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Late tissue reactions and degradation of biodegradable polylactide implants. An experimental study in rats

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SUMMARY AND CONCLUSIONS

Biodegradable poly(L-lactide) (PLLA) plates and screws are currently being used as an alternative for metal plates and screws for the fixation of bone fractures. Using these biodegradable implants, a re-operation for removal of the plates and screws can be avoided. However, in patients treated with as-polymerized poly(L-lactide) (PLLA), a swelling at the site of implantation was observed after three and more years following implantation. These swellings seemed to be related to the degrading PLLA and the formation of high crystallinity particles. For the detection of such late occurring events very long implantation periods are needed, periods which may even be longer than the normal life span of laboratory animals. To avoid long in vivo implantation periods, predegradation can be performed simulating long term physiological degradation.

In this study a comparison was made between the histopathological reaction to non-degraded and predegraded as-polymerized PLLA, and as a control, polyethylene (PE) disks implanted subcutaneously in rats. In addition, another recently developed poly(96%L,4%D-lactide) (PLA96) possessing lower crystallinity was tested that probably results in a faster and more complete degradation. In vitro predegradation was performed at elevated temperatures (90°C). Animals were sacrificed after a postoperative period varying from 2 to 52 weeks. A glycolmethacrylate (GMA) plastic embedding procedure was modified in order to obtain good 1.5 μm sections of the implants and surrounding tissue. Chemical analysis, light- and electron microscopical analysis and semi-quantitative histopathological evaluation were performed.

Chemical analysis of the predegraded implants revealed a difference in in-vitro degradation between PLLA, PLA96 and PE. PLLA had to be predegraded twice as long (336 hours versus 168 hours) as PLA96 to obtain a mass loss comparable to the implant material obtained from patients with a local swelling (about 50%). PE did not show degradation after in vitro incubation.

The histological reaction to non- and predegraded PE and to the non-degraded PLLA and PLA96 disks was very mild. It consisted of a capsule of fibroblasts and macrophages accompanied by a minor granulomatous reaction. The histological reaction to the predegraded implants was qualitatively similar to the reaction to the non-degraded implants, however, quantitatively an increase was noted, especially with regard to the granulomatous reaction surrounding the fibrous capsule. Also degradation and phagocytosis of implant material was increased. A number of predegraded PLLA and PLA96 disks showed an increase of volume with implantation time caused by the formation of fields of polymer debris accompanied by a granulomatous reaction. Especially for the predegraded PLA96 at week 52 after implantation the volume was more than doubled when compared to the volume observed at week 2 after implantation. The debris zone was found to consist of both polylactide polymer fragments and small remants of dead cells.

From our results it can be concluded that when compared to PLLA, the degradation of PLA96 is enhanced. Both predegraded PLLA and PLA96 disks, subcutaneously implanted in rats, induced a swelling with characteristics similar to that observed with PLLA implants in patients. Thus, although the degradation of PLA96 is enhanced, as-polymerized PLA96 will probably also induce local swelling when used in a clinical setting, and should therefore not be used as a new

ostheosynthesis material. The results of this study indicate that in vitro predegradation followed by in vivo implantation might be used as a model to predict late complications of biodegradable implants. In this regard the time of predegradation (168 versus 366 hours) seems to be less important than the extent (22% versus 50% mass loss) of degradation of the material itself. A mass loss of approximately 50% resulted after implantation in rats in similar observations as seen in explanted material of patients several years after the initial operation.

Conclusively, the implantation of partially degraded material using in vitro predegradation, might be a good model for studying local effects of biodegradable materials, thereby detecting possible late occurring events such as were observed for as-polymerized PLLA in patients.

This model might be suited as an implantation assay for biodegradable materials which are not yet included in the ISO/CEN 10993-6 guidelines: Biological evaluation of medical devices. Part 6 Tests for local effects after implantation.

SAMENVATTING EN CONCLUSIES

Biologisch afbreekbare implantaten van poly-L-lactide (PLLA) worden thans gebruikt als alternatief voor metalen platen en schroeven voor de fixatie van botbreuken. Door het gebruik van deze afbreekbare implantaten kan een heroperatie om de metalen platen en schroeven te verwijderen worden voorkomen. Tijdens het gebruik van deze afbreekbare implantaten zijn er echter locale complicaties waargenomen in de vorm van zwellingen vanaf ongeveer drie tot vier jaar na implantatie. De zwellingen worden waarschijnlijk veroorzaakt door het degraderen van de lactide polymeren en de locale vorming van hoog kristallijne deeltjes.

Om dit soort zeer laat optredende fenomenen preklinisch op te sporen zijn er zeer lange implantatie en observatie tijden nodig, die in een aantal gevallen zelfs langer zijn dan de levensduur van het proefdier. Om deze lange in vivo implantatie studies te vermijden, kan er een in vitro pre-degradatie worden uitgevoerd als simulatie van een fysiologisch degradatie proces middels hydrolyse.

In deze studie werd de histopathologische reactie van ongedegradeerd en pre-gedegradeerd "as-polymerized" PLLA, en als controle van polyethyleen (PE) onderzocht door middel vam de subcutane implantatie van schijfjes materiaal in ratten. Tevens werd in deze studie een recent ontwikkeld copolymeer van D- en L-lactide onderzocht, poly(96%L,4%D-lactide) (PLA96), een polymeer met een lagere kristalliniteit wat resulteert in een snellere en meer complete degradatie. In vitro predegradatie werd uitgevoerd bij een temperatuur van 90°C. Na een postoperatieve periode van 2 tot 52 weken werden de geimplanteerde materialen verwijderd voor histologisch onderzoek. Om goed beoordeelbare histologische preparaten te verkrijgen werd de standaard plastic "inbeddingsmethode" in glycolmethacrylaat aangepast. Na de in vitro predegradatie werd er chemisch, licht- en electronen microscopisch onderzoek verricht.

Chemische analyse toonde verschillen aan tussen de in vitro predegradatie van PLLA, PLA96 en PE. PLLA moest ongeveer twee keer zolang (336 versus 168 uur) in vitro geincubeerd worden dan PLA96 om eenzelfde massa verlies van ongeveer 50% te verkrijgen. PE vertoonde geen massa verlies na de in vitro predegradatie.

De waargenomen histologische reactie op het on- en pre-gedegradeerde PE en op de ongedegradeerde geimplanteerde materialen PLLA en PLA96 was zeer gering. De reactie bestond uit kapselvorming van fibroblasten en macrofagen met een minimale granulomateuze component. De histologische reactie op de pre-gedegradeerde implantaten was kwalitatief gelijk, maar kwantitatief veel sterker dan de reactie op de ongedegradeerde implantaten. Met name de rondom het kapsel gelegen granulomateuze ontstekingsreactie was sterk toegenomen. Ook werd er een verhoogde degradatie en fagocytose van het geimplanteerde materiaal gezien. Bij een aantal van de gepredegradeerde PLLA en PLA96 implantaten werd een volume toename waargenomen, welke gevormd werd door velden van polymeer debris en de granulomateuze ontstekingsreactie. De debris zone bleek te bestaan uit zowel polymeer fragmenten en restanten van cellen.

Geconcludeerd kan worden dat de degradatie van het copolymeer PLA96 verhoogd is ten opzichte van PLLA. De zwelling die in vivo waargenomen werd na in vitro predegradatie

van de PLLA en PLA96 polymeren vertoont histologisch dezelfde karakterestieken als de zwelling zoals deze bij patienten na enkele jaren werd waargenomen. PLA96 zal waarschijnlijk bij klinisch gebruik ook locale zwelling vertonen, en is daarom niet geschikt als een nieuw osteosynthese materiaal. De resultaten van deze studie geven aan dat in vitro predegradatie gevolgd door in vivo implantatie als een model gebruikt kan worden om laat optredende reacties van afbreekbare implantaten te voorspellen. Voor "as-polymerized" PLLA lijkt de tijd van in vitro predegradatie (168 versus 336 uur) hierbij minder belangrijk dan de mate (in termen van massa verlies, 22 % versus 50%) van degradatie van het materiaal zelf. Massaverlies van het polylactide in de orde van grootte van 50% gaf na implantatie in ratten vergelijkbare resultaten als bij materiaal verkregen van patiënten enkele jaren na de primaire operatie.

Concluderend kan gesteld worden dat de implantatie van gedeeltelijk, middels in vitro predegradatie, gedegradeerd materiaal een goed model kan zijn voor het bepalen van de locale effecten van afbreekbare implantaten. Hierbij kunnen mogelijk laat optredende bijwerkingen zoals deze gevonden worden bij patiënten na behandeling met "as-polymerized" PLLA, reeds in een preklinisch stadium gedetecteerd worden.

Dit model is mogelijk geschikt als implantatie model te dienen voor afbreekbare materialen. Afbreekbare materialen zijn tot op heden nog niet opgenomen in de ISO/CEN richtlijnen voor wat betreft de locale effecten na implantatie (ISO/CEN 10993-6, Biological evaluation of medical devices. Part 6 Tests for local effects after implantation).

1. INTRODUCTION [1]

General aspects

Metallic bone plates and screws have been used successfully in oral and maxillofacial surgery for internal fracture fixation[1,2]. Although good fracture healing is achieved, there are several disadvantages of the use of these metallic implants, including bone atrophy due to stress-shielding. This is one of the main reasons why these devices have to be removed in a second operation[2-4]. In addition, in the long run inflammatory reactions may occur due to loosening and corrosion of these metallic devices[5-7]. Metallic implants also cause problems in medical treatment; they cause backward scattering in patients needing irradiation because of a malignant tumor[8,9], and can also produce artifacts in computer tomography and magnetic resonance imaging.

Another problem in the use of metallic osteosynthetic devices is the possibillty of patients becoming sensitized. This goes especially for alloys that bear nickel, cobalt and chromium[10,11].

Bone plates and screws made of a biodegradable material are considered to be a good alternative for metallic ones. The main advantage of biodegradable plates and screws is that they gradually transduce the functional stresses to the bone because of the resorption of the material. Moreover, if the material fully degrades, a reoperation for the removal of the plate and screws can be avoided.

Biodegradable polymers

Biodegradable materials have already been used for biomedical applications for many years, especially for surgical sutures like Dexon^{R} [12] since 1970 and Vicryl^{R} [13] since 1975. Biocompatible and resorbable poly (a-hydroxy)-acids like polylactic acid have been proposed as a potential orthopaedic repair material. Lactic acid is optically active, it has a D- and L-form, and lactic acid polymers can therefore be poly (L-lactic acid), poly (D-lactic acid) or a co-polymer poly (D,L-lactic acid). Polymers made of the optically pure isomers are highly crystalline, whereas the racemic polymer is almost completely amorphous. In order to use polylactides for biomedical application, important factors, such as tissue tolerance, biodegradation, metabolism and toxicity of the lactic acid polymer, have to be determined.

