properties of this zone. Therefore, study of an interface will provide data concerning biocompatibility, biofunctionality, bioactivity, and biodegradation.

With respect to the reactions at the interface, a distinction can be made between bioinert, biotolerant, and bioactive materials. The differences observed between the interface of these three categories of material (6) are due to the surface chemistry of implant materials, which may affect, for example, protein precipitation (7) and osteogenesis (8).

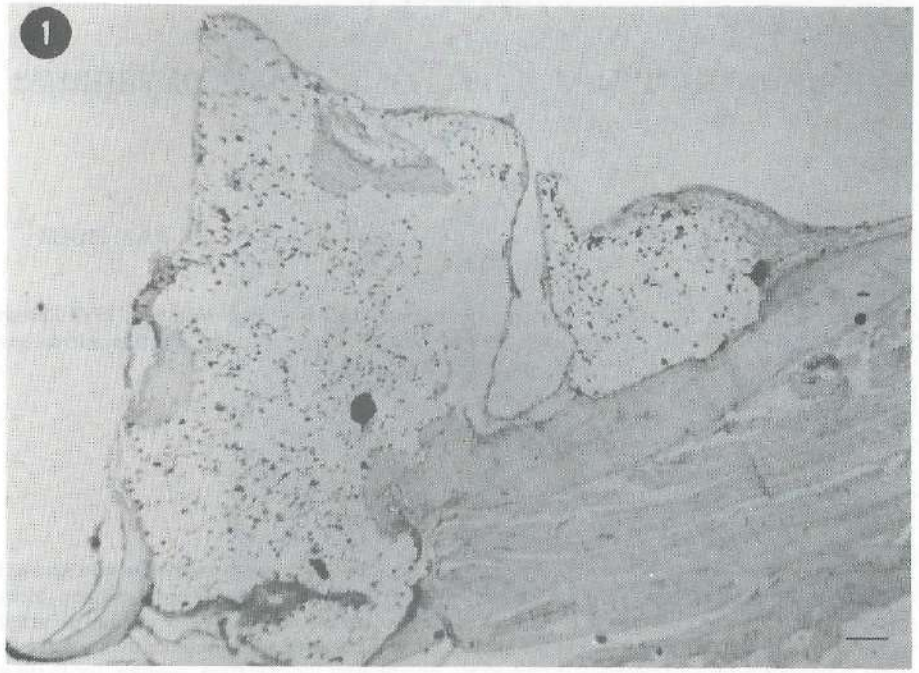


Fig. 1. Macroporous hydroxyapatite six months after implantation in the rat middle ear. The pores are filled with bony and fibrous tissue. Bone is in direct contact with the implant, which is covered by a thin mucosa-like layer. Bar: 100 μm.

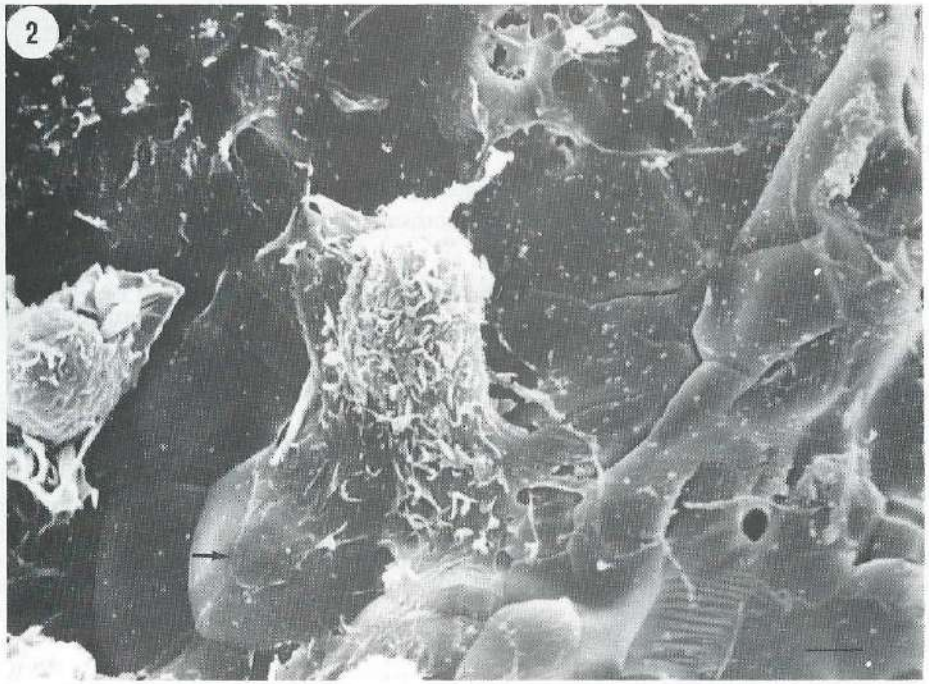
The interactions between cells adhering to the implant surface and the biomaterial are not only of significance for the connection of the implant with the surrounding tissue but may also lead to biodegradation, which can be caused by phagocytic and enzymatic activity of macrophages and the multinucleated cells resulting from their fusion (9, 10).

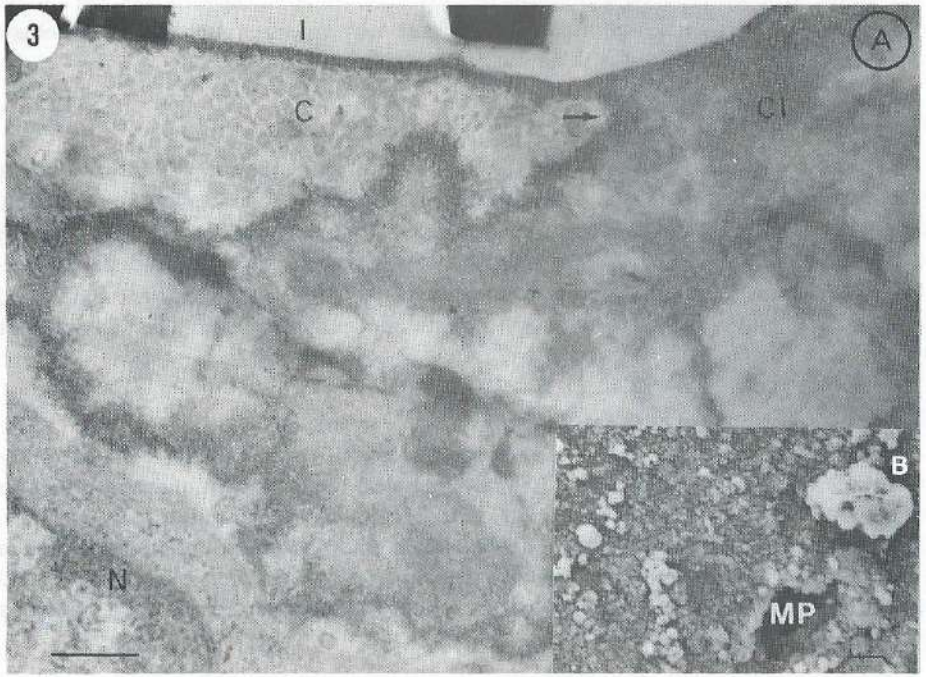
To obtain more insight into the interface phenomena associated with hydroxyapatite implant materials, we made use of routine histology, morphometry, autoradiography, and transmission and scanning electron microscopy. To determine the interface composition, X-ray microanalysis was applied.

Fig. 2. Scanning electron micrograph of the implant surface 1 week post-operatively. Note the adhering cell and the thin adherence zone (arrow). Bar: 3 μm.

Fig. 3a. Electron-dense layer covering the implant *(I)*. Note confluence (arrow) with the lamina limitans of the calcification island *(CI)*. *C* = collagen, *N* = nucleus. Bar: 0.5 μm.

Fig. 3b. Granular layer on the implant surface with dispersed protrusions. Scanning electron micrograph. *MP* = micropore. Bar: 3 μm.





MATERIALS AND METHODS

Implant materials

Two kinds of hydroxyapatite implant material were used, both with the stoichiometry $Ca_{10}(PO_4)_6(OH)_2$:

1) Macroporous sintered hydroxyapatite characterized by a macroporosity (pore diameter ca. 100 μm) of approximately 26% and a microporosity (pore diameter ca. 3 μm) of less than 5%.

2) Dense sintered hydroxyapatite having no macropores and a microporosity of less than 5%.

The technique used for sintering of these materials has been published elsewhere (11).

Animals and operation technique

Use was made of male Wistar rats (mean weight at operation 200 g). For implantation a small hole was burred in the bony middle-ear wall and a piece of hydroxyapatite was inserted into the middle-ear cavity, where it was in contact to the middle-ear mucosa, after which the defect was closed with another hydroxyapatite implant, in contact to the middle-ear bone.

The middle ears were investigated after various post-operative periods. Table I shows the distribution of the implant materials over the survival times.

Histology

The middle ears were fixed in a solution of 1.5% glutardialdehyde in cacodylate buffer (pH 7.4,4°C) for 16 hours. After decalcification (EDTA 10%, glutardialdehyde 1.5%, pH 7.4) for four weeks under constant agitation, the material was processed for routine histology. Paraplast sections were stained with haematoxylin eosin.

Transmission electron microscopy

Fixation and decalcification were performed in the same way as for histology. The specimens containing the implants were dissected, washed in phosphate-buffered physiological saline (pH 7.4) and post-fixed in a phosphate-buffered solution of osmium tetroxide (1%, pH 7.4) at room temperature, dehydrated in graded alcohols, and embedded for transmission electron microscopy. Ultrathin sections were cut on a Reichert Om U_2 ultramicrotome and stained with saturated aquous solutions of uranyl acetate and lead hydroxide. The sections were examined in a Philips 200 S electron microscope.

Back-scatter and scanning electron microscopy

The middle ears destined for scanning electron microscopy were carefully rinsed in phosphate-buffered physiological saline (pH 7.4, 0°C) to clear the epithelium of mucus prior to a two-hour period of fixation in 1.5% glutardialdehyde in cacodylate buffer (pH 7.4, 4°C). After fixation the material was washed, dehydrated in graded alcohols, critical point dried in carbon dioxide, and gold coated. The biomaterials were studied with a Cambridge S 180 scanning electron microscope. The hydroxyapatite used for back-scatter electron microscopy was prepared in the same way.

X-ray microanalysis

Some of the samples of implant materials, prepared as for scanning electron microscopy, were studied with a Philips 400 provided with a scanning (transmission) unit, equipped with a Tracor Northern (TN) 2000 X-ray analyser. Image formation, single-spot analysis, and X-ray mapping were performed at a standard accelerating voltage of 20 KV. Due to the gold coats, some of the counts in the Kα peak of the phosphorus had to be derived from the Mα gold peak.

Autoradiography

For autoradiography, some of the animals were killed by decapitation one hour after intraperitoneal injection of tritiated methyl thymidine (specific activity 25 curies/mmol) at a dose of 1 μCi per gram body weight. Routine autoradiographical techniques were used, and sections were stained with methyl-green pyronin.

Morphometry

Four hundred and twenty-seven histological sections of macropores, distributed over the survival periods, were photographed at a standard magnification. The macropore area was determined with an x-y tablet.

TABLE I.

Distribution of the implant materials over the survival time.

SUR	(w)	1	2	4	13	26	52	TOT
HIS	D	12	12	12	12	12	10	70
	M	12	12	12	12	12	12	72
TEM	D	4	4	4	4	4	4	24
	M	4	4	4	4	4	4	24
SEM	D	4	4	4	4	4	2	22
	M	4	4	4	4	4	4	24
TOT	D	20	20	20	20	20	16	116
	M	20	20	20	20	20	20	120

SUR = survival time in weeks, HIS = implants destined for histology, TEM = implants destined for transmission electron microscopy, SEM = implants destined for scanning electron microscopy, TOT = total number of implants, M = macroporous, D = dense.

RESULTS

Initially, part of the implant was covered by a fibrous envelope on which an epithelial layer could be seen. In these early post-operative periods the macropores were filled with exudate,

fibrous tissue, and a small amount of bony tissue. In the course of time, the envelope became thinner and came to resemble the normal middle-ear mucosa. During these longer survival periods exudate could hardly be distinguished in the pores, which were filled with bone and fibrous tissue in equal amounts (fig. 1).

No distinct differences were found between the obliterative and occlusive implant or between the dense and the macroporous hydroxyapatite with respect to the interfaces.

Interface characteristics

An ultrastructural analysis of the hydroxyapatite/tissue interface revealed the presence of four different structures which will be dealt separately with in the following.

1) Wadding-like material of a floccular and moderately electron-dense nature, which was seen regularly at the middle-ear lumen side of the implant. This structure was most prominent in the first post-operative month and decreased in both thickness and occurrence with increasing survival time. Its appearance suggested a similarity with the mucus normally covering the middle-ear mucosa, although it might also consist of precipitated proteins.

2) Adhering cells, which were observed at the implant/tissue interface. Some of these cells were characterized by extreme flatness, as revealed by scanning (fig. 2, 5) and transmission electron microscopy. The majority of these cells could be considered to be macrophages on morphological grounds. They will be dealt with further in the section on biodegradation.

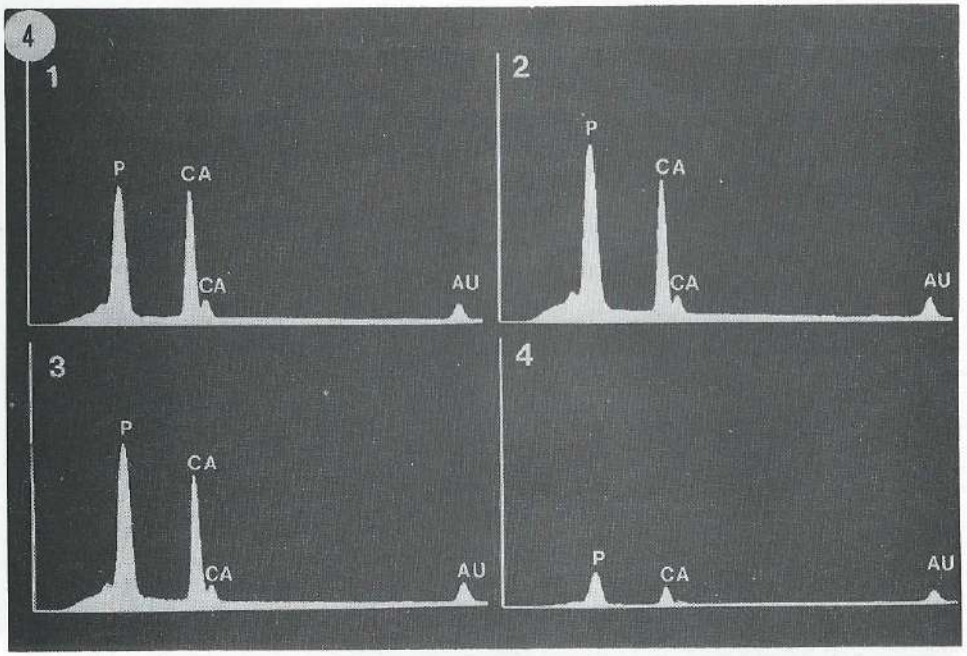
3) The third structure had the appearance in transmission electron microscopy of an electron-dense layer of variable thickness (fig. 3a) present at both the middle-ear wall and at the lumen side of the middle ear. This structure was continuous with the lamina limitans of bone and calcification islands. Scanning electron microscopy showed that the layer had a granular appearance with dispersed superficial protrusions (fig. 3b) representing the above-mentioned calcification islands, as shown by their identical dimensions. This structure will be referred to as the granular layer. With scanning electron microscopy the granular layer could only be studied at the lumen side of the implant, and even then only when the covering tissue was disrupted or absent. In some cases a more electron-lucent zone was seen between the lamina limitans-like structure and the implant surface.

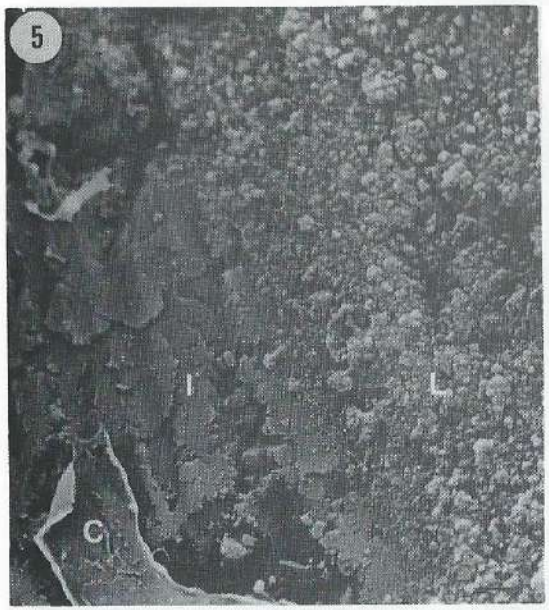
To find out whether the granular layer was partially composed of calcium salt, as suggested by the confluence with the calcification islands, X-ray microanalysis was performed. This approach was based on the assumption that unlike a layer of calcium phosphate, a structure of organic nature can be expected to inhibit the signal emitted by the hydroxyapatite implant material. The data acquired by single-spot analyses indeed showed that calcium counts ob-

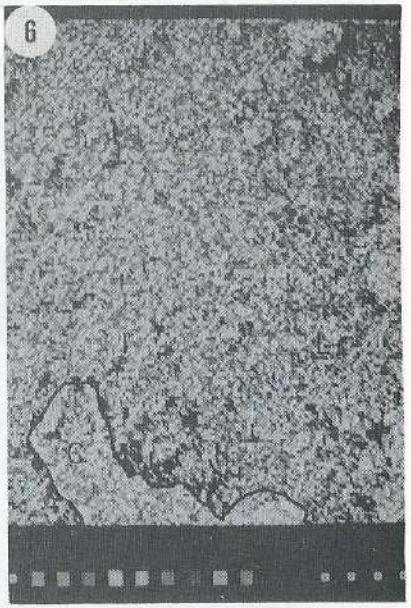
Fig. 4. Point analyses of the structures present on the implant surface. The four diagrams represent the implant surface (1), the granular layer (2), an adherent cell (3), the protrusions (4). The peaks for calcium (Ca) and phosphorus (P) are distinctly lower in the diagram of the adherent cell.

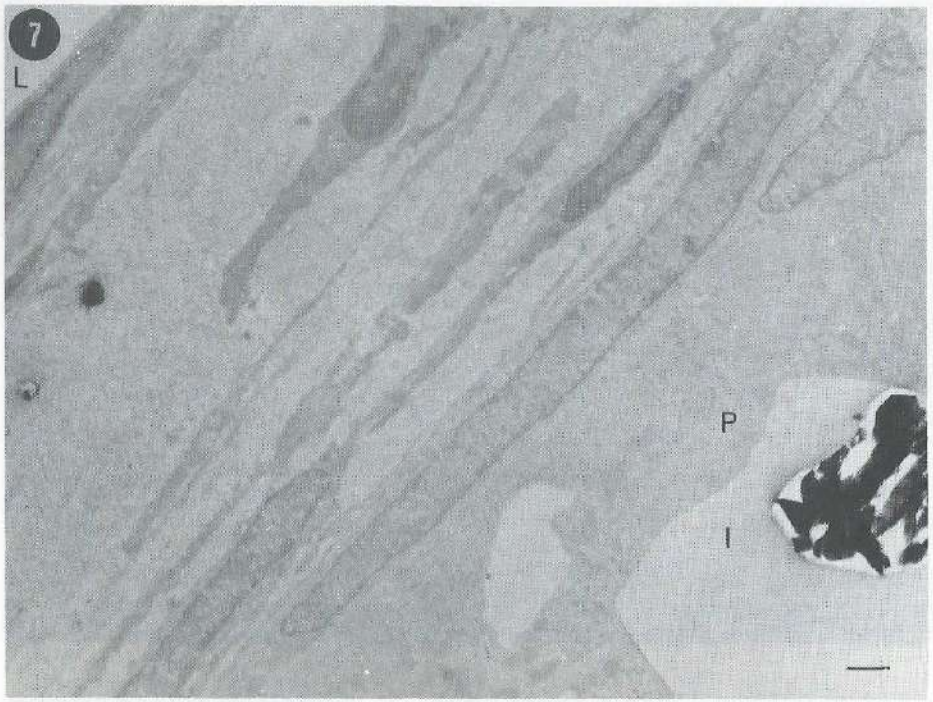
Fig. 5. Back-scatter image of implant surface. Note the adherent cell *(C)*, the bare implant surface *(I)*, and the granular layer *(L)*. Bar: 2 μm.

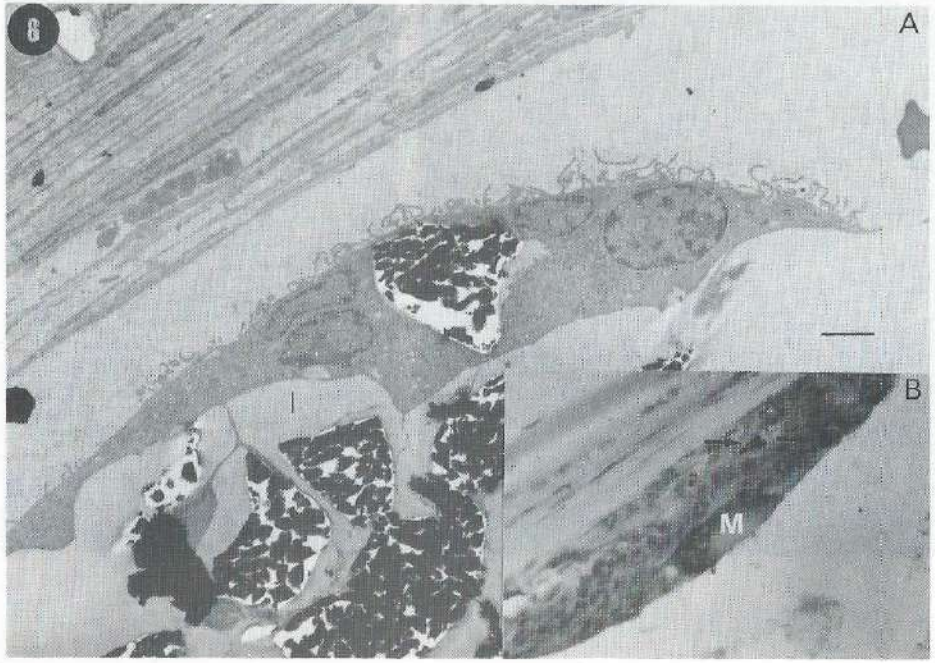
Fig. 6. Result of X-ray mapping of the same area as shown in fig. 5. The implant *(I)* and granular layer *(L)* show a higher intensity for the signal specific for calcium compared with the lighter cell *(C)*. The cell contours are indicated by the black line. The use of black and white photography unfortunately decreases the difference in signal when compared to the original colour prints.











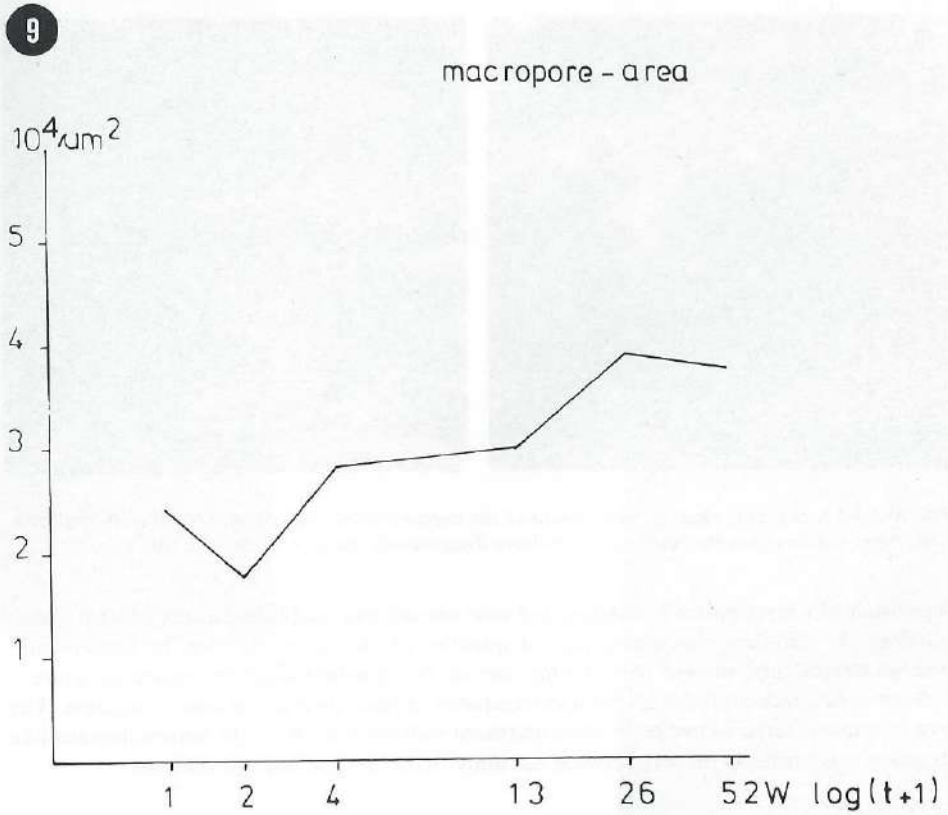


Fig. 9. Diagram in which the macropore area occupied by tissue, calculated by morphometry, is expressed as a function of time.

tained from the adhering cells were lower than those originating from the granular layer, the protrusions, and the implant surface (fig. 4). A similar phenomenon was found with X-ray mapping: this technique showed that the signal obtained from flat cells adhering to the granular layer and to the implant surface reflected a reduction of the calcium counts (fig. 5, 6), whereas no difference was found between the counts deriving from the implant surface and the granular layer. Furthermore, back-scatter electron microscopy showed that the latter two structures had a comparable density, whereas the adherent cells were transparent. Thus, the

Fig. 7. Transmission electron micrograph showing tissue covering the implant three months after the operation. Between the mucosa and the hydroxyapatite *(I)* a phagocyte can be seen *(P)*. *L* = middle-ear lumen. Bar: 1 μm.

