Enzymatic activity toward poly(L-lactic acid) implants

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Tissue reactions toward biodegradable poly(L-lactic acid) implants were monitored by studying the activity pattern of seven enzymes as a function of time: alkaline phosphatase, acid phosphatase, α-naphthyl acetyl esterase, β-glucuronidase, ATP-ase, NADH-reductase, and lactate dehydrogenase. Cell types were identified by their specific enzyme patterns, their morphology and location. Special attention was paid to the enzyme patterns of macrophages, fibroblasts and polymorphonuclear granulocytes (PMNs), being involved in foreign body reactions or inflammatory responses. One day after implantation, an influx of neutrophilic and eosinophilic granulocytes was observed, coinciding with activity of alkaline phosphatase (PMN's) and β-glucuronidase (eosinophils). From day 3 on, macrophages containing ATP-ase, acid phosphatase and esterase could be observed. From day 7 on, lactate dehydrogenase, the enzyme normally involved in the conversion of lactic acid, and its coenzyme NADH-reductase were observed in macrophages and fibroblasts. These two enzymes demonstrated more activity than expected on basis of wound-healing reactions upon implantation of a nonbiodegradable, inert biomaterial (as, e.g., Teflon). It is concluded that the biodegradable poly (L-lactic acid) used in these implantation studies is tissue compatible, and evokes a foreign body reaction with minor macrophage and giant cell activity, as observed during this 3-week implantation period. Most enzyme patterns were simply due to a wound-healing reaction. The slightly increased levels of LDH and NADH suggest the release of lactic acid from the implant, and thus confirms the biodegradable nature of this polymer.

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INTRODUCTION

Degradation of polymeric materials is a feature of considerable importance in the field of biomedical engineering and drug delivery. Degradation is sometimes desirable, for instance in the case of microcapsules for drug delivery,[1] or degradation needs to be avoided as for a polymeric cup of a hip prosthesis.[2] Degradation of polymers can occur by the uptake of energy causing cleavage of covalent bonds.[3,4] This may be caused by an increase in temperature, exposure to x-rays, uv, γ-radiation, or mechanical stress.

Polymers containing hydrolysable bonds can be degraded by hydrolysis. It has been reported in literature that hydrolysis of polymers in the physiological environment may be catalyzed enzymatically.[5,6] The role of locally produced enzymes in the degradation process of polymeric material has already been investigated for many materials, e.g., poly(L-lactic acid), poly(DL-lactic acid),[7–9] poly(glycolic acid),[10] polyglactin 910,[11] polystyrene,[12] polypropylene,[13] poly(methylmethacrylate) (PMMA), poly(ethyleneterephthalate) (PETP), Nylon 66,[8] and many others (e.g., Ref. 14).

Enzyme digestion of hydrolysable bonds of implanted polymers is usually not observed,[7,11] although Williams et al. demonstrated in vitro that enzymes could increase the rate of degradation of several nominally stable polymers as, e.g., PETP, PMMA, Nylon 66 or a poly(etherurethane).[7–10]

In general, two classes of enzymes are of interest to be studied in the immediate surroundings of the implantation site. First the hydrolases, hydrolytic enzymes like phoshatases, esterases, and aminopeptidases. These enzymes are predominantly lysosomal and are mostly contained within macrophages and giant cells. The second class of enzymes is represented by the oxidoreductases, providing a way for further hydrolytic breakdown.

It is interesting to know to what extent the cells in the immediate environment of an implanted biomaterial influence the in vivo degradation, e.g., by the production of specific enzymes.[15] Marchant et al.[16] described in detail the relations between different cell types present at an implanted poly-urethane interface as a function of time. Factors released by lymphocytes or PMNs can influence the number and activity of macrophages and giant cells at the polymeric interface.

In a previous paper, we have demonstrated[17] that the tissue reaction against biodegradable hollow fibers of poly(L-lactic acid) could be described as a very mild foreign body reaction. The initial presence of granulocytes and macrophages may suggest an active attack toward the implants. The absence of lymphocytes indicates a nonspecific inflammatory response. Many questions however, remain on the actual contribution of the different cell types to the degradation of poly(L-lactic acid).

In order to monitor tissue reactions toward subcutaneously implanted biodegradable poly(L-lactic acid) hollow fibers, we tested the tissue in the immediate surroundings of such an implant for the activity pattern of seven enzymes and compared these patterns with a normal wound healing process and with a nondegradable material to investigate the net effect of the poly(L-lactic) acid on its surroundings.

