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Cell response of cultured macrophages, fibroblasts, and co-cultures of Kupffer cells and hepatocytes to particles of short-chain poly[(*R*)-3-hydroxybutyric acid]

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The known biodegradability of poly[(*R*)-3-hydroxybutyric acid] (PHB) in certain biological environments has lead to its proposed use as biodegradable, biocompatible polymer. Recently, a new, rapidly biodegradable blockcopolymer has been synthesized that contains crystalline domains of PHB blocks. During degradation of these polymers, the PHB-domains are transformed in a first step into small crystalline particles of short-chain PHB. Therefore, particles of short-chain poly[(*R*)-3-hydroxybutyric acid] ($M_n \approx 2300$) (PHB-P), as possible degradation products, are investigated here for their effects on the viability and activation of macrophages, fibroblasts, and co-cultures of rat Kupffer cells and rat hepatocytes. Results obtained in the present study indicate that phagocytosis of particles of short-chain poly[(*R*)-3-hydroxybutyric acid] at high concentrations (higher than 10 μg/ml) is dose-dependent and associated with cell damage in macrophages but not in fibroblasts. At low concentrations, particles of PHB-P also failed to activate macrophages and are biocompatible. Besides the PHB phagocytosis by Kupffer cells, treatment of co-cultures of Kupffer cells and hepatocytes with 1 μg PHB/ml showed neither cytotoxic (lactate dehydrogenase activity) effects nor any change in albumin secretion by hepatocytes.

1. Introduction

Tissue injury, following implantation of a medical device or material, initiates tissue repair and regeneration processes. Cells such as fibroblasts, macrophages, and endothelial cells migrate into the implantation site (inflammatory site) and proliferate. Inflammatory mediators, cytokines, and growth factors released by these cells regulate the wound healing (for a review see [1]).

The appearance of degradation products *in vivo* correlates with an observed change of type or intensity (or both) of a pre-existing inflammatory response [2]. This may be caused by the toxic effects of the degradation products. It has been demonstrated that macrophages play a prominent role in the resorption process by phagocytizing fragments of polymeric materials [3, 4]. Phagocytosis of toxic degradation products is accompanied by activation and proliferation of macrophages and the foreign-body reaction. Phagocytosis of polymer fragments may also induce cell death. The release of the contents of dead cells may cause a renewed acute inflammatory response.

Macrophages are also involved in the biodegradation of foreign materials (for a review see [5]). After phagocytosis, the material may be totally digested or may persist in the form of indigestible residue; it may actually fill up the cell, and if toxic, may kill the cell.

Poly[(*R*)-3-hydroxybutyric acid] (PHB) is a biopolymer produced by various bacteria. It is regarded as a potentially useful biodegradable natural polymer and has been examined for its application in the medical field as a carrier matrix for the sustained release of drugs. Macrophages, fibroblasts, and mature vascularized fibrous capsula are typical of the tissue response. However, the cellular interaction of PHB, for example PHB-phagocytosis, alteration of the cellular functions, and the response of liver cells (hepatocytes and Kupffer cells), are less well documented.

Results obtained in this investigation indicate that phagocytosis of PHB-P causes dose-dependent cell activation, cell damage, and cell death in macrophages but not in fibroblasts. Although Kupffer cells showed PHB phagocytosis, co-cultures of Kupffer cells and hepatocytes were not affected by treatment with PHB.

2. Materials and methods

2.1. Preparation of PHB-P and DPHB-P

PHB-P, crystalline particles of OH-terminated telechelic oligomers of poly[(*R*)-3-hydroxybutyric acid], were obtained as described [6]. The particles are obtained by precipitation of the macrodiols in water

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from a dioxane solution. They are irregularly shaped and measure between 1 and 20 µm.

Dansyl-labelled PHB-P (DPHB-P) are essentially the same particles as PHB-P, except that the chains are labelled at their ends with dansyl sulphonamide fluorophores [6].

