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Late degradation simulation of poly(l-Lactide)

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High molecular weight as-polymerized poly(l-lactide) (PLLA) has been successfully used for fracture fixation and orbital floor reconstruction in animals and humans. As this PLLA takes more than 3 years to resorb, a method was developed to obtain insight into the final cellular degradation process of the PLLA by means of short-lasting animal experiments. Pre-degraded PLLA particles (< 500 μm) were implanted subcutaneously in the backs of 14 rats. Two different methods of sterilization (regular steamsterilization and gamma-irradiation) and implantation vehicles (gelatin capsules and hydroxypropyl-methylcellulose (HPMC)) were used to examine the biological behaviour of the pre-degraded PLLA. Two rats were sacrificed at 48 h, 3 days and 1, 2, 4, 8, 16 weeks following the operation. The tissues were examined using light microscopy and transmission electron microscopy. Gel permeation chromatography (GPC) and differential scanning calorimetry (DSC) were used to characterize the PLLA material. GPC measurements of the pre-degraded PLLA revealed a $\bar{M}_n$ of 5500. Upon hydrolysation the crystallinity of the PLLA increased by about 60% and the heat of fusion was 86 J g^{-1}. Deterioration of the mechanical and physical properties due to the two sterilization methods was negligible. No differences in cellular response were observed between the densely packed PLLA particles (gelatin capsules) and the particles scattered over the tissue (HPMC-gel). The present study enabled an early observation of the late degradation phase of PLLA.

1. Introduction

Poly(l-lactide) is a member of a group of biodegradable aliphatic poly(lactone)esters which have found important use as biomaterials, e.g. in prosthetics, sutures, and drug delivery systems.

As-polymerized, high molecular weight poly(l-lactide) (PLLA) synthesized according to Leenslag *et al.* [1] has been successfully used for fracture fixation and orbital floor reconstruction in animals and humans [2–5]. As-polymerized poly(l-lactide) is considered to be a fully resorbable, biocompatible material [6].

In the literature on PLLA and related aliphatic polyesters, different mechanisms of biodegradation are described. Most of the studies indicate that degradation of the PLLA has to be considered a hydrolytic process, initially occurring in the amorphous regions. This process in which chain scission [7–13] and mass loss [6, 14] occurs, seems to be independent of enzymatic or cellular activity of the body [7, 15–25]. Williams *et al.* [25, 26] however, suggest also an additional enzyme-accelerated component as part of the extracellular degradation process. The final part of the absorption process is believed to happen by enzymatic surface erosion or internalization (i.e. incorporation in cells) [26–28].

In a study on the late tissue response of PLLA bone plates in humans [29–31], all ten treated patients showed a mild intermittent swelling of the implant area after a period of approximately 3.5 years. The presence of particles smaller than 10 μm, incorporated in fibroblasts and macrophages which showed a "foamy" appearance in light microscopy, and numerous intracellular poly(L-lactide) crystallites in electron microscopy was correlated with the presence of this swelling [29–31].

Woodward *et al.* [28] observed in rats poly(ε-caprolactone) particles of 80–10 μm or smaller within the cytoplasm of macrophages and fibroblasts. Both cell types were secreting lysosomal enzymes and forming phagosomes. Bos *et al.* [6], in a study on rats, showed macrophages with a foamy appearance after 143 weeks of implantation of as-polymerized PLLA discs. However, the clinical manifestations as observed in the human study could not be detected in the rat study.

High molecular weight as-polymerized PLLA takes a long time to resorb. Extended observation time will be necessary to obtain insight into the whole course of the degradation process and the origin of the described swelling in patients. The aim of the present investigation was to develop short-lasting animal ex-

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periments in which insight into the final cellular degradation process of the as-polymerized PLLA could be obtained.

2. Materials and Methods

2.1. Preparation of pre-degraded poly(L-lactide)

The high molecular weight as-polymerized PLLA ($\bar{M}_n = 7.2 \times 10^5$) as used in this study, was synthesized according to the method described by Leenslag *et al.* [1]. An Ubbelohde viscometer (type Oa, ASTM D-445) was used for the determination of the intrinsic viscosity of the as-polymerized polymer at 25 °C. The viscosity-average molecular weight ($\bar{M}_v$) of the polymer was estimated using the relation $[\eta] = 5.45 \times 10^{-4} \bar{M}_v^{0.73}$ [32].