MATERIALS AND METHODS

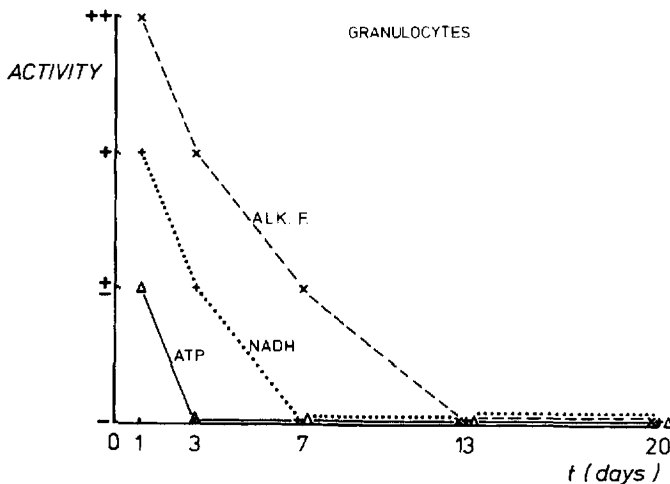
Poly(L-lactic acid) implants

Hollow fibers, intended for use as a drug release device, were spun of poly(L-lactic acid) (Initial M_w: 1.59 × 10^5) using a "dry-wet" coagulation spinning process.[17,18] The tube-in-orifice spinneret device contained an injection tube with an outside diameter of 0.6 mm, an inside diameter of 0.4 mm, and an orifice diameter of 1.0 mm. The spinning dope was composed of 80% dioxane, 15% poly(L-lactic acid), and 5% polyvinylpyrrolidine (PVP) at a temperature of 50°C; water was used as the internal and external coagulant. The dimensions of the implanted fibers were: length 1 cm, diameter 0.7 mm, wall thickness 0.1 mm. The fiber consisted of a porous matrix with a dense non-porous skin (approx. 0.3 μm) on both sides. The fibers were then heat-sealed at both ends.[17]

Gore-tex blood vessel prostheses (1-mm diameter) were used as a nondegradable reference material with minimal tissue reaction.

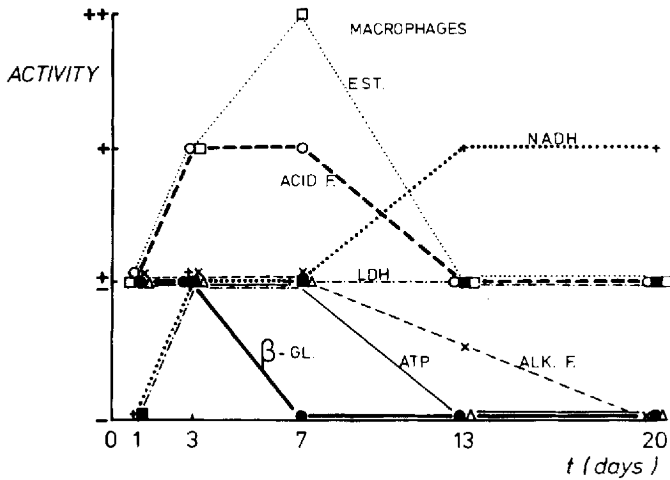
Implantation procedures

The poly(L-lactic acid) fibers were implanted subcutaneously (n = 3) in female AO/BN rats of approximately 3 months of age weighing 200–250 g. A specially adapted syringe was used for this purpose. Rats were ether-anesthetized, shaved on their backs, and subsequently ethanol desinfected



(a)

Figure 1. Enzyme activity profiles as a function of time. (A) Polymorphonuclear granulocytes. (B) Macrophages. (C) Fibroblasts.



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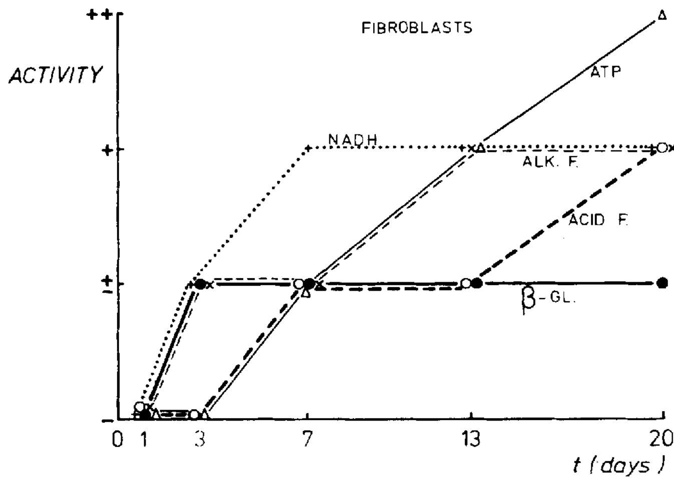


(c)

Figure 1. (continued)

before the fibers were implanted.[17] Two fibers were implanted in each rat. At day 1, day 3, 1 week, 2 weeks, and 3 weeks fibers were harvested including surrounding tissue. Wounds without the presence of a biomaterial were studied to achieve normal enzyme patterns.

Enzyme histochemical procedures and tissue preparation

After harvesting, the fibers with adherent tissue were snap frozen in freon at −80°C. Sections of 6 μm were cut using a Leitz 1720 digital cryostat. The

cryostat was operated at a temperature of −25°C. After cutting, the sections were stored at −20°C until staining. Alternating sections were stained with hematoxilin-eosin for cell identification and the determination of tissue organization.

To demonstrate alkaline phosphatase, acid phosphatase, esterase, and ATP-ase, sections were fixed in a mixture of formaldehyde-Macrodex-calcium chloride (0.9% sodium chloride and 1 g calciumchloride in 10 mL 40% formaldehyde and 90 mL 6% Macrodex) at 4°C for 10 min. For the demonstration of NADH-reductase, lactate dehydrogenase and β-glucuronidase, sections were fixed in acetone at −20°C for 30 min and subsequently incubated with the relevant substrates. The incubation procedures for the individual enzymes are summarized in Table I.