2.2. Cell culture

Cells were cultured in polystyrene flasks (NUNC, Roskilde, Denmark) in a humidified atmosphere with 5% CO_2. Macrophages (murine macrophage cell line, J774) were maintained in Dulbecco's modified eagle medium (DMEM medium) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 µg/ml gentamicin. Fibroblasts (mouse fibroblast cell line, 3T3) were cultured in RPMI 1640 medium supplemented with 2 g/l $NaHCO_3$, 25 mM HEPES, 10% FCS, 2 mM L-glutamine and 50 µg/ml gentamicin.

Peritoneal macrophages were harvested from adult male Sprague–Dawley rats with 50 ml DMEM medium. After centrifugation (500 g for 10 min), cells were seeded in culture medium (DMEM medium) supplemented with 10% FCS, 2 mM L-glutamine and 50 µg/ml gentamicin.

For purification of hepatocytes and Kupffer cells, 8-week-old male Sprague–Dawley rats (ZUR/SIV, Institute of Laboratory Animal Sciences, University of Zürich, Zürich, Switzerland, 240–270 g b.w., standard diet No 890 Nafag, Gossau Switzerland, water *ad libitum*) were used. The preparation of the cells included a standardized two-step collagenase perfusion technique. The viability of the cells as assessed by the trypan blue exclusion test was more than 85%. The enriched hepatocytes were suspended in Williams E medium (BioConcept, Allschwil, Switzerland) supplemented with 100 nM dexamethasone (Sigma), 10 nM insulin (Sigma), 30 nM selenium (Fluka, Buchs, Switzerland), 1 µg/ml aprotinin (Sigma), 2 mM L-glutamine (Gibco, Life Technologies, Basel, Switzerland), and 100 IU/ml streptomycin/penicillin (culture medium). The cells were plated on crude membrane fractions/collagen type I (100:1) (CMF/COL) coated tissue culture dishes (Petriperm, Heraeus, Zürich, Switzerland) at a density of 3×10^6 cells/20 cm^2, in 3 ml culture medium and incubated at 13% oxygen at 37 °C and 5% CO_2 [7, 8]. Kupffer cells (5×10^5/ml) were isolated from rats and added to the hepatocytes in 1 ml culture medium supplemented with 10% fetal calf serum. After 4 h the cells were washed and fresh medium was added. At 24 h the cells were stimulated with 1 µg PHB or DPHB/ml culture medium. The culture medium was then daily exchanged against the same culture medium containing 1 µg PHB/ml.

2.3. Quantification of albumin secretion and lactate dehydrogenase activity (LDH) in cocultures of Kupffer cells and hepatocytes

The amount of albumin in the culture supernatant of co-cultures of Kupffer cells and hepatocytes was measured using an enzyme-linked immunosorbent assay (ELISA) as described previously [7].

The LDH activity in cell homogenates and in culture supernatants was determined spectrophotometrically (COBAS Fara) with a commercially available kit (Boehringer Mannheim, Baar, Switzerland) following the manufacturer's instructions.

Protein concentrations were determined according to Bradford [9] using bovine serum albumin (BSA) as standard.

2.4. Determination of cytotoxicity using succinate dehydrogenase activity (MTT) assay

Fibroblasts (3T3) and macrophages (J774) were trypsinized using 0.25% Trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) for 5 min, centrifuged at 200 g for 10 min and then subcultured into 24-well flat-bottom culture plates (NUNC, Roskilde, Denmark) at a density of 2×10^5 cells/well and 1 ml medium. After 24 h, the culture medium was exchanged with the same medium containing increasing amounts of PHB-P (0–320 µg/ml). MTT assay was carried out one, two, four, eight, and 12 days later. All MTT determinations were performed in triplicate. For each experimental value, at least three independent experiments were carried out.