Fig. 8a. Multinucleated cell, situated at the hydroxyapatite *(I)*/tissue interface and showing a piece of implant-derived material in its cytoplasm. Bar: 2,5 μm.

Fig. 8b. Concentration of phagocytes on the borderline between the hydroxyapatite and muscle tissue. A multinucleated cell *(M)* can be clearly seen. Note the proliferative activity (arrows), light microscopic autoradiograph. Bar: 16 μm.

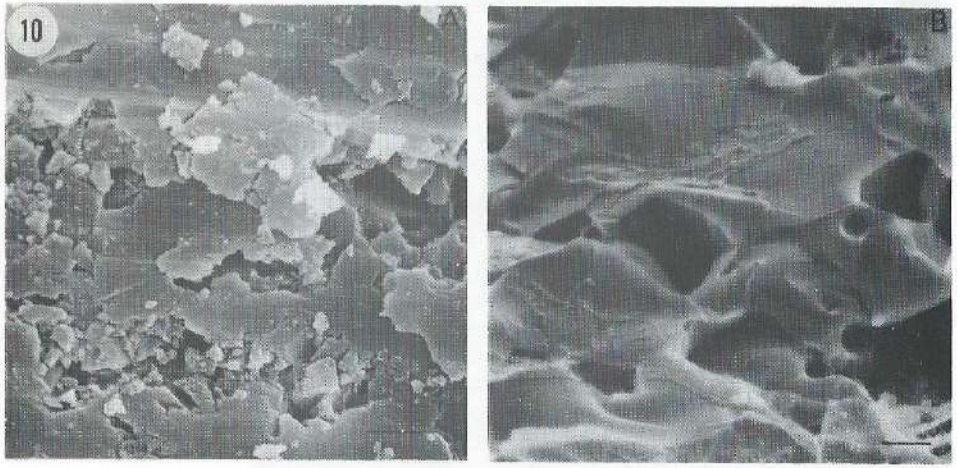


Fig. 10a and b. Scanning electron micrographs of the implant surface before *(A)* and after *(B)* implantation. After implantation the scaly structures have disappeared. Bar (A): 6 μm. Bar (B): 3 μm.

deposition of a layer partially composed of calcium salt was established at the implant/tissue interface. In addition, the ultrastructural qualities of this layer, as seen by transmission electron microscopy, showed that another part of this structure must be organic in nature.
4) Bone in intimate contact with the hydroxyapatite implant formed the fourth structure. The area of implant surface covered by bone increased with survival time. The lamina limitans-like structure was regularly present between the bony tissue and the implant material.

Biodegradation

As early as one week after implantation, macrophages were present at the implant/tissue interface. In the post-operative period extending from the end of the first week to the third month these cells formed a layer that partially covered the implant surface (fig. 7). The cells phagocytosed hydroxyapatite material, as indicated by the presence of numerous vacuoles in the cell cytoplasm. Although only a small proportion of these vacuoles contained partially decalcified implant material, the other vacuoles must originally have been filled with this material, because their structure was very similar to those vacuoles still containing hydroxyapatite. The composition of the contents of the vacuoles was determined by X-ray microanalysis. There were also multinucleated cells whose width frequently did not exceed that of the macrophages. In these multinucleated cells too a phagocytic activity could be shown by transmission electron microscopy. After 6 and 12 months the mononuclear and multinucleated cells were no longer present at the interface on the side of the middle-ear cavity. However, they were present at sites of evident mechanical irritation, as caused for example by bordering muscle tissue. In these zones the multinucleated cells showed a closer resemblance to the classic morphology of multinuclear giant cells (fig. 8a). The implant-derived material was still present in the cytoplasm of both mononuclear and multinucleated cells. Light-microscopical autoradiography revealed proliferative activity in these zones (fig. 8b), probably of mononuclear phagocytes. This activity persisted even after the twelfth post-operative month.

Both the multinuclear cells present at the side of the middle-ear lumen up to the third month and the cells at the sites of mechanical irritation after 6 and 12 months, were characterized by the presence of organelle-pore adherence zones at the implant surface. Sometimes sections showed plasma indentations suggesting an exocytic activity directed toward the implant.

The data obtained by morphometric analysis are shown in fig. 9. After an initial dip at the end of the second week, the macropore area increased up to six months post-operatively. At one year this increase had ceased. Hydroxyapatite was found to be degraded, which increased the macropore area by about 65%; this would decrease the implant area by about 17% if the original macroporosity of the hydroxyapatite of 26% is taken in consideration.

Correlation between this biodegradation and the observed change in implant morphology seems likely. The surface of the implant, which was scaly before the implantation, became smoother in places (fig. 10, a and b) due to loss of the scaly structures.

DISCUSSION

This discussion will deal with the general aspects of the observed interface interactions, but special attention will be given to the deposition of a layer of calcium salt on the implant surface and the associated biodegradation.

The first reaction observed at the middle-ear lumen side of the implant was the formation of a floccular covering at the implant/tissue interface, visualized by transmission electron microscopy. The origin of this covering could not be determined with certainty, but it is conceivable that it originated either from condensed mucus produced by secretory components of the middle-ear mucosa or from precipitated serum proteins. This would explain the thinning and disappearance of the floccular layer after longer intervals, for such substances would only be prominent during a wound reaction (5, 12) most of the effects of which will have ceased a week after the operation. The presence of proteins on a hydroxyapatite implant would be consistent with the results described by Klein (7), who observed absorbance of several serum proteins to calcium phosphate compounds. Klein (13) suggested that especially the absorbance of IgG and the complement factor C_3 to calcium phosphate might be of significance for phagocyte-mediated biodegradation. Unfortunately, the role of these proteins in hydroxyapatite resorption is not yet clear.

The granular layer seen in two places against the middle-ear wall and the middle-ear lumen, is interesting in several respects. The lamina limitans-like structure sometimes present above an electron-lucent zone or making up the total granular layer was of special interest, because it not only resembled the lamina limitans but even appeared to be confluent with the lamina limitans of calcification islands on the implant surface. This constitutes evidence that hydroxyapatite at least partially takes part in normal bone metabolism where a lamina limitans occurs between two zones of bone deposited at different times or on top of bone where osteogenesis has ceased temporarily of definitively (14, 15). The direct bonding of bone to hydroxyapatite implant material has been frequently described (16-19). Osborn (6) stated that this direct and firm bond finds its origin in bilateral crystal growth originating from both the bone apatite and the crystal phase of the ceramic. This bond becomes reinforced by the crystal-protein affinity. Bonding osteogenesis is considered to be a property of the bioactive calcium phosphates (20). In our study the combination of transmission and scanning electron micro-

scopy, together with X-ray microanalysis and backscatter electron microscopy, established the deposition of calcium, probably partially in the form of calcium phosphate, on the implant surface even without the presence of bone in the immediate vicinity. This observation and the presence of calcification islands on the surface of the granular layer indicate that encapsulation of hydroxyapatite by a mineral matrix is at least in part initiated by the material itself. Since a structure comparable to the granular layer was also seen at the bone/implant-material interface by transmission electron microscopy, the present results seem to support the above-mentioned theory (6, 20). It would be interesting to find out whether the composition of the granular layer along hydroxyapatite implants is similar to that of the layer along bioglass implants (4, 21).

Since the occurrence of biodegradation of hydroxyapatite, as observed by morphometry, was evident, our results on biodegradation are in conflict with the interpretation given to the findings of other investigators (2, 17-19), who concluded that biodegradation did not occur. This divergence might be explained by the use of different animals species, different implantation sites, or different methods for quantitation. It should be underlined that the hydroxyapatite used in all of these studies was of the same composition. With respect to biodegradation it can be stated that both mono- and multinucleated phagocytes play a role, as shown by the presence of implant-derived material in the cytoplasm of these cells, together with organelle poor adherence zones (22) and membrane indentations suggestive of exocytosis at the implant surface. Since hydroxyapatite is the main component of the mineral matrix of bone (1), some resemblance between the degradation of hydroxyapatite implant material and of bone may be expected. Studies on bone resorption have shown that multinucleated cells originating from macrophages, resorb more efficiently *in vitro* than their mononucleated precursors (23), a phenomenon whicht might be interesting to investigate for hydroxyapatite ceramics. The fact that the situation encountered at the sites where the implant is in contact with muscle tissue and where phagocytes are prominent, does not seem to change with respect to morphologic and autoradiographic qualities, even for the longest survival periods, suggests that these zones and their phagocytic activity persist over still longer periods.

The apparent decrease of implant-material resorption after six months can be explained by the disappearance of the majority of the phagocytes present at the interface, on the middle-ear lumen side, up to three months post-operatively. This argument is also true for the increasing amount of bone in and around the implant which will contribute to the reduction of resorption, because phagocytes are not present at these sites.

The fact that in this experiment no evident difference was found between interface characteristics of dense and macroporous hydroxyapatite is not surprising, since both materials possess the same surface properties.

The present results support the opinion that hydroxyapatite may be a useful material for application in reconstructive middle-ear surgery. The low compressive and tensile strengths of hydroxyapatite (24) compared to bone do not constitute a handicap, since the forces acting on the implant will be limited in the middle ear. In contrast, biodegradation can be a problem. However, if we take into consideration that the resorption ceases, at least to a high degree, six months post-operatively, complications due to degradation are not likely to occur. Furthermore, the fact that the pores become filled with bone and fibrous tissue indicates progressively greater strength of the implant (25). Further research on the effect of infection (often encountered in middle-ear surgery) on biodegradation and integration of the implant with the host tissue, is needed.

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CHAPTER VI

Macropore Tissue Ingrowth

A quantitative and qualitative study on hydroxyapatite ceramic

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SUMMARY

The aim of this study was to obtain more information about macropore tissue ingrowth into pores of sintered hydroxyapatite implanted in the rat middle ear, for assessment of the usefulness of this material in reconstructive middle-ear surgery. The exudate filling the pores during the early post-operative period was gradually replaced by equal amounts of fibrous tissue and bone. The percentage of the macropore area occupied by bone was directly correlated with the macropore size. Bone was deposited not only from the pore wall towards the pore center, but also in the opposite direction. Bonding osteogenesis was demonstrated. At sites of mechanical irritation, the presence of multinucleated cells and proliferatively active mononuclear phagocytes persisted for as much as a year.

Under appropriate conditions hydroxyapatite seems to be a promising material for bone substitution in reconstructive middle-ear surgery.

The use of porous implant materials (1, 2) has become common in reconstructive surgery. The presence of the pores promotes permanent fixation by ingrowth of living tissue. At constant volume fraction, the use of a small pore size results in a larger contact-surface between host tissue and implant material. Thus the smaller the pores the better the fixation. However, the pore size cannot be reduced too far, because the size of pores and the size of the interpore connections are known to have a significant effect on the kind and quantity of the tissue invading the pores (2, 3). Bone formation has been established for a variety of pore sizes in various materials (4-7). In general, a pore diameter greater than 100 μm allows bone to grow into the pores, whereas a diameter smaller than 20 μm results in insufficient ingrowth. Besides the pore dimensions, the physical and chemical characteristics of a biomaterial also influence tissue ingrowth. For instance, a low modulus of the implant is thought to be of importance (3), as is its surface chemistry (8, 9). In spite of the fact that sintered hydroxyapatite ceramics possess a high degree of stiffness (10, 11), excellent tissue ingrowth at various implant sites has been found in a number of studies (12-15). These results must be partially due to the composition of the material, which is almost identical to the mineral matrix of the human bone (16). Although hydroxyapatite has frequently been reported not to be subject to biodegradation, a recent quantitative study showed distinct resorption of the material when implanted in the rat middle ear (17). We found that the macropore diameter increased by 15-

20 μm during the first year. Hydroxyapatite is associated with bonding osteogenesis (,,bioactive"), whereas bioinert and biotolerant materials lead to contact and distance osteogenesis (18).

Although the mechanism of tissue ingrowth as a function of time and macropore size has been established for several materials (2, 3, 7), ingrowth into hydroxyapatite has not been investigated extensively. This led us to perform the present study to obtain more information by evaluating tissue ingrowth with the use of routine histology, transmission and scanning electron microscopy, bone fluorescence, autoradiography, and morphometry. Since we are primarily interested in the biocompatibility of hydroxyapatite in relation to reconstructive middle-ear surgery, where it is already being used to replace part of the meatus or of the ossicular chain (19), we implanted this material in the middle ear of rats.

MATERIALS AND METHODS

Implant materials and implantation techniques

Use was made of macroporous sintered hydroxyapatite with a macroporosity (pore size ca. 100 um) of 26% and a microporosity (pore size ca. 3 um) of less than 5%. The sintering technique has been described elsewhere (20).

One hundred and twenty implants measuring 3.4 mm^3 were inserted in the rat middle ear. The survival time of the animals ranged from one week to one year. The implantation method and exact distribution of the implants over the survival periods have been published elsewhere (17).

Routine histology, autoradiography, and transmission and scanning electron microscopy

The histological, autoradiographical, and electron-microscopical techniques were the same as those applied in earlier studies (17). Only the materials destined for scanning electron microscopy were not subjected to decalcification.

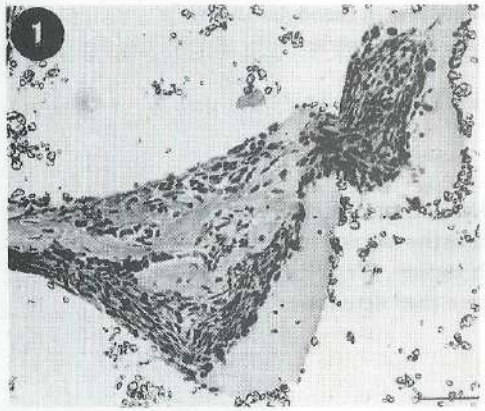
Bone fluorescence

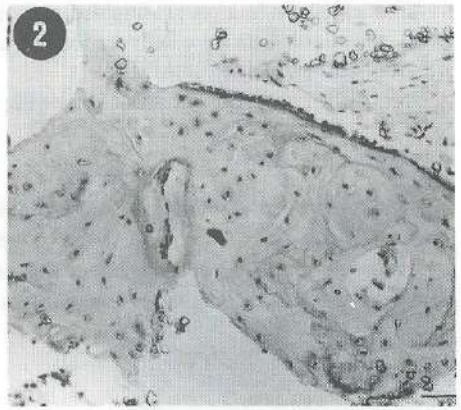
Some of the animals still alive at three months recieved a subcutaneous injection of alizarin after one month and an injection of calcein at two months. Animals still alive at six months were similary injected after three and five months. Thus, information was obtained about bone deposition at 1, 2, 3, and 5 months and about the amount of bone deposited between these periods.

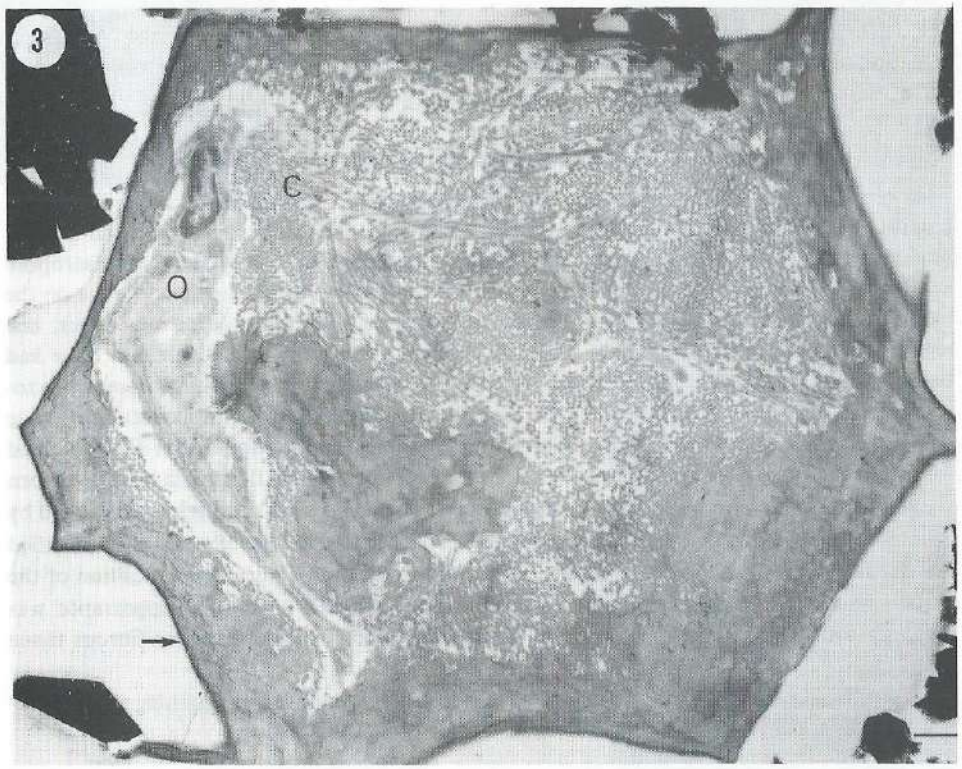
Fig. 1. Light micrograph of a pore filled with exudate, fibrous tissue, and bone one week after implantation. Bar: 30 μm.

Fig. 2. Histological section showing macropore filled with fibrous tissue and bone three months after implantation. Bar: 30 μm.

Fig. 3. Transmission electron micrograph of a small macropore partially filled with bone. An osteoblast (O), collagen (C), and an electron-dense granular layer (arrow) can also be seen. Bar: 1 μm.







After exposure to a paraformaldehyde solution (1%) in cacodylate buffer (0.14 M, pH 7.4, 4°C), the middle ears were dehydraded in a graded alcohol series, critical-point dried in carbon dioxide, and embedded in MMA.

Sections were cut 80 μm thick by sawing with a diamond blade and then ground smooth.

Morphometry

At a standard magnification, 427 histological sections (7 μm thick) of macropores were photographed. An x-y tablet was used to measure the macropore area and the areas occupied by exudate, fibrous tissue, and bone. For each section, the percentage of each type of tissue was calculated, and the average percentages were then determined.

RESULTS

To provide more insight into the ingrowth process, the results will be described with special attention to the activity of tissue in the pores of the implant material, new bone formation, and the activity of phagocytic cells around the implant.

Macropore tissue: light-microscopical and ultrastructural results

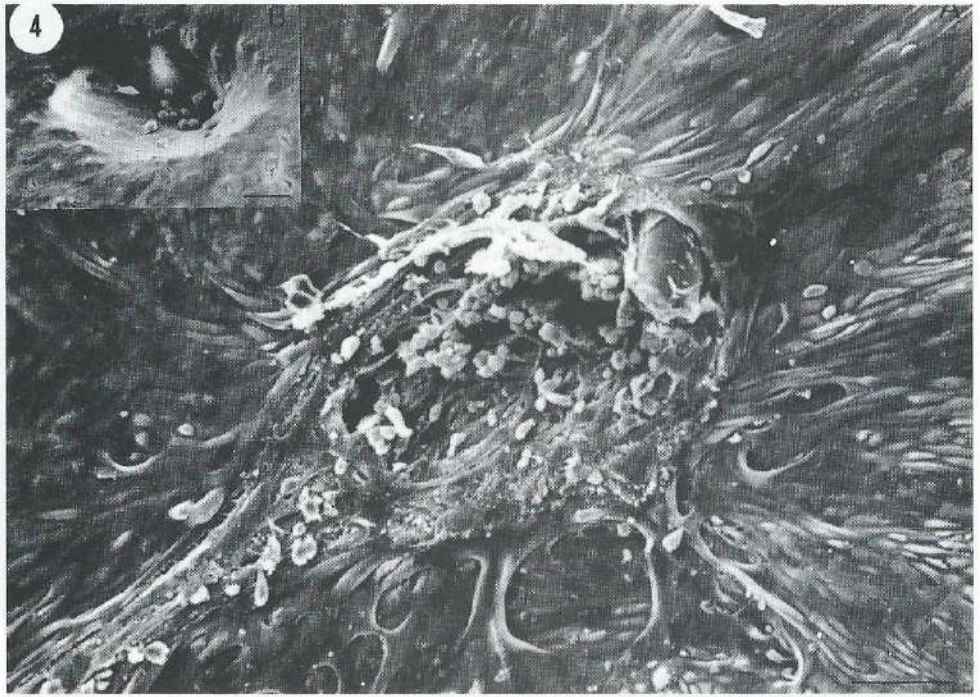
At one week post-operatively, light microscopy showed that some of the macropores were completely filled with exudative fluid. Other pores also showed fibrous tissue and a small amount of bone but these pores accounted for a smaller proportion of the total macropore area (fig. 1). The ratio between exudate on the one hand and fibrous tissue and bone on the other changed in favour of the latter with increasing survival time. After two weeks, one month, and three months, exudate could still be seen, but the fibrous tissue and bone had become more prominent and pores entirely filled with fibrous tissue or with fibrous tissue together with bone were present (fig. 2). Six and twelve months after the implantation, exudate was rarely observed and the area of fibrous tissue, which had predominated after one and three months, was about the same as that of bone. Pores completely filled with bone were seen frequently after these intervals. Many of these pores showed central capillaries surrounded by a small zone of fibrous tissue. Transmission electron microscopy showed collagen, a calcified matrix, fibroblasts, osteoblasts, and osteocytes, thus indicating a normal composition of the tissue present in the pores. An electron-dense structure with a granular appearance was regularly found at the implant/ macropore tissue interface, whether bone or fibrous tissue

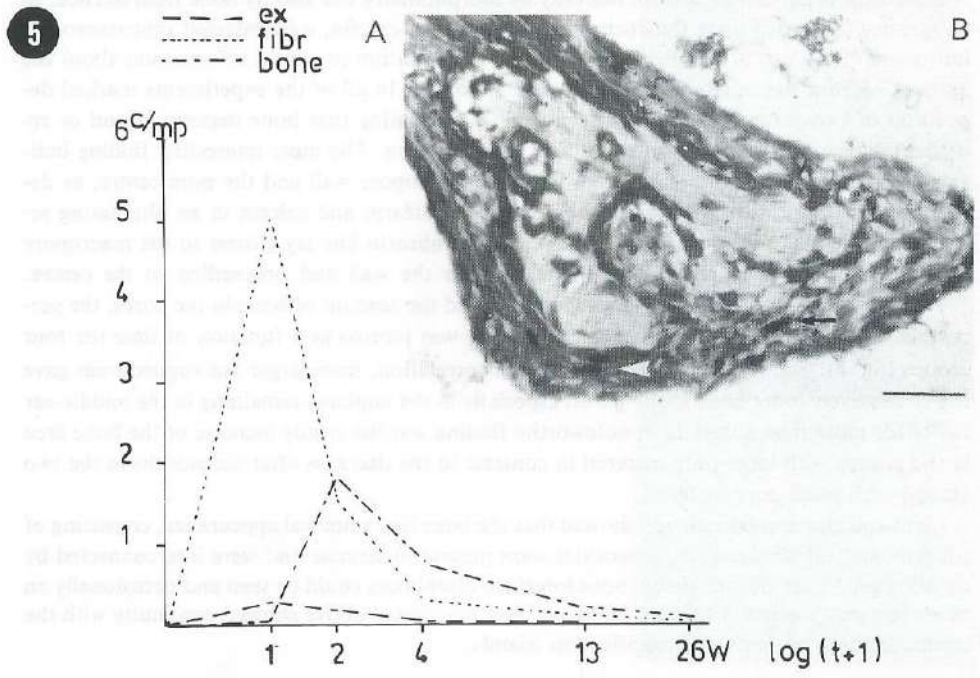
Fig. 4a. Outer surface of a macropore studied by scanning electron microscopy, suggesting migratory activity of cells at the pore periphery, 1 week after implantation. Bar: 50 μm.

Fig. 4b. Macropore surface six months after implantation. Scanning electron microscopy no longer shows any signs of migratory activity. Bar: 30 μm.

Fig. 5a. A graph showing proliferative activity of various tissues after various post-operative intervals, based on the autoradiographic findings.

Fig. 5b. Autoradiograph showing a macropore with bony tissue at the centre. In the peripheral area, cells showing marked proliferative activity (arrows) can be seen. Bar: 15 μm.





bordered the hydroxyapatite (fig. 3). Micropores became filled with calcified material, bone tissue, collagen, and cytoplasm.

Scanning electron microscopy showed that in the early stages after the operation the cells in pores formed a pattern resembling a migratory track between the periphery and the centre of the pore (fig. 4a), but these tracks were no longer seen after six months (fig. 4b). Autoradiographical analysis of the material (fig. 5a, b) from the group of rats injected with a solution of tritiated thymidine to label dividing cells, showed a peak of proliferative activity for exudative cells and fibroblasts one week after the implantation. In areas of bone formation this peak occurred after two weeks. The rather low activity of the exudative cells diminished to almost nil after longer periods. To obtain a more objective picture of the course of events, morphometry was performed in the light-microscopical sections. The results, which are shown in fig. 6, confirmed the initial light-microscopical findings. The area occupied by exudate showed a peak during the first two weeks and then gradually decreased until it had almost disappeared after six and twelve months post-operatively.