To create a pre-degraded PLLA the original synthesized PLLA was machined into thin chips under cooling, of which 20 g were refluxed for 30 h in a solution of 10 ml lactic acid 95% per 700 ml of distilled H_2O. During pre-degradation hydrolysis the PLLA disintegrated into particles with dimensions smaller than 500 μm (light microscopical measurements). No sieving was performed. The powder obtained was washed several times with distilled H_2O until a pH of 6–7 was reached. Characterization of the pre-degraded PLLA before and after the sterilization was carried out by measurements of gel permeation chromatography (GPC) at 35 °C using a Waters ALC/GPC 150C equipped with a PL gel 10 μm mixed column and calibrated with polystyrene reference materials. Chloroform was used as the eluent. Also, thermal analysis (differential scanning calorimetry; DSC) was performed by means of a Perkin-Elmer DSC-7, calibrated with ICTA (International Confederation for Thermal Analysis) certified reference materials, and operated at a scan-speed of 10 °C min^{-1}.

To investigate the tissue reaction to different methods of sterilization, half of the total amount of the powder was sterilized by means of regular hospital steam sterilization procedures (Rubber program, sterilization temperature of 120 °C for 20 min and a total cycle time of 50 min, while the other half was sterilized by γ-irradiation (1.8 MRad).

To examine the biological performance of PLLA particles the two types of differently sterilized powders were divided into (1) 10 mg portions placed into empty #0 gelatine capsules as dry powder (Capsugel, Belgium) simulating densely packed implantation areas; and (2) another portion, simulating scattered PLLA, which was mixed up to saturation with hydroxypropyl-methylcellulose (HPMC) in 2.0% Ringerlactate solution (used as injection vehicle) drawn up into 3.0 ml syringes.

The gelatine capsules were sterilized by exposure to 1.8 MRad γ-irradiation from a ^{60}Co source.

2.2. Animal study

Fourteen male Wistar Albino rats (TNO, Zeist, The Netherlands) ≈ 3 months old, weighing ≈ 275 g were anaesthetized with a nitrous oxide–oxygen–fluothane mixture. The dorsal hair was clipped and the skin was prepped with iodine solution. Aseptic surgical technique was used for 5 mm skin incisions at six locations on the back of each rat.

In each rat, separately mixed HPMC-gel and gelatine capsules filled with steam sterilized and γ-irradiated powder, were placed in bluntly created subcutaneous pockets. As a control, one additional empty gelatine capsule and one portion of pure HPMC-gel were similarly placed. After implantation the skin was closed with Dexon® resorbable sutures.

Regular clinical follow-up was planned up to 16 weeks. Two rats were sacrificed at 48 h, 3 days and 1, 2, 4, 8, 16 weeks following the operation.

After localization of the implantation sites the tissue was generously excised and sectioned for light microscopic analysis. After fixation in 2% glutaraldehyde/phosphate-buffered (0.1 M, pH 7.4), the tissue was dehydrated in graded series of ethanol and acetone and embedded in paraffin. Staining was carried out by hematoxylin and eosin.

Lead by light microscopic findings, ultrastructural analysis was performed. From selected areas of interest glutaraldehyde-fixed subcutaneous tissue material, oriented 50–100 μm Vibratome 1000 (Lancer/Sherwood, TPI Inc. USA) sections were made in such a way that cross-sections were obtained from the densely packed PLLA material, surrounded by a fibrous capsule (Fig. 1).

Three treatments were applied to such Vibratome sections:

(a) postfixation in 1% w/w osmium tetroxide in 0.1 M cacodylate/HCl buffer of pH 7.4, to which was added $K_4Fe(CN)_6.3H_2O$ to a final concentration of 0.05 M, for 1 h at room temperature; or

(b) a cytochemical reaction procedure to detect the enzyme acid phosphatase (AcPase) according to van Dort *et al.* [33]; or

(c) (b) followed by (a).

After acetone dehydration and infiltration in LX 112 epoxy resin the Vibratome sections were flat-embedded in epon (for details see van Dort *et al.* [33]) and cured at 60 °C for 48 h. Ultrathin sections were obtained at various sites from the 50–100 μm thick tissue layer, maintaining visual control over the places which were selected. The granulate material had not mechanically been removed prior to embedding.