2.5. Quantification of tumour necrosis factor-α(TNF-α) production and nitrite production

TNF-α levels in the culture supernatant of cells treated with PHB-P or lipopolysaccharide (LPS) were determined in a TNF-α specific bioassay using the WEHI cell line [10]. WEHI 164 clone 13 fibrosarcoma cells at a concentration of 2×10^4 cells per 100 µl were incubated with serially diluted samples in 96-well flat bottom microtitreplates for 48 h at 37 °C, under 5% CO_2. Then 100 µl of a 5 mg/ml MTT tetrazolium solution in phosphate-buffered saline (PBS) was added to the plate, which was further incubated for 2 h. The dye was then removed and cells were lysed by addition of 200 µl of isopropanol with 5% formic acid. Plates were read at 550 nm on a multiscan bichromatic ELISA Reader (Dynatech, MR 5000).

To measure the amount of nitrite in the culture, supernatant 50 µl aliquots of culture supernatant were mixed with 200 µl of the Griess reagent [11]. As positive control, NO was measured in the supernatants of LPS stimulated cells (10 µg LPS/ml).

2.6. PHB-P phagocytosis

Macrophages and fibroblasts were subcultured into glass chamber slides (NUNC, Roskilde, Denmark) at a density of 5×10^4 cells/well.

After 24 h, the culture medium was exchanged with the same medium containing 2 µg DPHB-P/ml medium (40 pg PHB-P/cell). After 2, 4, 24, and 96 h, cells were washed in PBS, fixed in 70% ethanol in PBS for 5 min at room temperature, and stained with standard

May Grünwald Giemsa solution. DPHB-P phagocytosis was then determined using fluorescence and light microscopy.

Fluorescence microscopy was carried out with an Axioplan Universal microscope (Zeiss, Oberkochen, Germany) equipped with an HBO 50 mercury lamp for fluorescence illumination, a 40 × objective operating in phase contrast, and a UV-G 365 filter set (G 365 excitation filter, FT 395 chromatic beam splitter and LP 420 barrier filter).

3. Results

The cell response of primary rat macrophages, mouse macrophage cell line J774, and mouse fibroblast cell line 3T3 to PHB-P treatment was characterized by measuring the PHB-P phagocytosis, cytotoxicity, production of nitric oxide (NO), and tumour necrosis factor (TNF-α). In addition, the cell response of co-cultures of rat Kupffer cells, the resident liver macrophages, and hepatocytes was determined by measuring the LDH activity and albumin concentrations.

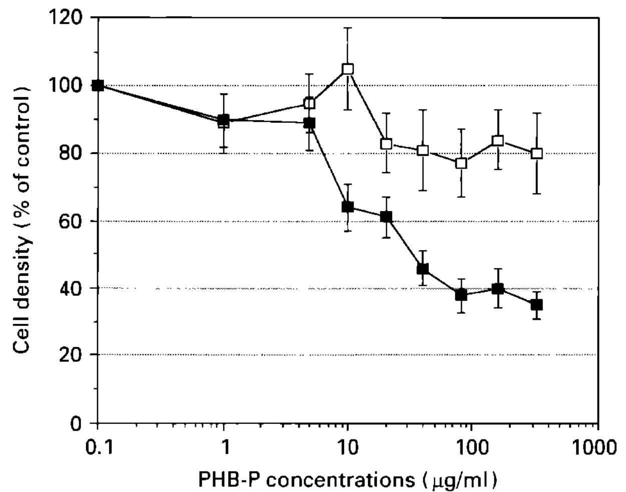

Figure 1 Effects of PHB-P-treatment on cell viability. For the determination of cell viability in cultured macrophages (■) and fibroblasts (□), cells ($5 \times 10^4/cm^2$) were treated with increasing concentrations of PHB-P (0–6.4 ng/cell). Succinate dehydrogenase activity was measured after 2 days using MTT assay. The plotted symbols represent the mean, the error bars indicate the standard deviation estimated.