TABLE I
Incubation Procedures for the Various Enzymes. The Enzyme, Its Substrate, Cofactors, and Coupling agent, the Buffer, pH, Incubation Time, and Temperature Are Shown

Enzyme	Substrate, Cofactors, and Coupling Agent	Buffer and pH	Incubation Time/Temp
Alkaline phosphatase	0.2 mM naphtol AS-MX phosphoric acid (sodium salt). 0.6 mg/mL fast blue BB 1.6 mM magnesium sulfate	0.1 M TRIS-HCl pH 8.9	1 h RT
Acid phosphatase	1.0 mM naphtol AS-B1 phosphoric acid (sodium salt). 1.6 mg/mL para-rosaniline and 24 mM sodium nitrite put together at 4°C before mixing with incubation medium	3.6 mM Michaelis barbiturate acetate pH 5.0	1 h 37°C
α-naphthyl acethyl esterase	1.2 mM sodium α-naphthyl acetate. 1.6 mg/mL para-rosaniline and 24 mM sodium nitrite put together at 4°C before mixing with incubation medium	0.2 M Phosphate pH 7.0	30 min RT
β-glycuronidase	0.25 mM naphtol-AS-B1 β-glucuronic acid. 0.6 mg/mL para-rosaniline and 8.7 mM sodium nitrite put together at 4°C before mixing with incubation medium	0.1 M Acetate pH 5.0	1 h 56°C
ATP-ase	1.0 mM ATP 0.01 mM magnesium sulfate 3.6 mM lead nitrate	0.08 M TRIS-maleic acid pH 7.2	45 min 37°C
NADH reductase	2.3 mM NADH 0.6 mM nitro blue tetrazolium	0.05 M Phosphate pH 7.6	15 min 37°C
Lactate dehydrogenase (LDH)	0.2 M sodium lactate 0.01 M potassium cyanide 0.005 M magnesium chloride 0.75 mM NAD	0.2 M Phosphate pH 7.4	45 min 37°C

The activity of the various enzymes tested and their possible role in wound healing and tissue reactions toward the implanted biomaterial are briefly summarized.

Alkaline phosphatase. This hydrolase is predominantly associated with the plasma membrane. PMN and fibroblast activity can be monitored by this enzyme. Blood vessel walls also stain with this enzyme. Angioblastic activity of sprouting endothelium can thus be shown.

Acid phosphatase is the most prominent lysosomal hydrolase, indicative of macrophage activity at the implant site. This enzyme hydrolyses phosphate esters.

α-Naphthyl acetyl esterase. This hydrolase catalyzes the hydrolysis of carboxylic acid esters and is contained within the lysosomes, predominantly in macrophages and giant cells. It is also present in fat tissue.

β-glucuronidase, a lysosomal hydrolase capable of hydrolysing esters of glucuronic acid, found diffusely in macrophages and fibroblasts, and in granular form in eosinophilic granulocytes at the implantation site.

ATP-ase. This hydrolase is associated with cell membranes and mitochondria and hydrolyses ATP to ADP; it functions in active transport across the cell membrane and phosphorylation. Cellular contents of this enzyme are increased with increasing cellular metabolic activity.

NADH-reductase. This oxidoreductase functions anaerobically and is associated with metabolic activity in mitochondria and endoplasmatic reticulum. It is not cell type specific. NADH coenzyme is converted to NAD+ along with the reduction of pyruvate to lactate (by lactate dehydrogenase (LDH)), and as such it can be helpful in monitoring low levels of LDH activity.

High *lactate dehydrogenase* activity is normally observed in ischemic areas. In the absence of oxygen it reduces pyruvate to lactate, using NADH as a coenzyme. This oxidoreductase is associated with mitochondria and endoplasmatic reticulum and is involved in cellular metabolic and respiratory functions. It is a useful indicator for cell metabolic activity but it is not cell type specific.

Quantitation of enzyme activity

The presence of enzymes at the implantation site was quantified morphologically by comparing the number of cells positive for a particular enzyme at different time intervals and comparing them with nonspecific background staining. Cellular morphology, enzyme pattern, and location were combined in the quantification and qualification of specific enzymatic activity for each cell type. The activity is represented by:

++ : very high activity > 50 cells positive
+ : high activity > 25 cells positive < 50
+/−: moderate activity < 25 cells positive
− : no activity : no cells positive

Only positive cells in the immediate vicinity of the implants (about 15 cell layers induced by the trauma or the presence of the material) were quantified (macrophages, fibroblasts, and PMNs). Eosinophils in surrounding connective tissue and other cells of specific interest with regard to the wound-healing or foreign body reaction were also monitored. Cells outside the fibrous capsule were not included in this study, unless they were of specific interest.

RESULTS

Polymorphonuclear granulocytes represent the major cellular component during the first few days after implantation. Alkaline phosphatase, NADH reductase, and ATPase activity can be demonstrated in these granulocytes (Fig. 1A). Highest numbers of positive cells were observed at day 1 (Fig. 2), slowly decreasing to day 7. After day 7 no more PMNs were observed.

Macrophages were observed at the implant site as soon as 1 day after implantation. Overall activity and number were highest from day 3 to 7 (Fig. 1B). Then, the number of macrophages generally decreased showing reduced enzymatic activity, except for NADH and LDH (Figs. 3 and 7). Acid phosphatase activity was present in the macrophages during the whole test period (Fig. 4). Esterase showed a similar profile, with a peak ac-