A great variety of animals have been used for experiments on tissue tolerance of biodegradable polymers like mice, rats, sheep, dogs and rhesus monkeys[14-16]. Tissue tolerance of polylactides and polyglycolides deals mostly with the surgical suture material Dexon^{R} and Vicryl^{R}. These proved to have good tissue tolerance in clinical use. Retrospective studies report the absence of antigenicity, the rarity of tissue reactions and the almost complete absence of abscesses and thread fistulae[17,18]. However, it should be noted that the tissue

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tolerance of a polymeric implant is not only determined by its chemical material properties. In addition, also the size, surface and shape of the implant and the site of implantation may have an influence on the tissue tolerance[19].

Biodegradation

Several possible degradation mechanisms are described, but for the degradation of PLLA hydrolysis seems to be the most likely process[14,15,20]. The only necessary condition for this degradation seems to be an aequous environment. Biodegradation is enhanced by an increasing hydrophilia and by a larger specific surface area. Water uptake is also affected by the crystallinity of the polymers: amorphous regions are more easily susceptible to hydrolytic attack. The relation between crystalline and amorphous regions in a copolymer depends on the ratio of monomer units: in contrast to poly (D,L-lactic acid) with an amorphous structure, copolymers of D- and L-lactic acid with a lower proportion of D- or L-units show an increased crystallinity.

In addition to hydrolysis, an intracellular degradation process of small particles occurring in macrophages and fibroblasts has been noticed when powders up to 500 µm were implanted in rats and rabbits[21]. Due to insufficiently defined composition and crystallinity of the polymer materials in most publications, there is no clear picture of degradation kinetics in a biological environment[13,22,23].

Williams et al.[24] have described the effects of enzymes on the degradation of polylactic acid. Schakenraad et al.[25] and Pitt et al.[21] did not observe significant differences between in vivo and in vitro degradation studies, suggesting no influence of enzymes on the rate of hydrolysis. Leenslag et al.[26] and Bos et al.[27] also compared the in vitro degradation of PLLA submerged in a saline solution with the in vivo degradation of PLLA implanted in sheep, dogs and rats. No difference between in vivo and in vitro degradation of PLLA was observed. Unfortunately, in none of these studies complete resorption of the pure PLLA was observed due to relatively short observation times.

The metabolism of D-lactic acid has long been subject of controversy, in particular the question of the existence of a D,L-lactate racemase[28]. Recent experiments on the metabolism of D- and L-lactic acid in several rat tissues revealed an oxidation of D-lactic acid in the liver exceeding the metabolism of L-lactic acid and showed equal rates in the heart and a definite but lower reaction in other tissues compared to L-lactic acid[29].

Toxicity

In accordance with its role in the intermediary metabolism of cells, the acute toxicity of lactic acid is low. Most mammals, including man, tolerate oral doses of more than 1,500 mg/kg[30]. Massive oral doses of D,L-lactic acid caused weight loss and anaemia in rats and lowered the level of carbon dioxide in the blood. Like other acids of moderate strength, the free acid is an irritant in particular on skin, eyes and mucous membranes. Decompensated metabolic acidosis with increased excitement, breathing difficulties and accelerated heart rate are the symptoms of lactic acid poisoning. High plasma concentrations of lactic acid were described in connection with fear and panic situations[31,32]. The few studies carried out with

subchronic and chronic applications of D,L-lactic acid showed no accumulation or cumulative effects[30,33]. L-lactic acid, the apparent non-allergenic, non-carcinogenic and non-toxic product of hydrolysis, can leave the body by normal excretory routes.

PLLA in osteosynthesis

Vert et al.[14] published a number of experiments on differently processed polylactides and copolymers with varying amounts of D-lactide and L-lactide used for bone plates and screws. They concluded that PLLA (100% L-lactide) had the best mechanical properties. Using the as-polymerization technique in combination with the semi-crystalline homopolymer PLLA, a biodegradable polymer was obtained which compared to other biodegradable polymers had good mechanical properties [34,35] .

PLLA plates and screws were used without complications for internal fixation of mandibular fractures in sheep and dogs [36,37]. In addition, PLLA sheets were successfully used for orbital floor reconstruction in goats [38].

Degradation and tissue reaction of PLLA was evaluated after subcutaneous implantation in rats [39]. After 26 weeks mass loss could be detected as an indication of resorption; no histologically detectable inflammatory reaction could be determined. During the observation period of 143 weeks, histological sections of the implantation site of one rat showed the presence of 'foamy' macrophages, suggesting a late degradation stage of the PLLA material. Based on these animal studies it was estimated that the implant would be fully resorbed in about 3.5 years[39,40]. These favourable results resulted in the start of a clinical trial in humans. Because of the smaller tensile strength of the as-polymerized PLLA[36] when compared to metal, it was decided to use the PLLA bone plates and screws only in a biomechanical favorable area in which the load would be minimal. Ten patients suffering from an unstable zygomatic fracture were treated in the period between 1986 and 1988 with specially designed PLLA bone plates and screws[41]. Healing was without complications in all cases. At the end of the initial 18 months observation period, however, the plates and screw heads were stil palpable through the skin, suggesting a slow resorption process[41,42]. Some patients showed after a post operative period of almost 3-4 years a clinically detectable swelling at the site of implantation (see Fig. 1)[43]. The patients felt uncomfortable about the presence of the PLLA plate which was still palpable and obviously still had not fully resorbed. At a recall, all remaining patients showed a similar local swelling. The postoperative implantation period in these patients varied from 3 years and 4 months to 5 years and 8 months[44].

The cause of this swelling remained unclear, but a possible explanation might be a massive granulomatous reaction induced by small PLLA particles resulting after degradation and disintegration. In a study with in vitro predegraded polylactide particles, a positive correlation was noted between the mean particle size and the intensity of the cellular and tissue reaction [45,46]. Another explanation might be swelling of the implant itself, as in a cross-section of an explanted swelling, 3.5 years after treatment, up to 65% of the sectioned area consisted of PLLA remnants[44]. This would suggest that the PLLA implant itself, through an increase in volume during degradation, could induce a swelling after years.

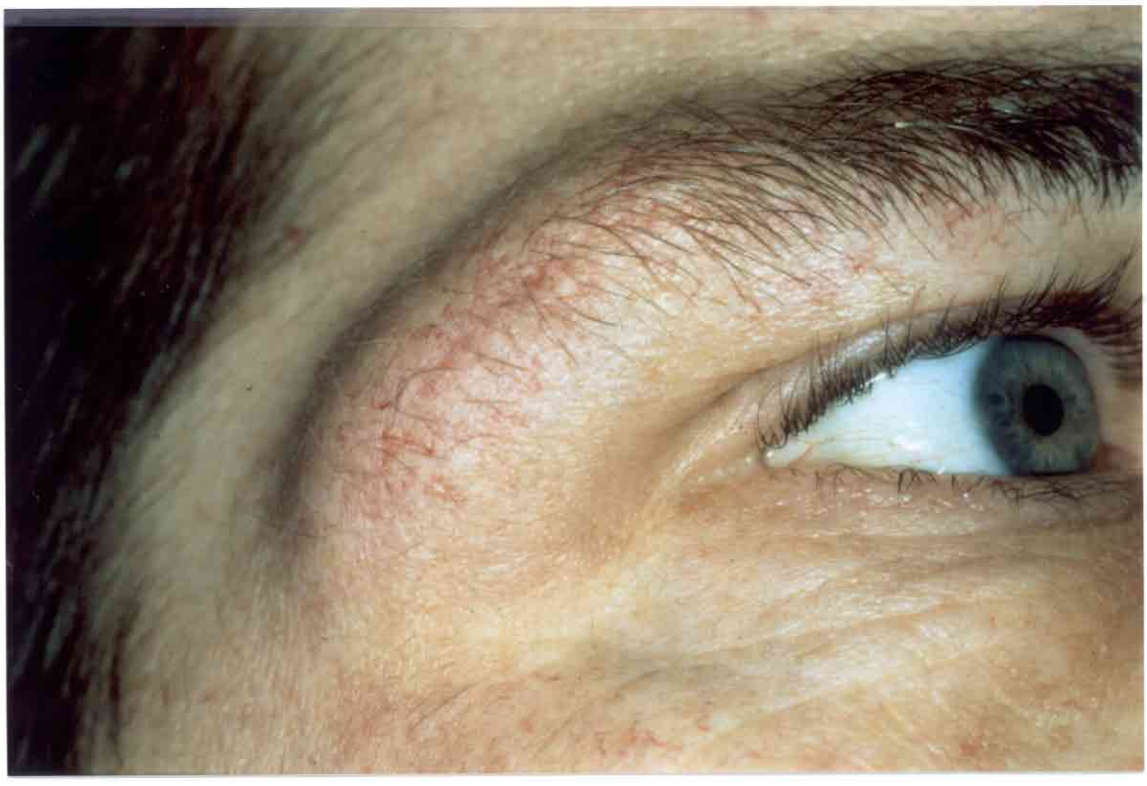


Figure 1. Subcutaneous swelling at the site of implantation

Experimental study

To study the mechanism by which the implants induce the described swellings, implants with dimensions comparable with the zygomatic bone plates of the patients should be used that have to be implanted for three years and longer. In order to avoid these long in vivo implantation periods, in vitro predegradation at elevated temperatures can be used to simulate the physiological degradation in vivo. This method can be used to provide PLLA implants comparable to the material obtained from re-operated patients. With in vivo implantation of these predegraded discs, which have a mass loss comparable to the implants taken from the patients at the time the complications occurred, an experiment was performed to study the origin of the local swelling, and the tissue reaction and degradation pattern.

It is possible that PLLA particles, or macrophages with internalized PLLA material, migrate to lymphoid nodal tissue from the implant site [47]. So, lymph nodes were included for the detection of PLLA particle transport to the lymph nodes draining the implantation site.

The degradation kinetics in bone seem to be the same as in soft tissue[48]. However, in a study on poly (L-lactide-co-glycolide) and polyglycolide for material implanted in various areas, different degradation rates were described[49]. This difference in degradation might be caused by different vascularization in the different areas of the skin. In this study the implants wil be implanted in six different subcutaneous implantation sites to study a possible influence of the site of implantation on the tissue reaction to the implants.

Other polymers have already been developed as an alternative material for PLLA. One of these materials is a copolymer of 96%L- and 4%D-lactide (PLA96)[50]. By varying the percentage of D-lactide, the degradation rate and mechanical properties can be altered.