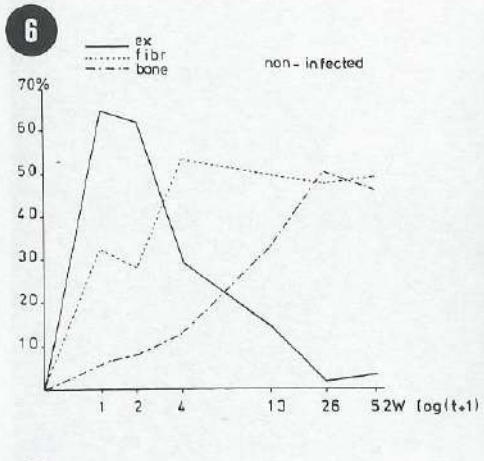
The amount of fibrous tissue increased to about half of the macropore area during the first month, and remained at that level until the end of the study period. It is striking that according to this graph, the surface area of fibrous tissue was smaller at two weeks than after one week. This phenomenon might be related to the contracted morphology of the fibroblasts observed with the electron microscope. The area occupied by bone tissue showed a gradual increase up to six months and then remained at that level.

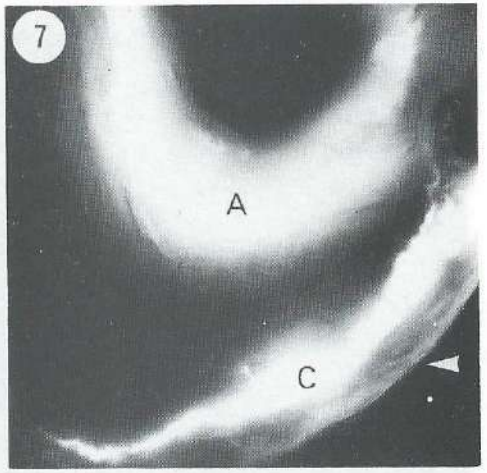
Osteogenesis

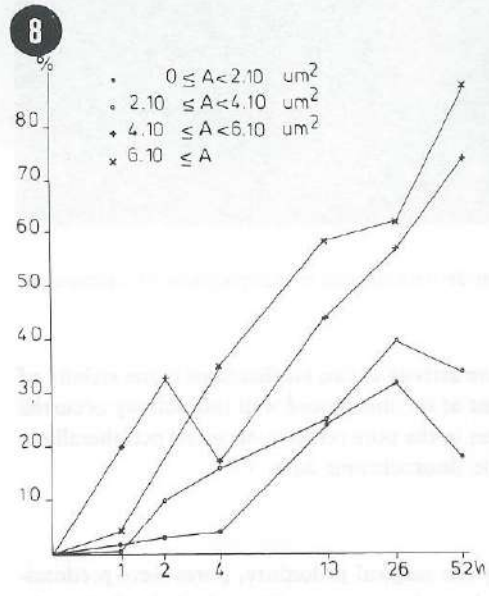
Bone deposition can be studied not only by morphometry but also by bone fluorescence. In the present study two bone fluorochromes, alizarin and calcein, were injected subcutaneously into some of the rats at various times after the implantation to obtain information about the amount of bone deposited after 1, 2, 3, and 5 months. In all of the experiments marked deposition of two fluorochromes was seen (fig. 7), indicating that bone deposition and or remodelling occurred even five months after the operation. The most interesting finding indicated that bone deposition starts from both the macropore wall and the pore centre, as deduced from the presence of fluorochrome lines of alizarin and calcein in an alternating sequence. However, in the majority of the cases the alizarin line lay closest to the macropore wall, corresponding with deposition starting from the wall and proceeding to the centre.

To detect correlation between macropore size and the amount of bone in the pores, the percentage of the macropore area occupied by bone was plotted as a function of time for four groups (fig. 8). The results clearly showed such correlation, since larger macropore areas gave rise to relatively more bone in the pores, especially in the implants remaining in the middle-ear cavity for more than a month. A noteworthy finding was the steady increase of the bone area in the groups with large-pore material in contrast to the decrease after six months in the two groups with small-pore material.

Light and electron microscopy showed that the bone had a normal appearance, consisting of collagen and calcified matrix. Osteocytes were present in lacunae and were interconnected by canaliculae. At the fibrous tissue/bone interface osteoblasts could be seen and occasionally an osteoclast was present. The electron-dense layer mentioned above showed continuity with the lamina limitans of bone and calcification islands.







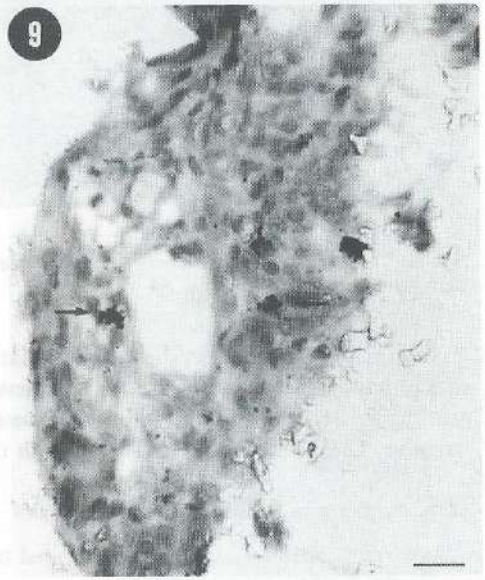


Fig. 6. Diagram based on morphometric data concerning the kind and amount of tissue present in the macropores expressed as a function of time.

Fig. 7. Micrograph showing lines of alizarin *(A)* and calcein *(C)*. The macropore wall is indicated by an arrow. This pore shows bone deposition starting from the centre and progressing toward the wall.

Fig. 8. Diagram showing the correlation between macropore size and the relative amount of bone found at four pore-diameter intervals.

Fig. 9. Concentration of phagocytes at the implant/muscle tissue interface. Proliferatively active cells shown by autoradiography are prominent. Bar: 20 μm.

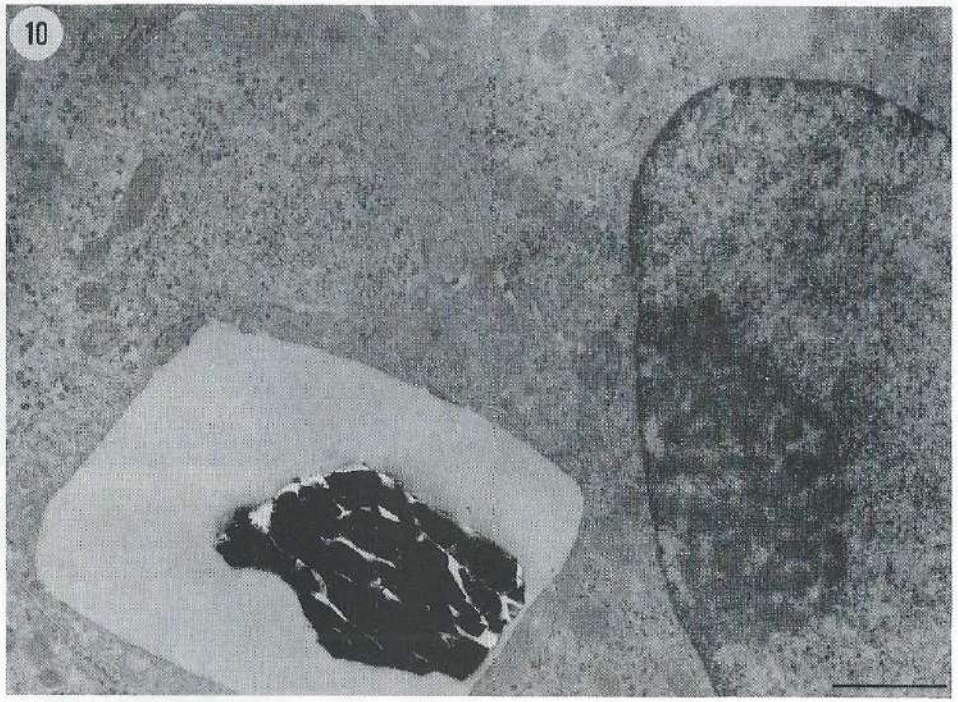


Fig. 10. Transmission electron micrograph of implant-derived material in the cytoplasm of a phagocyte. Bar: 1 μm.

Autoradiography (fig. 5b) showed proliferative activity at two localizations in the vicinity of sites of bone formation: where bone was present at the macropore wall this activity occurred near the pore centre, and where bone was present in the pore centre it occurred peripherally in the pore. These results correspond well with the fluorochrome data.

Mononuclear and multinucleated phagocytes

As a result of the wound reaction elicited by the surgical procedure, pores were predominantly filled with exudate containing mononuclear phagocytes at the end of the first week. These cells gradually disappeared from the macropore lumen, but persisted at the macropore wall/tissue interface. On the basis of the electron-microscopical findings, these cells were considered to be macrophages. Multinucleated cells were also seen. Both types of cell were rather flat. When these mononuclear and multinucleated cells adhered to the macropore wall, the above-mentioned electron-dense layer was absent. During the first three months a large part of the macropore wall was covered by these phagocytes, but after longer periods they disappeared almost completely. However, phagocytes persisted at sites where muscle tissue bordered the implant material (fig. 9). Autoradiography revealed proliferative activity at this interface even after twelve months.

An interesting observation was the presence of vacuoles containing electron-dense implant-derived material (fig. 10) in the cytoplasm of the phagocytes in the macropores. In view of the rather rectangular shape of many of the empty vacuoles in the cytoplasm, it seems likely that some of them must have contained implant material before decalcification. These vacuoles were found after all of the intervals studied and occurred together with organelle-poor adherence zones, at the interface, and together with cell cytoplasm protruding into the macropores. Apparently phagocytosis had occurred.

DISCUSSION

The use of macroporous biomaterials has been reported frequently (1, 2). The advantages of these macropores are threefold. One is the better fixation of an implant with the host tissue as a result of tissue ingrowth (1, 5); a second advantage is thought to be reduction of the fibrous capsule size; and lastly, the presence of pores apparently reduces the carcinogenic properties of an implant (21, 22). The first material found in the pores is the direct result of the wound reaction (23, 24) after the operation and therefore consists of exudate. In exudate, depending on the duration of the post-operative period, polymorphonuclear granulocytes, lymphocytes, monocytes, macrophages, and multinucleated cells become prominent in various proportions (25). Since in this study the implants were not recovered earlier than the eighth day, the first effects of the wound reaction were not studied.

After the first week, exudate still covered the major part of macropore area, but fibrous tissue was already present and, in smaller qualities, bone. One month after the operation fibrous tissue occupied more than 50% of the macropore area and had become more prominent than exudate. In spite of this decrease of exudative fluid, some cells characteristic of exudate still played a significant role, especially during the first three months. These exudative cells were macrophages and multinucleated cells, which were found at the macropore wall/tissue interface. At the interface, phagocytosis of implant material by these cells occurred, as indicated by the presence of vacuoles that contained, or must once have contained, implant-derived material. Furthermore, the presence of organelle-poor adherence zones together with protrusion of cell cytoplasm into the micropores fits well with active phagocytes (26, 27). Identical features were found after six and twelve months, when the majority of the phagocytes had disappeared and the remainder were only present where muscle tissue bordered the implant. In our experimental model the implants became almost free of phagocytes on the lumen side of the middle ear, but phagocytes were still present along the border between implant and muscle tissue. It therefore seems advisable to avoid contact with muscle tissue as much as possible in reconstructive middle-ear surgery. The finding of endocytosis by macrophages and multinucleated cells corresponds with earlier findings showing an increase of the pore size of the hydroxyapatite/tissue interface. These observations differ from those of other authors, who did not see biodegradation of hydroxyapatite material (7, 13, 19, 28). Whether this divergence is due to the use of different animal species, different implantation sites, or different techniques, remains a question.

As already stated, fibrous tissue was seen in the pores so early at the end of the first week, and the amount increased to 50% of the total macropore area after one month. Of interest was the decrease after two weeks, relatively to the first week. This was probably due to shrinkage

of the fibroblasts during the wound reaction, an assumption which is supported by the electron-microscopical demonstration of contracted fibroblasts during this stage. On the basis of the scanning electron-microscopical observations and tritiated methyl thymidine labeling it may be concluded that macropores became filled due to a combination of migratory and proliferative activity of cells in the vicinity of the pores. This activity was strongest during the first week after implantation.

Although the ingrowth of fibrous tissue itself leads to fixation of a porous implant, the fixation established by ingrowing bone seems to be firmer due to the modulus of this tissue, which has a closer resemblance to that of hydroxyapatite. A firm bond between bone and hydroxyapatite has been reported by several authors (10, 11, 18). With respect to the quality of fixation, our finding of bone in the macropores was of significance. Bone occupied about 50% of the macropore area after six and twelve months. This presence of bone in the macropores of hydroxyapatite in our experimental model agrees with the findings made in earlier studies (6, 12, 13).

With respect to the mechanism of bone deposition characteristic for bioactive ceramics (18, 29) it has been supposed that bone deposition starts from the macropore wall and progresses toward the centre. In the present study this direction of deposition was confirmed. However, bone deposition starting from the centre was also seen, albeit less frequently. This means that distance and contact osteogenesis are not specific for biotolerant and bioinert implant materials, respectively. However, bonding osteogenesis is specific for bioactive materials.

Besides the surface properties of a material (9), the macropore size has been shown to contribute to the determination of the features of the tissue present in the pores (1, 3-5). As shown by this study, the latter also holds for hydroxyapatite. In histological sections smaller than 2×10^4 um^2, bone tissue ingrowth amounted to less than 15% as against 87% for larger surfaces, 6×10^4 um^2. A striking finding was that the amount of bone tissue in the smallest pores increased after six months, possibly due to the effects of biodegradation (17), during which some pores shifted to a group with larger pores. Why macropore size is of such great significance for the determination of the kind of cells in the pore lumen is not clear. The most obvious explanation would be that a pore size too small to permit capillaries to grow into the pores would inhibit bone formation, whereas a larger pore size leaving space for one or more capillaries in the pore would guarantee adequate nutrition for the tissue in the macropore. Determination of the minimal macropore size needed for the presence of bone in the larger part of the pore is of direct importance for optimal fixation of a biomaterial, because use of this size will yield bone ingrowth combined with a relatively large adherence area.

The granular layer observed at the interface of hydroxyapatite deserves special attention (17). This layer is partially composed of calcium, as shown by X-ray microanalysis, and is very similar to the lamina limitans of bone (30, 31). Because of its location, this layer could be responsible for the firm bond between the hydroxyapatite and the host tissue. However, when macrophages and multinucleated cells were present along the implant, this layer disappeared.

Because infection usually leads to the presence of macrophages (32) and the middle ear is vulnerable to infection, the present findings underscore the importance of investigating the biocompatibility of hydroxyapatite in the presence of infection. Except for this reservation, the results obtained with hydroxyapatite in this and earlier studies performed in the middle ear (6, 7, 14, 17, 19) and with different experimental set ups (10, 13, 18, 28) justify the conclusion that hydroxyapatite seems to be a highly suitable material for use in reconstructive surgery.

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CHAPTER VII

Biocompatibility Of Hydroxyapatite During *Staphylococcus aureus* Infection

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SUMMARY

In the present study the biocompatibility of macroporous and dense hydroxyapatite after implantation in the rat middle ear was evaluated during an induced *Staphylococcus aureus* middle-ear infection. The course of the infection was similar to that in the absence of an implant. Hydroxyapatite was frequently integrated with fibrous ingrowths in the middle-ear lumen, originating solely from the infection. Good epithelial covering of the implant with all types of epithelial cell of importance for middle-ear defence was found. Increase of the exudate in the pores due to the infection was relatively small, and most of the exudate was restricted to pores on the implant surface. The bony tissue in the pores was not influenced significantly by the induced infection. Degradation of hydroxyapatite was consistent with earlier results obtained in the non-infected middle ear.

The results obtained so far suggest that hydroxyapatite is highly suitable for middle-ear implantation.

INTRODUCTION

The implantation of a biomaterial into host tissue implies the occurrence of a wound and a corresponding wound reaction (1). Such a wound promotes susceptibility to bacteremic infection. The presence of an implant, whether percutaneous, subcutaneous, intramuscular, intravascular, intra-osseous, or of any other kind, has long been recognized as a factor which increases the risk of infection, as established by several studies (2-4). Implant characteristics associated with bacteremic infection have been the subject of many studies (5-10). An implantation site might be more susceptible to infection than a normal wound due to an inhibition of the wound reaction or a change of oxygen tension in the vicinity of the implant (11). According to the literature, the structural properties of biomaterial may (5, 9) or may not (4) affect the occurrence of infection. Despite some controversy it may be considered as established that some characteristics, for example rapid integration with the host tissue (including covering of the total implant) and limited bacterial adherence to implant materials, are of significance for reduction of the risk of infection.

In some fields of surgery where infection is likely to occur, the behaviour of a biomaterial in infected surroundings is of great importance. This category includes reconstructive middle-ear

surgery, where the surgical field is contaminated frequently and becomes infected. Surprisingly, despite the appreciable number of implant materials used in otology (12-18), experimental studies on the behaviour of these materials in the infected middle ear have not been performed, although some critical reports have appeared in casuistic form (19, 20).

A material recently introduced in otologic surgery is hydroxyapatite, a calcium phosphate ceramic whose composition closely resembles that of the mineral matrix of bone (21). In the non-infected middle ear, positive results have been obtained with this material in both man (22, 23) and the rat (24, 25). To assess the quality of this material further, studies in the infected middle ear are indispensable. This led us to investigate the behaviour of hydroxyapatite under the conditions associated with a *Staphylococcus aureus* infection induced in the rat middle ear.

MATERIALS AND METHODS

Implant material: A dense and a macroporous form of hydroxyapatite were tested. The former material had no macropores (∅ ± 100 μm) and less than 5 volume per cent consisted of micropores (∅ ± 3 μm). In the latter material approximately 26 volume per cent was accounted for by macropores (∅ ± 100 μm) and less than 5% by micropores. The sintering technique and some properties of these forms of hydroxyapatite have been published elsewhere (26).

Implantation technique and distribution over survival time: A total of 70 dense and 72 macroporous implants were inserted into rat middle ears. For this purpose, a hole was drilled in the bony middle-ear wall; one implant was placed in the middle-ear cavity and the other was used to close the defect. The implants were equally distributed over 4 groups representing intervals of 1, 3, 6, and 12 months. For each of these periods 10 dense and 10 macroporous implants were examined by light microscopy, 4 of each type by scanning electron microscopy, and 4 of each by transmission electron microscopy. In the first post-operative year only 2 dense implants were studied by scanning electron microscopy. However, for the other techniques the number was the same as for the other periods.

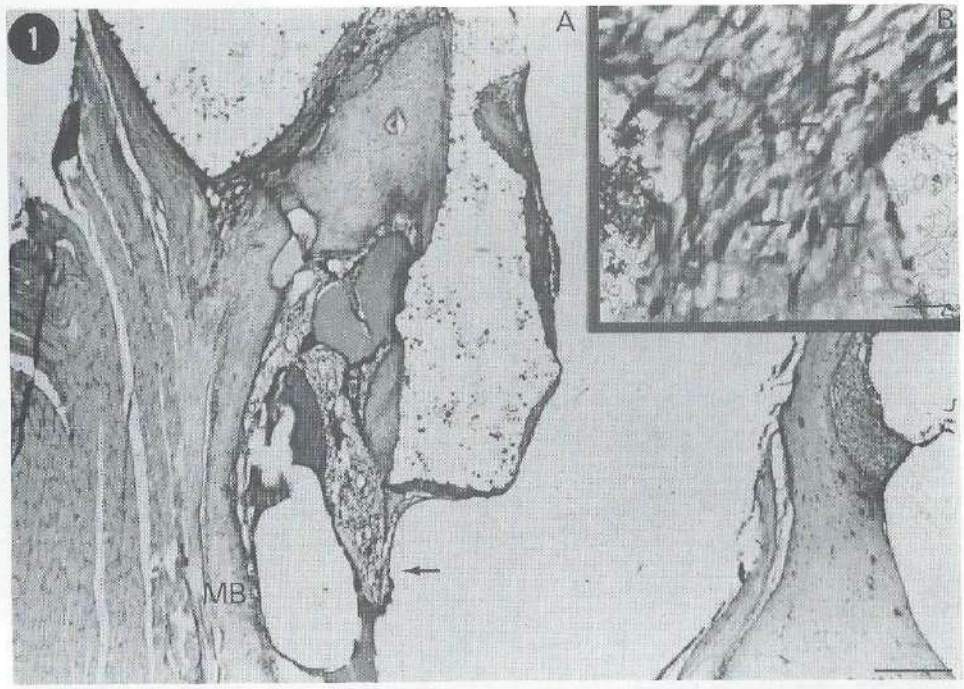
Induction of infection: Infection was induced three weeks before the animal was to be killed. Approximately $1.4.10^6$ *Staphylococcus aureus* in physiological saline were injected intratympanically.

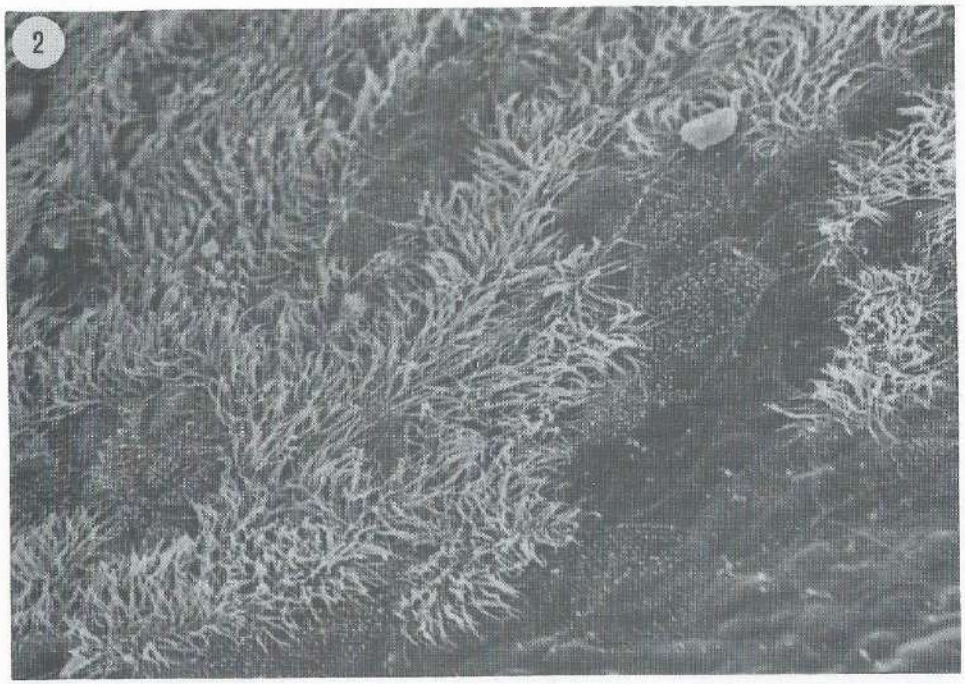
Fig. 1. Hydroxyapatite implant after one month in the middle-ear cavity. Infection was present after the first week.

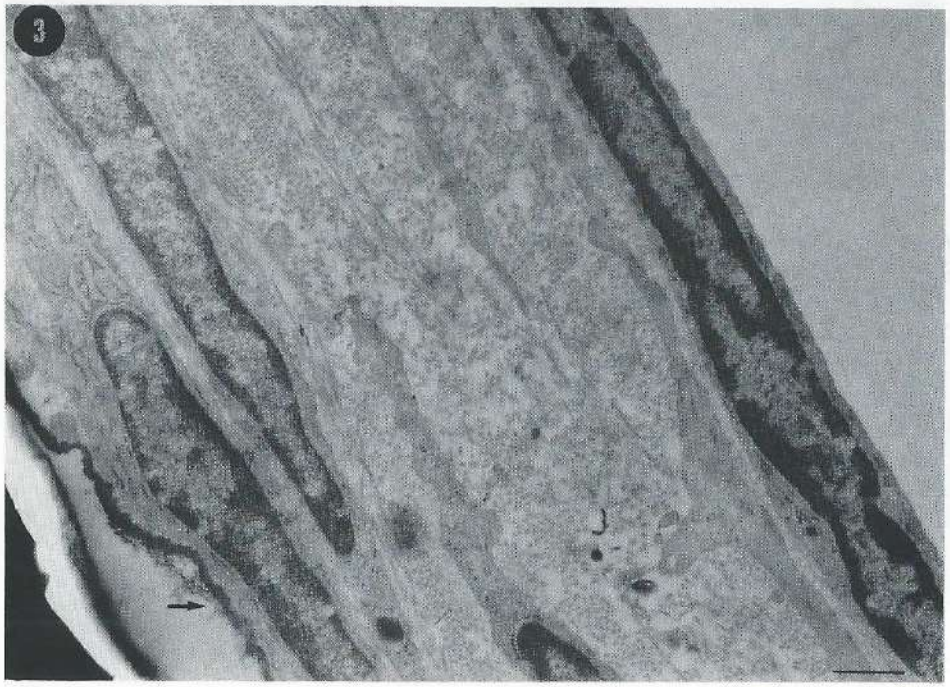
Fig. 1a. Section showing fibrous tissue ingrowth (arrow). MB = Middle-ear bulla. Bar: 100 μm.