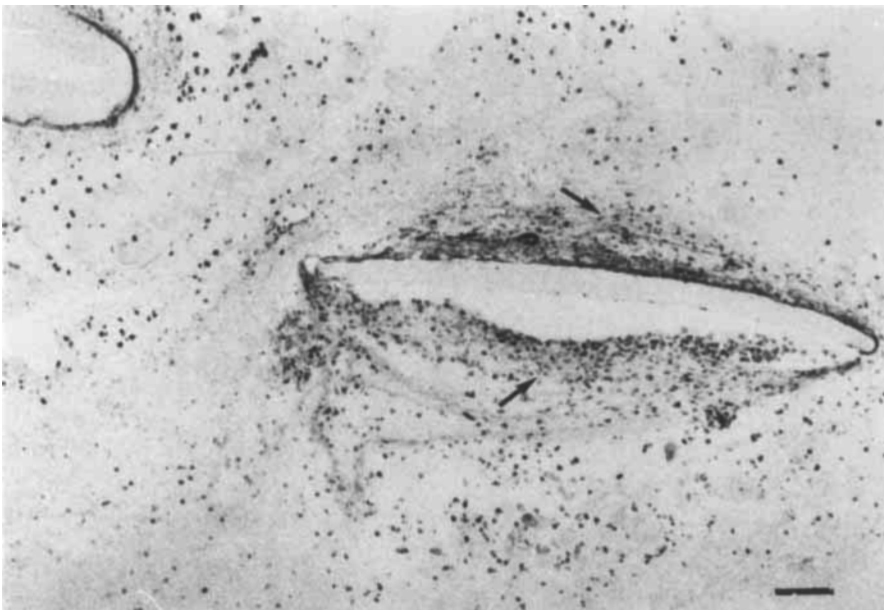


Figure 2. Alkaline Phosphatase at day 1 after implantation; PMNs are visible throughout the section, though concentrated around the implant (arrows). Bar = 1.3 mm.

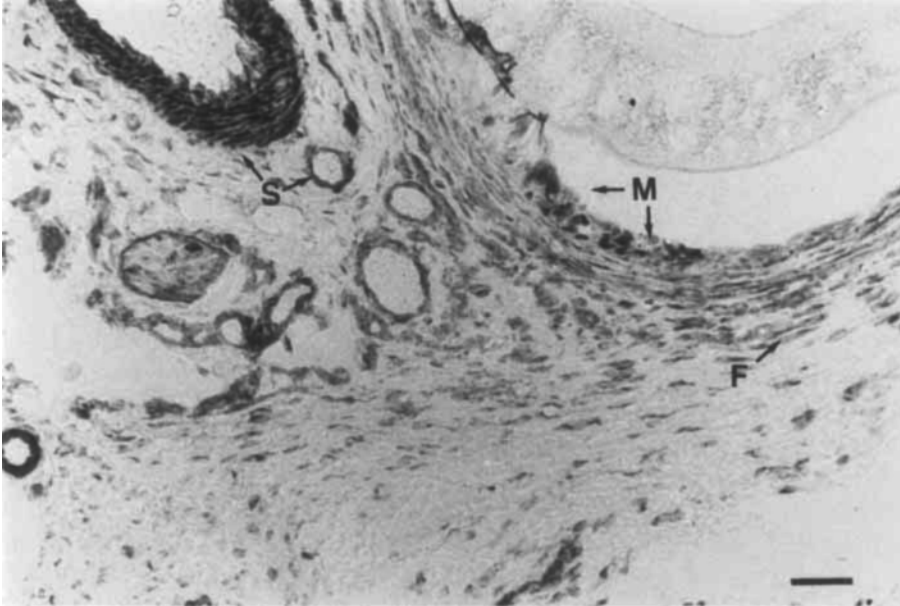
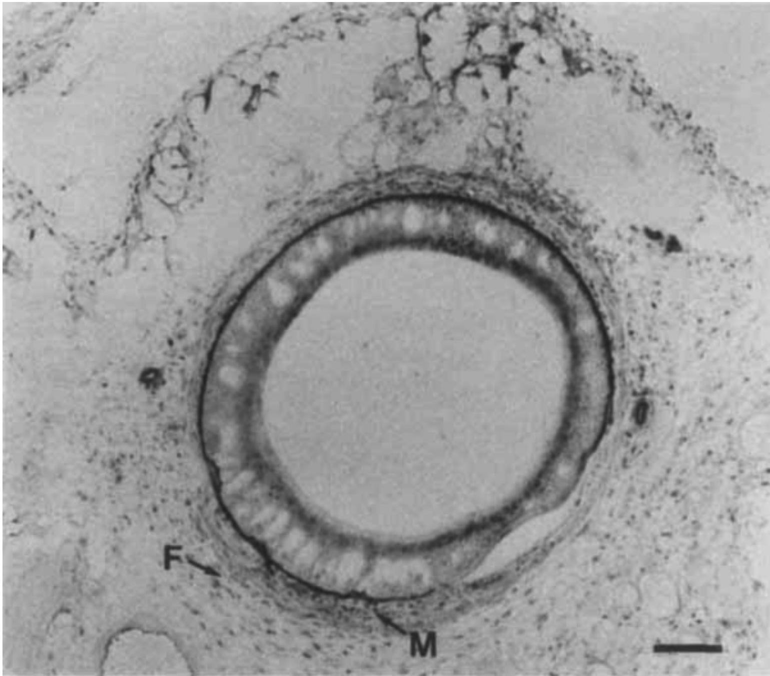


Figure 3. Lactate dehydrogenase at 3 weeks after implantation; M = macrophages, F = fibroblasts, S = smooth muscle cells of blood vessels. Bar = 520 μm.