3.1. Effects of PHB-P on cell viability of cultured macrophages, fibroblasts, and co-cultures of Kupffer cells and hepatocytes

In macrophage cultures, the mitochondrial succinate dehydrogenase activity (MTT test), an indirect estimate for the number of attached, viable cells, was found to be affected, depending on dose and time, by PHB-P treatment (Figs 1 and 2). A significant decrease in the number of viable cells was observed following incubation with PHB-P concentrations higher than 10 μg/ml. This significant decrease was seen in all cases 24 h after PHB-P treatment and a further decrease with increasing PHB-P concentrations and incubation time. Compared to untreated cells, only 50% of the cells were found at day 12 after treatment with PHB-P for concentrations > 10 μg/ml (Fig. 2). In contrast, low PHB-P concentration (0–5 μg/ml corresponding to 0–100 pg PHB-P/cell) failed to induce relevant cytotoxic effects even after long-time treatment (up to 12 days). To ensure that the observed effects are induced directly by PHB-P and not by possible impurities found in the PHB-P used, PHB-P was washed three times with the culture medium and incubated for at least 2 days in culture medium before use.

In contrast to the effect on macrophages, high concentrations of PHB-P (up to 320 μg/ml corresponding to 6.4 ng PHB-P/cell) failed to lead to significant decrease in succinate dehydrogenase activity in fibroblasts culture (Fig. 1). These results were underscored by light microscopy upon incubation with high PHB-P concentrations (> 10 μg/ml); macrophages demonstrated signs of cell damage, cell death, and cell lysis due to phagocytosis of PHB-P. In contrast, fibroblast showed only limited PHB-P-phagocytosis, but no sign of any cellular damage, even after treatment with high concentration of PHB-P (up to 320 μg PHB-P/ml). No difference in the observed effects induced by PHB-P and DPHB-P was seen (data not shown).

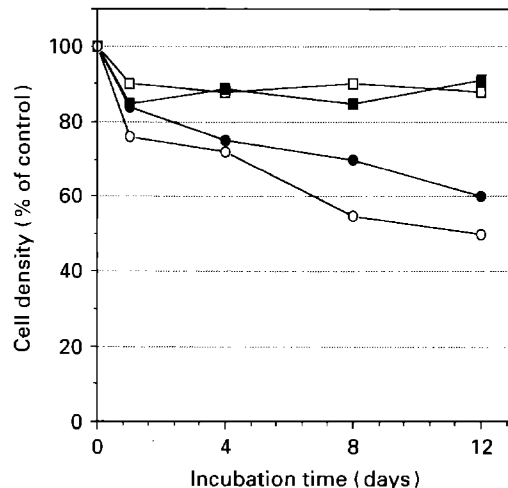

Figure 2 Time dependence of PHB-P effect on cell viability. For the determination of the effects of PHB-P on cell viability after long-term treatment, macrophages ($5 \times 10^4/cm^2$) were treated with various concentrations of PHB-P for the times indicated (-□- 2 μg/ml; -■- 4 μg/ml; -●- 10 μg/ml; -○- 20 μg/ml). Succinate dehydrogenase activity was measured using MTT assay. The plotted symbols represent the mean, the error bars indicate the standard deviation estimated.

In co-cultures of Kupffer cells and hepatocytes, the extracellular LDH activity as a fraction of the total (intra- and extracellular), used as indication for plasma membrane integrity, was not increased by the treatment with 1 μg PHB/ml (Table I), indicating that PHB failed to induce cytotoxic effects in hepatocytes and Kupffer cells.

3.2. Determination of phagocytosis using light and fluorescence microscopy

Macrophages (cell line and primary cultured peritoneal macrophages), fibroblast cultures and co-cultures of Kupffer cells and hepatocytes were treated with

2 μg DPHB-P/ml to investigate the process of PHB-P phagocytosis. At this DPHB-P concentration, no signs of cellular damage or cell death were observed. Fig. 3 shows that the DPHB-P were well phagocytosed by macrophages and Kupffer cells, but not by hepatocytes within 4 h. Similar results were obtained with primary cultures of peritoneal macrophages. In contrast to macrophages, fibroblasts showed only limited phagocytosis of DPHB-P.