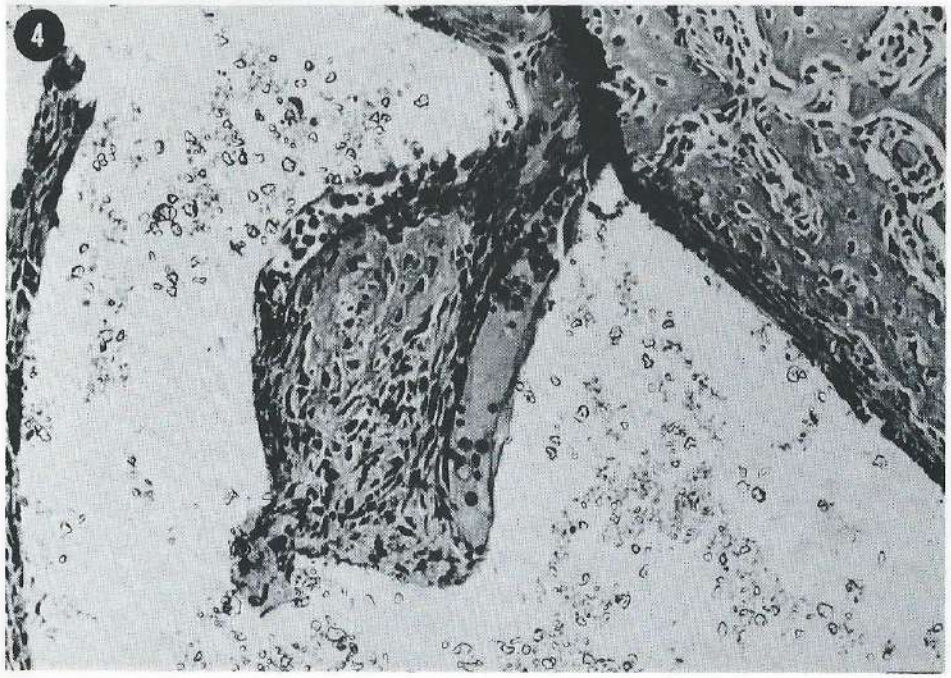
Fig. 1b. Light microscopical autoradiograph of the transitional area between fibrous tissue ingrowth and macropore contents. Excessive proliferative activity is present (arrows). Bar: 15 μm.

Fig. 2. Scanning electron micrograph of the epithelial cells over hydroxyapatite near the mucociliary tracks. Flat polygonal and ciliated cells are visible. Bar: 10 μm.









Light- and electron-microscopical techniques: Use was made of routine light microscopy and scanning and transmission electron microscopy. For fluorescence microscopy, at each time-point 2 dense and 2 macroporous implants were exposed to the bone fluorochromes alizarin and calcein, which were injected at 1 and 3 months and 2 and 5 months, respectively. The same number of implants were exposed to tritiated thymidine and prepared for autoradiography. The techniques are described elsewhere (27).

Morphometry: 295 histological sections (7 μm thick) of macropore material were measured with an x-y tablet. A distinction was made between total macropore area, area occupied by exudate, area occupied by fibrous tissue, and area occupied by bony tissue.

RESULTS

Several effects of *Staphylococcus aureus* initiated middle-ear infection during implantation can be distinguished. The present study concerned reactions of the middle ear in the vicinity of hydroxyapatite implant material, the epithelial reactions to the hydroxyapatite, the macropore tissue characteristics, and the effects of infection on the biodegradation of implant material. The results pertaining to these four points will be discussed in that order with respect to the infected middle ear of the rat. Since the findings showed no significant difference between dense and macroporous hydroxyapatite, no distinction will be made between these two materials, and the two implantation sites will be treated together.

Middle-ear tissue reactions: Three weeks after the introduction of a *Staphylococcus aureus* suspension into the middle-ear cavity, several phenomena characteristic for this stage after the induction of infection were seen. This similarity in the reactions occurring in each post-operative period holds for all of the phenomena under study. An interesting feature shown by the bone of the middle-ear bulla was the deposition of a layer of new bone on the inner side of the bony wall (easily recognized by the presence of a dark line between the two zones of bone). The amount of new bone varied considerably depending on the site in the middle ear. Furthermore, compared with the pre-infectious situation the fibrous tissue envelope showed swelling. This swelling was accompanied by high proliferative activity of the fibroblasts, as shown by light-microscopical autoradiography (fig. 1b), which demonstrated the incorporation of injected tritiated thymidine. The rise of proliferative activity was more or less equal in each of the post-operative periods and was therefore independent of survival time. Fibrous tissue ingrowth into the middle-ear lumen was also observed, and regularly reached impressive proportions; large parts of the implant surface were covered (fig. 1a). Inside these ingrowths, pockets containing exudate were surrounded by what looked histologically like active secretory epithelial cells.

Epithelial reactions: All implants were covered by epithelium composed predominantly of flat polygonal cells, as revealed by scanning electron microscopy. These flat polygonal cells

Fig. 3. Transmission electron micrograph of pseudomucosa covering the implant. A granular layer can be seen at the interface (arrow). Bar: 1 μm.

Fig. 4. Macropore filled with exudate, fibrous tissue, and bone, one month after implantation of hydroxyapatite. Bar: 20 μm.

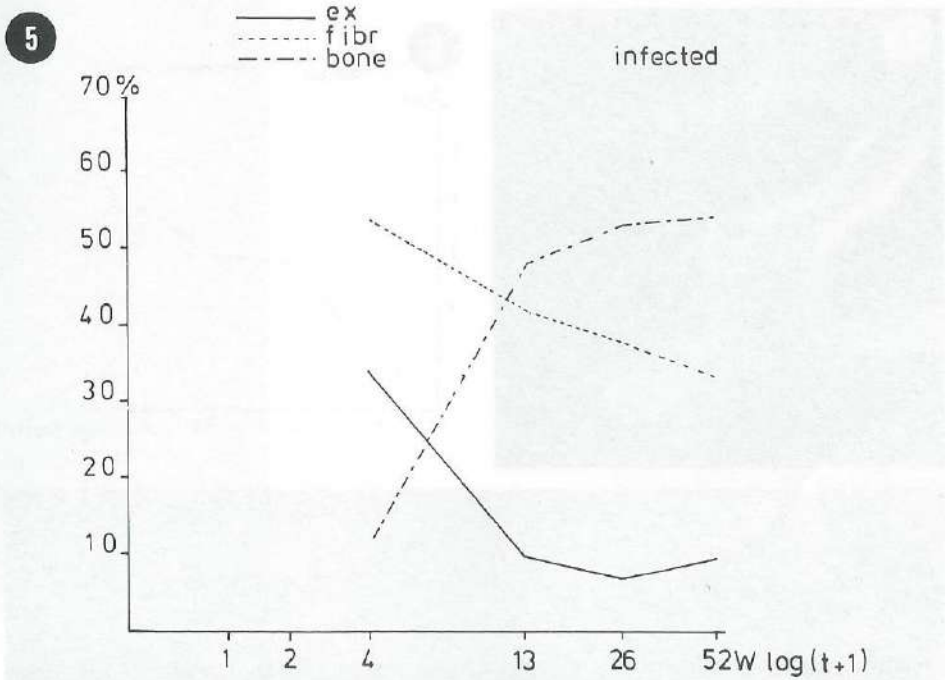
frequently bore a marginal row of microvilli and variable numbers of these structures on the cell surface, but cells without microvilli were also seen. Apart from the already-mentioned polygonal cells, a ciliated cell and a goblet-like cell were found, mainly when the implant was situated in the vicinity of the mucociliary tract, but also at other sites on the implant surface (fig. 2). Light and transmission electron microscopy showed that most of the epithelial cells with superficial microvilli were relatively thin and separated from the interface by a layer of fibrous tissue varying in thickness (fig. 3). Injection of tritiated thymidine followed by light-microscopical autoradiography showed some proliferation of epithelial cells in the one-month period and almost negligibly low activity in the longer periods.

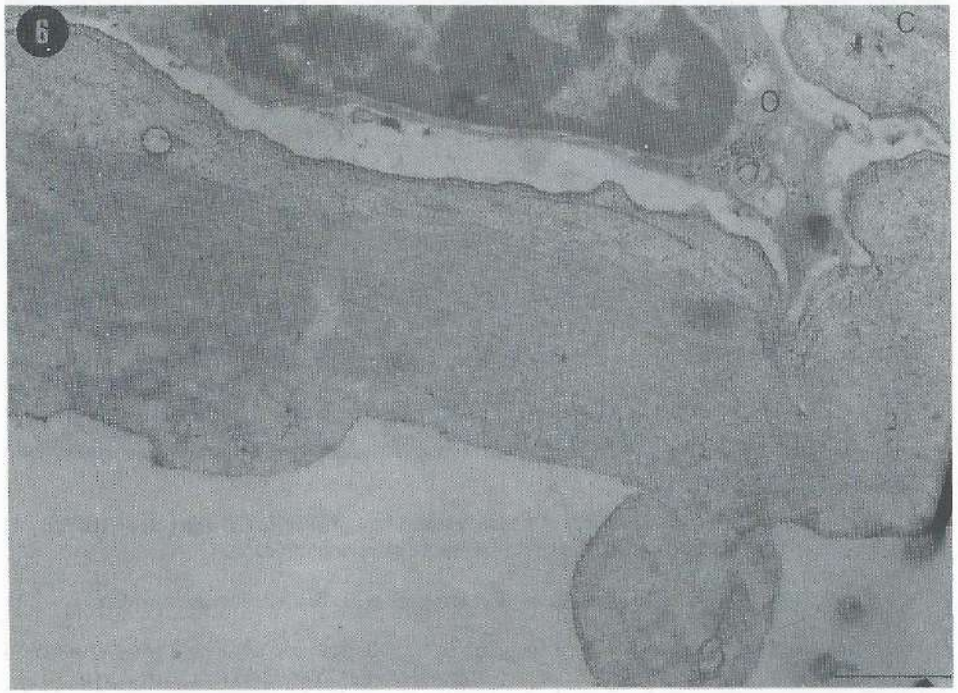
Macropore tissue characteristics: Light microscopy showed that in the one-month period the pores in the hydroxyapatite acquired a content of fibrous tissue, exudate, and bone (fig. 4). In all further post-operative periods up to one year the amount of bone increased and the relative area occupied by fibrous tissue decreased. The amount of exudate in the pores also seemed to be smaller than that seen after the shortest interval. Most of the exudate was present in macropores along the outer side of the implant. Morphometry, which was used to determine the macropore area occupied by each of the three types of tissue distinguished, confirmed these findings and showed that the amount of exudate present in the pores remained approximately constant between the third and the twelfth month (fig. 5). The incorporation of tritiated thymidine in zones of bone formation was most pronounced after one month; with longer post-operative periods this proliferative activity decreased. The fibrous tissue was characterized by a relatively high, constant tritiated thymidine uptake. Occasionally, areas with debris were found. Bone in the pores showed normal light-microscopical and ultrastructural characteristics, i.e., collagen, osteoblasts, osteocytes, and a single osteoclast (fig. 6). The bone fluorochromes alizarin and calcein gave information about the amount of bone deposited after 1, 2, 3, and 5 months post-operatively. Infection was induced at the earliest one week after the fluorochrome injection. The animals used in the fluorescence experiment were injected with the two fluorochromes at different times, which gave two separate fluorochrome lines in bony tissue. Since lines of both alizarin and calcein were always present, it may be concluded that there was no appreciable resorption due to the infection (fig. 7). A lamina limitans-like electron-dense granular structure was regularly found at the hydroxyapatite/tissue interface (fig. 3) but was absent where phagocytes bordered the implant.

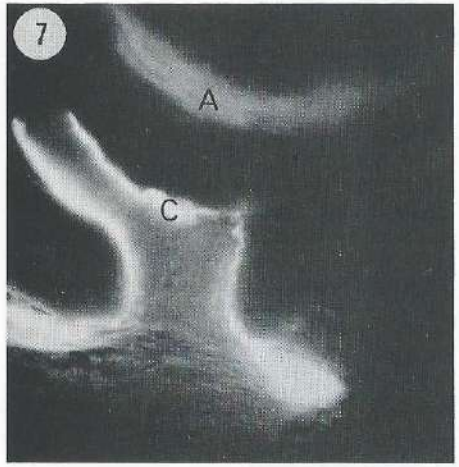
Biodegradation and phagocyte activity: To establish degradation of implant material, if any, the average macropore area in histological sections was determined by morphometry for each post-operative interval. The results of this morphometric analysis are given in fig. 8, which shows a 75% increase of the macropore area after one year relative to the area at one month. Apparently, this degradation was partially due to phagocytic activity of macrophages and multinucleated cells at the interface. This phagocytic activity was indicated by the presence of implant-derived material in the cytoplasm of these cells (fig. 9). Since the material studied

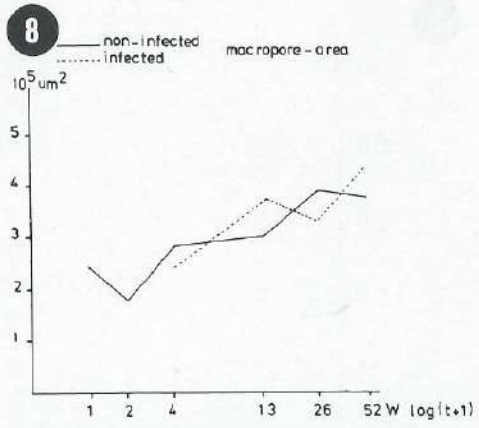
Fig. 5. Results of morphometric analysis of tissue present in the pores of the hydroxyapatite implant.

Fig. 6. Transmission electron micrograph of bone bordering the hydroxyapatite implant. An osteocyte (O) and collagen fibres (C) are visible. Bar: 1 μm.









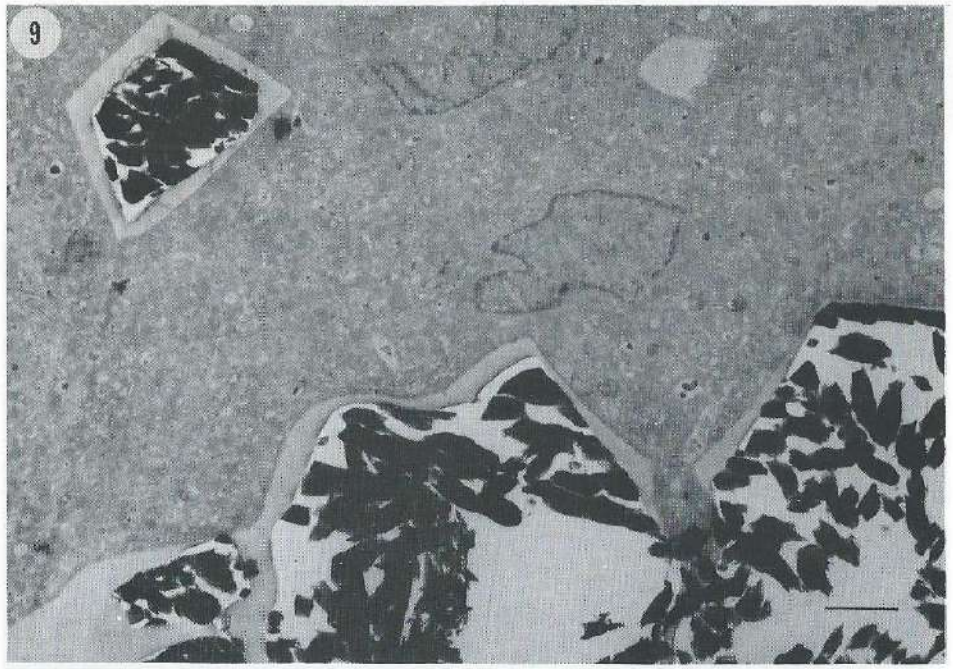


Fig. 7. Non-decalcified section (ca. 80 μm) of a macropore. Lines of alizarin (A) and calcein (C) can still be seen, indicating little or no resorptive activity of the bone during infection.

Fig. 8. Morphometric result for the average total macropore area. Biodegradation is evident.

Fig. 9. Low-power transmission electron micrograph of a multinucleated cell with a piece of implant-derived material in the cytoplasm. Note absence of granular layer at the interface. Bar: 2 μm.

by routine light and transmission electron microscopy was decalcified before being embedded, most of the phagocytes showed apparently empty, rather rectangular vacuoles. Occasionally, however, the original implant material was still present, and in such cases, there was similarity between the shapes of these vacuoles and the empty decalcified ones, indicating that hydroxyapatite had been present in both. Furthermore, phagocytosis was suggested by the presence of organelle-free adherence zones and the protrusion of cell cytoplasm into the micropores.

DISCUSSION

Staphylococcus aureus is not the most common pathogen in otitis media, but its presence in the infected middle ear has been reported (28, 29). Although another micro-organism, for example *Streptococcus pneumoniae* or *Haemophilus influenzae,* both of which are cultured more often from middle-ear effusions (28), might have seemed to be a more obvious choice, the use of *Staphylococcus aureus* offered two great advantages. In the first place, *S. aureus* is one of the main micro-organisms cultured from infected wounds (30), and secondly, the experimental animal used in this study, the rat, is rather resistant to it (31), which means that an experimentally induced infection of the rat middle ear will be rather mild, thus facilitating study of the effects. Furthermore, the effects of an intratympanic injection of *Staphylococcus aureus* had already been studied (31-34) and the findings could serve as control data. Compared with the earlier observations during infection, the infected middle ear with hydroxyapatite implants showed the same characteristics as the infected middle ear without an implant three weeks after the introduction of *Staphylococcus aureus.* The most prominent features seen after three weeks were fibrous-tissue ingrowth into the middle-ear lumen and the deposition of a layer of new bone on the middle-ear wall. Hydroxyapatite present in the infected middle ear was frequently partially embedded in this ingrowth of fibrous tissue. On the basis of the control experiment as reference, it may be stated that such ingrowth is not the result of the presence of implant material but seems to be due entirely to the infection.

An important finding concerning a biomaterial in the infected environment is that it can become covered with host tissue. Studies on hydroxyapatite in the middle ear (24, 25) revealed the presence of an epithelial layer very similar to the original epithelial lining (36, 37). The present study showed a comparable epithelium consisting of flat polygonal cells and, of more importance, the presence of ciliated and goblet-like cells. The latter two cell types play an important role in middle-ear defence in that the former provide mechanical transport and the latter have a secretory function (38).

Although the presence of macropores promotes good fixation of the implant into the host tissue, these pores may also serve as foci for the development and persistence of infection. However, Kiechel (39) did not find a negative influence of macropores on the genesis of infection.

The presence of exudate in the pores may be regarded as a sign of inflammation or infection. However, the amount of exudate encountered in the pores in this experiment was not much greater than that seen in the earlier experiments performed in the non-infected middle ear. Thus, the effects of infection in macropores were not impressive, and potentially negative effects of the presence of pores on the persistence of infection are not likely to occur. Further-

more, most of the exudate in the pores was seen in those along the edge of the implant, an area which seems to be susceptible to antibiotic treatment.

It has been postulated that infection can affect the bone present in macropores of an implant material (7, 40). To investigate this phenomenon in relation to hydroxyapatite, use was made of fluorescence studies and morphometry. Fluorescence showed unequivocally that if resorption of bone had occurred it must have been very limited, because lines derived from separate fluorochrome injections of alizarin and calcein could always be clearly distinguished. Morphometry confirmed this result, since comparison of the amount of bone present in the pores after the various post-operative intervals with the amount found in the implant in the non-infected middle ear (35), showed no noticeable differences. In a way this is surprising, because in the control infection experiment (34) bone resorption was seen one day after the introduction of *Staphylococcus aureus* into the tympanon. This might be explained by absence of resorption in the pores due to milder inflammation at those sites or by such weak resorption that the fluorochrome lines did not dissolve. The control infection experiment suggested that bone deposition was to be expected, but this process was not observed either.

Another factor of possible importance for biomaterials in an infected area is an effect of the inflammatory process on the degradation of implant material. Three mechanisms of degradation can be distinguished: a pH shift during inflammation, a phagocyte-induced process, and microbial decomposition. The first of these mechanisms deserves consideration, here because Klein (41) had described *in vitro* degradation of hydroxyapatite in acid surroundings. The second has been reported to occur in hydroxyapatite (27), and the third has been described in dental resins (42). Since some bacteria are known to adhere to hydroxyapatite (43, 44) this third mechanism might also play a role with respect to hydroxyapatite. The degradation of hydroxyapatite found in the present study corresponds well with findings in the non-infected middle ear (27). Therefore, a pH shift and bacterial-induced degradation are not likely to occur in this experimental set up. Macrophages and multinucleated cells, however, provided clear evidence of phagocytosis, since they displayed not only vacuoles that contained or must formerly have contained hydroxyapatite, but also cytoplasm protrusions in macropores and organelle-pore adherence zones characteristic for endocytosis (45). It is of interest that, in contrast to the findings in the non-infected middle ear (30), continuing degradation was established at twelve months for implants left in middle ears which had been infected for the preceding three weeks. This persistance may have been coincidental since it is certainly not significant, but could have been due to phagocytic activity of exudate cells present as a result of the inflammatory process. If the latter hypothesis proves to be valid this might indicate a temporary degradation during infection caused by an influx of exudate macrophages, a type of cell that is to become prominent during infection.

From the foregoing, the effects of a *Staphylococcus aureus*-initiated infection on hydroxyapatite seem to be limited and usually temporary. It should be kept in mind, however, that this study was limited to only one of the micro-organisms present during otitis media. Nevertheless, the results support the view that hydroxyapatite is a very promising material for application in reconstructive middle-ear surgery.

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CHAPTER VIII

Biological Evaluation Of Hydroxyapatite During Short-term Infection

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SUMMARY

Macroporous hydroxyapatite was implanted submucosally in the rat middle ear and studied after intratympanic injection of a *Staphylococcus aureus* suspension. The middle-ear infection was evoked one week after the implantation, and the effects of infection on the middle ear and the implant material were evaluated after one, three, seven, and fourteen days by light and electron microscopy. The findings in the infected middle ear with an implant corresponded well with those described for the infected middle-ear cavity without an implant. The reactions of the tissue over the implant were similar to those of the original mucosa of the middle ear.

Bone was deposited on the implant and in its pores in relatively large quantities. Biodegradation, due at least partially to phagocytic activity of macrophages and multinucleated cells, was more prominent than previously found. This higher degree of biodegradation may be attributed to the use of the submucosal implantation technique, because this was the only point of divergence as to material or methods from earlier work reported by our group.

The present results together with those published earlier suggest that this material has promising features for use as a bone substitute in reconstructive middle-ear surgery. Definitive conclusions on biocompatibility and biofunctionality will, however, have to await long-term clinical trials.

INTRODUCTION

Chronic middle-ear infection can lead to the destruction of vital parts of the sound transmission system. Although these parts can be replaced by autologous or homologous grafts (1), such materials are relatively scarce and their preservation and antigenicity are still controversial subjects. Therefore, allogenous biomaterials lacking these disadvantages have come into use in reconstructive middle-ear surgery. Among the allogenous biomaterials, bioactive ceramics such as bioglasses (2, 3) and calcium phosphates (4, 5) as well as the bioinert aluminum oxide (6) seem to be promising for reconstruction of the ossicular chain. The favourable features in question have been established by experimental and clinical findings (3, 4, 6-10). The calcium-phosphate ceramics resemble the mineral matrix of bone (11, 12), and this holds especially for hydroxyapatite, which constitutes 80% of matrix. This high degree of similarity suggests good biocompatibility, as has been demonstrated in several studies in the rat and human middle ear (5, 8, 13-15).

However, most assessments of the biocompatibility of biomaterials considered or in use for

reconstructive middle-ear surgery have ignored the frequent occurrence of infectious middle-ear disease. The effects of infection vary widely and may be expected, for instance, to exert an influence on bone formation (16-18) and/or material biodegradation (19). Only hydroxyapatite has been studied in the infected middle ear, i.e., in an experimental study done in the rat (20), and the findings showed no significant effects on either the biomaterial or the surrounding tissue. Because that study was limited to the effects of infection three weeks after experimental induction, the short-term effects remained to be investigated.

In the present study the effects of an experimentally evoked rat middle-ear infection on macroporous hydroxyapatite were studied on days 1, 3, 7, and 14. *Staphylococcus aureus* was chosen as infectious micro-organism because of its known effects on the middle-ear tissue of the rat (18) and its frequent presence in contaminated wounds (21).

MATERIALS AND METHODS

Implant material: Macroporous sintered hydroxyapatite was used. This material is characterized by *ca.* 26% macroporosity (pore ∅ *ca.* 100 μm) and 5% microporosity (∅ *ca.* 3 μm). The sintering technique has been described elsewhere (22). The hydroxyapatite was from the same sintering batch as used previously (13).

Implantation technique: For the introduction of hydroxyapatite implants into the middle ear of male Wistar rats (mean weight at operation: 200 g), the bony middle ear bulla wall was carefully opened superio-posteriorly, leaving the mucosa intact. Each middle ear received two hydroxyapatite implants, some of which were inserted between the bony wall and the mucosa, the other being used to close the resulting defect. Thus, both implants were in contact with the mucosa.

Infection: One week after the operation, infection was induced by intratympanic injection of 1.4×10^6 *Staphylococcus aureus* in saline. Rats were killed on days 1, 2, 7, and 14 after the injection. Light microscopy and scanning and transmission electron microscopy were used as investigative techniques. A total of 64 implants were studied. For the exact distribution of the implants over time and experimental technique, see table I.

Histology: Use was made of routine scanning and transmission electron microscopy techniques described elsewhere (13, 18). The material destined for transmission electron microscopy and histological studies was decalcified in an EDTA solution (EDTA 10%, glutardialdehyde 1.5%, pH 7.4).