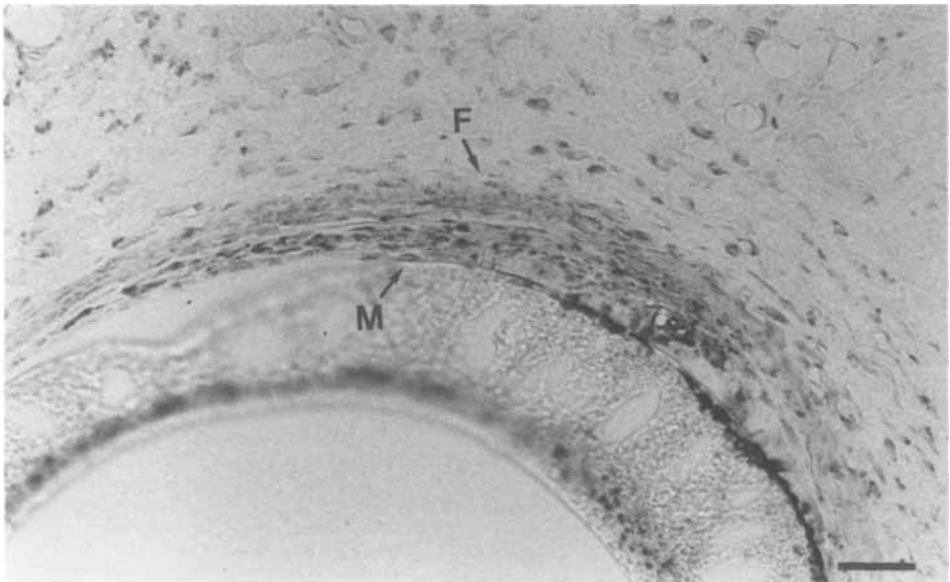
tivity between 3 and 7 days (Fig. 5). LDH and NADH reductase activity was observed from day 3 and was maintained or slightly increased until day 21 (Figs. 3 and 7). Macrophage ATP-ase activity was only observed between days 3 and 7. Alkaline phospatase and β-glucuronidase, two enzymes not specific for macrophages, showed moderate to low activity during the entire test period. Occasionally giant cells were observed, demonstrating similar enzymatic activity.

Fibroblasts were identified at the implant site from approximately day 3 on (Fig. 1C). They were positive for alkaline phosphatase (Fig. 6). In accordance with increased cellular activity during the process of fibrous encapsulation (from day 7 on) fibroblasts showed increased ATP-ase activity. Some acid phosphatase as well as lactate dehydrogenase activity was observed from day 7 to 21 (Fig. 3). The number of fibroblasts around the implant increased until the formation of the capsule was completed.[17] The number of fibroblasts then slowly decreased, whereas the amount of collagen slowly increased.

Blood vessels. Sprouting endothelial cells showed alkaline phosphatase activity in the immediate surroundings of the implant, only at day 3 (Fig. 6). By day 7 this activity had disappeared. Vascular smooth muscle cells contained normal levels of LDH (Fig. 3), NADH (Fig. 7), and ATPase (Fig. 8) throughout the entire test period.



(a)



(b)

Figure 4. Acid phosphatase at 3 weeks after implantation (A, overview); macrophages (M) around the implant are intensely stained, fibroblasts (F) stain diffusely (B, detail). Bar = 1.3 mm (A), resp. 520 μm (B).

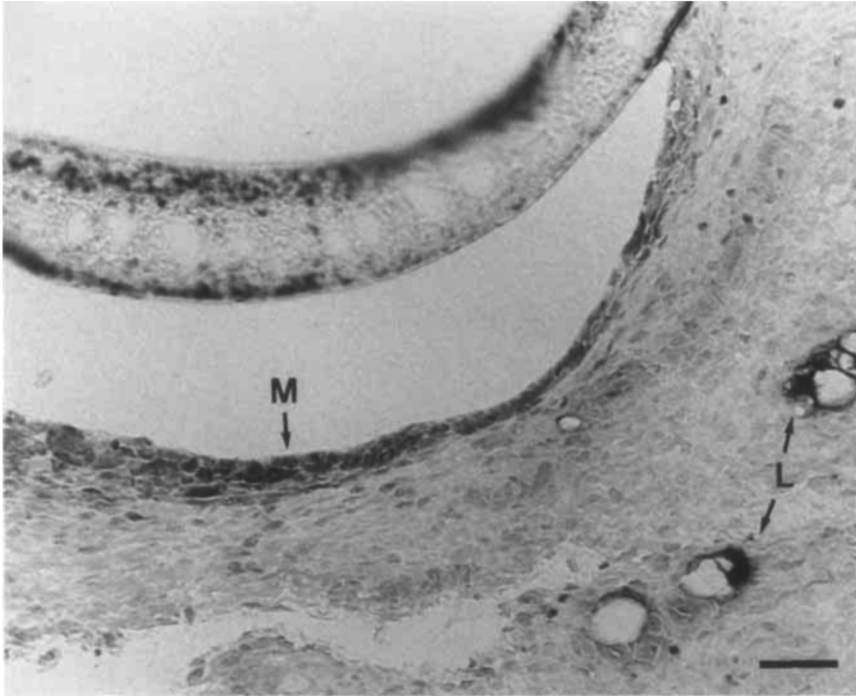


Figure 5. Esterase at 1 week after implantation; macrophages (M) around the implant and lipid compounds (L) have been stained. The implant is disrupted from its original place. Bar = 520 μm.