Copolymerization of L-lactide with 4%D-lactide will provide a semicrystalline polymer with a lower initial crystallinity and smaller and less perfect crystalline domains as PLLA, which will facilitate hydrolytic degradation, when compared to the homopolymer PLLA. The mechanical properties of PLA96 are comparable to PLLA implants which could make PLA96 a good alternative polymer to PLLA.

In none of the degradation and biocompatibility studies of high molecular weight PLLA complete degradation of the polymer or the tissue response in the final degradation phase have been described, simply because the follow-up periods for these kinds of polymers are too short. Considering the above mentioned findings there is a strong need for the description of the final stage of degradation and late tissue response of the PLLA implants. Therefore, in vitro predegradation and in vivo implantation were combined in one experiment.

In summary, the aims of this study are to investigate if in vitro predegradation in combination with in vivo implantation can describe and predict the late tissue reaction and degradation of the polylactides PLLA and PLA96. In addition, the results might give an explanation for the origin of the observed swelling in patients after three years of implantation with PLLA plates and screws.

Another reason to develop such a test is that the ISO/CEN guidelines for the biological evaluation of medical devices do not (yet) include an implantation test for local effects of biodegradable materials[51].

2. MATERIALS AND METHODS

2.1. Implants

Poly(100%L-lactide) (PLLA) and poly (96%L,4%D-lactide) (PLA96) were as-polymerized according to the method described by Leenslag et al[34]. Polymerization of L-lactide and D-lactide was performed after purification of the monomer (CCA/Purac Biochem, Gorinchem, the Netherlands) by recrystallization from toluene under N_2 atmosphere. Both PLLA and PLA96 were polymerized under vacuum at 110°C with 0.0015 wt% stannous-2-ethyl-hexanoate as a catalyst. Molecular weights (weight average molecular weight $\overline{M}_w$, and number average molecukar weight $\overline{M}_n$) were determined by gel permeation chromatogrphy (GPC). Thermal properties were evaluated by differential scanning calorimetry (DSC) on a Perkin Elmer DSC-7. Five to 10 mg samples of PLLA and PLA96 were measured at a heating range of $10^{o}C\ min^{-1}$ to determine the heat of fusion, the melting temperature and the glass transition temperature.

PLLA disks (mean weight 193 ± 1.6 mg, n=116), PLA96 disks (mean weight 197 ± 2.1 mg, n=76) with a diameter of 10 mm and a thickness of 2 mm were prepared from blocks of PLLA and PLA96 (Fig. 2). As a non-degradable control material, ultra high molecular weight polyethylene (PE, Goodfellows, England) disks were used (mean weight 147 ± 2.5 mg, n=76). All disks were sterilized by regular hospital steam sterilization.

Figure 2. Disks and the instrument used to insert the disks subcutaneously.

The mass loss of the predegraded disks was to be in the same range as the mass loss of the implants obtained from patients at the time the swellings occurred, in order to investigate the late degradation and tissue reaction of the implants. The mass loss of material explanted from patients was about 50%. The predegradation periods were based on results of previous studies[43,44]; for the PLA96 this was obtained after a predegradation period of 168 hours, for the PLLA a predegradation period of 336 hours was needed. For a good comparison between PLLA and PLA96, part of the PLLA disks were also predegraded for 168 hours.

80 PLLA disks, 40 PLA96 and 40 PE disks were predegraded, the remaining 36 disks of each polymer (PLLA, PLA96 andPE) were implanted as non-degraded disks. For predegradation, the disks were separately immersed in phosphate buffer of pH 7.4 in a glass tube with a permeable cap. Subsequently the glass tubes were placed in a basin, set at 90°C, containing 20 litres of phosphate buffer of pH 7.4. Of the 80 predegraded PLLA disks, 40 were predegraded for 168 hours ($PLLA_{168}$), the second group of 40 PLLA disks was predegraded for 336 hours ($PLLA_{336}$). The 40 PLA96 and PE disks were also predegraded for 168 hours ($PLA96_{168}$ and PE_{168},respectively).

Four disks of each predegraded group were dried to constant weight and mass loss was determined.

A total of seven different implants were used in this study, designated as follows:

PLLA	non-degraded poly-L-lactide
PLA96	non-degraded poly-D,L-lactide (96%L, 4%D)
PE	non-degraded poly-ethylene
$PLLA_{168}$	predegraded poly-L-lactide (168 h at 90°C)
$PLA96_{168}$	predegraded poly-DL-lactide (168 h at 90°C)
PE_{168}	predegraded poly-ethylene (168 h at 90°C)
$PLLA_{336}$	predegraded poly-L-lactide (336 h at 90°C)

2.2. Animal Study

A total of 42 male, SFP Wistar albino rats, 6 to 8 weeks of age, weighing approximately 250-300 g were operated on. Animals were kept in macrolon type III cages, at a 12/12 hours light/dark regimen, and water and food was provided ad libitum. The rats were anaesthetized with a nitrous oxide-oxygen-fluothane mixture. The dorsal hair was clipped and the skin was treated with iodine. Subsequently six incisions were made on the back of the rat and in bluntly created subcutaneous pockets one sample of each implant as mentioned above was inserted. The disks were inserted with a specially designed instrument to prevent damage of the feeble predegraded disks during insertion in the pocket (Fig. 2).

To detect a possible influence of the site of implantation on the tissue reaction, the implant position was rotated for each group of implants, with the exception of $PLLA_{336}$ of which six samples were implanted into one animal. The implantation sites were (left and right) inguinal, (left and right) median and (left and right) axillar. After implantation the wounds were closed with Dexon^R sutures.

At the intervals, 2, 4, 8, 12, 26 and 52 weeks postoperatively, animals were anaestesized with a CO_2/O_2-mixture and were killed by exsanguination. The dorsal hair was

clipped and the implants were generously excised, bisected and one part was subsequently fixed in 4% commercial formaldehyde/1% glutaraldehyde (4CF1GA, Merck, Darmstadt, Germany) in 0.1 M NaCacodylate buffer at pH 7.4 for at least one week at room temperature. The other half was deepfrozen in liquid nitrogen.

Lymph nodes, draining the implantation sites (Lnn inguinalis, Lnn brachialis and Lnn axillaris) were also excised and fixed in 4CF1GA.

Before excising the implants, the size of the implants plus capsule (width, length and thickness) and the thickness of the skin was measured with vernier callipers. For the $PLLA_{336}$ implants the dimensions of the implants were not measured. This part of the experiment was performed at the University Hospital Groningen. The volume of the implants plus surrounding capsule was determined as: width x length x (thickness of (implant + capsule + skin) - thickness of the skin) in mm^3. A swelling was noted if an implant showed a volume over 400 mm^3, the differences in volume were tested statistically with a student's t-test.

2.3. Patient material

Excised material was obtained from one patient approximately 8 years after fracture fixation at the Department of Oral and Maxillofacial Surgery, University Hospital Groningen, Groningen, The Netherlands. This material was processed for LM and TEM histological analysis (see below).

2.4. Histology

Excised implants and surrounding tissue were processed and embedded in GMA (glycol methacrylate; Technovit 7100, Heraeus Kulzer GmbH, Wehrheim, Germany) after modification of the standard procedure as described by the supplier[52]. See Appendix A. In short after fixation the implantation sites were dehydraded in graded series of ethanol, infiltrated with Technovit 7100 solution, embedded in glycolmethacrylate, and sectioned. Tissue sections of 1-2 µm were prepared and stained with Toluidine blue 0.1% (Fluka Chemika, Buchs, Switzerland).

After evaluating the implants light microscopically, specific implants and areas of implants were selected for transmission electron microscopy.

Lymph nodes were routinely processed, embedded in paraffin and sectioned, and stained. Sections of the lymph nodes were examined for the presence of implant material.

2.4.1. Light Microscopy (LM)

Sections were evaluated by light microscopy. Implant material is easily recognized; under crossed Nicol Prisms the foreign implant material is brilliantly birefringent.
Tissue reactions to the implants were scored semiquantitatively (- no reaction, + mild reaction, ++ moderate reaction and +++ marked reaction) according to the following criteria:

Capsule

\+ Presence of predominantly macrophages directly against implant plus fibroblast connective tissue layers

++ Capsule consisting of three layers, macrophages and fibroblasts next to the implant, several layers of fibroblasts, and an outer layer of macrophages
+++ Presence of extensive outer layer of macrophages

Phagocytosis
+ A few macrophages containing polymer
++ A few areas of macrophages containing polymer
+++ Many and large conglomerates of macrophages containing polymer

Infiltrate
+ A few foamy macrophages around fibrous part of capsule
++ A few areas with groups of macrophages around fibrous capsule
+++ Around whole implant granulomatous reaction of macrophages and giant cells

Implant
+ A few pieces of polymer detectable outside implant degradation
++ Several pieces of polymer outside implant
+++ Major part of implant degraded, presence of debris zone

Cellular invasion
+ A few cells within implant
++ Several groups of cells in implant or deep infiltration in implant
+++ Many groups of cells in implant or deep and broad tissue cord formation in implant

After scoring each implant separately, the mean score for each criterium and time interval was obtained by adding all the scores of the implants (+=1, ++ = 2 etc.) and divide this by the number of implants investigated in the group.

2.4.2. Transmission Electron Microscopy (TEM)

Based on light microscopy ultrastructural analysis (TEM) was performed on tissue explanted from one of the patients treated with PLLA bone plates and screws, and 4 $PLLA_{336}$ implants 26 weeks postoperatively. Small pieces of 1 mm^3 were cut out of each implant and fixed at 4°C in 4CF1GA solution in 0.1 M NaCacodylate/HCl buffer at pH 7.4.

After rinsing with buffer, all pieces were post-fixed for 1.5 hours in 1% buffered OsO_4 + 1.5% $K_4Fe(CN)_6$ in 0.1 M NaCacodylate/HCl buffer at pH 7.4, at 4°C. They were routinely dehydrated through a graded series of ethanols and subsequently, pieces were immersed in mixtures of propylene oxide and Spurr epoxy resin (Biorad) in different ratio's; 100:1, 50:50, and 25:75, to infiltrate the tissue completely with the resin. Afterwards tissues were embedded in Spurr resin for 8 hours at 70°C and cut into 1 µm sections on a LKB 8800A ultratome III using glass knives with an angle of 45° (LKB knifemaker 7800 B). Thin sections were stained with Toluidine blue (Merck) and evaluated light microscopically in order to make a selection of the areas which were investigated at the ultrastructural level.

Ultrathin section of 60-70 nm of these areas were cut using a diamant knife. Subsequently these sections were stained with 2% uranyl citrate and lead nitrate (LKB 2168 ultrostainer). The stained sections were evaluated with a Phillips EM 201 transmission electron microscope.