Light and fluorescence microscopy revealed that the phagocytosed DPHB-P are found in intracellular vacuoles. These observations were verified with transmission electron microscopy (data not shown).

TABLE I Intracellular and extracellular lactate dehydrogenase activity (LDH_{int} and LDH_{ext}) and albumin secretion of control and PHB-stimulated (1 μg PHB/ml for 2 and 6 days) co-cultures of Kupffer cells and hepatocytes

	LDH_{ext} (% of total LDH)		LDH_{int} (U/mg total protein)		Albumin (μg/mg total protein)	
	control	+PHB	control	+PHB	control	+PHB
2 days	6	7	1	0.9	70	67
6 days	15	13	0.8	0.9	65	63

3.3. Effects of PHB-P on NO production by cultured macrophages

Fig. 4a shows the NO release into the culture supernatant of untreated or PHB-P treated macrophages 96 h after treatment. A significant increase is evident with high concentrations of PHB-P (> 20 μg/ml). The NO levels after PHB-P treatment are about 50% of those obtained after LPS treatment. These observations indicate that only toxic concentrations of PHB-P are able to stimulate the activation of macrophages. This activation may be due to a direct or to an indirect (i.e. induced by damaged cells) cell response.

3.4. Effects of PHB-P on TNF-α production by cultured macrophages

Production of TNF-α by cultured macrophages was tested in the culture supernatants using sensitive bioassays. It was found that macrophages produce detectable amounts of TNF-α after treatment with PHB-P or stimulation with LPS (as positive control). Fig. 4b illustrates the dose response of TNF-α production into the culture supernatant by untreated