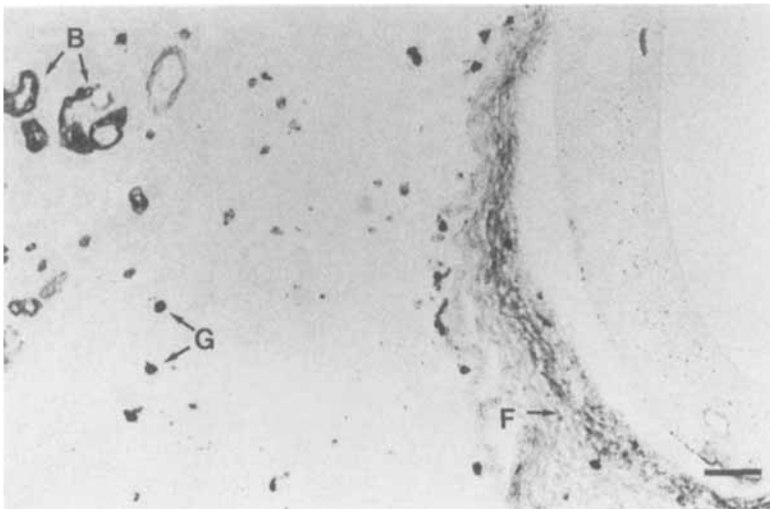


Figure 6. Alkaline phosphatase at 3 days after implantation; fibroblasts (F) around the implant stain diffusely, few remaining granulocytes (G) and sprouting blood vessels (B) can be observed. Bar = 520 μm.

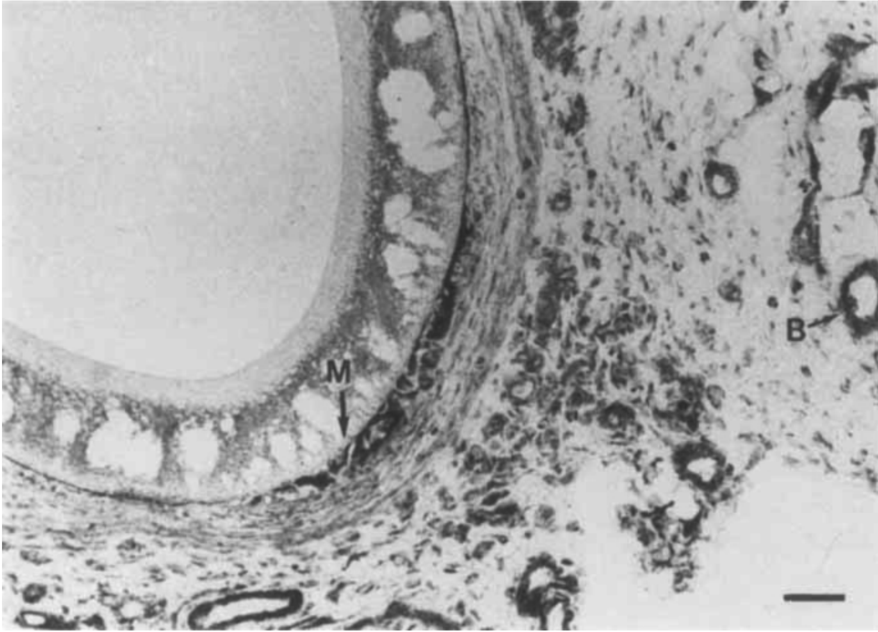


Figure 7. NADH at 3 weeks after implantation; macrophages (M) at the implant surface demonstrate elevated levels of NADH, blood vessels (B) show normal NADH activity. Bar = 520 μm.

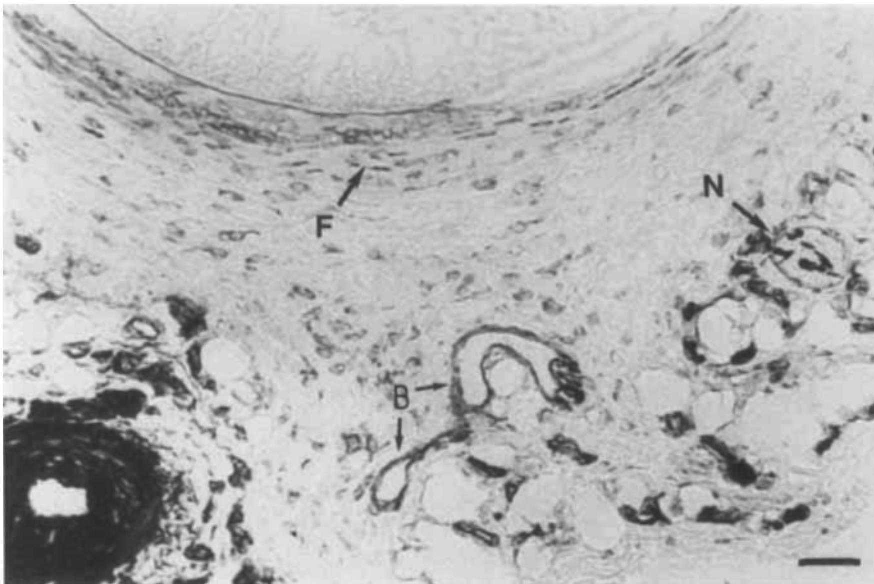


Figure 8. ATPase at 3 weeks after implantation; fibroblasts (F) around the implant demonstrate increased activity; blood vessels (B) and nerves (N) are also shown. Bar = 520 μm.

Eosinophilic granulocytes were only observed on the first and third day after implantation, and could be monitored by their granular appearance of β-glucuronidase. At day 7 and after, no eosinophils were observed. This profile was also observed in normal wound healing.

Lymphocytes were hardly observed in any stage of the study. Apparently lymphocytes do not play a dominant role in the cellular response at the implant site of this synthetic polymer.