The untreated or lead-citrate stained ultrathin sections were observed with a Zeiss EM 902 transmission electron microscope (TEM). The system geometry and the observation conditions are described elsewhere (Sorber *et al.* [34, 35]). The presence of the in-column electron energy-loss spectrometer (EELS) in such a TEM instrument allows, in addition to the acquisition of contrast-related images, elemental distribution images to be obtained. This type of analytical image analysis is used to identify the acid phosphatase (AcPase) activity related cerium precipitates in lysosomes. For details of this technique the reader is referred to Sorber *et al.* [35] and the literature therein.

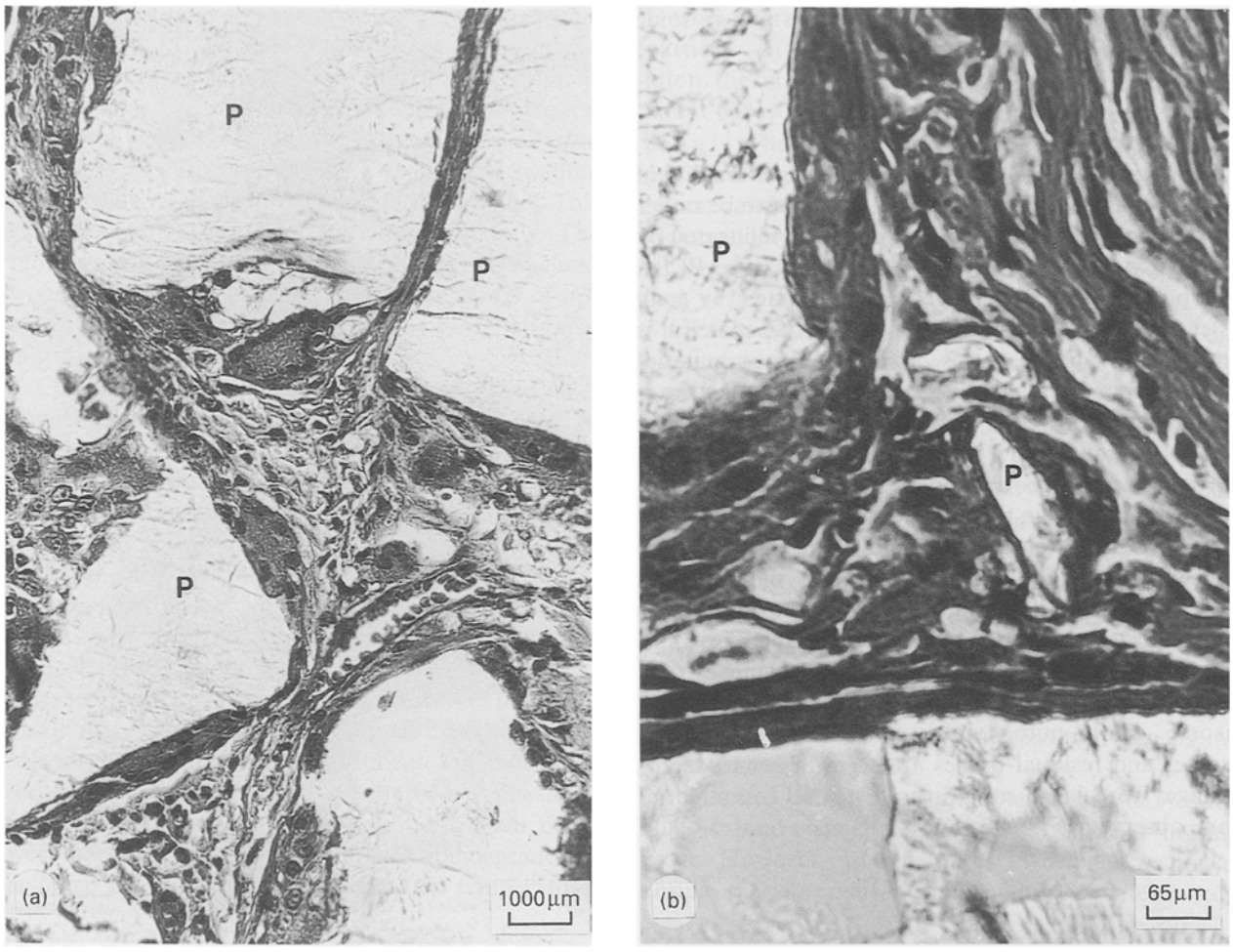

Figure 1 (a) Low power micrograph of PLLA particles (P) under crossed Nicol prisms, showing the invasive cells and connective tissue components. H&E stained paraffine section. (b) High power view of giant cells and macrophages in which birefringent material (P) can be seen to be internalized. H&E stained paraffine section.