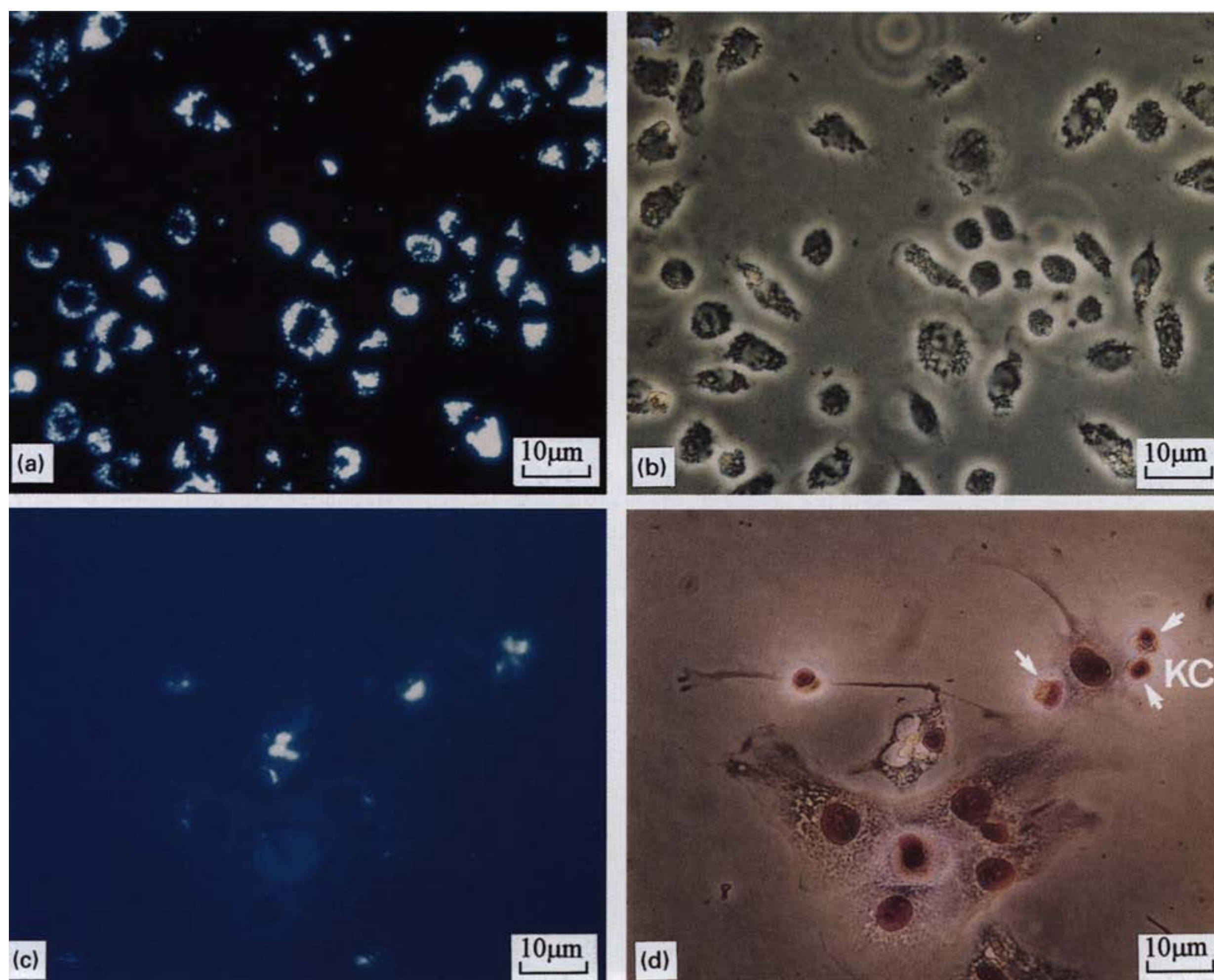

Figure 3 DPHB-P phagocytosis in macrophages and Kupffer cells (KC) co-cultured with hepatocytes. Fluorescence analysis of cultured macrophages (a) and Kupffer cells (b) after 4 h of DPHB-P treatment (1 μg/ml). (b) and (d) are the phase contrast micrographs corresponding to fluorescence images (a) and (c), respectively.

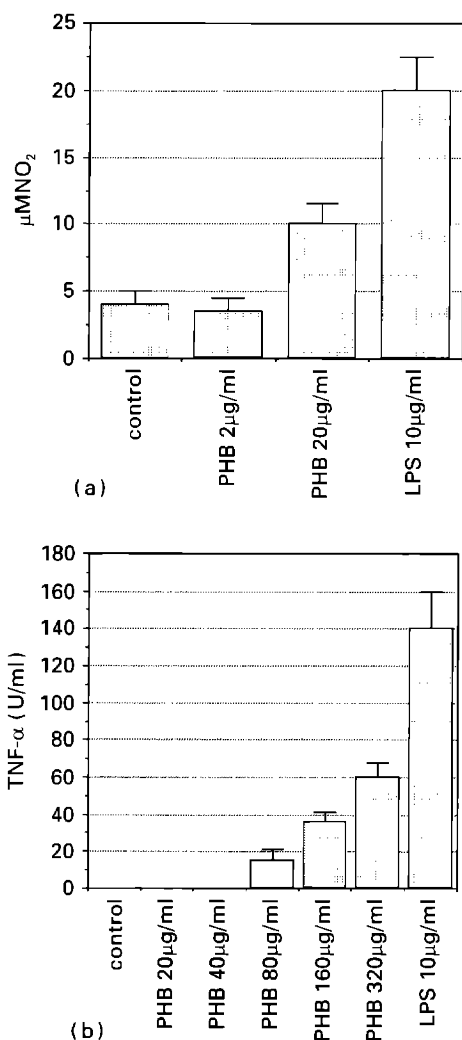

Figure 4 PHB-P effects on macrophage activation. For the determination of NO release (a) and TNF-α production (b) in cultured macrophages, cells were treated with 10 µg LPS/ml (as positive control) or with various concentrations of PHB-P. TNF-α concentrations were measured after 4 h using a specific bioassay and NO release was determined after 96 h using the Griess reaction. The plotted symbols represent the mean, the error bars indicate the standard deviation estimated.