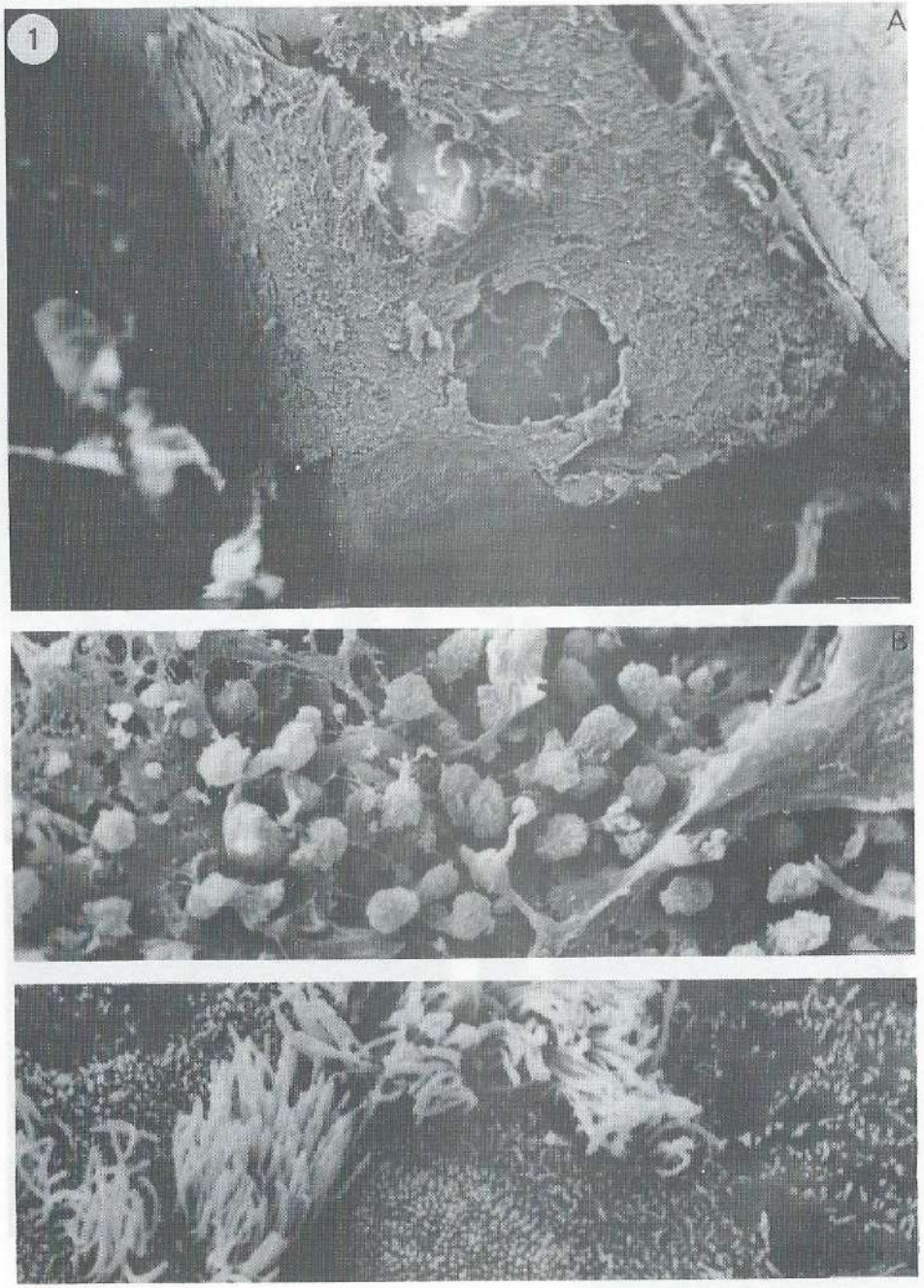
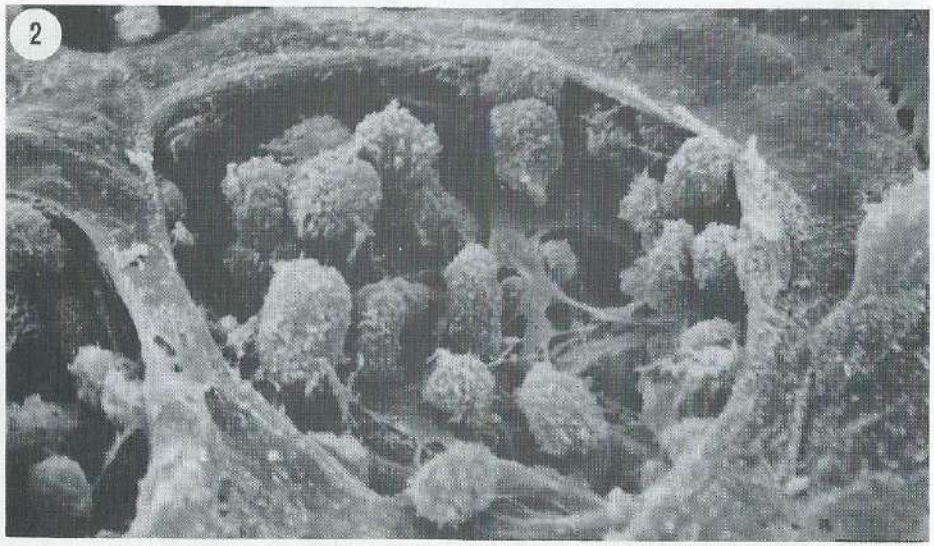


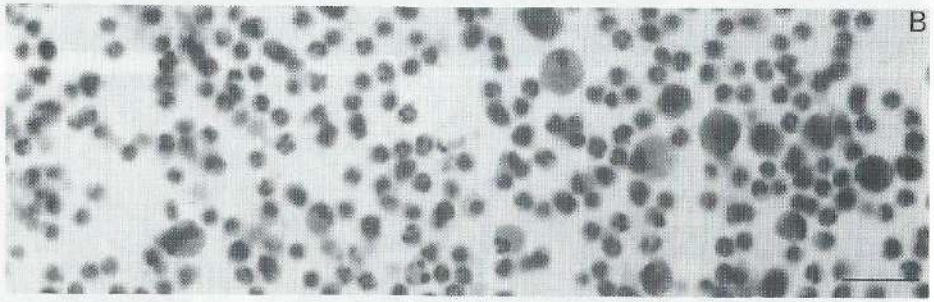
Fig. 1a-c. Scanning electron micrographs of hydroxyapatite three days after induced infection.

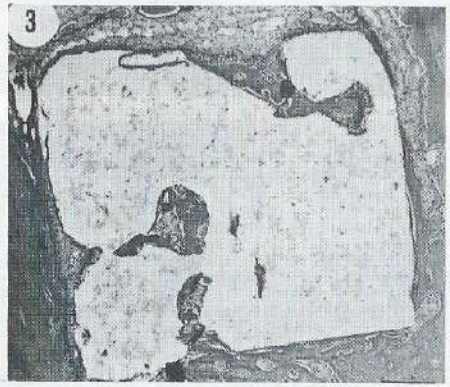
Fig. 1a. Note the irregular structure partially covering the epithelium. Bar: 150 μm.

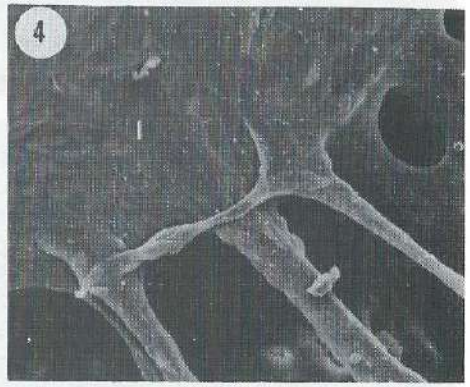
Fig. 1b. Detail of the same irregular structure showing the presence of exudative cells. Bar: 10 μm.

Fig. 1c. Ciliated cells on the implant shortly after induction of infection. Bar: 3 μm.









Morphometry: For the morphometrical analysis of the tissue in the macropores and estimation of the macropore area, 344 histological sections cut 7 μm thick were photographed at standard magnification and measured on an x-y tablet.

	1d	3d	7d	14d	TOT
HIS	8	8	8	8	32
SEM	4	4	4	4	16
TEM	4	4	4	4	16
TOT	16	16	16	16	64

Table 1: Distribution of hydroxyapatite implants according to time after the induction of infection and the three techniques applied.

RESULTS

In the following, the events observed in and around macroporous hydroxyapatite in the infected middle ear are described, with special attention to the implant surface and middle ear cavity, the macropore reactions, and biodegradation and phagocytic activity. Since no significant differences were found between the two implantation sites, they will discussed together.

Implant surface and middle-ear cavity

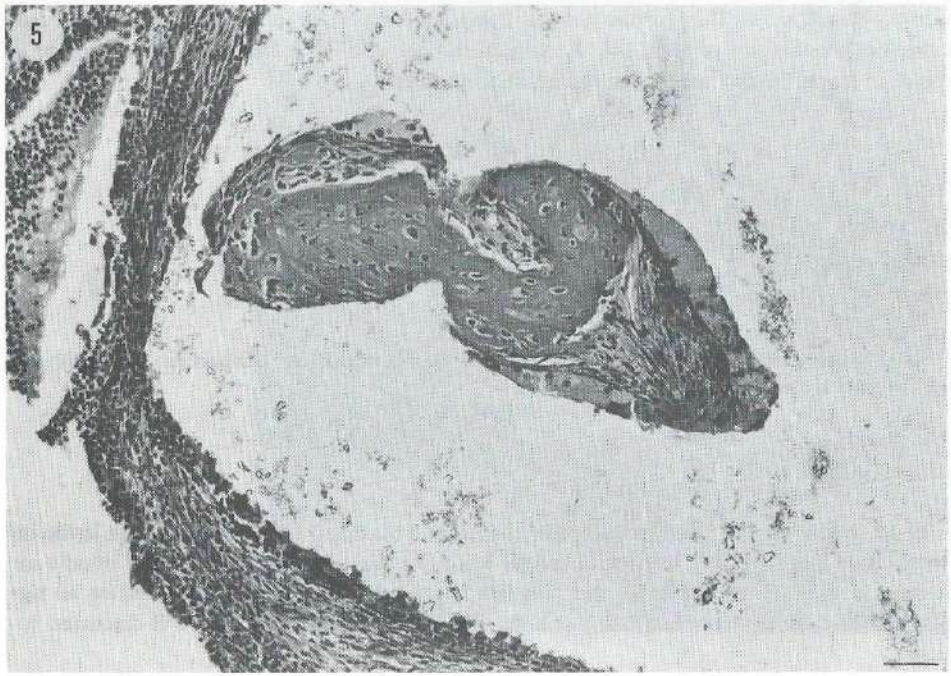
Shortly after the introduction of *Staphylococcus aureus* into the middle ear, several phenomena were observed in the tissue at the implant surface. For instance, scanning electron microscopy showed that part of the implant was covered by a structure containing numerous exudate cells (fig. 1, a and b), whereas flat polygonal and ciliated cells were present at other sites (fig. 1c). The flat polygonal cells had varying numbers of microvilli on the cell surface and occasionally had a swollen appearance. The exudate cells were seen on the epithelial layer but also on the fibrous tissue or on a layer of apparently mucoid composition. Frequently, exudate cells were found in what appeared to be a gap in the epithelial covering (fig. 2a). Apparently, some of the exudate cells on the implant had originated from the exudate which, in the form of mucopurulent fluid, almost completely filled the middle-ear lumen after day 1. After that the

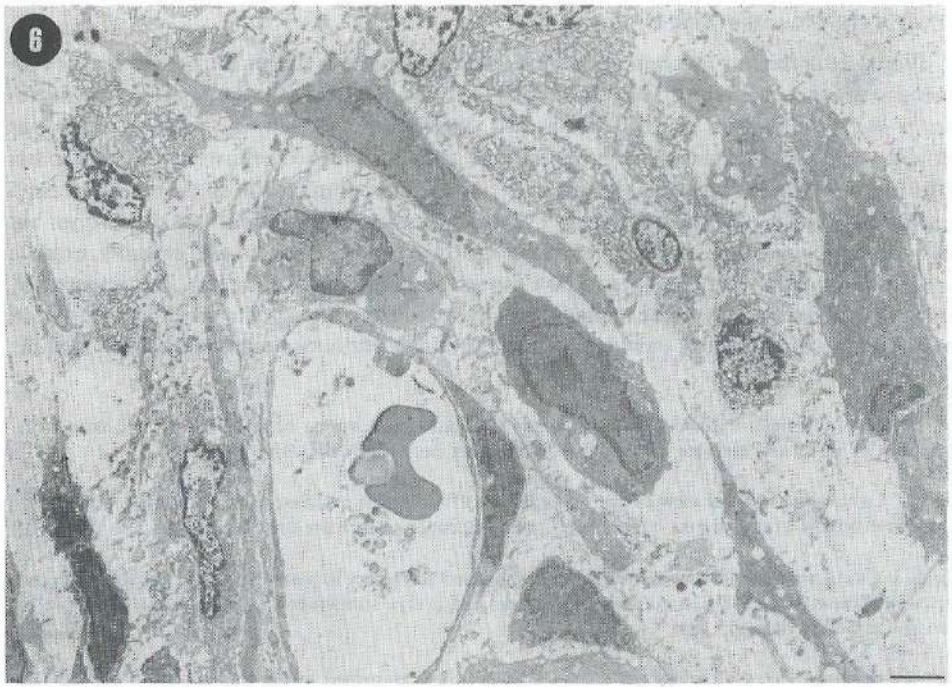
Fig. 2a. Scanning electron micrograph of part of the epithelial covering of an implant three days after induction of infection, showing perforations containing exudative cells. Bar: 5 μm.

Fig. 2b. Light-microscopical view of the mucopurulent fluid in the middle ear on the third day of infection. Neutrophilic granulocytes, lymphocytes, and macrophages are present. Bar: 10 μm.

Fig. 3. Light micrograph of macroporous hydroxyapatite 3 days after implantation. The implant is in contact with fibrous tissue at the middle-ear lumen side, and is now surrounded by bone. Bone tissue is also seen in the pores. Bar: 125 μm.

Fig. 4. Scanning electron micrograph of the fibrous tissue connected to the implant (I). Note the flat polygonal cells. Bar: 30 μm.





amount of this fluid decreased, and after a week it was only found in pockets of pus in the fibrous tissue. The distribution of the types of cell in the exudate varied with time. The histological studies showed that the exudate was predominantly filled with neutrophilic granulocytes and lymphocytes after day 1; after three days macrophages were also present (fig. 2b) and their number increased in the decreasing amount of fluid after one and two weeks. The exudate cells in the tissue on the implant also included neutrophilic granulocytes, lymphocytes, and macrophages, and the fibrous tissue around the implant showed multinucleated cells as well. All of these types of exudate cell were easily recognized from their morphological characteristics in transmission electron microscopy. Transmission electron microscopy also showed debris in the fibrous tissue together with normal-looking collagen and fibroblasts. Capillaries were more prominent in the early stages. Light microscopy showed another characteristic feature of infection, the ingrowth of fibrous tissue into the middle-ear lumen, which assumed impressive proportions with the prolongation of infection time. These ingrowths were sometimes in contact with the tissue envelope around the implant (fig. 3). Pockets of exudate were even seen in the fibrous ingrowths two weeks after the onset of the infection. Scanning electron microscopy also showed these structures (fig. 4) and their covering layer of flat polygonal epithelium.

As the time after infection increased, the exudate cells at the implant surface gradually disappeared and the number of swollen epithelial cells decreased. Flat polygonal epithelial cells became more prominent and ciliated cells were seen, especially near the mucociliary tracks of the middle ear. After one and two weeks, these mucociliary tracks had become much larger than in the normal non-infected ear.

Another interesting observation at the implant surface was the presence of bone. As early as day 1, part of the implant was covered by large amounts of bone, as shown by light microscopy (fig. 3). Bone deposition associated with infection was also seen at other sites on the middle ear wall. This bone deposition was preceded by bone resorption at the border between the wall and lumen of the middle ear after one day, as shown by lacunae in the bony middle-ear wall.

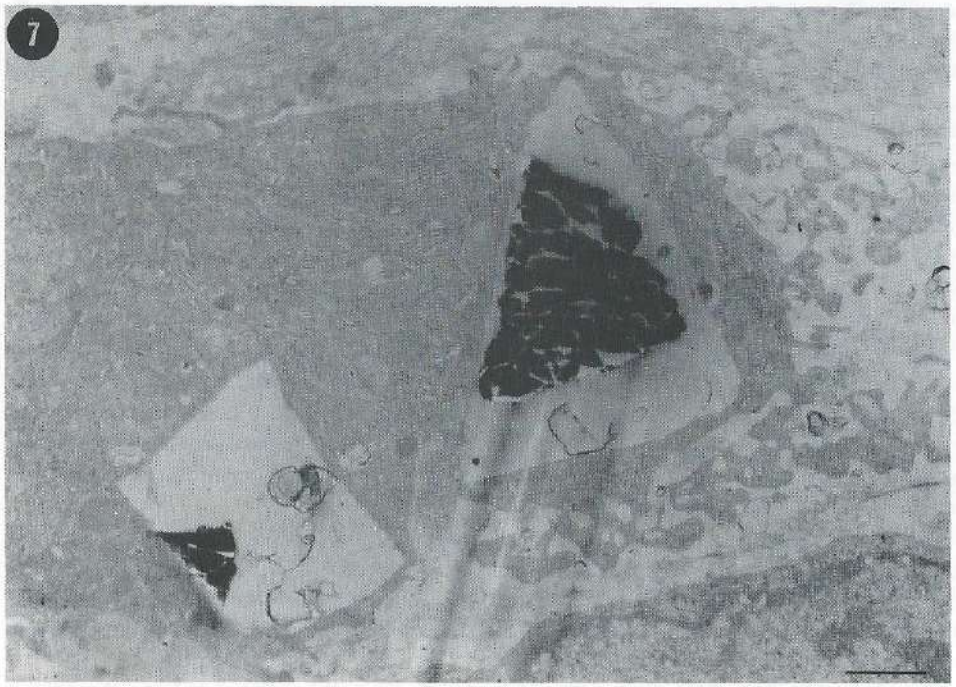
Macropore reactions.

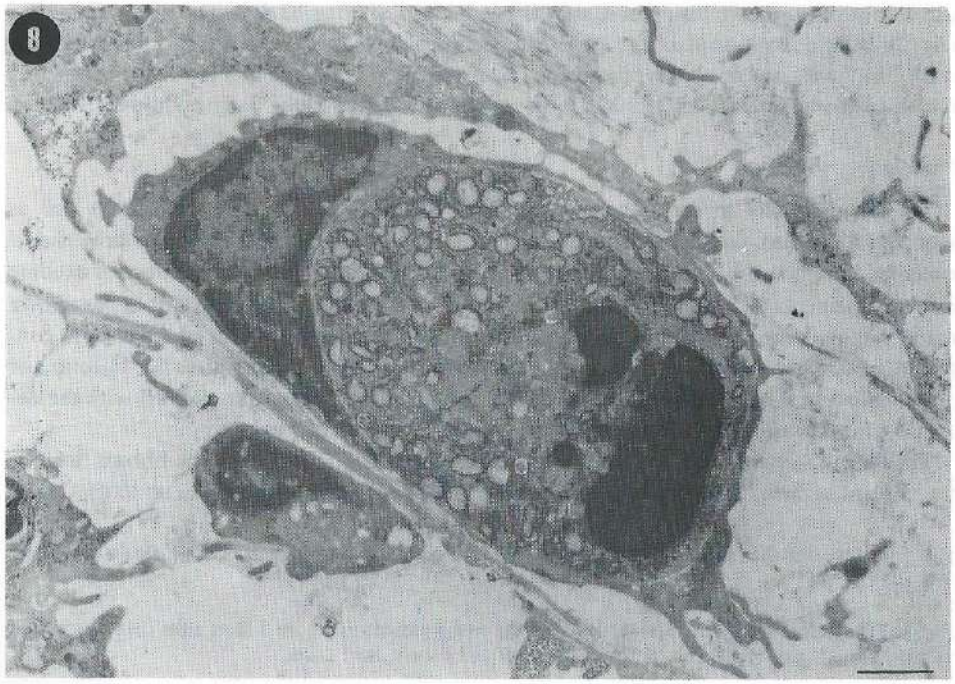
The tissue present in the macropores of hydroxyapatite was studied by light as well as scanning and transmission electron microscopy. Light microscopy revealed the presence of exudate, fibrous tissue, and bone in the pores (fig. 5). With respect to the amount of exudate on the implant surface and in the middle ear lumen, it is interesting to note that the exudative reaction in the pores appeared to be weaker and that pores near the surface of the implant contained more exudate than those near the centre. With increasing time after the injection of *Staphylococcus aureus* the amount of exudate decreased in the pores in parallel with the decrease of mucopurulent fluid in the middle-ear lumen.

The morphology of the fibrous tissue in the pores was similar to that of the fibrous tissue at the implant surface, and the amount did not seem to undergo major changes up to day 14. In

Fig. 5. Macropore after 3 days of infection. Exudate fibrous tissue and bone are visible. Eosin haematoxilin. Bar: 30 μm.

Fig. 6. Transmission electron micrograph showing tissue present in a pore 3 days after the induction of an infection. A striking feature is the presence of cell debris. Bar: 2 μm.





the fibrous tissue, cell debris was most prominent on days 1 and 3 (fig. 6). The morphology of the bone in the pores was normal, showing calcified matrix, collagen bundles, and osteocytes. In the early phase of the infection osteoblasts frequently showed signs of lysis. After that, however, the amount of cell debris decreased and the number of osteoblasts with normal morphology increased.

The morphometric results confirmed the initial light-microscopical and transmission electron-microscopical findings. Analysis of 344 histological sections of macropores showed that the amount of exudate decreased from 35% of the macropore area at day 1 to 16% at two weeks. Fibrous tissue remained rather constant at 30% at day 1 and 32% after two weeks, whereas bone initially occupied 43% of the macropore area, which increased to 49% two weeks after the infection was induced.

Biodegradation and phagocytic activity.

The morphometric results were also used to assess possible biodegradation of implant material by estimating the average macropore area at each of the time-points. The macropore area was found to have increased by 69% after day 14, which corresponded to an 18% decrease of total implant area.

Transmission electron microscopy revealed that part of this biodegradation must have been at least partially caused by the phagocytic activity of macrophages and multinucleated cells. These cells frequently showed vacuoles containing implant-derived, electron-dense material as well as many vacuoles with a rectangular shape suggesting that implant material had been present before decalcification (fig. 7). The phagocytic activity of these cells was not limited to implant material, since they also contained mucus, bacteria, cell debris, and occasionally complete cells (fig. 8).

DISCUSSION

Surprisingly, the only otologic biomaterial which has been tested during experimentally induced infection is hydroxyapatite. (20). Evaluation of biomaterials used, for example, for sutures and vascular prostheses under the conditions of infection has been reported more often (23-28), and the material properties appeared to exert an influence on the incidence of infection. Differences in material properties which may be considered to be of significance for the incidence and course of an infection include the chemical composition, surface structure, and macropore volume. For hydroxyapatite it is known that bacteria can adhere to the surface (29, 30), and no special effects attributable to the presence of macropores were found (20).

The micro-organism used in this study, *Staphylococcus aureus,* is not frequently encountered in middle-ear infections. However, it offered some advantages, such as the rat's relative resistance to it (31), its frequency in contaminated wounds (21), and the availability of comparable control experiments without biomaterials (18). Although the relative resistency of

Fig. 7. Transmission electron micrograph showing partially decalcified implant-derived material in the cytoplasm of a phagocyte 1 day after the induction of an infection. Bar: 1 μm.

Fig. 8. Transmission electron micrograph of a macrophage phagocytosing a cell, 3 days after the injection of *Staphylococcus areus.* Bar: 1 μm.

the rat to *Staphylococcus aureus* seems at first to be a disadvantage rather than an advantage, it should be kept in mind that the use of large numbers will induce an infection whose effects can be adequately studied just because of the relatively low severity of the reaction.

Comparison of the effects evoked by *Staphylococcus aureus* in the middle-ear cavity containing an implant with those found in the absence of a biomaterial in an earlier study showed no significant differences with respect to the cellular, mucociliary, fibroblastic, and bone responses. As to the cellular response leading to the presence of neutrophilic granulocytes, lymphocytes, and macrophages, the proportions of these cells corresponded with those of the cell population in mouse peritoneal exudate (32). Besides these three cell types, multinucleated cells were also found. Since these cells have not been reported for the infected middle ear without an implant, their presence must be attributed to the presence of the implant, which is consistent with the findings for hydroxyapatite implants in the non-infected middle ear. The latter results showed that these cells were especially dominant within the first three months after implantation and at sites of obvious mechanical friction. The occurrence of a mucociliary response was indicated by the mucociliary tracks covering a large part of the middle ear in the longer periods of infection. Fibroblastic ingrowths in the middle-ear lumen were demonstrated, and occasionally showed contact with the implant. The fourth mechanism, bone response, was seen in the form of osteoresorption at the bony middle-ear wall after day 1 and as osteogenesis at the later time-points. Apparently, events in the middle-ear cavity during a *Staphylococcus aureus*-induced infection do not differ essentially in the absence and presence of implanted macroporous hydroxyapatite.

The tissue reactions in or over hydroxyapatite were also quite similar to those seen in middle-ear tissue during an experimentally induced acute infection. In both cases the epithelium initially showed some swelling and damage. The reaction in tissue over hydroxyapatite, however, seemed more severe, in all probability due to mucosal damage caused by the surgical intervention. Despite the damage done to the epithelium, large parts of the implant were covered with flat polygonal epithelial cells, the predominant cell type in the normal rat ear (33), and ciliated cells were also found.

The impressive occurrence of bone around the implant and in the pores differs from the findings in earlier studies on hydroxyapatite in the infected and non-infected middle ear for such short implantation periods (3, 8, 13, 20). Since there was only one divergence from the technique used in our earlier studies, i.e., the use of submucosal implantation, this effect must be attributed to the different implantation technique, which establishes close contact between the periosteum of the middle-ear bony wall and the implant. This contact might explain the intensity of bone deposition. Furthermore, the immediate covering of an implant with mucosa might lead to early stabilization of the amount of fibrous tissue, as found in the present study.