3. Results

3.1. Material characterization

DSC measurements of the untreated PLLA chips revealed a heat of fusion (ΔH_m) of 56 J g^{-1}. The predegraded PLLA powder showed a ΔH_m of 86 J g^{-1} and the $\bar{M}_n$ was 5500 with a polydispersity ratio ($\bar{M}_w/\bar{M}_n$) of 6 as determined by GPC. After sterilization only negligible difference of crystallinity and molecular weight could be determined.

3.2. Light microscopic analysis

Microscopic findings showed no difference in host response between the gamma-sterilized and steam-sterilized PLLA powders. Also, no difference in tissue response was found between the HPMC-gel and the gelatin capsules insertion vehicle. There was a slight difference in distribution of the PLLA particles in the implantation area between the gelatin capsule and the HPMC-gel implants. The gelatin capsule group showed a dense packing of the PLLA particles while the HPMC-gel group showed a loose scattering of the particles.

After 48 h no remaining gelatine capsule and HPMC gel could be detected. Normal wound healing had occurred with only a few mononuclear cells present.

After 3 days, fibrous tissue with young loose collagen between the particles had been formed. The ventral side showed less formation of fibrous tissue than the skin side.

After 1 week only a mild inflammatory reaction with a lining of macrophages around the various PLLA particles was found. At the periphery of the implantation area a first attempt at encapsulation of the bulk PLLA particles by fibrous tissue was observed.

After 2 weeks a tissue encapsulation of two layers of fibrous tissue in apposition to the lining of macrophages was shown. The capsule at the periphery was thicker than the tissue surrounding each particle at the central part of the bulk of the PLLA powder. Between the particles blood capillaries were observed. A mild tissue response with giant cells covering the smaller individual particles (< 10–20 μm) could be detected.

The 16-week samples showed also the more prominent particles surrounded by macrophages. In the mass of the large particles, invasive cells were seen to occupy the spaces in between. Fibrocytic cells, macrophages and giant cells were regularly observed between extracellular connective-tissue components. When observed under crossed Nicol prisms it was noticed that at some places birefringent material was

present, apparently inside macrophages and in some giant cells (Fig. 1a and b).

3.3. Electron microscopic analysis

The large PLLA particles present in the sections could not be examined. The material turned out to be rather beam sensitive and apparently is poorly infiltrated by the epoxy resin. In the area close to the large PLLA particles, sheets of collagen are separated by long slender fibrocytic cells, in which no PLLA material is detected. In between this connective tissue material some true macrophages with internalized PLLA fragments are observed (Fig. 2). At the sites further away from the granulated PLLA material a more mixed cell population is encountered between sheets of extracellular connective-tissue material. Giant cells, monocytes, some neutrophils and macrophages are observed. Inside the giant cells and macrophages PLLA material is present as irregular-shaped particles in the order of 1–2 μm or less in diameter (Fig. 3). In addition to the normal cytoplasmic organelles, like rough endoplasmic reticulum (RER), Golgi zones and (some giant) mitochondria, numerous phagolysosomes and residual bodies are seen. In some macrophages very large conglomerates of PLLA material are observed (Fig. 4a) consisting of several 1–2 μm