2.4.3. Lymph nodes

Lymph nodes were routinely dehydrated in alcohol and xylene (Merck) with a Vacuum Infiltration Processor (Tissue Tek, Miles Scientific, Elkhart, USA) and embedded in paraffin (Paraplast, Sherwood Medical, St.-Louis, USA) using a Paraffin Embedding Processor (Tissue Tek II, Miles Scientific). Subsequently sections of 5 µm thickness were cut on a Reichert Jung 2030 microtome using disposable knifes (Feather R35, Surth, Germany). Sections were stained with haematoxylin and eosine (H&E) for histological observation.

3. RESULTS

Directly after surgery one of the animals died. Therefore one group of animals consisted of n=5, these animals were sacrificed at 52 weeks after implantation.

3.1. Mass Loss

After in vitro predegradation for 168 hours the mass loss of the $PLLA_{168}$ was about half compared with the $PLA96_{168}$. After 336 hours the mass loss of the $PLLA_{336}$ was similar to the $PLA96_{168}$. (Table 1). The molecular weight showed a decrease for all three materials, although the values for the PLLA remained slightly higher. Opposite to the molecular weight, the heat of fusion showed an increase to respectively 78.8 Jg^{-1} , 92.4 Jg^{-1} and 101 Jg^{-1} for the $PLA96_{168}$, the $PLLA_{168}$ and the $PLLA_{336}$. The melting temperature of the PLA96 and the PLLA samples decreased with degradation time and is presented in Table 1.

Table 1 Molecular weight and thermal properties

Polymer	Degradation in hours	Mass loss %	$\bar{M}_W$	$\bar{M}_n$	$\bar{M}_W/\bar{M}_n$ (Jg^{-1})	ΔH (^0C)	T_m (^0C)	T_g (^0C)
PLLA	0	-	$88\ 10^5$	$35\ 10^5$	2.5	60	187	59
PLA96	0	-	$13\ 10^5$	$62\ 10^5$	2.1	26	152	55
$PLLA_{168}$	168	22.5	4000	2800	1.6	92.4	183	55
$PLA96_{168}$	168	46.5	1400	800	1.6	78.8	140.5	42
$PLLA_{336}$	336	50.2	1800	900	2	101	177	47

$\bar{M}_W$, molecular weight ; $\bar{M}_n$, number average molecukar weight; ΔH heat of fusion; T_m melting temperature; T_g glass transition temperature.

3.2. Volume of Implants

At autopsy the dimensions of implant plus capsule were determined in order to detect a possible swelling during degradation. Macroscopically a capsule of connective tissue around the implant was observed for all groups of implants.

No significant changes in volume of the non-degraded implants could be observed during follow-up (Table 2). Incidentally, at several explantation times in each group of implants for one implant an increase in volume (volume $\geq 400\ mm^3$) was measured. The $PLLA_{168}$ implants did not reveal any changes in volume with implantation time. The $PLA96_{168}$ disks showed 52 weeks after implantation a significant increase in volume compared with $PLA96_{168}$ disks at week 2 ($p<0.001$, student's t-test) and with PLA96 disks at week 52

($p<0.01$, student's t-test). For the predegraded $PLA96_{168}$ disks even a doubling in volume was observed (Table 2).

Although the $PLLA_{336}$ implants were not measured it was noted that from 8 weeks onwards, some $PLLA_{336}$ implants showed a clinically detectable increase in volume. At each follow-up interval some disks had clearly swollen, although some disks still had a normal appearance.

Table 2 Volume of implants at several time points after implantation

Time weeks	n PLLA non-degraded	n PLA96 non-degraded	n PE non-degraded
2	6 262 ± 62 (0)[a]	6 258 ± 20 (0)	6 259 ± 14 (0)
4	6 330 ± 145 (1)	6 316 ± 89 (1)	6 260 ± 34 (0)
8	6 286 ± 39 (1)	6 291 ± 19 (0)	6 270 ± 28 (0)
12	6 339 ± 50 (1)	6 311 ± 56 (1)	6 326 ± 77 (1)
26	6 290 ± 84 (1)	6 356 ± 64 (1)	6 266 ± 47 (0)
52	5 245 ± 81 (1)	5 277 ± 80 (0)	5 262 ± 50 (0)
Time weeks	**n $PLLA_{168}$ pre-degraded**	**n $PLA96_{168}$ pre-degraded**	**n PE_{168} pre-degraded**
2	6 249 ± 25 (0)	6 240 ± 16 (0)	6 272 ± 47 (0)
4	6 318 ± 163(1)	6 251 ± 57 (0)	6 267 ± 27 (0)
8	6 269 ± 22 (0)	6 251 ± 45 (0)	6 293 ± 39 (0)
12	6 256 ± 6 (0)	5 286 ± 75 (0)	6 282 ± 35 (0)
26	6 261 ± 33 (0)	6 319 ± 97 (1)	6 283 ± 87 (1)
52	5 287 ± 63 (0)	5 565 ± 115(5)[b]	5 270 ± 36 (0)

[a] Mean volume in mm^3 ± standard deviation, within parentheses number of animals with volume of implant ≥ 400 mm^3

[b] Statistically significant (Student's t-test), $p<0.001$ compared to $PLA96_{168}$ week 2, and $p<0.01$ compared to PLA96 week 52

3.3. Tissue reactions to the implants

A semiquantative score (see Materials and Methods) of the histologic evaluation with implantation time is presented in Table 3. In this table the scores of both 2 and 4 weeks are presented as one time interval because the difference in time between 2 and 4 weeks compared with 2 and 52 weeks is considered negligible, and the results at both implantation times were similar.

Table 3 Semiquantitative evaluation of local reactions of PLA polymers after s.c. implantation in rats.

Implant	Time (wks)	N	Capsule	Phagocytosis	Degradation	Cell ingrowth
PLLA	2+4	12	+/++	-/+	-	+/++
0 h	8	6	+	-/+	-	+
Pre-	12	6	+	-	-	+
degra-	26	6	+	-/+	-	+
dation	52	5	+	-	-/+	+/++
PLA96	2+4	11	+	-	-	-
0 h	8	6	+/++	-/+	-/+	+
Pre-	12	6	+/++	-/+	+	+/++
degra-	26	6	+/++	-/+	+/++	++
dation	52	5	+/++	+	+/++	++/+++
PE	2+4	12	+/++	-	-	-
0 h	8	5	+	-/+	-	-
Pre-	12	4	+	-	-	-
degra-	26	5	+	-	-	-
dation	52	4	+	-	-	-
PLLA	2+4	12	++	++	+	+/++
168 h	8	6	++	++	+	+/++
Pre-	12	6	++	++	+/++	++
degra-	26	6	++	++	+/++	++
dation	52	5	++	++	+/++	++/+++
PLA96	2+4	12	++	++/+++	++	+/++
168 h	8	5	++/+++	++	++	+/++
Pre-	12	5	++	++	+/++	+/++
degra-	26	5	+++	+++	+++	++
dation	52	5	++/+++	+++	+++	+/++
PE	2+4	11	+	-	-	-
168 h	8	5	+	-	-	-
Pre-	12	4	+	-	-	-
degra-	26	5	+	-	-	-
dation	52	4	+	-	-	-
PLLA	2+4	12	++/+++	+++	++	++
336 h	8	6	++	++/+++	++/+++	++/+++
Pre-	12	5	++/+++	++/+++	++/+++	+/++
degra-	26	4	+++	+++	++	++
dation	52	-	nd	nd	nd	nd

3.3.1. Non degraded implants

The histologic reaction towards the non-degraded implants was characterized by a fibrous tissue capsule with an intermediate layer of predominantly macrophages between implant surface and capsule. With longer implantation periods the fibrous capsule increased somewhat in thickness and had a more mature appearance with long slender fibrocytes. For the PLLA and especially the PLA96, the formation of small cracks and fissures was associated with infiltration of fibroblasts and macrophages (Fig 3). Occasionally, at 26 and 52 weeks with the PLA96 implants, under polarized light a layer of foamy macrophages with internalized birefringent polymer fragments was observed in the periphery of the capsule. Indications for a beginning of further degradation/dissolution are present for PLA96 at 52 weeks after implantation (Table 3, Fig. 4). For the reference material, at each interval investigated a mild histologic response was found consisting of a fibrous encapsulation with a few macrophages directly adjacent to the implants (Table 3). No indications for a preference of the implantation site with regard to the degree of capsule formation or infiltrate was noted.

3.3.2. Predegraded implants

For the predegraded $PLA96_{168}$ and the $PLLA_{336}$ a similar fibrous encapsulation was seen but the number of macrophages directly adjacent to the implants and in the periphery of the capsule was higher when compared with the non-degraded implants (Table 3). $PLLA_{168}$ induced a moderate histological reaction, of which the composition was qualitatively comparable to the non-degraded PLLA disks, but quantitatively an increase was noted. In addition, degradation and particle formation were observed after 12 weeks and onwards. Figure 5 shows the moderate reaction to $PLLA_{168}$ at 52 weeks after implantation.

For the $PLA96_{168}$, $PLLA_{168}$ and $PLLA_{336}$, fragmentation, particle formation and internalization by cells, as indicated by microscopy using polarized light, was already observed in the first weeks of implantation (Table 3). Larger particles of degraded material were surrounded by macrophages, which sometimes seemed to form multinucleated cell syncitia/giant cells (Fig. 6).

As shown in Table 3 (score +++ for degradation) for the $PLLA_{336}$ from 8 weeks on, and for the $PLA96_{168}$ at weeks 26 and 52, fields of polymer debris surrounding the fragmented implants were noted (Figs. 7 and 8). Generally there was a sharp boundary between the debris and the original implant, a similar sharp boundary was also observed in the sections of explanted material obtained from a patient.

For the $PLA96_{168}$ at 52 weeks, and $PLLA_{336}$ from 8 weeks and onwards indications were found for a more complete breakdown of the implant, as besides the debris zones also fragmentation of the implant in large pieces was noted (Fig. 9). The cellular influx around these implants and the number of cells with internalized polymer fragments had increased. Light microscopically, the debris material seemed to be acellular (Fig. 10), only incidentally cells were noted in such areas. Major tissue reactions surrounding the fibrous part of the capsule were only noted with the predegraded ($PLLA_{168}$, $PLLA_{336}$ and $PLA96_{168}$) polymers with the exception of PE_{168}.

For PE_{168} a reaction similar to the tissue response to undegraded PE was observed (Table 3).