Concerning the exudate in the macropores, it was interesting to note that in accordance with an earlier study with longer infection and implantation times (20), the peripheral pores in the implant contained much more exudate than those in the centre. In addition, morphometry showed that the amount of exudate decreased rapidly with time, which corresponds with the findings in infections induced after longer implantation periods and in implants in normal ears (8, 20). Morphometry showed another significant event, i.e., a 69% increase of the macropore area by day 14, which corresponds with the amount of biodegradation reported for hydroxyapatite in the non-infected middle ear (13) after six and twelve months. However, this increased degradation cannot be attributed to the occurrence of infection, since we did not observe it in earlier infection experiments. The only remaining explanation for the increase must be the difference in implantation technique. This might explain the conflicting reports on the degree of biodegradation of hydroxyapatite, some authors having found degradation (13, 34) and others none (35, 36). At first sight the high degree of biodegradation found seems disquieting, but it must be kept in mind that the enlarged macropores became filled with large amounts of bone and fibrous tissue (49% and 32%, respectively). Apparently, biodegradation need not mean an essential decrease of biocompatibility and/or biofunctionality.

While we wait for the results of long-term clinical studies making use of different implantation sites and different techniques, it seems justified to use hydroxyapatite as a material for reconstructive middle-ear surgery on the basis of the findings in the present and other experimental studies (5, 8, 13, 20) and the preliminary clinical results (9, 10).

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CHAPTER IX

Biological Performance Of ß-whitlockite

A Study In The Non-infected And Infected Rat Middle Ear

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SUMMARY

The biological performance of macroporous ß-whitlockite implanted in the rat middle ear was evaluated. The material was studied in the non-infected middle ear and in middle ears infected by *Staphylococcus aureus*. ß-whitlockite was quickly covered by a normal mucosa. One week post-operatively the macropores were filled with exudate, fibrous tissue, and a small quantity of bone. Six months after the operation the greater part of the macropore area was filled with bone (74%); fibrous tissue accounted for 20% and exudate for 5%. In histological sections the macropore area of ß-whitlockite had increased by 68% after six months, indicating biodegradation. Macrophages and multinucleated cells were present in the vicinity of the implant and played a role in this biodegradation. Besides cytoplasmic vacuoles containing calcium phosphate, the cells showed smaller granules containing trace elements originally present in the implant material, such as silicium, titanium, aluminium, iron, and magnesium.

INTRODUCTION

A variety of biomaterials have been applied in otologic surgery (1-8). Among these materials, ceramics have recently attracted interest. At the moment two kinds of ceramic materials used in otologic surgery can theoretically be distinguished, the bioinert ceramics based on aluminumoxide and the bioactive ceramics represented by the bioglasses and the calcium phosphate ceramics. So far, only the calcium phosphate ceramics have been fabricated in a porous form, a structure which can contribute to the successful integration of these materials. Another advantage offered by the calcium phosphate ceramics lies in their composition, which is rather similar to that of the mineral matrix of human bone; this holds especially for hydroxyapatite, which even has a crystal structure similar to that of the human middle-ear ossicles (9). Some of the past confusion concerning ß-whitlockite and hydroxyapatite originated from the terms tricalcium phosphate (ß-whitlockite) and calcium triphosphate (hydroxyapatite). It is conceivable that this confusion is partially responsible for the divergent reports about biodegradation. With respect to hydroxyapatite several authors found no degradation (7, 10-12) whereas others did (13, 14). In view of the subject of the present paper it should be mentioned that degradation has been found in the middle ear (15). The controversy is similar concerning ß-whitlockite, with reports claiming limited or no degradation (16) and others describing

distinct degradation (11, 12). Apart from differences in biomaterial composition, the use of different animal species and different implantation sites may contribute to this divergence in results.

The present study was performed to compare the behaviour of ß-whitlockite and hydroxyapatite in the rat middle ear. The latter material showed mild biodegradation (15, 17). Both materials have been investigated extensively by Klein et al. (11, 12) in the tibia and subcutaneously in the rabbit. According to their observations, hydroxyapatite was much less susceptible to resorption than ß-whitlockite.

In reconstructive middle-ear surgery it should be kept in mind that the chance of postoperative infection is relatively large. It has been reported that biomaterials may promote the occurrence of infection (18, 19), and their structure and composition may exert an influence on the severity of such an infection. For this reason, ß-whitlockite, a material intended for reconstructive middle-ear surgery, was tested in both the non-infected and the infected middle ear of the rat.

MATERIALS AND METHODS

Implant material: The material used in this study was characterized by a ß-whitlockite lattice and a stoichiometry of $Ca_3(PO_4)_2$. The material has a macroporosity (pore ∅ *ca.* 100 μm) of approximately 37% and a microporosity (pore ∅ *ca.* 3 μm) less than 5%. The exact preparation and properties have been published elsewhere (20).

Surgical procedure: The middle-ear bulla of the male Wistar rat (± 200 g) was reached via a superio-posterior approach. A small hole was drilled in the bulla with a diamond drill, after which the defect was obliterated by insertion of a piece of ß-whitlockite measuring about 1 mm^3.

Implant distribution: A total of 75 ß-whitlockite implants were used for obliteration of the middle-ear defect, 46 being placed in non-infected middle ears and 29 in the infected middle-ear cavity. The time-points selected for assessment ranged from one week to six months postoperatively. For a detailed description of implant distribution over these intervals and the techniques applied, see table I.

Induction of infection: Three weeks before decapitation, infection was induced by intratympanical injection of a suspension of *Staphylococcus aureus* in physiological saline. Approximately 1.4×10^6 bacteria were injected, as in the study on hydroxyapatite (21, 22).

Fixation: Immediately after decapitation, the middle ears destined for histology and transmission electron microscopy were filled with glutardialdehyde 1.5% in cacodylate buffer (0.14 M, pH 7.4, 0°C) injected intratympanically. After dissection, the middle ears were kept in this solution for 16 hr. The specimens intended for scanning electron microscopy were carefully rinsed with phosphate-buffered physiological saline (pH 7.4, 0°C) before fixation for two hours in the same fixative.

Histology: All material for the histological studies except the fluorescence material, was decalcified by immersion in a 10% EDTA solution (pH 7.4) containing 1.5% glutardialdehyde for four weeks after fixation. Decalcification was followed by routine paraffin embedding after which 7 μm sections were stained with haematoxylin eosin.

Autoradiography: Some of the animals received an injection of tritiated methyl-thymidine (specific activity 25 curies/mmol at a dose of 1 μCi per gram body weight) one hour before decapitation. Routine light-microscopical autoradiography was applied, and 7 μm-thick sections were stained with methyl green pyronin.

Bone fluorescence: Information on bone deposition 1, 2, 3, and 5 months after implantation was obtained by subcutaneous injection of alizarin and calcein. Animals decapitated after three months had received a subcutaneous injection of alizarin after one month and of calcein after two months. In the group to be studied at 6 months these injections were given after 3 and 5 months, respectively. After fixation in paraformaldehyde 1% (pH 7.4, 0°C) for 16 hr, the material was dehydrated, critical point dried in carbon dioxide, and embedded in MMA. Sections were cut 80 μm thick by diamond-blade sawing, ground, and subjected to fluorescence microscopy.

Transmission electron microscopy: After the same decalcification procedure as applied for histology, followed by rinsing for 24 hr, the material for transmission electron microscopy was post-fixed in phosphate-buffered osmium tetroxide 1% (pH 7.4) for 30 min. at room temperature, dehydrated, and embedded in Epon. Gold- to silver-coloured ultrathin sections were mounted on copper grids, contrasted with uranyl acetate and lead hydroxide, and studied with a Philips EM 200 transmission electron microscope.

Scanning electron microscopy: After fixation, the middle ears were dehydrated, critical point dried, and coated with gold. The implant material was studied with a Cambridge S 180 scanning electron microscope.

X-ray microanalysis: Some of the ß-whitlockite material prepared as for transmission electron microscopy was studied with a Philips EM 400 connected to a Tracor Northern (TN) 2000 X-ray micro-analyser. Both single-spot analysis and X-ray mapping were performed.

Morphometry: 312 histological sections more or less equally distributed over the various time-points were photographed at a standard magnification. Measurements were made on photographs with an x-y tablet. A distinction was made between total macropore area and areas occupied by exudate, fibrous tissue, or bone tissue. Unfortunately, the two-week interval could not be used for quantification because of the small number of implants available.

Table I: Distribution of implants over the various time-points and techniques applied. LM = light microscopy, EM = scanning and transmission electron microscopy.

	6 m		3 m		1 m		2 w		1 w		
	LM	EM	LM	EM	LM	EM	LM	EM	LM	EM	TOT
non-infected	5	5	6	4	6	4	2	4	5	5	46
infected	4	5	6	4	6	4					29

RESULTS

The implantation of ß-whitlockite in the rat middle ear evokes a variety of phenomena some of which are of interest with respect to the biological performance of this material. We made a

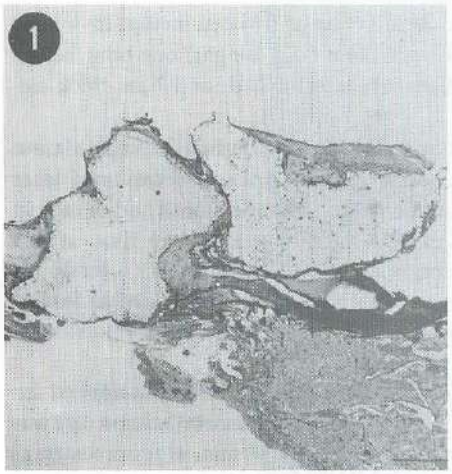


Fig. 1. Micrograph of ß-whitlockite after a 3 months stay in the middle ear. Note the direct contact with bone and the thin epithelial covering. Bar: 125 μm.

distinction between several aspects which in our opinion are relevant for the evaluation of the suitability of this implant material for application in reconstructive middle-ear surgery. These aspects will be discussed first for the experiment without infection, and the infection experiment will be dealt with in the second part of this section.

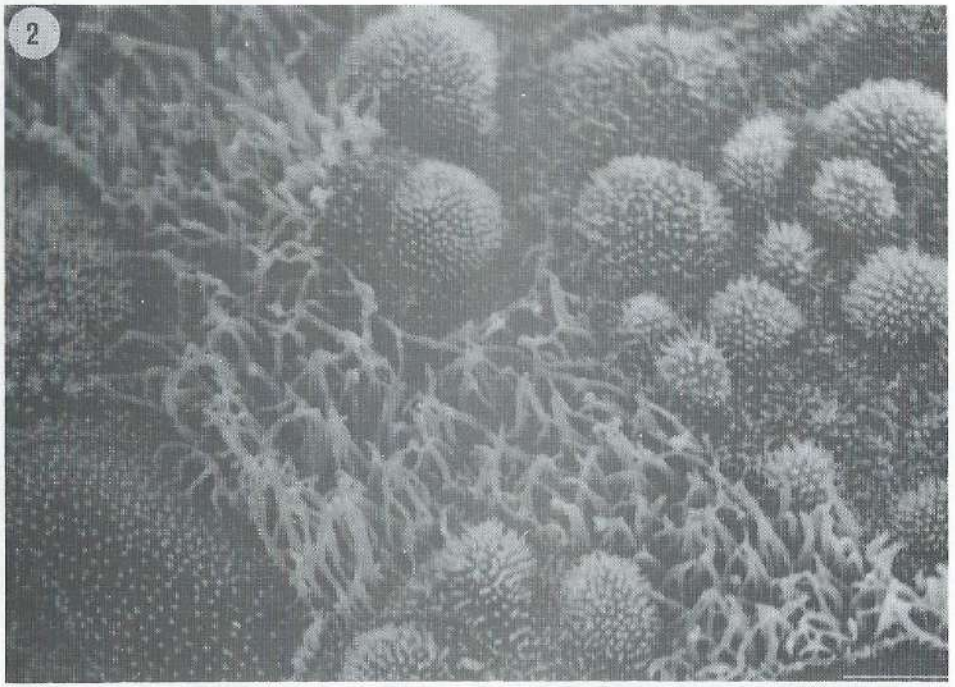
Epithelial reactions: Between the middle-ear lumen and the bony middle-ear wall, the first biological structure of interest is the epithelial lining of the tissue envelope. Light microscopy revealed that one week post-operatively the greater part of the implant surface was already covered by an epithelial layer. After six months the biomaterial was completely covered by such tissue (fig. 1). In the early phase after the operation, incorporation of tritiated thymidine into the epithelial cells was observed autoradiographically. This incorporation, which indicates proliferative activity, disappeared with increasing time. Scanning electron microscopy showed that the greater part of the epithelial covering was composed of flat polygonal cells together with ciliated epithelial cells (fig. 2, a and b). Some of the polygonal cells were characterized by a row of microvilli at the cell border and by a variable number of these structures on the cell surface. Transmission electron microscopy demonstrated the rather thin structure of the flat epithelial cells (fig. 3a). In the early post-operative periods the morphology of the epithelial cells appeared to be more variable than it was after the longer periods. Initially, too, a more cuboidal-like epithelium was observed as well as some migratory activity, which was suggested by what looked like tracks of epithelial cells revealed by scanning electron microscopy.

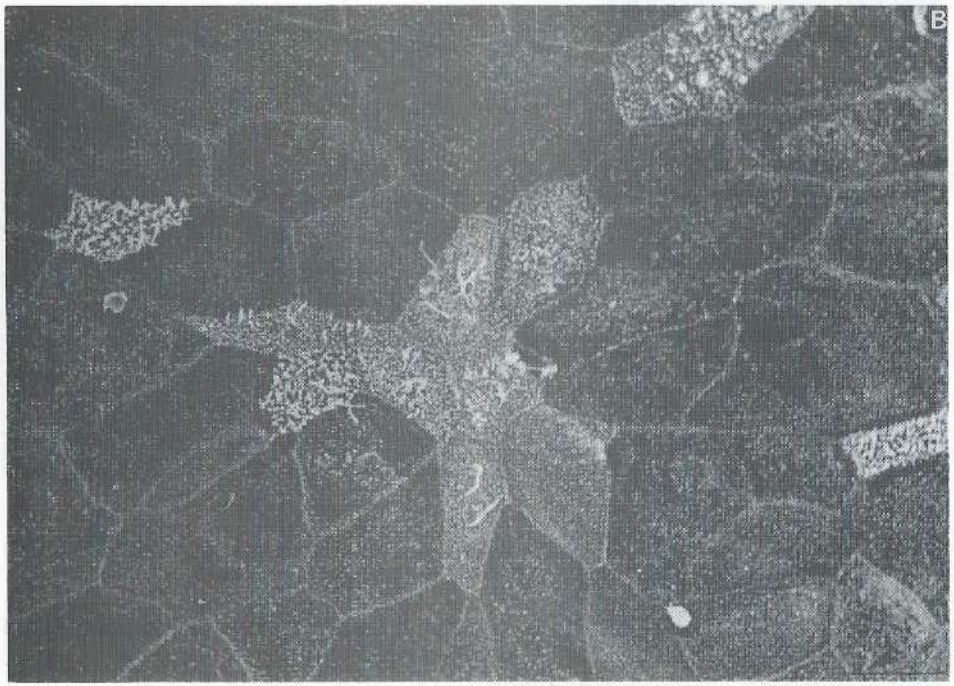
Pseudo lamina propria: Normally, the middle-ear mucosa consists of an epithelial layer separated from the middle-ear wall by a thin lamina propria with fibroblasts, collagen fibres, and capillaries. The thickness of this layer depends on the location in the middle ear. The layer

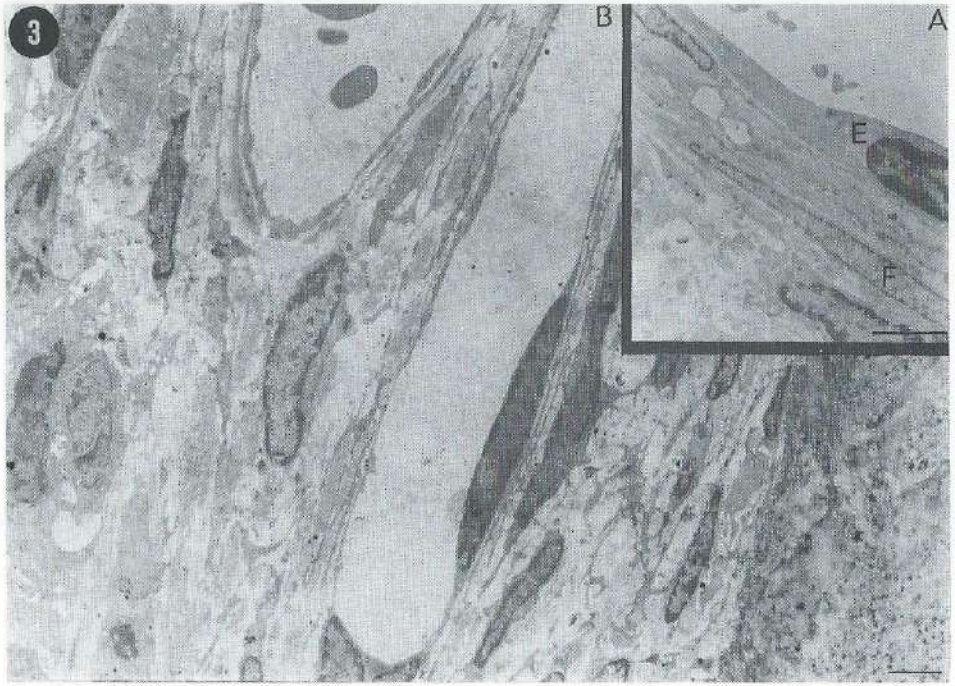
Fig. 2. Scanning electron micrographs of epithelial covering after 1 week.

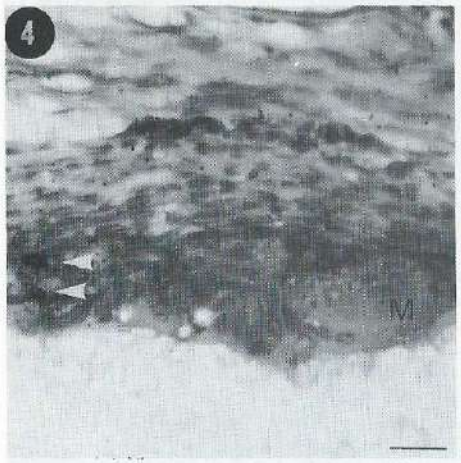
Fig. 2a. The cells have a "cobblestone" appearance and abundant microvilli. The presence of ciliated epithelium is prominent. Bar: 3 μm.

Fig. 2b. At this site, predominantly flat polygonal cells are present. A similar picture was found at later time-points. Bar: 10 μm.









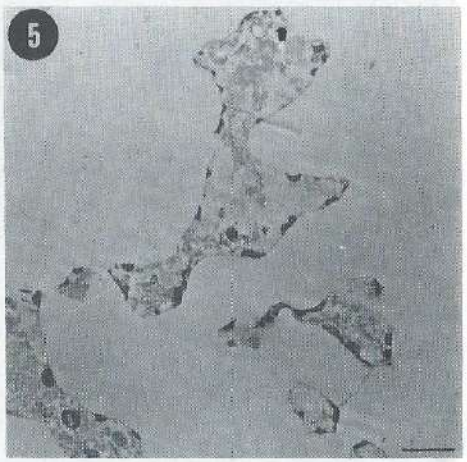
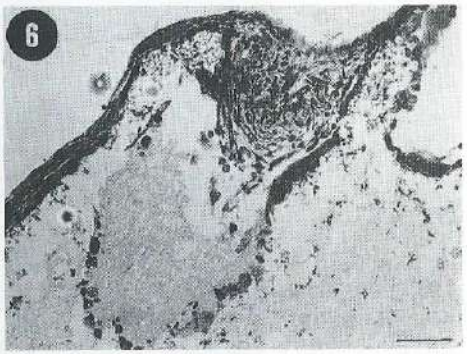
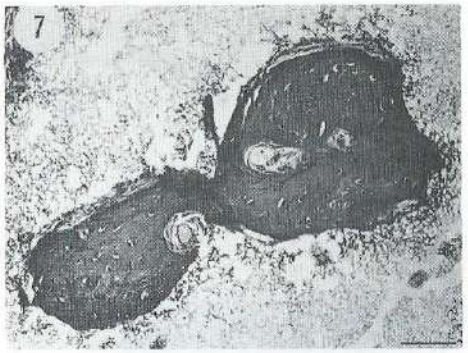
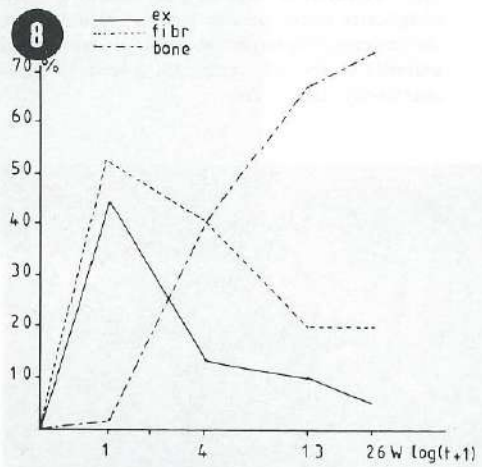


Fig. 3a. Transmission electron micrograph of the upper part of the tissue covering ß-whitlockite after 6 months. An epithelial cell (E), a fibroblast (F), and some collagen can be seen. Bar: 2 μm.

Fig. 3b. Transmission electron micrograph showing pseudo lamina propria containing capillaries and fibrous tissue covering ß-whitlockite. Some bone is also present. Bar: 2 μm.







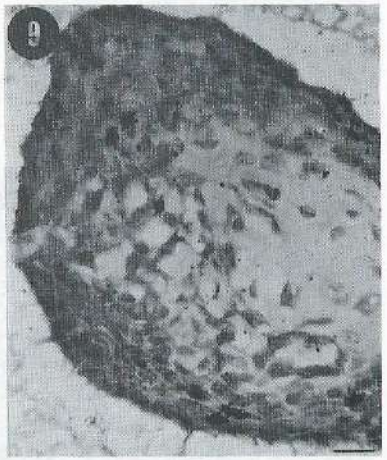


Fig. 6. Micrograph showing a macropore 1 week after implantation. Fibrous tissue and exudate can be seen. Bar: 30 μm.

Fig. 7. Light-microscopical view of a macropore after 6 months. The pore is almost completely filled with bone. Two capillaries are present. Bar: 30 μm.

Fig. 8. Graphic representation of the relative proportions of exudate, fibrous tissue, and bone in the macropores. The various time-points are shown on the x-axis.

Fig. 9. Autoradiograph of a macropore. Bone is present in the middle of the pore and distinct signs of proliferative activity can be seen in the peripheral region of the bone. Bar: 10 μm.

Fig. 4. Autoradiograph of the ß-whitlockite/muscle tissue interface. Phagocytes and a multinucleated cell (M) are visible. Note the marked signs of proliferation (arrows). Bar: 10 μm.

Fig. 5. Electron-microscopical survey of some micropores in ß-whitlockite after 6 months. The micropore wall is characterized by the presence of electron-dense structures. Bar: 2 μm.

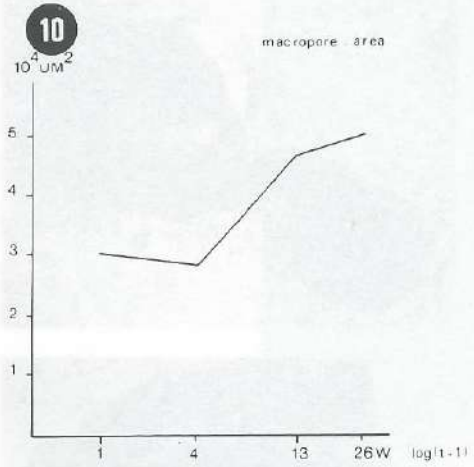
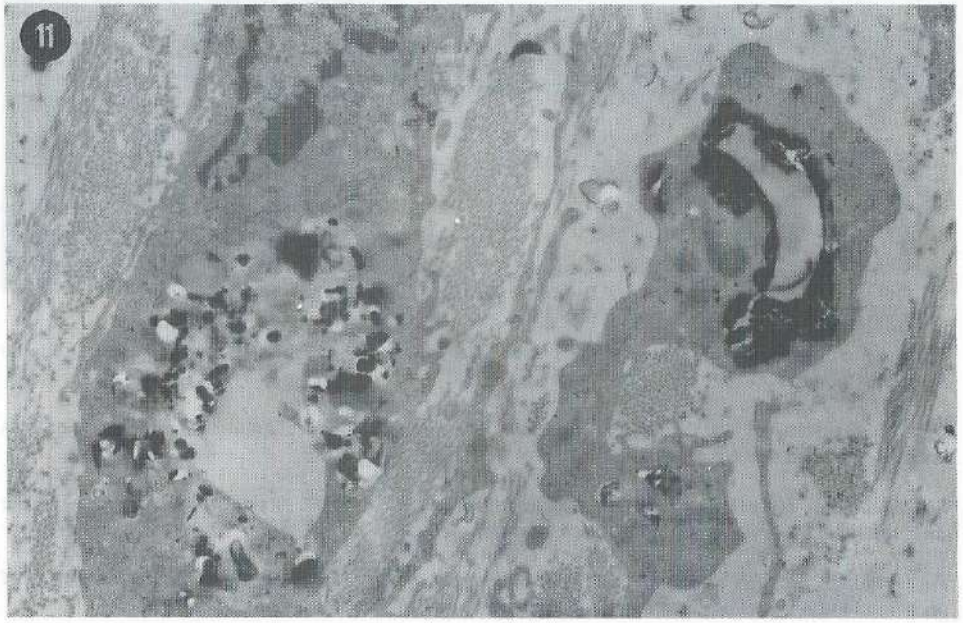


Fig. 10. Graph showing the increase of macropore area as a function of time.