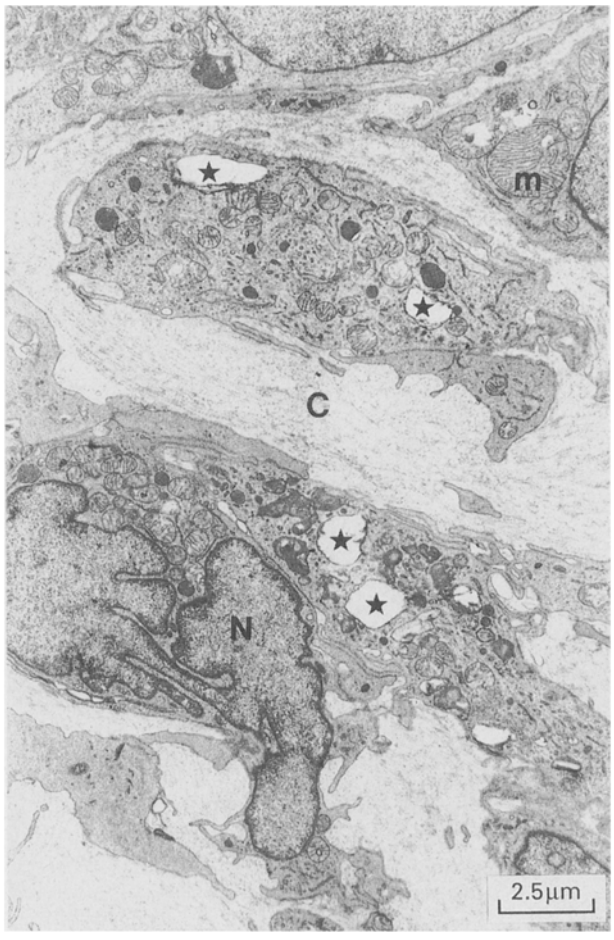

Figure 3 Small PLLA particles (stars) inside a true macrophage (N = nucleus). Numerous phagolysosomes and residual bodies are present, and in some cells giant mitochondria (m) were observed. Cells are embedded in a mature fibrous caspsule (C). Uranyl acetate plus lead citrate stained section.

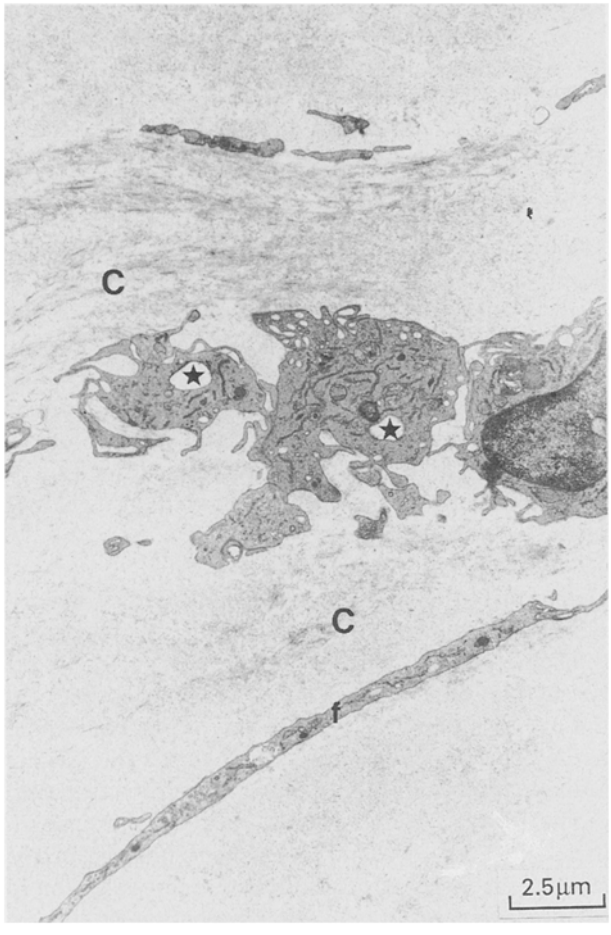

Figure 2 Slender fibrocytic cell (f) and some true macrophages amidst massive collagen sheets (C). The stars indicate the non-electron dense PLLA particles. Uranyl acetate plus lead citrate stained section.

fragments. In some mitochondria the mitochondrial matrix is swollen and has partially lost its electron scattering capacity. In a number of macrophages numerous residual bodies are present in which small gritty fragments can be observed mixed with lipid-like osmiophilic material (Fig. 4b).

At some places multinucleated giant cells are present, in which sometimes also conglomerates of PLLA material are seen. Although most mitochondria have a normal appearance, also in these cells some mitochondria have the empty-looking matrix seen in macrophage mitochondria (Fig. 5).