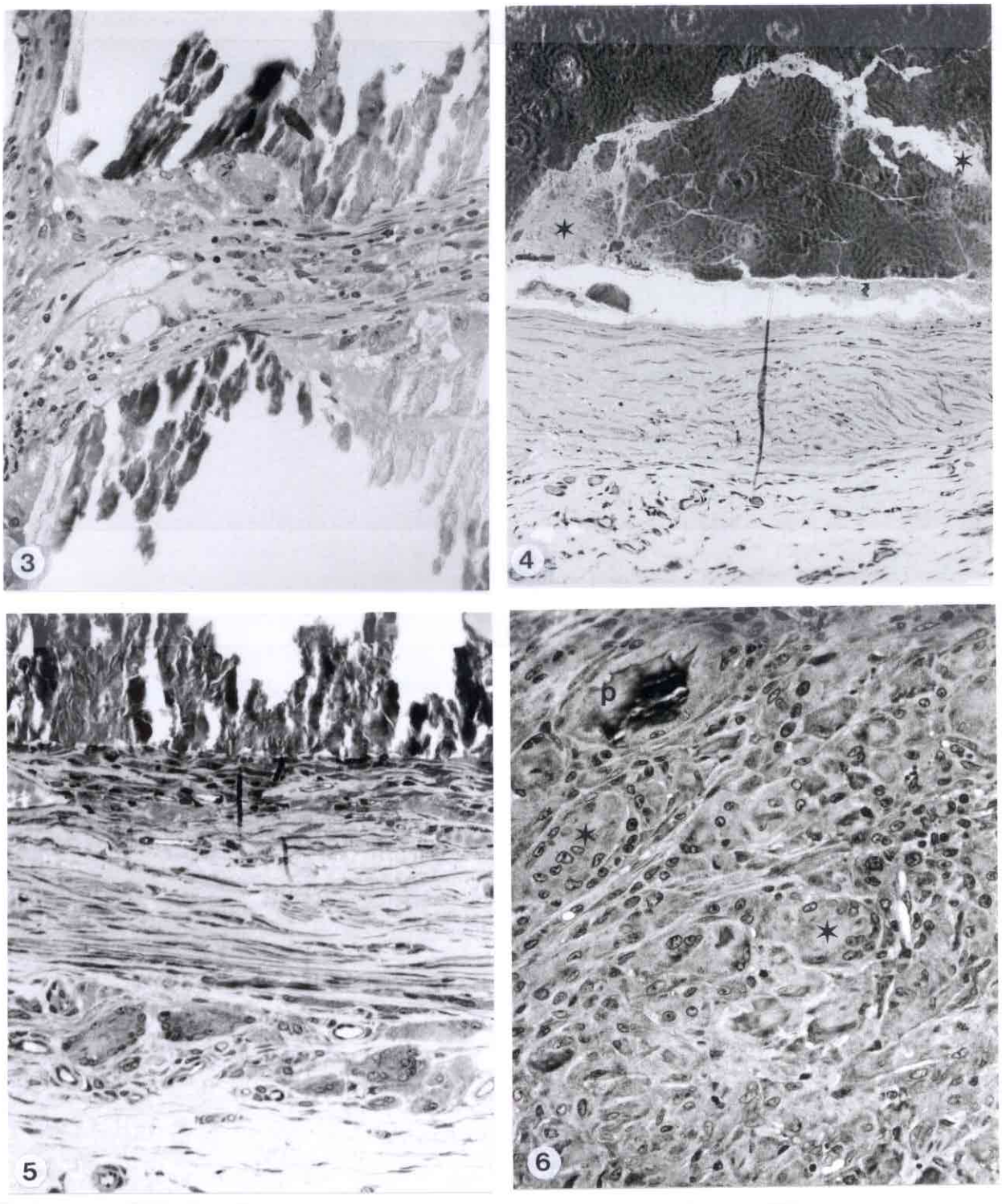
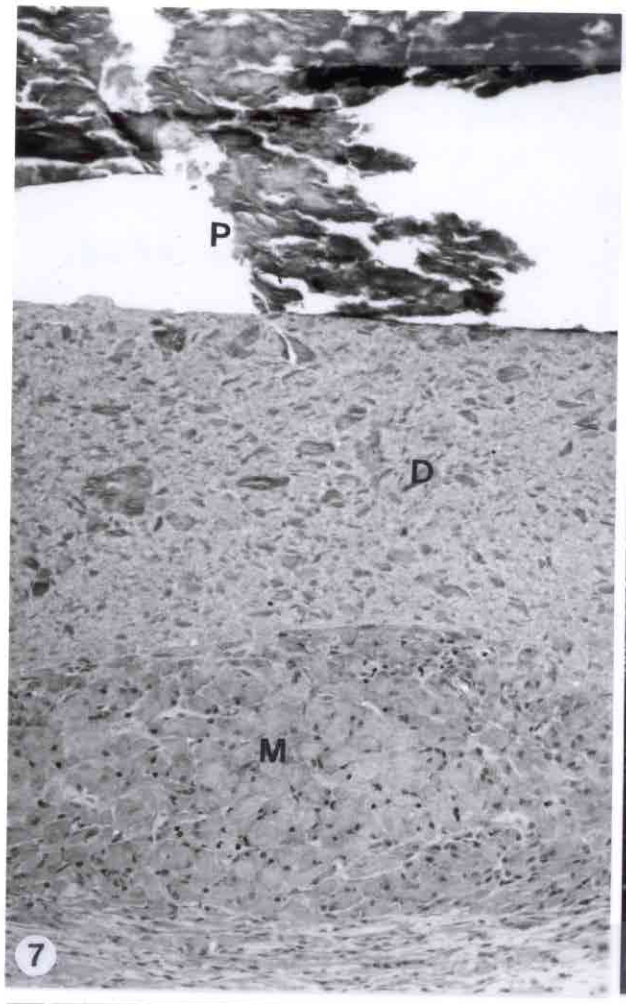


Figure 3. PLA96 implant 26 weeks after implantation with cellular invasion in fissure of implant by macrophages and connective tissue (including blood vessels). x20.
Figure 4. PLA96 implant 52 weeks after implantation, limited degradation and disintegration (asterisks) of implant, presence of connective tissue capsule. x4.
Figure 5. $PLLA_{168}$ implant 52 weeks after implantation with a mature (limited) connective tissue capsule and multinucleated giant cells. x20.
Figure 6. A marked macrophage infiltrate including multinucleated giant cells (asterisks) around a $PLLA_{336}$ implant 2 weeks after implantation. Polymer fragments (P) surrounded by macrophages. x20.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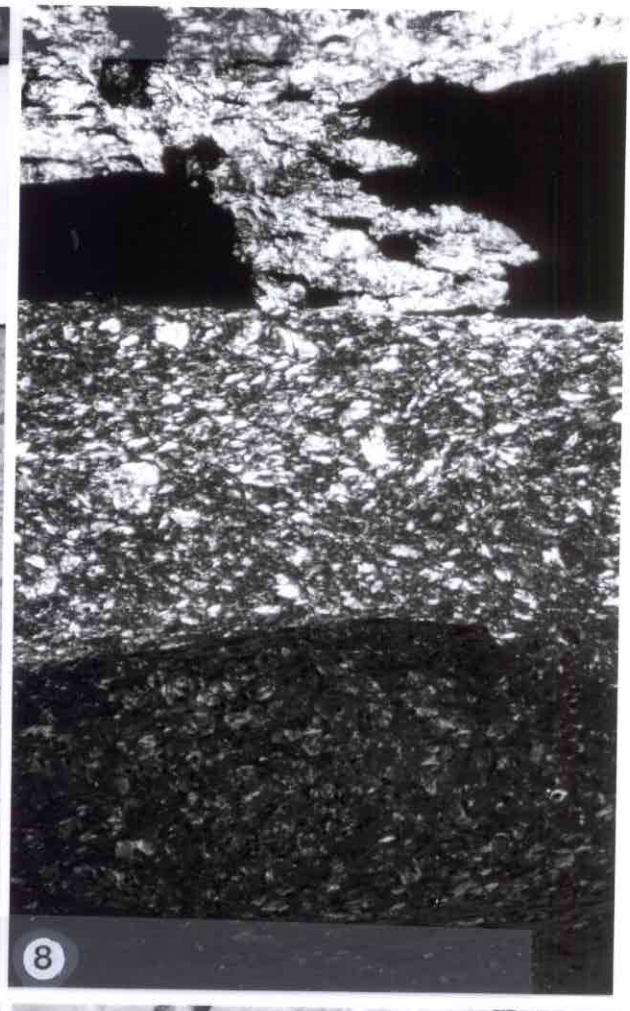
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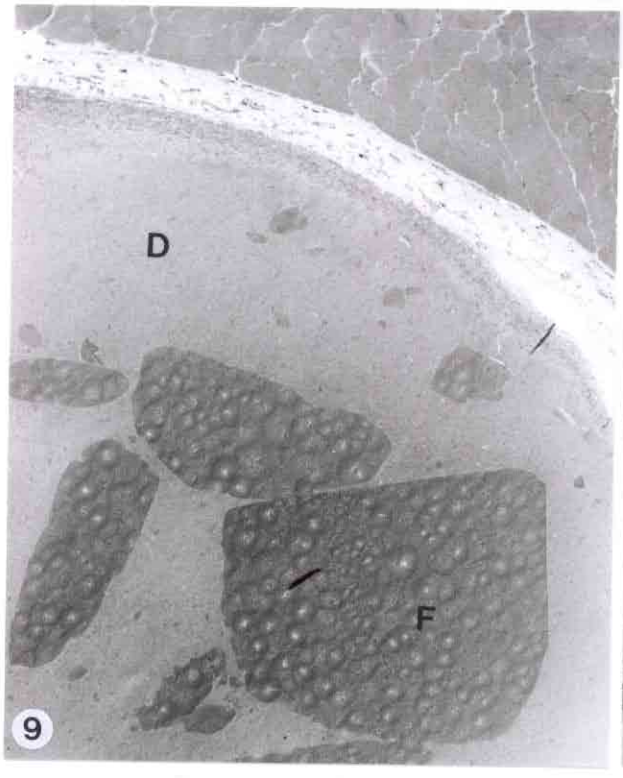
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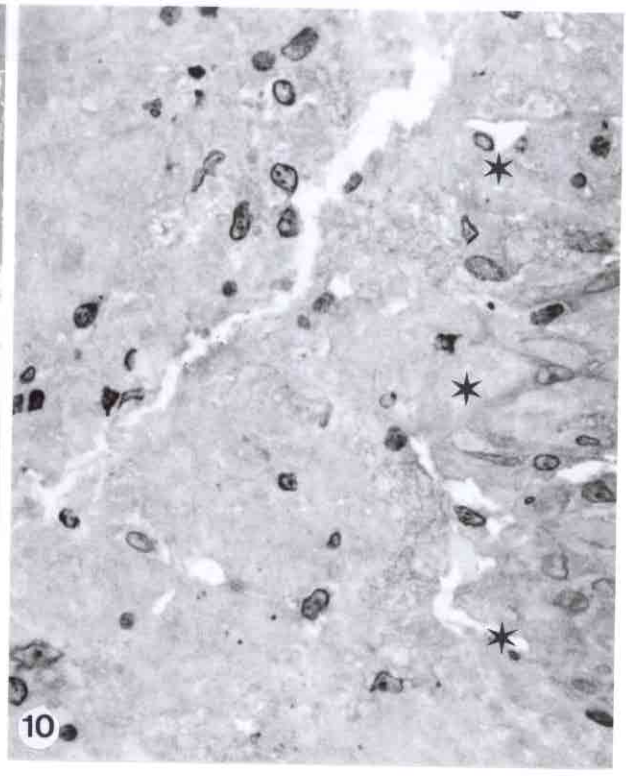
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Figure 7. $PLLA_{336}$ implant (P) after 12 weeks of implantation with zone of debris (D) with polymer fragments and macrophage infiltrate (M) with connective tissue. x10.
Figure 8. $PLLA_{336}$ implant 12 weeks after implantation (see also Fig. 7). Presence of birefringent material (PLLA) in debris zone and macrophages of infiltrate. x10.
Figure 9. Disintegration of $PLA96_{168}$ disk into small fragments (F) with large zones of debris (D) surrounding the implant at 52 weeks after implantation. x4.
Figure 10. $PLA96_{168}$ implant at 26 weeks after implantation. Interface (asterisks) of macrophage infiltrate and debris zone with remnants of degenerated cells. x40.