DISCUSSION

The application of enzyme histochemistry offers an additional tool to evaluate the polymer–tissue interaction in addition to the traditional morphologic and histologic evaluation.[5]

A combination of cellular enzyme patterns, cell localization, and morphology resulted in enzyme profiles for the respective cell types. Enzyme profiles possibly different from those observed in normal wound healing[20] could thus be studied.

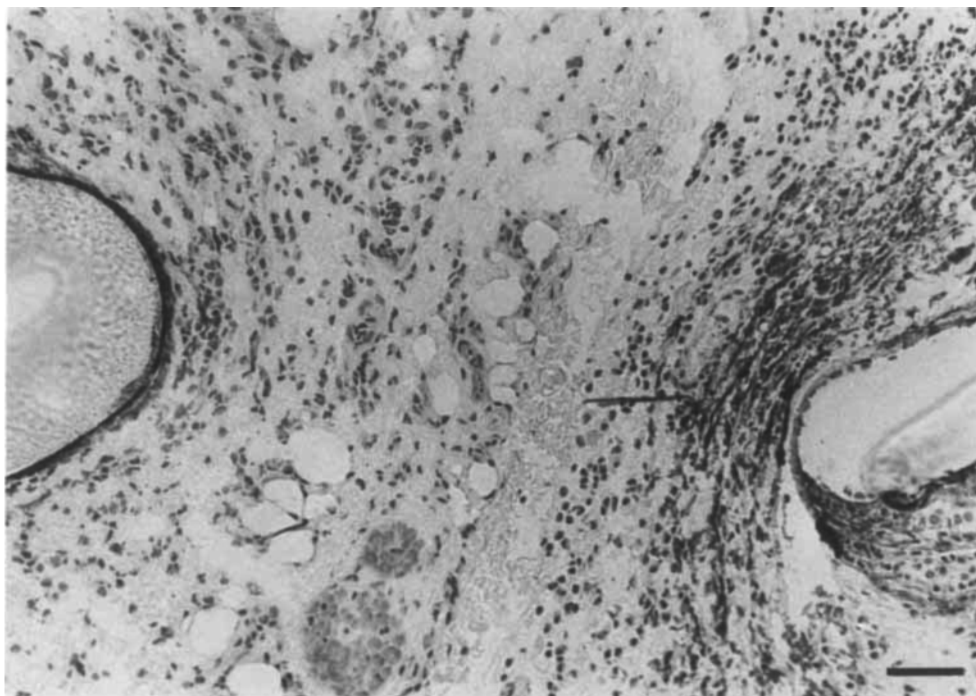
In studying the tissue response after implantation of a biomaterial, it has to be evaluated whether the cellular reactions are caused by the implant or by a simple wound healing process, i.e., would they also occur if the implant was not there or was completely biotolerant.[25,26]

The introduction of a biomaterial into the body results in tissue trauma. A sequence of cellular and humoral events is then set in motion, i.e., vascular and capillary dilatation, diapedesis of PMN's and formation of an exudate can be observed.[19,22] PMN's migrate toward the site of implantation, followed by monocytes, transforming into macrophages.[23] Proliferation of resident tissue macrophages[24] is the alternative source for macrophage accumulation. Macrophages release factors that cause fibroblast migration and proliferation in an early stage;[19] in a later stage these macrophages produce factors that stimulate fibroblasts to synthesize collagen.

It has been demonstrated by Ross[27,28] that from the three classical stages in wound healing (inflammation (Fig. 9), proliferation, and reorganization) the first two stages are comparable for normal wounds and nonirritating implant sites but that the tissue repair time is usually longer for the latter.

Enzymatic activity

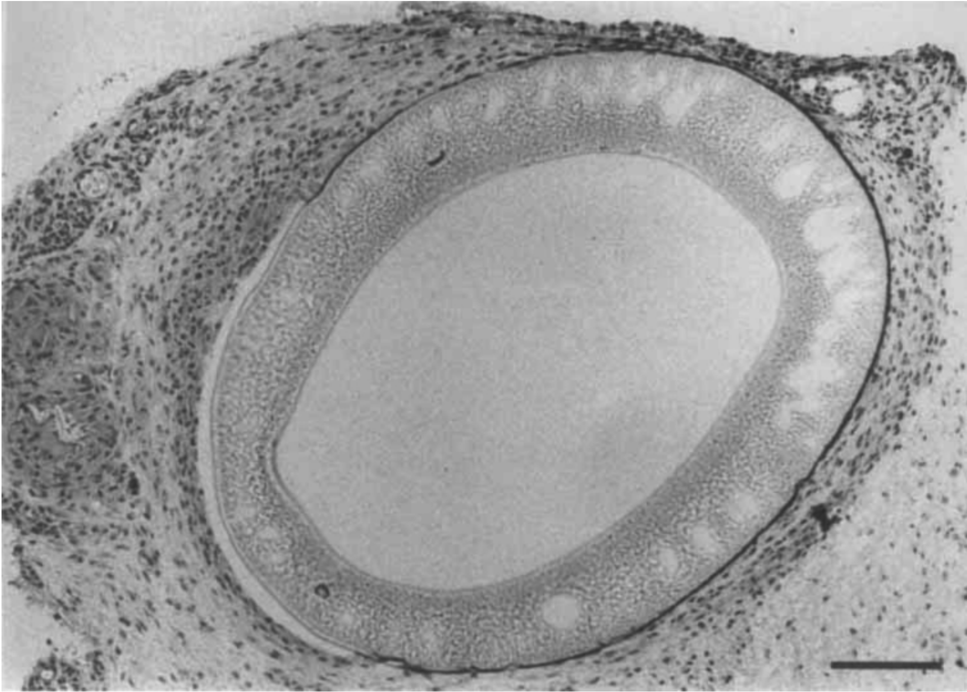
During the first days after implantation neutrophyls showed alkaline phosphatase, ATP-ase, and NADH reductase activity (Fig. 1A). Other investigators have also demonstrated the presence of collagenase[29] and elastase[30] in neutrophils. These two enzymes are active in the removal of connective tissue debris from traumatized tissues. Neutrophils disappear after approximately 3 days if healing is uneventful and the implant is not toxic or causing mechanical stress.[21]