Fig. 11. Electron micrograph showing two phagocytes in the pseudo lamina propria. Note the presence of a large number of electron-dense granules in the cell cytoplasm 6 months post-operatively. Bar: 1 μm.



of fibrous tissue seen just below the epithelial lining of a ß-whitlockite implant in this experiment had characteristics similar to those of the original middle-ear lamina propria. Light microscopy and transmission electron microscopy showed that this layer of fibrous tissue varied in thickness and was made up of collagen, fibroblasts, and capillaries (figs. 1 and 3b). The proliferative activity of the cells in this tissue, as determined by incorporation of tritiated thymidine, resembled that of the epithelial cells. This activity was highest during the first two weeks after implantation and then gradually decreased.

Cells of exudate origin, such as polymorphonuclear leucocytes and macrophages, occurred in the pseudo lamina propria in the earlier periods after the operation, but their number gradually decreased in the course of the later periods.

Bone covering: In the tissue envelope around the ß-whitlockite two other structures were observed, the first being bone tissue and the other an electron-dense zone at the implant surface. The properties of the latter zone will be described under *Interface phenomena.* Much of the bone present on the implant surface originates from the middle-ear bulla, i.e., from the site of the defect made for implant insertion, as determined by fluorescence, routine light microscopy, and transmission electron microscopy. It remains possible, however, that bone deposition started from other foci at the implant surface as well. This was suggested by the sequence of alizarin and calcein lines found not only in the order of administration from the bulla wall toward the implant but also in the opposite direction. The bone had a normal appearance, and osteoblasts, osteocytes, lacunae, and canaliculae were present. Proliferative activity was demonstrated at sites of bone formation, and here too the activity was highest in the earlier periods.

Interface phenomena: At the implant/tissue interface three basically different contact zones were found. In order of increasing mechanical friction at the interface, these are the ß-whitlockite/bone interface, the ß-whitlockite/fibrous tissue interface, and lastly the highest level of friction at the ß-whitlockite/muscle interface where the implant borders the muscle outside the bulla. The ß-whitlockite/muscle interface differed from the other two. It was characterized by the presence of a zone of mononuclear and multinucleated phagocytes. The thickness of this zone varied considerably. Both cell types showed distinct phagocytic activity, as will be discussed below, and also extensive incorporation of tritiated thymidine even in the six-month period (fig. 4). The ß-whitlockite/bone interface had a strongly dominant feature i.e., the direct contact between bone and the implant. At this interface and at the ß-whitlockite/fibrous tissue interface an interesting feature was seen that was especially characteristic for the micropores of the implant (fig. 5). This feature consisted of foci of electron-dense material deposited on the implant surface. Frequently, semicircular concentric circles characteristic for calcification islands were found on these sites.

Macropore tissue: During the first weeks after implantation the macropores were filled with a relatively large amount of exudate and fibrous tissue, and to a minor extent with bone (fig. 6). With increasing time the amounts of exudate and fibrous tissue decreased and bone became dominant in the pores (fig. 7), occasionally filling the cavities completely. Frequently, a central blood vessel could be seen. To quantitate these phenomena, the proportions of the various types of tissue in the pores were determined, a distinction being made between exudate, fibrous tissue, and bone. The results are shown in fig. 8. This graph confirms the initial histological findings and reflects the continuing increase of bone up to the sixth post-operative month. Of equal importance is the decrease of exudate, which after the sixth post-operative month occupied only 5% of the macropore volume. The number of exudate cells at the macropore/tissue interface also decreased during the successive intervals. The cells showed signs of phagocytic activity, as will be discussed below.

A subject of persisting interest with respect to macroporous implant materials is how they eventually become filled with bony tissue. The use of light-microscopical autoradiography and fluorescence microscopy in combination with routine histological and electron-microscopical techniques, yielded results which shed some light on this mechanism. Autoradiography showed proliferative activity at the centre of pores when bone covered the macropore interface, but there were also signs of proliferation at the periphery of the pore when bone covered the centre, though less frequently (fig. 9). The results of fluorescence microscopy were comparable to the autoradiographic findings. The lines of alizarin and calcein after injection at different times were found both in order of administration from the pore periphery toward the centre and in the opposite direction. As in the autoradiography experiment, the latter occurred less frequently. In light-microscopical and transmission electron-microscopical sections both directions of bone deposition were found.

Biodegradation: To find out whether degradation of ß-whitlockite took place the average macropore area in the histological sections was plotted against time, as shown in fig. 10. From this graph it can be concluded that the ß-whitlockite macropore area had increased by 68% after six months. Apparently, degradation had indeed occurred. The question was whether this biodegradation was partially the result of the phagocytic activity described above. Cells with the morphology of macrophages and multinucleated cells were seen near the ß-whitlockite/tissue interface. In the cell cytoplasm transmission electron microscopy revealed vacuoles whose rectangular shape indicated that they must have contained implant material before decalcification. This was confirmed by the presence of the original material in similar vacuoles, as determined by X-ray microanalysis. Another interesting phenomenon was the presence of a fair number of granules varying in electron density (fig. 11) in the cytoplasm of the cells representing the 6-month post-operative period. In these granules X-ray microanalysis revealed the presence of a variety of elements, including silicium, titanium, aluminum, iron, chromium, calcium, phosphorus, and magnesium. The composition of the contents of the granules was determined in two ways, i.e., single spot X-ray microanalysis (fig. 12, a and b), which showed which elements were present in the area captured by the spot, and X-ray mapping (fig. 12, c and d), which provided supplementary information about the distribution of the elements over a certain area. One of the most interesting characteristics was the relatively pure state of these elements. Most of the granules contained mainly silicium, a smaller number mainly titanium and some calcium and phosphorus, or other mixtures of elements. Apparently, some storage or purification of elements occurred in the phagocyte cytoplasm. Besides these granules, a relatively large amount of ferritin was present both in the cytoplasm itself and in some of the granules.

Infection experiment: Infection developed in all middle ears studied in the infection experiment, as shown by the presence of mucopurulent fluid in some niches in the middle-ear cavity, fibrous tissue ingrowth into the lumen, and the deposition of a layer of new bone on the original wall. All of these phenomena are characteristic for *Staphylococcus aureus*-induced infection in the three-week stage. In general, the phenomena seen in and on the ß-whitlockite implants in the infected middle ear did not differ greatly from those seen in the non-infected middle ear without an implant. The implants were characterized by a mucosal covering very similar to the one on the implants in the non-infected middle ear, although the submucosa was sometimes thicker. Scanning electron microscopy showed the presence of both flat polygonal and ciliated epithelial cells with a normal morphology in transmission electron microscopy (fig. 13). Most macropores were well filled with bone and fibrous tissue, and the implants were firmly integrated into the middle-ear tissue (fig. 14). However, some differences could be distinguished. For example, the exudate in the pores showed an elevated number of exudate cells, such as neutrophilic granulocytes, lymphocytes, and macrophages. This change was seen most frequently in pores bordering the implant surface. The amount of exudate present in the macropores at all time-points was approximately the same as in the non-infected middle ear, and accounted for about 6% of macropore area in the sixth post-operative month.

The amount of bone and fibrous tissue found in the pores also corresponded well with the findings in the non-infected group (after 6 months 69% and 25%, respectively). An implant phenomenon that pointed to a difference between the non-infected and infected middle ear was the presence in the latter of fibrous tissue ingrowths and the numerous connections between these structures and the biomaterial.

Because the application of fluorochromes always led to two separate fluorochrome lines, resorption of bone seems unlikely. Increased implant resorption ascribable to the infection could not be established morphometrically.

DISCUSSION

From the available reports concerning the performance of hydroxyapatite (calcium triphosphate) middle-ear implants (7, 15, 17, 23, 24), this material appears to be useful for reconstruction in middle-ear surgery. In the present study the biological performance of ß-whitlockite (tricalcium phosphate) was investigated for comparison of the qualities of this material with those of hydroxyapatite and to determine its suitability for application in reconstructive middle-ear surgery.

Biomaterials intended for this purpose must fulfill several biofunctional criteria. In the first place, when implanted in the middle-ear cavity they should become covered by a lining of epithelium that can participate in middle-ear defence during otitis media. Second, when applied as a canal-wall prosthesis (24), good fixation with the surrounding tissue should be guaranteed. Furthermore, a material intended to replace a middle-ear ossicle (25) or the tissue eventually replacing the ossicle should retain its biofunctionality to insure good, lasting sound transmission. Finally, it need hardly be said that biomaterial-derived elements must not enter either the circulation or the middle ear-cavity in a form that could cause systemic damage or damage to the middle- or inner-ear tissue.

With regard to the epithelial cover, the ß-whitlockite implant was already covered by epithelium a few weeks after implantation. This is of obvious value for the reduction of postoperative infection, as has been postulated for the pseudo-intima development of vascular prostheses (26). Furthermore, the completeness of the epithelial covering suggested that it would take part in middle-ear defence if otitis media occurred (20).

The amount and kinds of tissue present in the pores of ß-whitlockite are important for the fixation of this material. In the present study ß-whitlockite and the fibrous tissue initially present in the pores were replaced by bony tissue in the course of time, and after six months 74%

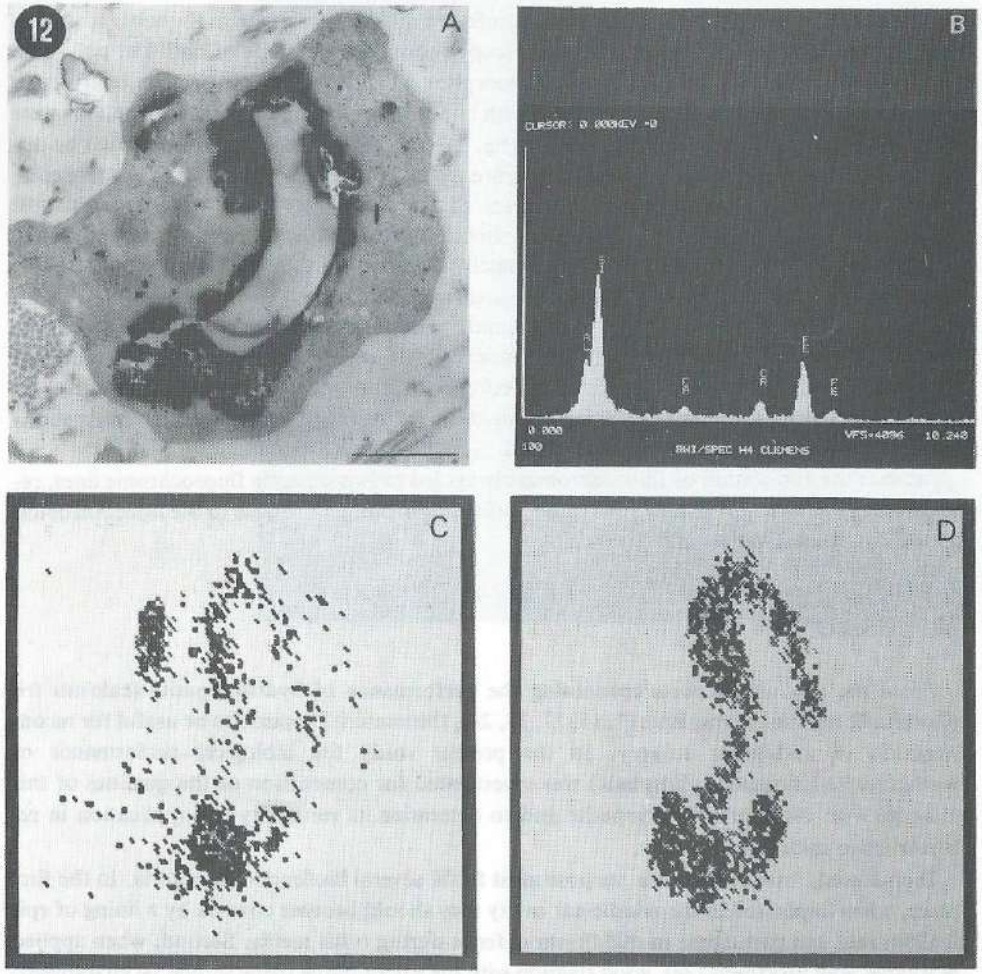


Fig. 12a. Transmission electron-microscopical view of a phagocyte with apparently implant-derived material in the cytoplasm. Bar: 1 μm.

Fig. 12b. Schematic representation of a single-spot analysis of the large vacuole in the same phagocyte. Note the presence of several elements.

Fig. 12c. X-ray mapping for silicium in the area shown in fig. 12a.

Fig. 12d. X-ray mapping for iron in the area shown in fig. 12a.

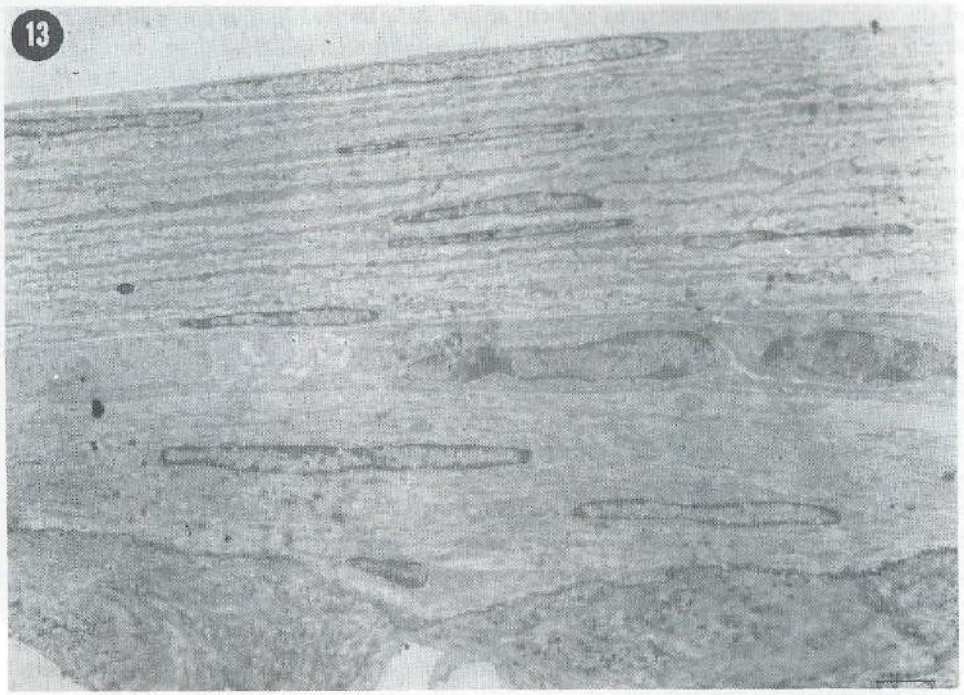


Fig. 13. Tissue covering ß-whitlockite 1 month after implantation in the middle-ear cavity. Infection was present after the first week. A thin epithelial layer, a relatively thick neo lamina propria, and bone can be seen. Bar: 2 μm.

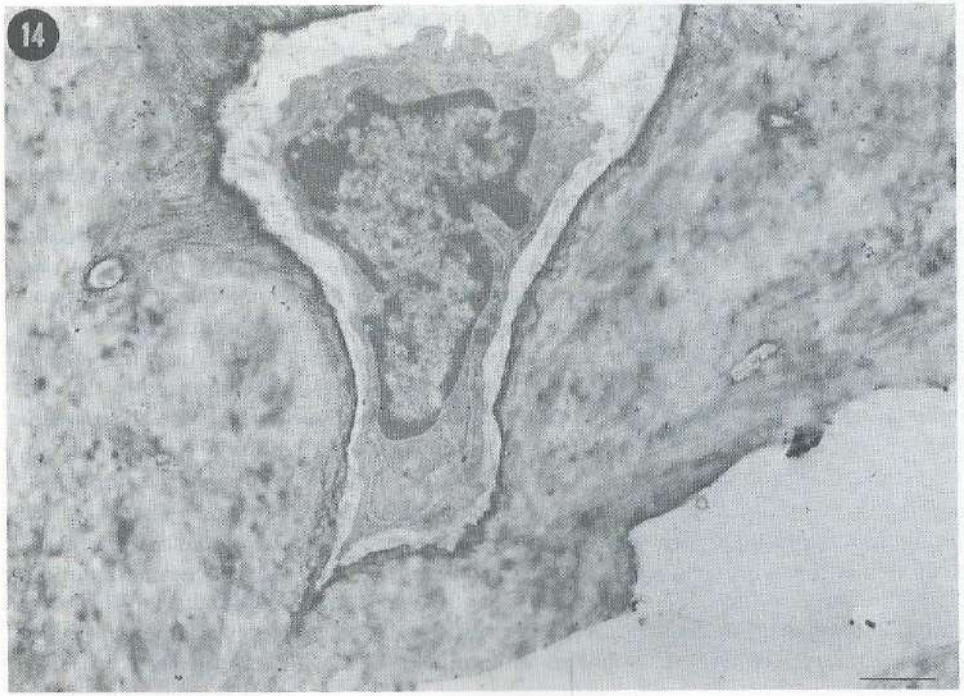


Fig. 14. Transmission electron micrograph of the implant/bone interface after infection, showing direct contact. Note the osteocyte and the collagen fibres. Bar: 1 μm.

of the macropore area was covered by bony tissue, 20% showed fibrous tissue, and 5% had exudate, which seems to fulfill the conditions for adequate fixation. Bone deposition was seen to occur, starting from the macropore wall and proceeding toward the centre as well as in the opposite direction, the former being a characteristic of bioactive material. The adherence of bone to bioactive calcium-phosphate ceramics such as hydroxyapatite may be expected to be firm due to their ionic composition. For hydroxyapatite this was confirmed by push-out experiments (27). Although this test was not performed in the present study, the light-microscopical and transmission electron-microscopical observations of the implant/ tissue interface revealed deposition of bone directly to the implant surface, suggesting firm integration. The differences between bioactive and bioinert materials are considered to be interface dependent (28, 29). For the hydroxyapatite interface, deposition of calcium salts in the form of a granular, lamina limitans-like layer has been established (15). This structure, although varying in thickness, was continuous over relatively long distances. In contrast, the ß-whitlockite interface was characterized by electron-dense material in the form of semicircular local foci which, on the basis of their resemblance to the lamina limitans and small calcification islands, must also be composed at least partially of a calcium salt. A lamina limitans (30, 31) is defined as a structure between two layers of bone or on top of bone where the formation of new bone has come to a temporary or definitive stop. Since the lamina limitans-like structure at the ß-whitlockite surface is less dominant and occurs less frequently than is the case for hydroxyapatite, it may be concluded that ß-whitlockite showed a higher level of bioactivity at the surface. Apparently, high bioactivity is not necessarily associated with stronger biodegradation, because in the present study the degradation of ß-whitlockite was the same as had been found for hydroxyapatite (15). The degradation of ß-whitlockite found in the middle ear was smaller than that reported by Klein (11, 12) for the same material at another location in the rabbit. This divergency too supports the hypothesis that the rate of biodegradation is partially dependent on the implantation site.

As established for hydroxyapatite (15), macrophages and multinucleated cells played a part in the degradation of ß-whitlockite. However, one distinct difference was found between these cells near the hydroxyapatite/tissue and ß-whitlockite/tissue interfaces: besides the rectangular vacuoles sometimes filled with implant-derived material, electron-dense granules of great morphological variety were found in the phagocyte cytoplasm in the ß-whitlockite material. X-ray microanalysis showed the presence of a variety of elements in these granules, including silicium, titanium, aluminum, iron, magnesium, chromium, calcium, and phosphorus. All of these elements except the last two and chromium, were present as trace elements in the original bulk material in concentrations between 0.15% (silicium, magnesium) and 0.005% (titanium) (20). Apparently, some storage of these elements occurred in the cell. Several mechanisms underlying this partial purification of the trace elements can be proposed. First, conglomerates of the elements already present in the bulk material might be phagocytosed and then remain in the cell cytoplasm. Second, a fragment of implant material containing a single or several trace elements might be phagocytosed, followed by removal of the calcium and phosphate in some way. Third, some kind of sorting of the stored elements might occur after either of the first of these two mechanisms. The difference between ß-whitlockite and hydroxyapatite with respect to the presence of these granules suggests that stoichiometry and/or a crystal structure could be factors of significance for these processes. The fact that we found migratory activity of macrophages with granules filled with trace elements raises the question as to whether any systemic effects (32) are to be expected. Although such effects have not been established and may even not occur, high purity of biomaterials must be recommended, since storage or trace elements probably occurs.

From the observations made in the infection experiment it may be concluded that neither ß-whitlockite nor the surrounding tissue is significantly affected by the events evoked by *Staphylococcus aureus*-induced infection. A similar conclusion was reached for hydroxyapatite (22).

With respect to the four criteria for a biomaterial destined for application in reconstrutive middle-ear surgery, ß-whitlockite meets the first three, since it develops a good epithelial lining and good fixation and does not permit excess material resorption, the observed degree of absorption being combined with replacement by healthy tissue. Concerning the fourth point, however, some doubt is justified on the basis of the presence in the phagocyte cytoplasm of granules containing stored or purified trace elements. Because the effects of such storage must certainly be extensively investigated before ß-whitlockite can be accepted for application in reconstructive middle-ear surgery, it seems advisable to use only hydroxyapatite calcium-phosphate implants while awaiting further assessment of ß-whitlockite.

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CHAPTER X

GENERAL DISCISSION

A wide variety of biomaterials have been used for reconstruction of the sound transmission system in reconstructive middle-ear surgery. Although some of these materials are reported to have been used successfully, this work has concerned mainly short-term clinical studies. The performance of biomaterials particularly during long-term implantation and infection is still under discussion and the search for new biomaterials with better properties is therefore still going on. Recently, a new group of biomaterials, the bio-ceramics, has attracted interest in otology and the resemblance between the calcium phosphate implant materials and the mineral matrix of the human bone makes hydroxyapatite and β-whitlockite seem especially promising, particularly the former. This material not only resembles the mineral matrix in its ionic composition but also has a similar crystal structure. One of the first objectives of the present study on the biocompatibility of hydroxyapatite and β-whitlockite was to develop techniques for evaluating the behaviour of these two calcium phosphate ceramics under middle-ear implantation. The implantation technique described by Grote & Kuijpers (1-4) seemed highly suitable as standard method for this study particularly when the prescribed light microscopy was combined with scanning and electron microscopy. However, optimal evaluation of the epithelial reactions required the development of an *in vitro* testing system. Furthermore, an animal experimental infection model had to be developed, because infection is frequently encountered in the surgical treatment of middle-ear disorders. These techniques (see Chapters 2 and 3) not only provided control information concerning the calcium phosphate ceramics but also proved useful for the study of various aspects of middle-ear pathology.

Culture of rat middle-ear epithelium: Rat middle-ear epithelium was cultured according to Rheinwald's (5) technique, which can be applied to a variety of epithelia (6,7). Terminal differentiation of epithelial cells in culture is easily observed (8,9) and is characterized by a relatively high cytokeratin level and the formation of cross-linked envelopes (10-13). Our culture method did not allow the demonstration of changes in the cytokeratin content accompanying terminal differentiation but cross-linked envelope formation was established and was found to be promoted by addition of ionophore X537 to the culture medium. The culture of explants of rat middle-ear epithelium and serial cultivation of middle-ear epithelium make it possible to test biomaterials destined for otology rapidly and reproducibly. This technique like the cultivation of cholesteatomous tissue (14-16), may also prove to be of value for the study of the pathogenesis of middle-ear disease.

Experimentally induced middle-ear infections: Middle-ear infections have usually been investigated with emphasis on the epidemiological (17-20) and bacteriological aspects (21-27) rather than in experimental studies in animals (28-32). This is surprising, because the latter approach seems highly appropriate for the study of the pathogenesis of infectious middle-ear disease. Although in our study the use of *Haemophilus influenzae* or *Streptococcus pneumoniae* might seem more logical for the induction of a middle-ear infection, we wished to test biomaterials and gave preference to *Staphylococcus aureus* on the grounds of the frequent occurrence of this micro-organism in contaminated wounds (33). Furthermore, the

relative resistance of the rat (34) to *Staphylococcus aureus* leads to an infection whose effects are clearly expressed and may be well studied. The observation in acute infections showed that middle-ear resistance is realized by five defence mechanism during acute otitis media, i.e., the cellular response, the humoral response, the mucociliary response, the fibroplastic response, and the bone response (see Chapter 3). Although this experimental model provides information on the effects of infection on biomaterials and vice versa, these interactions are restricted to the effects of *Staphylococcus aureus*, and the investigation of otologic biomaterials with other micro-organisms is to be recommended.

Tissue reactions

We examined hydroxyapatite and β-whitlockite with special attention to the tissue reactions in general and the epithelial covering and the tissue in the macropores in particular, since the phenomena seen on and in β-whitlockite and hydroxyapatite were similar in many ways, the two materials will be discussed together.

Epithelial reactions: The middle-ear cavity of the rat is predominantly covered with flat polygonal epithelium, with ciliated cells concentrated in the mucociliary tracks above and below the promontory. Furthermore, goblet cells are present near the orifice of the Eustachian tube and in the vicinity of the oval-window niche (35-37). The same cell types are present in the human middle ear (38-41). It has been postulated that the middle-ear epithelium is of significance for middle-ear defence during infection, as suggested by for example, the mechanical transport function of the mucociliary tracks (42). An epithelial covering resembling that in the normal middle ear seems indicative for good biocompatibility of an implant material. In our experiments, a large proportion of the calcium phosphate implant was covered by an epithelial layer within the first two weeks, when the level of proliferative activity was highest. With increasing implantation time the implant gradually became completely covered by this epithelial layer in which all cell types characteristic for the middle-ear could be distinguished. The predominant component was flat polygonal cells, many of them showing a marginal row of microvilli and similar structures on the cell surface. Besides these ciliated epithelial cells, goblet-like cells were seen on the implant surface, the former most often seen when the mucociliary tracks bordered the implant. In the *in vitro* experiments hydroxyapatite did not affect the morphology or proliferation of the epithelial cells in explants or serial cultures. The thickness of the fibrous capsule around an implant is considered to be strongly dependent on the characteristics of the material of which the implant is made and may therefore be seen as an indicator for the biocompatibility of that material. Generally, a thin fibrous capsule is considered to indicate good compatibility. In our study on two calcium phosphate ceramics a thin fibrous layer formed over the implant on the middle-ear lumen side. This layer varied in thickness and its morphology did not differ from that of the normal lamina propria.