3.4. TEM

TEM analysis of $PLLA_{336}$ implants retrieved 26 weeks after implantation revealed that the cellular layer surrounding the debris zone consisted mainly of macrophages, some fibroblasts and vascular structures. Macrophages contained variable amounts of internalized polymer material varying from needle-like lamellar structures, regularly in parallel arrangement, to large fragments (Figs. 11 and 12). Fibroblasts occasionally showed internalized polymer fragments.

The major part of the debris zones consisted of degraded polymer material ranging in size from small lamellar fragments to small and large blunt pieces. In this area also numerous small dense particles, residual bodies and other cytoplasmatic components derived from degenerated cells were observed (Fig. 13). At the boundary between the debris zone and the cellular layer, but also deeper in the cellular layer, macrophages and cellular compartments were present showing signs of degeneration, with residual (myeloid) bodies (Fig. 14). With progressive degeneration plasma membranes were no longer observed, leaving the intracytoplasmatic remnants and phagocytized polymer materials free.

No major differences were noted between the material which was implanted in the rat or material which was obtained after approximately 8 years of implantation in a patient. Similar observations were made regarding the presence of polymer and cellular fragments in the debris zone surrounding the implant.

3.5. Lymph Nodes

Lymph nodes draining the implantation sites (Lnn inguinalis, Lnn brachialis and Lnn axillaris) were investigated under polarized light using crossed Nicol prisms. No polymer material was detected in the lymph nodes.

3.6. Implantation site

After evaluating the scores per implantation site no difference in tissue reaction to each implant could be observed for the various implantation sites. An example of this evaluation is shown in Table 4, in which the scores for the capsule formation are presented For $PLLA_{336}$ a possible effect could not be evaluated, as the numbers of the six implant samples (at each time point) were designated at random. The implantation site of these implants was unknown.

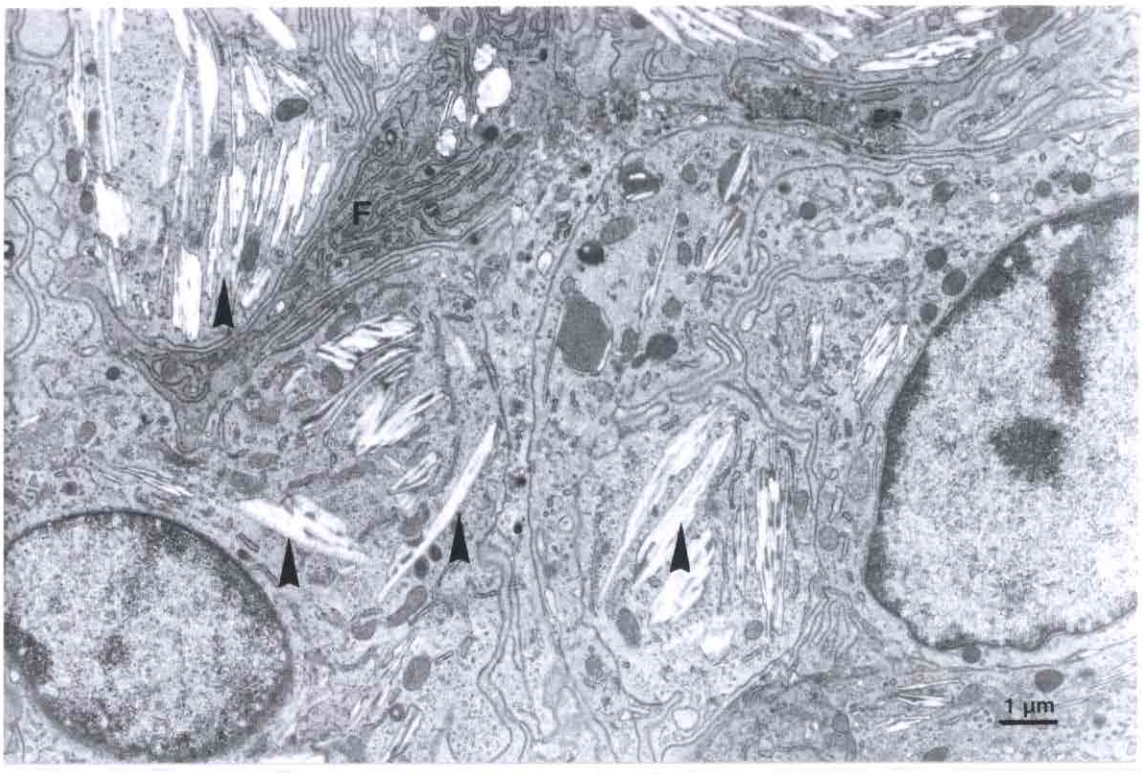

Figure 11. Macrophages in the substantial cellular layer lining the debris zone, containing various amounts of phagocytized $PLLA_{336}$ material (arrows) 26 weeks after implantation. The cellular layer also contains various fibroblasts (F.). x7.600.

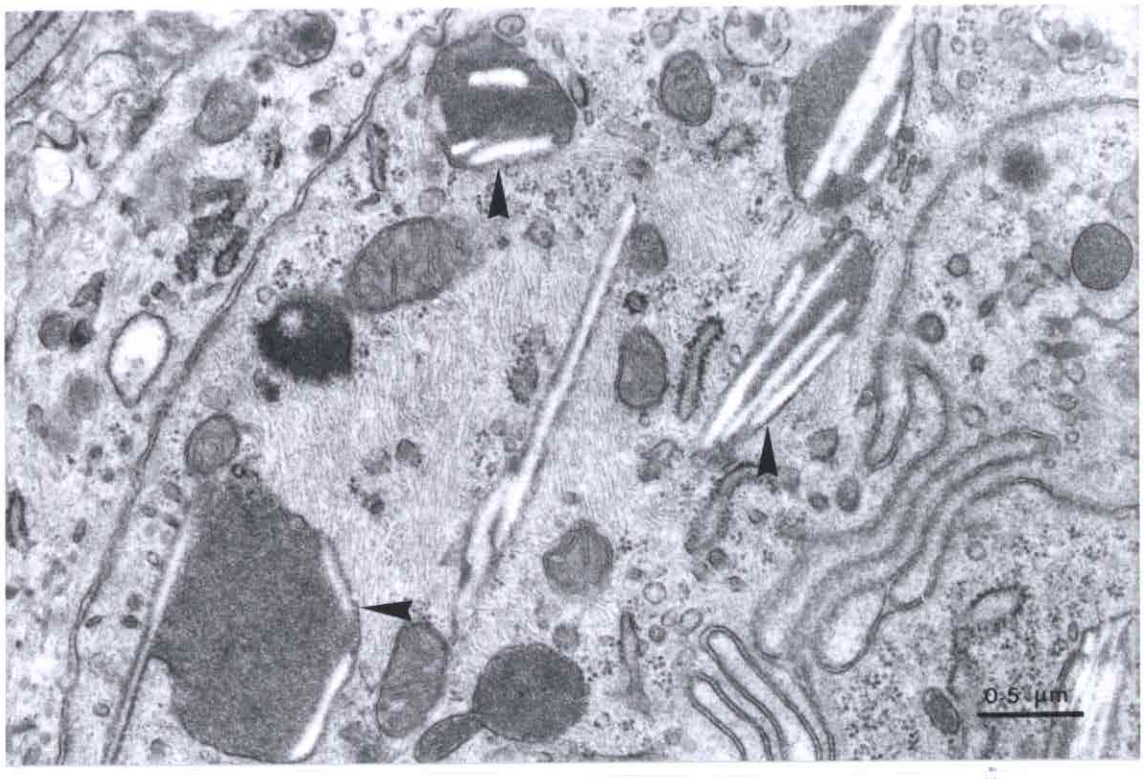

Figure 12. Detail of macrophage cytoplasm showing phagocytized PLLA fragments in lysosomes (arrows), suggesting that phagosome/lysosome fusion has taken place. $PLLA_{336}$ disk 26 weeks after implantation. x28.000.