The presence of AcPase related cerium again is established in fibrocytic cells. In these lysosomes there is no sign of any internalization of PLLA fragments seen in the macrophages and giant cells at other places between the PLLA granulate material.

4. Discussion

Until now, in none of the previous *in vivo* experiments [2–5] with high molecular as-polymerized PLLA, has complete resorption occurred. Based on the mass and molecular weight loss over time, in these experiments the total resorption time was estimated at approximately 3.5 years. This is very long for an experiment. In

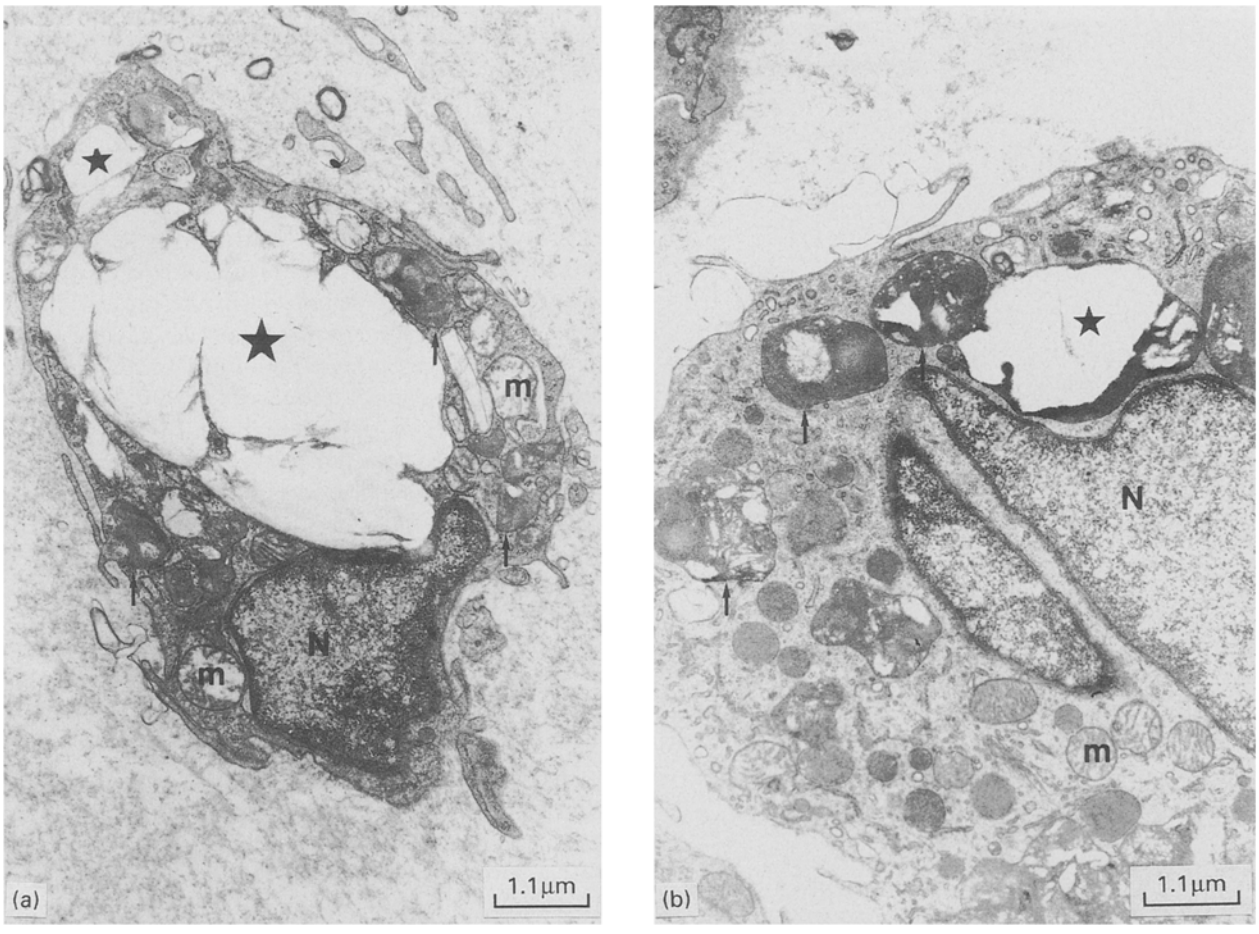

Figure 4 (a), (b) In some macrophages (N = nucleus) several PLLA fragments (stars) are agglomerated inside large phagolysosomes. The arrows point to giant mitochondria. Uranyl acetate plus lead citrate stained section.