macrophages and others treated with PHB-P and LPS. Maximal TNF-α levels were detectable in the culture supernatants 4 h after PHB-P or LPS treatment. TNF-α levels were increased with increasing PHB-P concentrations, and maximal TNF-α levels were found after treatment with 320 µg PHB-P/ml: PHB-P-stimulated macrophages produced 50% of the amount of TNF-α obtained after LPS stimulation. These results indicate that phagocytosis of PHB-P at cytotoxic concentration stimulates cell activation directly or indirectly.

3.5. Effects of PHB-P on the production of albumin by cultured hepatocytes

The amount of albumin secreted into the culture medium (Table I), an indicator for the metabolic activity and secretory capacity of the cultured hepatocytes, was not affected by PHB treatment after either 2 days or 6 days.

4. Discussion

One of the most important factors in the development of degradable biocompatible polymers is cellular interaction, in particular the inflammatory response and the cytotoxicity of the prospective degradation products. When a foreign substance, such as a polymer, comes in contact with the human body, the system initiates its host-defence mechanisms involving the inflammatory response. The infiltration of macrophages and fibroblasts and the formation of a mature vascularized fibrous capsule are typical for the tissue response. Macrophages play a major role in all these kinds of inflammation (for a review see [1]).

PHB is a polyester made by many micro-organisms. The use of PHB and its copolymers as biocompatible and degradable materials for surgical use is showing increasing promise. PHB-oligomers are thought to be a degradation product of PHB-homopolymers and copolymers with hydroxyvaleric acid as well as the new blockcopolymers, mentioned in the introduction, which contain PHB blocks in the backbone. However, the cellular interaction of these degradation products, e.g. PHB-P phagocytosis, alteration of the cellular functions and the response of liver cells, e.g. the hepatocytes and Kupffer cells (the resident liver macrophages), are not well documented.

Results obtained in this study clearly indicate that macrophages and Kupffer cells and, to a lesser degree, fibroblasts have the ability to take up (phagocytose) PHB-P (size 1–20 µm). In contrast, hepatocytes demonstrated no signs of PHB-phagocytosis. These results were verified by light, fluorescence, and confocal microscopy and by transmission electron microscopy. The phagocytosis is accompanied by toxic effects and alteration of the functional status of the macrophages but not the fibroblasts. Cell activation, cell damage, and cell death were found to be dose- and time-dependent. At high concentrations of PHB-P ($>$ 10 µg/ml), this process is accompanied by cell damage and cell death. Therefore, the elevated levels of NO and TNF-α may be due to a direct, PHB-P dependent cell activation or, more likely, to an indirect activation as a result of cell damage and cell death. The PHB-P-induced effects may be due to:

1. direct effects of PHB-P which may be toxic at high concentration; this possibility can be excluded since no toxic effects were observed in fibroblast and hepatocyte cultures;
2. impurities found in the PHB-P preparation used; however this possibility can be excluded since PHB-P was washed three times and incubated in culture medium for at least 2 days before use;
3. phagocytosis of large amounts of PHB-P; after phagocytosis, PHB-P may persist in the form of indigestible residue, fill up the cells and cause cell damage and cell death;
4. after phagocytosis, PHB-P may be totally or partially biodegraded and the produced degradation products may cause the observed toxic effects.

5. Conclusions

The rate of degradation of an implanted biomaterial determines the rate of release of degradation products. Particles of low-molecular PHB-P were found to cause dose-dependent cell damage and cell activation in macrophages, but not in fibroblasts. At low concentrations, PHB-P is biocompatible. Therefore, controlling the degradation rate is important for controlling the inflammatory reaction and biocompatibility. Although Kupffer cells showed a clear PHB-phagocytosis, co-cultures of Kupffer cells and hepatocytes were not affected by the treatment with 1 μg PHB/ml.

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