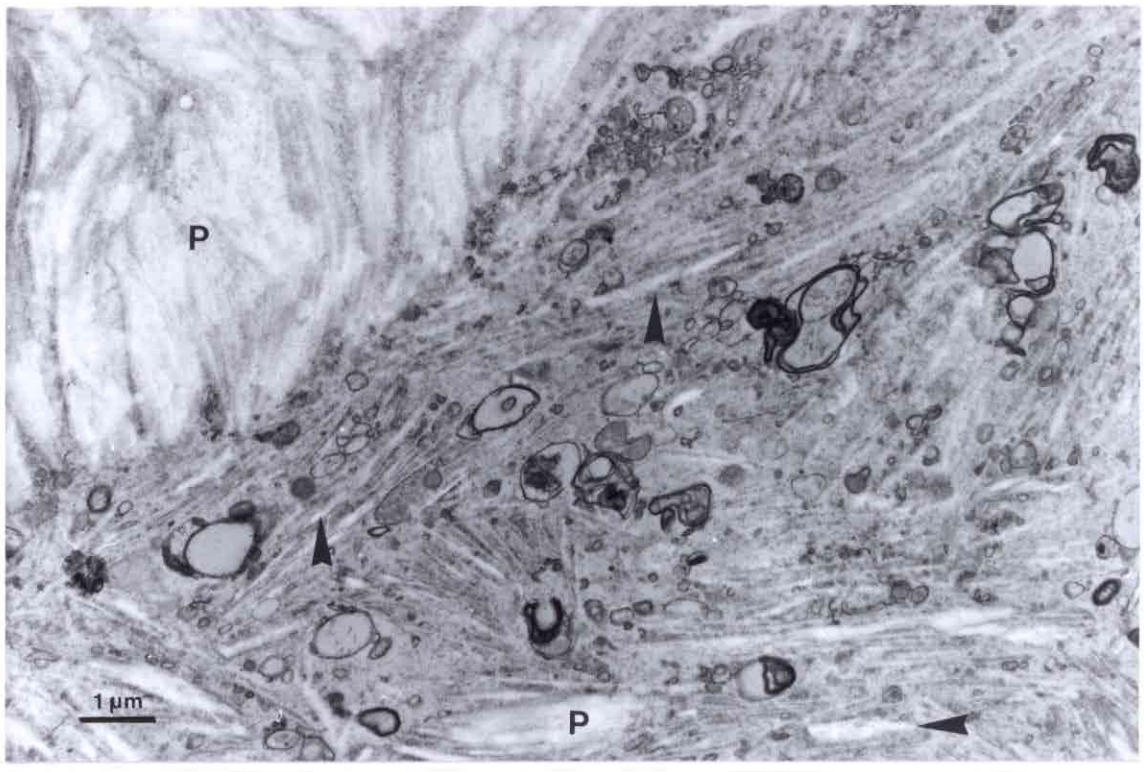

Figure 13. Centre of a large debris zone showing degraded polymer fragments (P) ranging from large blunt to small thin lamellar structures (arrows), intermingled with numerous small cellular degradation debris. $PLLA_{336}$ disk 26 weeks after implantation. x10.000.

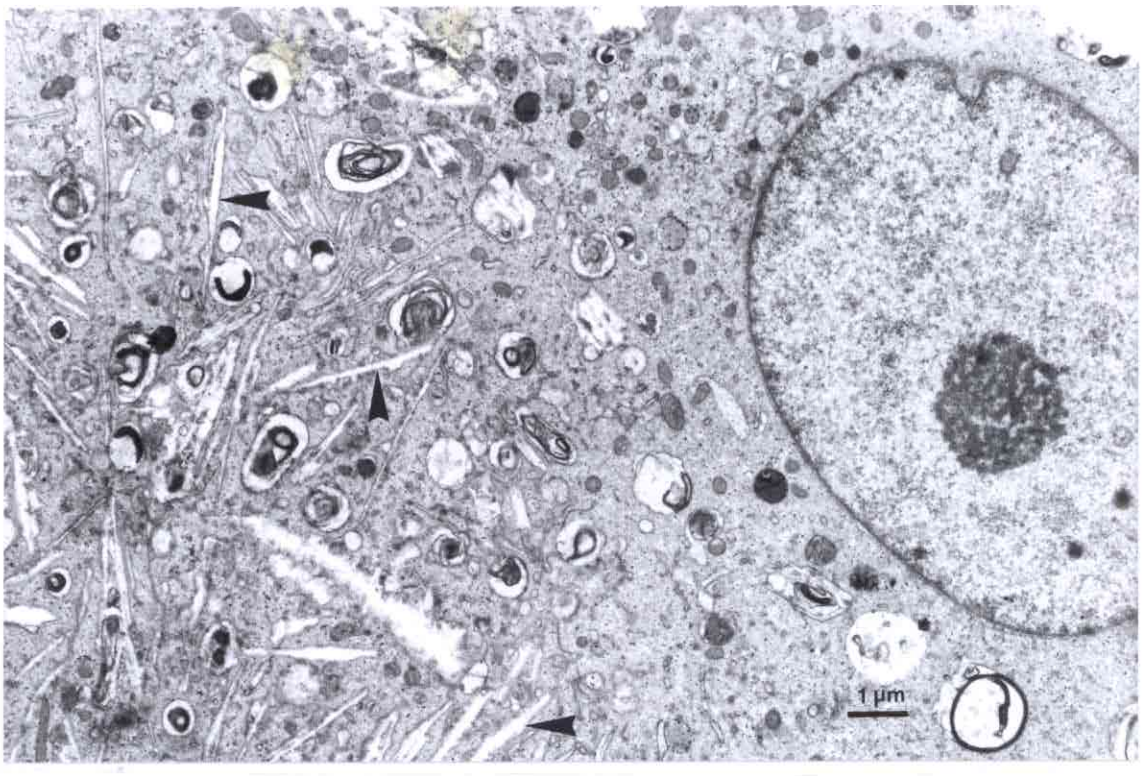

Figure 14. Transition of debris zone of $PLLA_{336}$ to the cellular layer consisting mainly of macrophages, showing cell compartments with phagocytized PLLA material (arrows) but also autophagic vacuoles and residual bodies, indicative for cell degeneration. x7.600.

4. DISCUSSION AND CONCLUSIONS

After several reports on biocompatibility, biological performance and successful fracture healing, the tissue response to as-polymerized PLLA seems to be well described. Up to now, however, in none of the previous studies late or complete degradation of the as-polymerized PLLA used in bone fixation has been described. Bos[39,40] estimates the total resorption time to be at least 3.5 years, based on mass and molecular weight loss of the high molecular weight as-polymerized PLLA during in vivo experiments.

Still these polylactides are considered to be fully biodegradable and clinical use was started based on the results of these experiments[39,40,53,54]. Three years after the treatment of ten patients with PLLA plates and screws, however, complications occurred in the form of swellings at the site of implantation [43,44].

In the present study in vitro predegradation was used to simulate long-term physiological degradation of implants. It is rather difficult to define the exact in vitro predegradation period needed to study the final degradation phase of a polymer in an in vivo experiment. In the present study we have used predegraded $PLLA_{336}$ with physical characteristics that were comparable with PLLA material obtained 3 years postoperatively from patients that were treated with PLLA bone plates and screws for zygomatic fractures[43,44]. PLA96 disks were predegraded for 168 hours which provided, based on the mass loss, disks that were comparable with $PLLA_{336}$ implants. A mass loss of approximately 50% was obtained. Our results show that this is sufficient to induce a local tissue reaction similar to that observed in the clinical situation. For a good comparison between PLLA and PLA96, part of the PLLA disks were also predegraded for 168 hours.

The chemical analysis of the predegraded disks showed a very low molecular weight with a normal polydispersity when compared with physiologically degraded material [11,12]. There was an increase in the heat of fusion for all disks indicating that the crystallinity had also increased. The melting temperature had decreased with degradation indicating that the crystalline domains become less perfect or smaller [55]. The heat of fusion and the melting temperature of the PLA96 however, remains much lower compared with the PLLA. The lower crystallinity and greater imperfection caused by the incorporation of 4% D-lactide has a substantial influence on the chemical characteristics of the polymer.

Histopathologic examination showed that the local tissue response to non-degraded polylactide and the PE disks was very mild during the entire implantation period. The histologic reaction towards the non-degraded PLLA and PLA96 disks consisted of a minor local reaction, which remained approximately constant from 12 weeks onwards. This mild tissue reaction is in good accordance with literature, as in many studies only a minimal inflammatory response to PE but also to the PLLA and the copolymer PLA96 is reported[53,54,56]. The slightly elevated histologic response to the PLA96, is probably due to sterilization procedure. Steam sterilization of moisture labile biodegradable polymers will influence the mechanical and physical properties, and can be considered as a kind of predegradation[57]. During sterilization the chemical properties might be significantly altered,

which can explain the early cracking and particle formation and the accompanying increased cellular influx in the implant.

In contrast with the non-degraded PLLA and PLA96 disks, the $PLLA_{168}$ showed a moderate, and the $PLLA_{336}$ and the $PLA96_{168}$ a marked reaction, including degradation, disintegration, and cellular infiltration with longer implantation periods. Although there is a clear tendency for these reactions to increase in time, our results show that the characteristic features of each implant are already present during the first 8 to 12 weeks after implantation. Thus the differences between non-degraded and predegraded material could already be established in that period.

Some predegraded implants clearly showed a swelling, similar to what was observed in the patients. This increase in volume of the implant plus surrounding tissue could be ascribed only partly to the granulomatous reaction surrounding the implant. In addition, large zones of degraded and disintegrated polymer debris and acellular debris were observed around the implants.

Between the seemingly intact implant and the debris zone a rather sharp boundary was present. A similar sharp boundary between PLLA bone plates and surrounding polymer debris was observed in a cross-section of material explanted from patients implanted with PLLA plates and screws in the zygomatic region[45]. These polymer fragments and debris were surrounded by a granulomatous reaction, including cells with internalized birefringent material, similar to our observations for $PLLA_{336}$ and $PLA96_{168}$ disks. Although in the present study predegradation was used to simulate long-term physiological degradation, the results i.e. the histopathological reaction, suggests that the degradation and swelling occurs according to a similar mechanism as seen physiologically in patients.

Light microscopically, the debris zones contained only a minor amount of cells or were virtually acellular. As TEM examination revealed the presence of subcellular structures in the debris zones, these zones consist of polymer fragments and debris from degenerated cells. It is possible that the environment in the debris is cytotoxic for cells. Lactic acid can cause a decrease of pH resulting in necrosis of cells and the release of the internalized particles. Lactic acidosis has been described to be a major mechanism promoting cell damage or death[58,59]. In vivo pH maesurement might provide some additional insight in what really occurs locally. On the other hand the debris may also cause a continuous attraction of macrophages that may again phagocytize the PLLA particles and thus repeating the intracellular cycle.

The origin of the observed swelling is not quite clear. Maybe the swelling is initiated by a gradual disintegration of the PLLA bone plate and screws into fragments. Bergsma et al.[60] described how during degradation the PLLA plates and screws disintegrate in small fragments. These small fragments or particles are possibly the last stable and visible manifestation of the PLLA material[61]. The presence of these particles, which can be internalized by various cells, may evoke a foreign body reaction[15,62,63]. In a study on the connective tissue reaction to polymer implants, Sevastjanova et al.[19] reported that the biomechanical conditions created by the implanted material in the adjacent tissues are directly dependent on a number of factors, the most important being the particle size and geometrical shape. The changes in biomechanical properties and morphology due to the degradation of the PLLA material are factors that may intensify the foreign body reaction.

Besides biomechanical alterations, disintegration of the PLLA disks into small fragments leads to an increased volume, in comparison with the volume of the original implant. In a cross section of excised tissue specimens of a patient after three years of implantation, the sectioned area occupied by the PLLA particles was estimated 65% of the total surface area[44]. The remaining 35% of the cross section was occupied by the enveloping fibrous capsule. It therefore can be concluded that a substantial part of the swelling is formed by fragmented particles of the polylactic acid material.