a study describing the late tissue response to the as-polymerized PLLA bone plates and screws used for internal fixation of zygomatic fractures in humans [29–31], the final stage of degradation and resorption seemed to be observed. This final stage of degradation caused in all of the 10 patients a slight local swelling which enforced surgical removal of the implants. This enabled us to carryout further investigations. The remnants of the PLLA bone plates and screws used were characterized. A molecular weight ($\bar{M}_n$) of approximately 5000 was measured using NMR-spectrography and GPC.

Cellular changes and internalization of small PLLA particles were noticed which were assumed to be correlated with the final stage of degradation.

Other investigators who have been working with pure PLLA in humans [25] did not mention any clinical complications. Unfortunately, in their study the biological behaviour and degradation process of the implanted materials in humans was not described because no histological data and material characterization were available.

In an attempt to study long-term effects of physiologically degraded PLLA in an animal experiment and to shorten the time of the experiment, low molecular weight PLLA comparable to long-term physiologically degraded PLLA [29] was used. Thin chips were machined from a block of PLLA and degraded in acidified (lactic acid) water at elevated temperature. During degradation the thin chips disintegrated into particles with a size up to 500 μm. Upon hydrolysation the crystallinity of the material increased by about 60%. The heat of fusion of the final powder was 86 J g^{-1}. The increase in crystallinity resulted from the preferred degradation of the PLLA in the amorphous regions and a partial recrystallinization [4]. This increase in crystallinity is also observed in physiologically degraded PLLA [29] which has an ultimate heat of fusion of 96 J g^{-1}.

GPC measurements revealed a $\bar{M}_n$ of 5500 for the pre-degraded PLLA with a $\bar{M}_w/\bar{M}_n$ ratio of 1.2. Since a pilot study showed no difference in crystallinity and molecular weight between particle sizes of < 200 μm and 200–500 μm pre-degraded PLLA, as obtained after hydrolysis, a suitable material to study tissue response in comparison with physiologically degraded PLLA was obtained.

The commonly applied hospital sterilization methods are known to cause deterioration in the physical and mechanical properties of high molecular weight polylactones [36–40]. In the case of the pre-degraded PLLA, changes in crystallinity and molecular weight due to the applied hospital sterilization procedure appeared negligible.

Implanted gelatine capsules filled with PLLA particles caused densely packed particle areas in the tissue. However, no differences in cellular response during the observation time could be determined,

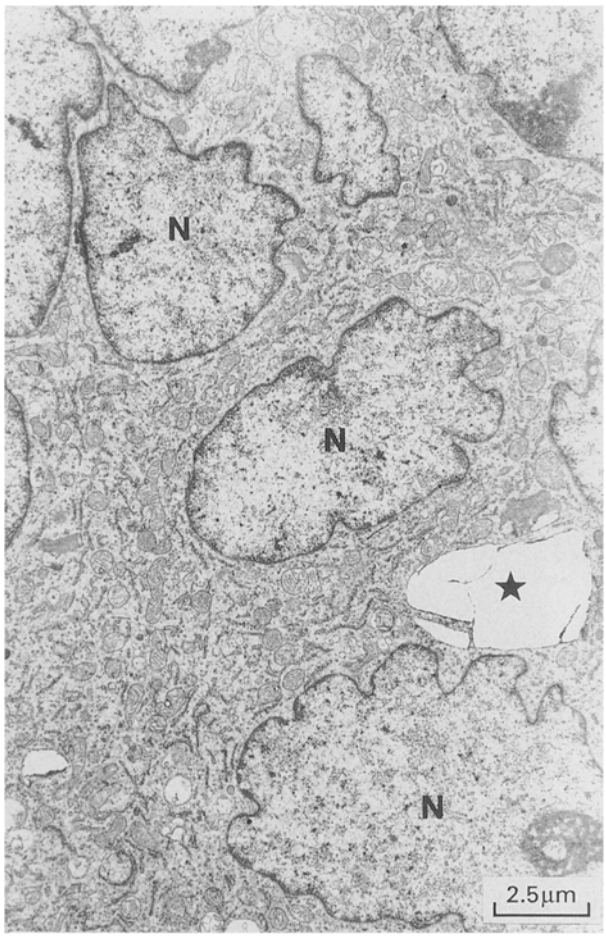

Figure 5 Multinucleated (N = nucleus) giant cell with some small PLLA fragments (stars) inside a phagolysosome. Uranyl acetate plus lead citrate stained section.