On the basis of the epithelial and pseudosubmucosal characteristics of calcium phosphate implant materials, good biocompatibility and functionality can be expected.

Macropore tissue: The introduction of porous implant materials marked a great step forward in biomaterial science, because firmer fixation and a smaller fibrous capsule could now be obtained. Furthermore, macropore diameter appeared to exert a direct influence on the amount and kind of tissue that grew into the pores (43-48). Generally, a pore size of 30 μm allows fibrous tissue ingrowth, whereas a pore size larger than 100 μm favours the ingrowth

of bone. Because no data were available for hydroxyapatite on the correlation between macropore size and tissue ingrowth and more exact data were needed on the chronological order of events during the filling of the pores, morphometry was applied. The morphological findings confirmed the initial light-microscopical results, which showed that pores were filled with exudate, fibrous tissue, and bone successively. This ingrowth into the pores appeared to be the result of simultaneous proliferative and migratory activity. The following correlation was found between macropore size and tissue ingrowth: a pore with a cross-section of less than 2.10^4 μm^2 showed bone ingrowth in less than 15% of total macropore area as against 87% for cross-sections larger than 6.10^4 μm^2. This correlation could only be established for hydroxyapatite, probably because of the smaller number of implants used in the β-whitlockite studies. Not surprisingly, the published data on macropore size and tissue ingrowth also hold for macroporous hydroxyapatite.

Interface reactions

The biomaterial/host tissue interface is considered to be of great importance for the functionality and compatibility of a biomaterial (49-53). Hydroxyapatite and β-whitlockite are as already mentioned, bioactive materials, which means that a calcium phosphate ion exchange occurs at the interface, resulting in a direct bond with bone. In the case of hydroxyapatite this concerns a physico-chemical bonding between the bone tissue and the biomaterial made possible by the great similarity between the chemical composition and crystal structure of both (54-58). Since the structure of β-whitlockite (59) differs from that of the bone matrix, another bonding mechanism could be expected. This difference in the bond with bone at the interface probably reflects the divergence in interface morphology revealed by transmission electron microscopy; large parts of the hydroxyapatite/tissue interface showed a continuous electron-dense granular layer containing calcium, as indicated by X-ray microanalysis. The β-whitlockite/tissue interface lacked this continuous structure but showed local semicircular foci of electron-dense material. The electron-dense structure at the hydroxyapatite interface resembled the lamina limitans of bone and sometimes showed a continuity with this structure, which occurs between two layers of bone deposited at different times or on top of bone whose formation has been permanently or temporarily arrested (60,61). If the lamina limitans may be considered a relatively stable structure, β-whitlockite is apparently more active at the interface with tissue.

Biodegradation

Mechanical friction can affect the implant-tissue interaction (53). In our studies this was seen at the borderline between calcium phosphate implants and muscle tissue, cohert concentrations of mononuclear and multinucleated cells persisted even after the longest intervals. These cells appeared to play a role in the biodegradation of these ceramics. There is some controversy as to whether hydroxyapatite and β-whitlockite are (62-64) or are not (63-66) subject to biodegradation. Our morphometric results show comparable biodegradation for both materials, corresponding to an increase in pore diameter by approximately 10-15 μm six to twelve months postoperatively. Although the biodegradation found for hydroxyapatite and β-whitlockite did not differ greatly between the infected and the non-infected middle-ear (Chapters 5, 7 and 9)

a much more intense biodegradation of hydroxyapatite occurred shortly after the induction of infection (Chapter 8). Since the only essential difference between this experiment and the other was in the implantation procedure i.e., submucosal implantation was used in the short-term infection experiment, it may be concluded that the implantation procedure is of great importance with respect to biodegradation. This might explain the differences in degradation behaviour of the same calcium phosphate ceramics in different studies.

Mononuclear and multinucleated phagocytes: With respect to the mechanism underlying the biodegradation of calcium phosphate implants, Klein et al. pointed to the significance of the ultrastructural geometry of the material (67) for dissolution *in vitro*. Although this factor may also have exerted an influence in our *in vivo* experiments, part of the degradation must be due to phagocyte activity. Within a week after implantation, macrophages and multinucleated cells could be seen at the implant/tissue interface and in the tissue around the implant. This was to be expected, because these cells migrate to sites where a wound reaction occurs (68-72). Their number declined the longer the post-operative interval. After implantation periods of six and twelve months they were predominantly found at sites where the implant bordered muscle tissue. Although macrophages were recognized relatively easily by their light-microscopical and ultrastructural chracteristics, it was difficult to determine whether the multinucleated cells represented foreign body giant cells or inactive osteoclasts, due to the great morphological resemblance between these cells (73-75). In biomaterial science the surroundings of these cells might be considered with respect to their nomenclature. Multinucleated cells at the implant interface would thus be designated as foreign body giant cells, i.e., with the exception of cells with a ruffled border, and similar cells near bone osteoclasts. For calcium phosphate implants, however, such nomenclature would be arbitrary due to the strong resemblance between the mineral matrix of bone and the composition of the material. Therefore, the neutral term multinucleated cell seems preferable for calcium phosphate ceramics. With respect to multinucleated cells, many studies have been done on the formation and function of polykaryons. The multinucleated cell was at first thought to originate by the fusion of freshly arrived macrophages with older macrophages or polykaryons at granuloma sites (73), and later by the fusion of macrophages and polykaryons during simultaneous endocytosis (76-78). These opinions may well be related, since multinucleation was associated with a decrease in phagocytic capacity due to cytoplasmic interiorization of receptors (79-81). This would decrease the incidence of phagocytosis by cells remaining longer at an implantation site and thus lead to relatively more frequent fusion of freshly arrived cells with other phagocytes compared with fusion between cells present for a longer time (82). The function of polykaryons is not completely understood. Although their association with macrophages and their presence at sites of chronic inflammation and near foreign bodies suggests a phagocytic function, this does not seem to be their main function, because the relative phagocytic capacity of multinucleated cells is lower than that of macrophages. A function in extracellular degradation has been suggested on basis of a relatively higher acid phosphate production compared with mononuclear cells (83,84). However, this enzyme is also known to be active intracellularly. This activity recently became interesting when macrophage-derived multinucleated cells were found to resorb bone more efficiently than their mononuclear precursors (85), which were found to resorb bone and even seemed to be influenced by chemotactic factors and factors inducing or inhibiting bone resorption (86-95). This behaviour

of macrophages and multinucleated ceels seems consistent with our finding that phagocytes play a role in the degradation of both hydroxyapatite and β-whitlockite. The degree of degradation of hydroxyapatite and β-whitlockite was quite similar but the cytoplasm of the macrophages and multinucleated cells involved in the degradation process showed a striking divergence. In the vicinity of β-whitlockite these cells differed from those near hydroxyapatite in two ways. In the first place transmission electron microscopy and X-ray microanalysis showed a relatively large amount of ferritin in the cytoplasm and vacuoles in the former cells. This relatively high intracellular concentration of ferritin may be part of the inflammatory response induced by the biomaterial. Activated macrophages are thought to have a relatively high ferritin content, which partially explains the decreased serum iron concentration seen during inflammation (96,97). This ferritin pattern was also found in the hydroxyapatite studies but less frequently. The second point of intracellular divergence was more prominent and was only seen with implanted β-whitlockite: electron-dense inclusion bodies occurred in the phagocyte cytoplasm. X-ray microanalysis established the presence of relatively pure silicium, aluminum, titanium, magnesium, iron, and chromium in these vacuoles, all but chromium trace elements originally belonging to the bulk material (59). The intracellular storage of elements has been reported by others (98,99), but our results suggest that purification of elements occurs as well as storage. The only other conceivable explanation of the presence of relatively pure trace elements in the cytoplasm of phagocytes would be the occurrence of areas where such elements occur in the implant material in a concentrated form.

Infection

As stated in the introduction, biomaterials can affect the incidence of infection but contrariwise, infection can influence the biocompatibility and biofunctionality of implant materials. To investigate both phenomena we studied the short-term and long-term effects of a *Staphylococcus aureus*-induced infection in the middle ear with and without an implant. No marked effect of the biomaterials on the course and characteristics of infection was found. The events in the middle ear with an implant did not differ significantly from those in the controls. The most dominant feature of the infected middle-ear with an implant was the contact between the fibrous ingrowths, originating from the infection, and the tissue covering the implant. The osteoresorption seen in the infected middle-ear without an implant did not significantly affect the bone around and in the porous implant. The macroporous implants showed a slightly increased amount of exudate during the infection. Increased biodegradation of implant material attributable to the infection did not occur. The higher intensity of biodegradation and osteogenesis in the short-term infection studies compared with the long-term studies (infected and non-infected) must have been due to the use of a different (submucosal) implantation technique.

A *Staphylococcus aureus*-induced middle-ear infection does not seem to have any influence on the biocompatibility and biofunctionality of hydroxyapatite and β-whitlockite, nor does the presence of these biomaterials change the course of an infection.

GENERAL CONCLUSIONS

It may be concluded that both hydroxyapatite and β-whitlockite have features suggesting good biocompatibility. Both materials were soon covered by an epithelium resembling that of the original rat middle ear. Macropores became filled with fibrous tissue and bone and for hydroxyapatite bone became more dominant with increasing pore size. Both materials allowed bonding osteogenesis, and although β-whitlockite seemed to show higher bioactivity, the rate of biodegradation of both materials was similar, i.e. about 15 microns in 6-12 months. Infection did not have any strongly negative effect on either of the biomaterials. With respect to the properties of β-whitlockite and hydroxyapatite the former was associated with storage of trace elements in the cytoplasm of phagocytes, which must be considered a negative characteristic. Since the effects of such storage are unknown, clinical application of β-whitlockite cannot be recommended. The biodegradation observed for both materials does not mean decreased biofunctionality, because the biomaterial is replaced by fibrous tissue and bone. In view of the present and earlier results concerning hydroxyapatite (2-4, 62-64, 100-102) as well as the results of long-term clinical follow up (103-105), hydroxyapatite seems to be a highly suitable material for use in reconstructive middle-ear surgery.

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SUMMARY

Chronic middle-ear disease may necessitate the removal of part of the sound transmission system, but partial restoration of the hearing capability can often be achieved by reconstructive middle-ear surgery. Biomaterials have proven useful for reconstruction of the sound transmission system but in spite of frequent application the success rates are still under discussion, which explains the continued attempts to find new and better biomaterials for otology. Among these new biomaterials the calcium phosphate ceramics seem promising because their composition closely resembles that of the mineral matrix of bone. The investigation reported in this thesis was performed to assess the suitability of two of these materials, hydroxyapatite and β-whitlockite, for application in reconstructive middle-ear surgery. Hydroxyapatite was studied in more detail because its stoichiometry and crystal structure showed greater similarity to those of the bone mineral matrix. Hydroxyapatite was tested in both dense and macroporous forms whereas β-whitlockite was only investigated in its macroporous form. The materials were tested in the middle ear of the rat, which was known to be appropriate for the testing of otologic biomaterials. Light microscopy and scanning and transmission electron microscopy were used throughout the study. A method to induce middle-ear infections was developed to facilitate testing in infected as well as non-infected areas, because reconstructive middle-ear surgery is often preceded or followed by infection. Induction by intratympanic injection of *Staphylococcus aureus* was chosen. The effect was observable as early as one day later, oedema and vasodilation being prominent and the middle-ear cavity completely filled with mucopurulent fluid. Osteoresoprtion followed by osteogenesis were seen as well as an increase of the area occupied by the mucociliary tracks. Ingrowth of fibrous tissue into the middle-ear cavity also occurred. One to three weeks after the infection had been induced, the state of the middle ear appeared to be stabilized. In the tissue-culture model developed for the present study, middle-ear mucosa and epithelium were directly exposed to the biomaterial under study. In control experiments, the explants showed good outgrowth of both flat polygonal and ciliated epithelium, whereas only flat polygonal epithelium was found in serial cultures.

In animal experiments, hydroxyapatite was rapidly covered by a mucosalike coat, i.e., in the first week after implantation. This coat was the result of both proliferative and migratory activity of the tissue on and near the implant, and its surface layer was composed of all cell types characteristic for the rat middle ear, i.e., flat polygonal, ciliated, and goblet cells. The cultured tissue showed no significant effect of the presence of hydroxyapatite.

Examination of the hydroxyapatite/tissue interface in the *in vivo* material showed the presence of an electron-dense layer which was continuous with the lamina limitans of calcification islands. X-ray microanalysis showed that calcium was present in this layer. At the interface, macrophages and multinucleated cells were also observed. These cells showed distinct phagocytic activity in response to the biomaterial which must at least partially explain the biodegradation amounting to about 15 μm a year, as shown by morphometry. The macropores of implanted hydroxyapatite were initially filled with exudate and fibrous tissue, but after that the amount of exudate decreased and the pores were predominantly occupied by fibrous tissue and bone in about equal amounts. Morphometry revealed a distinct correlation between macropore size and the relative amount of ingrowing bone.

The infection experiment showed that the presence of hydroxyapatite implant had hardly any influence on the course of infection and vice versa: the infection had no marked effect on the implant or the newly formed tissue covering it. The observed effects included a slight increase of exudate in macropores and a contact between the implant material and the ingrowing tissue. We found no negative effect of infection on the amount of bone in or over the implant, but the biodegradation of hydroxyapatite, presented a more complicated situation due to divergence between the results concerning short- and long-term infection, the former leading to distinctly more biodegradation. Since infection was induced in the same way in both cases, this divergence must be ascribed to the use of a different implantation technique in the short-term infection experiment. Thus, the site of and technique used for implantation are both important for the behaviour of an implant material.

Our experimental results concerning β-whitlockite were quite similar to those obtained for hydroxyapatite, but differed in one important respect. In the studies on β-whitlockite the cytoplasm of phagocytes in the vicinity of the implant showed elctron-dense inclusion bodies and X-ray microanalysis revealed that a number of elements including silicium, iron, magnesium, aluminum, titanium and chromium were present in these structures. Since all these elements except chromium had initially been present as trace elements in the implant material, it may be concluded that storage of trace elements in the cytoplasm of phagocytes had occurred in the β-whitlockite studies. Such storage was not found in the hydroxyapatite experiment.

In view of the present and earlier results, hydroxyapatite seems promising as a material for use in reconstructive middle-ear surgery. A final opinion on the value of this material must await the results of long-term clinical studies. The clinical usefulness of β-whitlockite will be indicated by the results of studies on the effects of the storage of trace elements in relation to the biocompatibility of the material.

SAMENVATTING

Het optreden van chronische middenoorpathologie kan leiden tot aanzienlijke beschadigingen van het geluidsgeleidingssysteem. Chirurgische ingrepen ter behandeling van dergelijke chronische afwijkingen maken het soms noodzakelijk de geluidsoverdracht nog verder te beperken. Reconstructieve middenoorchirurgie echter biedt de mogelijkheid dergelijke beschadigingen gedeeltelijk te herstellen, het gebruik van biomaterialen kan daaraan een bijdrage leveren. Het aantal biomaterialen dat inmiddels in deze chirurgische discipline is en wordt toegepast is groot, de verkregen resultaten zijn echter niet optimaal, wat een voortdurend voortgaand onderzoek naar alternatieve materialen tot gevolg heeft. Door de overeenkomst in samenstelling met de minerale matrix van het bot lijken calciumfosfaat keramieken een veelbelovende categorie biomaterialen te vormen. Met als doel de geschiktheid van twee van deze keramieken, hydroxyapatiet en β-whitlockiet, voor toepassing in de reconstructieve middenoorchirurgie te bepalen, werd het in dit proefschrift beschreven onderzoek opgezet. Daar hydroxyapatiet de grootste overeenkomst vertoont met de minerale matrix van bot, zowel wat stoichiometrie als wat kristalstructuur betreft, kwam het zwaartepunt van het onderzoek op dit materiaal te liggen. Van hydroxyapatiet werd zowel een dense als macroporeuze versie beproefd, terwijl het experiment bij β-whitlockiet tot de macroporeuze versie beperkt bleef. Als dierexperimentele testomgeving werd het rattenmiddenoor gekozen, deze keuze vond zijn oorzaak in het feit dat deze plaats in eerdere studies geschikt was gebleken voor het bepalen van de biocompatibiliteit van implantatiematerialen bestemd voor de middenoorchirurgie. Ter verkrijging van goede evaluatiemogelijkheden werd in alle experimenten gebruik gemaakt van zowel licht microscopie, scanning- en transmissie electronen microscopie.

Het dient als onvoldoende te worden beschouwd indien een biomateriaal, bestemd voor otologische toepassing, slechts in het ongeïnfecteerde middenoor wordt getest. In de middenoorchirurgie is immers regelmatig sprake van infectie. Dientengevolge dienen biomaterialen bestemd voor de reconstructieve middenoorchirurgie eveneens in een geïnfecteerde omgeving getest tē worden. Bij gebrek aan gegevens betreffende een goed reproduceerbare wijze tot opwekken van infectie in het middenoor van de rat diende een dergelijke methode allereerst te worden ontwikkeld. Een intratympanale injectie met *Staphylococcus aureus* suspensie bleek hier voor geschikt. Reeds na een dag waren de effecten van deze injectie duidelijk waarneembaar, er trad oedeem en vasodilatatie op en het gehele middenoor was gevuld met mucopurulent vocht. Voorts was er sprake van initiële osteoresorptie gevolgd door osteogenese in een later stadium, tevens namen de trilhaarbanen in omvang toe en was ingroei van fibreus weefsel in de middenoorholte zichtbaar. Een relatieve stabilisatie van de situatie in het middenoor trad op in de periode gelegen tussen een en drie weken na de inductie van de infectie. Een ander probleem ten aanzien van de te gebruiken testmethodes was het ontbreken van een bruikbaar weefselkweek model. Om aan deze behoefte te voldoen werd een experimentele opzet ontwikkeld waarmee zowel explants van middenoor mucosa als seriekweek van middenoorepitheel aan de implantatiematerialen konden worden blootgesteld. In deze opzet vertoonden explants zonder de aanwezigheid van implantaat goede uitgroei van vlak polygonaal en trilhaarepitheel, in de seriekweek was slechts vlak polygonaal epitheel aanwezig.

Aan de hand van voornoemde experimentele modellen kon worden vastgesteld dat hydroxyapatiet na implantatie, deels door proliferatieve en deels door migratoire activiteit van het omringende weefsel, snel door een mucosa-achtige laag werd bedekt. De bovenzijde van deze laag vertoonde alle karakteristieke cellen van het middenoorepitheel, zoals vlak polygonaal epitheel, trilhaarepitheel en slijmbekercellen. In het weefselkweekexperiment bleek hydroxyapatiet geen aantoonbare nadelige invloed op het weefsel uit te oefenen. In het dierexperiment werd aan de hydroxyapatiet/weefsel interface regelmatig een electronendichte laag aangetroffen welke continuïteit vertoonde met de lamina limitans van calcificatie eilandjes. Met behulp van X-ray microanalyse werd de aanwezigheid van calcium in deze laag vastgesteld. Aan de interface kon nog een ander verschijnsel worden waargenomen en wel de aanwezigheid van makrofagen en multinucleaire cellen. Deze cellen vertoonden een duidelijke fagocytose van biomateriaal welke in elk geval gedeeltelijk verantwoordelijk geacht moet worden voor de met morfometrie aangetoonde biodegradatie van hydroxyapatiet met ca. 15 μm per jaar. De macroporiën van hydroxyapatiet werden aanvankelijk gevuld met exudaat en fibreus weefsel, naarmate de implantatie periode toenam werd de hoeveelheid exudaat echter aanzienlijk minder en werden poriën voornamelijk gevuld met fibreus weefsel en bot in ongeveer gelijke verhoudingen. Een duidelijke correlatie tussen macroporiegrootte en de relatieve hoeveelheid botingroei kon door toepassing van morfometrie worden vastgesteld.

Betreffende de opgewekte infectie kan worden gesteld dat enerzijds de aanwezigheid van hydroxyapatiet nauwelijks invloed uit bleek te oefenen op het verloop van de door *Staphylococcus aureus* geïnduceerde infectie, terwijl anderzijds de invloed van infectie op implantaat en bedekkend weefsel eveneens als gering beschouwd kon worden. De verschijnselen beperkten zich voornamelijk tot een lichte toename van de hoeveelheid exudaat in de poriën en tot de aanwezigheid van contact tussen de ingroei van fibreus weefsel en het implantaat. Een negatieve invloed van de infectie op de hoeveelheid bot in en rond de implantaten kon niet worden aangetoond. Ten aanzien van de invloed van infectie op de biodegradatie van hydroxyapatiet was de situatie betrekkelijk gecompliceerd. Het bleek namelijk dat het korte termijn infectie experiment relatief een aanzienlijk hogere biodegradatie te zien gaf dan het langere termijn infectie experiment. Daar de wijze van opwekken van infectie identiek was moet dit zijn oorsprong hebben gevonden in de verschillende implantatietechnieken die in beide experimenten werden toegepast. Klaarblijkelijk zijn plaats en wijze van implanteren van groot belang voor het gedrag van een implantatiemateriaal.

De bevindingen betreffende β-whitlockiet weken niet in grote mate af van die ten aanzien van hydroxyapatiet, desalniettemin was er sprake van ten minste een significant verschil. Dit verschil uitte zich in de aanwezigheid van electronendichte insluitingen in het cytoplasma van fagocyten rond het β-whitlockiet. In deze insluitsels konden met behulp van X-ray microanalyse elementen als silicium, ijzer, aluminium, magnesium, titanium en chroom worden aangetoond. Al deze elementen, op chroom na, vormden spore-elementen in het oorspronkelijke bulk materiaal. Hieruit kan worden geconcludeerd dat er een ophoping van spore-elementen in het cytoplasma van de phagocyt plaatsvindt. Een dergelijk fenomeen werd bij hydroxyapatiet niet waargenomen.

Samenvattend kan worden gesteld dat hydroxyapatiet aan de hand van eerder verschenen studies en de in dit proefschrift vermelde resultaten een veelbelovend materiaal voor toepassing in de reconstructieve middenoorchirurgie lijkt te zijn. Lange termijn klinische studies zullen verder uitsluitsel moeten geven over de definitieve geschiktheid van het materiaal. Aangaande β-whitlockiet kan worden gesteld dat het materiaal verder bestudeerd dient te worden om aldoende meer inzicht te verkrijgen betreffende de effecten van de ophoping van spore-elementen op de biocompatibiliteit van het implantaat, alvorens tot eventuele klinische toepassing kan worden overgegaan.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 07-07-57 geboren te 's-Gravenhage. Vanaf 1969 volgde hij het middelbaar onderwijs op het atheneum B van het Thomas More College in dezelfde plaats, alwaar hij medio 1976 slaagde voor het eindexamen. Hier op aansluitend startte hij de biologie studie aan de Rijks Universiteit Leiden. Op 29-01-80 werd het kandidaatsexamen B1W behaald en hier op volgend het doctoraal examen op 23-02-82. Per 01-03-82 werd een aanvang gemaakt met het in dit proefschrift beschreven onderzoek op de afdeling Keel-Neus-Oorheelkunde van het Academisch Ziekenhuis Leiden (begeleider: Prof. Dr. J.J. Grote). Het onderzoek werd grotendeels gefinancierd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO). Op 01-03-85 trad de auteur in dienst van de voornoemde vakgroep als universitair docent.

STELLINGEN
behorende bij het proefschrift
CALCIUM PHOSPHATE MIDDLE-EAR IMPLANTS

1. De experimenteel vastgestelde biocompatibiliteit van hydroxyapatiet rechtvaardigt klinische toepassing.
2. Bij hydroxyapatiet implantaten bestaat er een positieve correlatie tussen macroporiegrootte en de relatieve hoeveelheid botingroei.
3. De biodegradatie van hydroxyapatiet wordt ondermeer veroorzaakt door fagocytaire activiteit van macrofagen en multinucleaire cellen.
4. Implantatie van β-whitlockiet leidt tot cytoplasmatische ophoping van sporeelementen.
5. Daar biocompatibiliteit en biofunctionaliteit direct voortvloeien uit de interacties tussen gastheer en implantaat, dient een eventueel falen niet louter als ongeschiktheid van het implantaat te worden beschouwd.
6. De aanwezigheid van keratine in middenoorepitheel dient niet voorshands als een vorm van metaplasie te worden beschouwd.
7. De uitstoting van trommelvliesbuisjes is een treffend voorbeeld van een onvoldoende begrepen verschijnsel dat klinisch toepassing vindt.
8. Het begrip van de pathogenese en etiologie van de ziekte van Menière zou kunnen worden verruimd door toepassing van X-ray microanalyse in combinatie met electronen microscopische immunocytochemie.

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9. Zowel het onderzoek naar de evolutie van de mens als dat naar de origine van de resident macrofaag ontleent zijn charme aan de missing-link.
10. Het is niet ondenkbaar dat per toegekend project meer wetenschappelijke staftijd wordt geïnvesteerd dan er aan wetenschappelijk assistententijd wordt uitgekeerd.
11. Ook het denkraam van een promovendus dient tijdig gewist.
12. Het standpunt dat het gezin de hoeksteen van de samenleving is gaat voorbij aan het gegeven dat een hoeksteen in kwantitatieve zin slechts een klein onderdeel van een degelijk gebouw vormt.

C.A. van Blitterswijk
Leiden, 12 juni 1985.