Also the quantity of the implanted material, in relation to the degradation characteristics of the material may be a factor influencing the clinical manifestation of the foreign body reaction. Bioresorbable sutures made of copolymer polyglycolide/polylactide can also provoke a foreign body reaction, but due to the relatively small dimensions of these sutures, there is no, or only a minor clinical manifestation of this reaction[64].

In the patients treated with the PLLA bone plate and screws, the foreign body reaction was localized in soft tissue with good vascularization. Because the PLLA degradation rate is relatively slow, it makes little demand on the clearing capacity of the tissue. Nevertheless, all patients showed a clinical manifest foreign body reaction. This may imply that the long lasting presence of a bulk of PLLA-fragments exceeds the local tissue tolerance. Also the polymer debris might be toxic for cells, as in our study, in the debris zone also cellular residual bodies were observed.

The mechanism of the debris formation is not fully understood. A possibility might be that mechanical damage to the degraded disks causes fragmentation and particle formation which can lead to debris formation and swelling of the implant. In a study with intraosseously used polyglycolide pins, Böstman et al.[65] observed osteolytic areas which seemed to be related with the formation of degradation products and debris formation. They also suggest that this debris would be very hydrophillic which causes an increased osmotic pressure and thereby osteolytic changes. In the present study polylactide was used but perhaps a similar mechanism may account for the subcutaneous swellings. The formation of a PLLA debris probably depends on the degradation stage, fragmentation, and the hydrophillicity of these fragments. Non-degraded PLLA is a less hydrophillic polymer when compared with polyglycolide, but during degradation the hydrophillicity of the low molecular weight degradation products increases. This increase can be explained by the fact that these low molecular weight particles have polar and free carboxylic endgroups. At some point PLLA debris will be formed that can cause an osmotic pressure within the enveloping fibrous capsule prompting an increase in volume if the resistance of the surrounding tissue does not exceed the osmotic pressure. Our results show that in contrast to $PLLA_{336}$ the $PLLA_{168}$ discs did not show debris formation and swelling. So, it is likely that a certain level of degradation is needed to result in debris formation followed by osmotic swelling within the fibrous capsule.

Possibly a combination of all these factors, disintegration of the PLA material in small particles and an increased osmotic pressure caused by these particles, overloading the local tissue tolerance and transport potential, and the, compared to bone, low resistance of the subcutaneous tissue can explain the origin of the decribed swelling.

Another mechanism that may induce or maintain the swelling is given by Fornasier et al.[66] who described a correlation between the presence of birefringent polyethylene particles, the density of histiocytes, and the thickness of a fibrohistiocytic capsule all of which showed

an increase with time. A section obtained from the material of one of the patients with an implantation period of 5.7 years[44], hardly any PLLA material could be found in the extracellular space, in contrast with the material that was implanted for 3.3 years. The majority of the PLLA crystals has been internalized by phagocytizing cells in membrane bound vacuoles. These results may lead to the conclusion that with longer implantation periods there is a gradual shift of PLLA particles from extra- to intracellular in phagocytic cells that are embedded in a fibrous matrix. Indigestable foreign body particles may cause a continuous attraction of macrophages that may again phagocytize the PLLA particles and thus repeating the intracellular cycle. In addition the occurring cell death provides another mechanism which attracts cells. The continuous process of attracting cells which obviously can not degrade the PLLA material, contributes to the granulomatous reaction and thus to the observed local swelling.

In summary, the disintegration of PLLA into particles with the accompanying increase in volume of the PLLA material itself plus the granulomatous reaction/fibrous tissue explains the origin of the described swelling.

It is possible that PLLA particles or macrophages with PLLA particles internalized are transported to lymph nodes[47]. In this study, lymph nodes draining the implantation sites have been studied for the presence of the implanted material. In none of these lymph nodes PLLA particles or macrophages with PLLA material could be observed, indicating that migration to lymphoid nodal tissue had not occurred during the observation period of this study.

Also no influence of the implantation site could be observed. It is possible that the implantation sites with a better vascularization develop a more severe tissue reaction because (inflammatory) cells can reach these sites more easily. The difference in tissue reaction between implantation in bone versus subcutaneous tissue seems to be more obvious than the differences in tissue reaction between the various subcutaneous implantation sites.

The biocompatibility, of the non degraded PLLA material has been established in a number of studies. The (pre-) degraded material does not cause any major cell injury but can induce and maintain a clinically detectable swelling. This implies that the PLLA material used for bone fixation can no longer be considered to be fully biocompatible.

The degradation pattern and the formation of particles and debris and the histological reaction towards the $PLA96_{168}$ implants was comparable to the histologic reaction of the $PLLA_{336}$ disks. The $PLA96_{168}$ disks did show debris formation and after 52 weeks of implantation a significant increase in volume was detected. Based on these reults it can be predicted that PLA96 can induce a swelling similar to the PLLA implants. The results of this study suggest that clinically detectable swellings can be expected when PLA96 is used as bone plates and screws for the fixation of zygomatic fractures in patients. It can therefore be concluded that although the degradation of PLA96 is enhanced, as-polymerized PLA96 should not be used as a new osteosynthesis material.

In conclusion the results of this study show that in vitro pre-degradation in combination with in vivo implantation is a good method to simulate long term physiological (clinical) degradation. Although there is a clear tendency for the tissue reactions to increase in time , our results show that the characteristic degradation features of each implant are already

present during the first 8 to 12 weeks after implantation. Thus the differences between non-degraded and predegraded material could be established in a relatively short term experiment. However, the implantation of a polymer with a certain predegradation time does not exclude the possibility of a swelling when used in a clinical setting. The level of degradation obtained in the predegradation process is, in view of our results, likely to be of more importance than the predegradation period. By varying the pre-degradation periods the simulated implantation period can be increased thereby decreasing the risk of missing essential stages in the polymer degradation and accompanying tissue response. The model presented here may be appropiate to study possible effects occurring late after implantation of biodegradable materials.

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6. APPENDIX

Modification of the Technovit 7100 (GMA) embedding procedure.

Since the introduction of glycol methacrylate as an embedding medium in light microscopic research in 1966/1967[67,68] this medium has become increasingly popular. The method has many inherent advantages: well preserved morphology and easy thin sectioning in the range of 1-4 mm providing high quality sections that show fine morphological detail[69].

In this study Technovit 7100[52] was used as a GMA-polymerization system. The polymerization of GMA is initated by means of a barbituric acid derivate in combination with chloride-ions and benzoyl peroxide. The catalyst system does not contain aromatic amines, which is an advantage compared with most systems which are based on peroxide/aromatic amines. The components used in the Technovit 7100 system are significantly less toxic compared to other systems[52].

The standard procedure as described by the supplier/manufacturer[52] was used as our initial procedure. Because of the properties of the implants, e.g. tissue size and hardness of the implants, and for practical reasons, this method had to be modified.

Because of the size of the halved explanted tissue specimens (15x5x5 mm), each step had to be carried out for hours instead of minutes. Furthermore an extra dehydration step in acetone had to be included to obtain complete dehydration. It was impossible to cut the tissue specimens into smaller pieces because this would damage the implant-tissue interface, which was the area we wanted to observe.

Another problem due to the size and hardness of the materials was observed in the polymerization. The embedding solution polymerizes very fast at room temperature, even before complete infiltration of the whole tissue had occurred. Therefore we placed the tissue at -20°C for 2 hours in order to obtain a better infiltration of the tissues.

Because of the hardness of implant material, the GMA could not infiltrate into the implant itself, therefore we had problems cutting 1-2 µm sections. Especially with the undegraded, and therefore harder, implants we were not able to obtain sections with the whole implant in the section. When the implants were predegraded, it was easier to cut the tissue specimens. We have worked with different kinds of disposable and glass knives to improve the quality of the tissue sections. Best results were obtained with disposable knives from Spikker. When we were able to obtain tissue sections that included the implant, there may remain some wrinkles in the surrounding tissue because the hardness of the GMA and the implant material are different.

A short description of the final method used is presented below.

Procedure used for sample preparation

*** Fixation:**
At each time interval 36 tissue specimens (6 animals x 6 implants) were processed. Halved implants plus surrounding tissue (15 x 5 x 5 mm) were fixed by immersion in 4CF1GA in 0.1 M NaCaCodylate buffer at pH 7.4 for at least one week at room temperature.

*** Dehydration:**
Tissues were dehydrated using ascending concentrations of ethanol (Merck); ethanol 70% for 8 hours, ethanol 96% for 4 hours, ethanol 100% for 4 hours followed by dehydration with acetone (Merck) for 8 hours, at room temperature under continuous shaking.

*** Pre-infiltration:**
A pre-infiltration step in a solution consisting of acetone and Technovit 7100 infiltration solution in equal amounts was used as the final step prior to complete infiltration for 8 hours at room temperature under continuous shaking.

*** Infiltration:**
Tissues were placed in the infiltration solution which was made by mixing 100 ml Technovit 7100 infiltration solution with 1 g of hardener I (Dibenzoyl peroxide, H_2O content 20%). Tissues were left in the infiltration solution up to 3.5 days at room temperature under continuous shaking.

*** Embedding:**
After infiltration tissues were placed in peel-away-cups (22 x 30 mm, Klinipath, Duiven, the Netherlands). Embedding solution was made by mixing 100 ml infiltration solution with 6 ml of Hardener II (Accelerator which contains a barbituric acid derivate). The embedding solution was kept at -20 °C to avoid early polymerization.
Approximately 2.5 ml of embedding solution was poured into each cup. Subsequently tissue pieces were placed in the cups, and another 5 ml embedding solution was poured into the cups.
The cups were placed at -20 °C for 2 hours in order to obtain good infiltration of the whole tissue specimen with GMA. Following the cups were placed in a flow chamber, tissues repositioned and left for complete polymerization for 24 hours at room temperature.

*** Mounting:**
To attach the specimen adaptors (LKB, Bromma, Sweden) to the GMA block, Technovit 3040 (Heraeus Kulzer GmbH, Wehrheim, Germany) was used. Technovit 3040 was prepared in the mixing ratio of 2-3 parts powder to one part liquid and poured into the recess of the peel-away-cup. LKB-specimen adaptors were placed into the Technovit 3040, due to polymerization for at least 2 hours in a flow chamber at room temperature the adaptors get firmly attached to the embedded block.

*** Further processing:**
The peel-away-cups were removed and the GMA blocks were cut into the right shape. Tissue sections of 1-2 µm were obtained on a LKB-2218 historange using disposable knives. Sections were stretched at room temperature in a water bath containing distilled water and mounted on slides, which were dried at 60 °C. Subsequently, sections were stained during 80 sec with 0.1% $^{w}/_{v}$ Toluidine blue (Fluka Chemika, Buchs, Switzerland) and dried on air.