when compared to PLLA mixed with HPMC-gel causing scattering of the PLLA particles over the tissue.

The gelatin capsules could no longer be detected with light microscopy after 48 h. This is similar to the findings of Woodward *et al.* [28] who carried out degradation studies of poly(ε-caprolactone). Also, no HPMC was detected after 48 h of implantation. Hydroxypropyl-methylcellulose 2% solution has been used successfully as a substitute for sodium hyaluronate (Healon)® in eye surgery [41–43]. In these studies HPMC showed to be biocompatible, non-toxic and degradable [41–44]. According to the findings in the present study HPMC-gel seemed to be also suitable as an implantation vehicle.

After 2 weeks the implanted PLLA particles were surrounded by a clearly visible fibrous tissue capsule. Experiments on polymer implants in general showed similar encapsulation patterns [6, 20, 45–47]. Light microscopy showed no substantial resorption of the implanted PLLA particles during the observation period of 16 weeks. No disturbances in capsule formation took place.

The smaller PLLA particles (10–20 μm) sometimes were surrounded by giant cells. These findings were apparently a homologue on a light microscopic level to the tissue response to PLLA bone plates and screws in humans after around 3 years [29, 30]. In a study on rats, in which PLLA plates were implanted in the back of rats, no giant cells could be detected after 143 weeks [6]. The 'foamy' macrophages containing intracellular PLLA particles occurring in the rat tissue [6] were not detected in the present study.

The intracellular PLLA particles as seen in the human tissue [29–31] and in the rat study [6] were also seen light microscopically in the present study. The occurrence of internalized particles may be a sign of the final stage of degradation of PLLA. However, electron microscopical findings showed differences in the appearance of the internalized PLLA particles. The internalized lamellar packed crystals present in the phagosomes of fibrocytes in the human study [29] do not resemble the internalized PLLA particles of the present study. Probably, the phase of internalized 'single crystal' polymers is not yet present. In the previous rat study [6] no ultrastructural analysis was available.

The early tissue response to the pre-degraded PLLA powder, comprising fibroblasts, histiocytes and lymphocytes, was similar to several studies on PLLA [5, 6, 25, 47] and also on other α-hydroxy-acids [7, 9, 20, 25, 28].

In the present study the cells with internalized PLLA particles are well recognizable as macrophages. These macrophages contain many active lysosomes, phagolysosomes and residual bodies. Similar findings were seen in a study on the degradation of poly-(ε-caprolactone) (PEC) [28]. At day 6 particles from 80 down to 10 μm were already internalized by macrophages and giant cells. The smaller particles were within the phagosomes of the activated macrophages. Enzymatic degradation with the help of lysosome-derived acid hydrolyses is suggested.

In contrast with the fibrocytes seen in the human tissue [29], the fibrocytes in the present study do not contain PLLA particles. The multinucleated giant cells also contain relatively little PLLA material.

In giant cells containing internalized PLLA particles, several mitochondria with a swollen mitohondrial matrix could be observed suggesting active digestion of lactates. In a 3-week implantation study of hollow fibres of pure PLLA [48, 49], slightly increased levels of lactate dehydrogenase and NADH indicated the same assumption of release of lactates during degradation.

In the present implantation study pre-degraded PLLA was internalized in a few macrophages, indicating a late cellular degradation. The deviating aspect of the internalized particles, however, did suggest that the final degradation phase of the PLLA had not yet been encountered. In spite of these findings, we may yet conclude that by pre-degrading PLLA, the observation period of the final degradation phase could be successfully decreased from around 3.5 years to months.

Studies are in progress now in which pre-degraded particles are delivered with smaller polymer integrity in order to obtain more insight into the final cellular phase of the degradation process.

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