(a)

Figure 9. Hematoxilin–Eosin staining at day 1 (A) and day 14 (B) after implantation; The inflammatory phase of the wound-healing reaction is demonstrated; many neutrophils and macrophages are visible around the implant at day 1 (A). At day 14 (B) several layers of macrophages and fibroblasts can be seen around the implant. Bar = 520 μm (A), resp. 1.3 mm (B).

Macrophages arriving at the implant site showed hydrolytic enzymatic activity from day 3 onward. By day 13, the enzyme profiles decreased to remain low thereafter (Fig. 1B). If the implant had been toxic or caused mechanical stress, macrophages would have remained at the implant site for extended periods, to eventually fuse to foreign body giant cells.[22] At day 21 only LDH and NADH showed levels above normal (Figs. 3 and 7). This might be related to the fact that the implanted material is made of poly(L-lactic acid). Upon degradation, oligomers of L-lactic acid and monomeric L-lactic acid will be released and may cause an increase in macrophage LDH activity, in which NADH is operating as a coenzyme. In a separate study[17] it was demonstrated that significant giant cell activity could be observed when the implanted biomaterial was desintegrating at a high rate. In the case of this poly(L-lactic acid) implant, significant degradation could only be observed after approximately 1 year. The absence of giant cell activity 3 weeks after implantation can therefore be expected.



(b)

Figure 9. (continued)

We observed endothelial cells in the vicinity of the implant at day 3 showing high levels of alkaline phosphatase (Fig. 6). This suggests that capillaries form vascular sprouts, growing toward the implanted foreign body. The macrophage angiogenic factor[31] may be responsible for this phenomenon.

Mast cells were observed, activated by tissue damage. Mast cells are known to produce histamine, serotonin, heparin, and hydrolytic enzymes. These release products induce vascular permeability and can be associated with slight edema at the implant site at very early stages. Mast cells do not play a direct role in the tissue reaction against the implanted biomaterials.

Matlaga et al.[32,33] already indicated that the shape and size of the implanted biomaterial is of crucial importance with regard to the degree of tissue reaction. They found that rod-shaped implants with a diameter smaller than 1 mm were causing the least activation of surrounding tissue. The fact that an increased activation was found with larger implants may possibly be explained by an increase of ischemia around implants with a larger diameter.[22] The implants used in this paper were also rod-shaped and had a diameter of 0.7 mm. We observed that our hollow fiber implants were slightly compressed by the pressure of the skin causing the spherical diameter to become ovoid. This resulted in a slightly higher mechanical activation of surrounding tissue at the more convex lateral sides of the implant, showing somewhat higher enzymatic activity.

The implants studied are to be used for the controlled release of drugs, especially steroid hormones. The macrophages with their content of lysosomal enzymes present directly adjacent to the implanted polymer are therefore directly in contact with the released drug. Allison[34] already mentioned the negative effect of lysosomal enzymes on drug efficiency. He studied the uptake of drug in lysosomes. The enzymes were either stabilizing or labilizing the drug release rate. In some cases lysosomal hydrolases were degrading the drug molecule and the degradation products were exocytosed. It is unknown in what way hormones do influence tissue reactions, but the steroid hormones used in separate studies[17] are not metabolized in lysosomes or by lysosomal enzymes, and we therefore do not expect a negative influence of enzymatic activity on the efficiency of the drug.

The main degradation mechanism involved in the breakdown of these poly(L-lactic acid) implants is simple hydrolysis, taking place in tissue fluids; intracellular breakdown of small debris however can not be excluded. The lactic acid used in these studies starts to degrade slowly; an exponential increase in degradation is only observed after 1 year, when the molecular weight of the bulk of the polymer material has dropped to approximately 5000. The observation of intracellular debris, 3 weeks after implantation is therefore unlikely.

Events as described in this paper concerning the tissue reaction upon implantation of poly(L-lactic acid) hollow fibers have been referred to by others as a low turnover foreign body granuloma.[18,22] This classification, however, implies[22] that the macrophages present are persistent for longer periods of time and form giant cells. A zone of macrophages, lymphocytes, and granulocytes should persist around these giant cells with an encapsulating membrane of fibrous tissue. Since we did not observe most of these phenomena, the term "granuloma" does not fit the observations and should not be used in this context. We only observed an inflammatory reaction as a host response upon the traumatizing implantation of a biomaterial, followed by a wound-healing process that is somewhat prolonged, possibly as a result of mechanical irritation of tissue by the implant.

It is concluded that the poly(L-lactic acid) hollow fibers used in these implantation studies are completely tissue compatible, comparable with a normal wound-healing process and have a very low inflammatory potential. The slightly increased levels of LDH and NADH suggest the release of lactic acid, and thus confirms the biodegradable nature of this polymer.

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