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Phagocytosis and biodegradation of short-chain poly [(*R*)-3-hydroxybutyric acid] particles in macrophage cell line

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The biocompatibility and the degradation mechanisms of block co-polyurethanes containing crystallizable telechelic poly [(*R*)-3-hydroxybutyric acid] (PHB) segments have found recently growing interest for the possible biomedical applications of these new materials. The random hydrolytic cleavage of the amorphous part of these polymers might result, *in vivo*, in the production of small crystalline particles of low molecular weight PHB that could then undergo phagocytosis and biodegradation inside phagosomes. To test this possibility, a fluorescent-labelled PHB segment was synthesized, precipitated in the form of crystalline particles, and used for an *in vitro* investigation of its interaction with macrophage cell line. Light and fluorescence microscopy performed in the present study clearly show that the fluorescent particles are well internalized in phagosomes already after 1 h of incubation. The number of phagocytized particles decreases notably after 8 days of incubation.
A quantitative determination of the time dependence of the phagocytosis was obtained through laser cytometry and fluorescence-activated cell sorting (FACS). Fluorescence spectroscopy and high-performance liquid chromatography (HPLC) analysis of extracts of cell supernatants revealed the presence of supposed degradation products of PHB after 8 days of incubation, suggesting that macrophages could degrade low molecular weight PHB.

1. Introduction

Poly [(*R*)-3-hydroxybutyric acid] (PHB), long known in microbiology as a natural storage material, has emerged only recently as a biomaterial. Films obtained from this polymer do not show any inflammatory activity [1], the degradation of fibres, sheets, and granules made of high molecular weight PHB, however, has been shown, with small rates, to always take place [1, 2], and the rate of degradation is higher for copolymers with hydroxyvaleric acid [3].

Low-molecular-weight hydroxy-terminated PHB polymers have been used as crystallizable segments in the synthesis of bioresorbable block-copolyurethanes, that show phase segregation in amorphous and crystalline domains [4]. "Bioresorbable" polymers are materials that maintain a well-defined property for a period of time (e.g. a desired E-modulus), and then, after having fulfilled their duty, lose their properties under the influence of the environment and are finally cleaved through biological processes into their non-toxic building-blocks. Considering the possible application of these polymers, it is clear that their interaction with the biological environment and the possible mechanisms of degradation should be investigated and understood as far as possible.

In analogy with the mechanisms proposed for several kinds of degradable polymers [5] the first stage of degradation should be restricted, *in vitro* as well as *in vivo*, to a random hydrolytic cleavage of the amorphous part of the material. This process might result in the production of small crystalline particles of low-molecular-weight PHB, which could then undergo phagocytosis and biodegradation inside macrophages.

Macrophages are in fact always involved in degradation of foreign materials [9]. Following phagocytosis, the material can be either digested or remain as an indigestible residue. In the latter case the material can fill up the cell and kill it. Macrophages are able, *in vitro*, to phagocytize microspheres of non-degradable (polystyrene) and biodegradable (polylactic and polyglycolic acid) polymers [7] and low molecular weight poly-ε-caprolactone particles [8].

In order to simulate this situation and to investigate the process, macrophages cell line was incubated

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with non-toxic concentrations [6] of crystalline particles of dansyl-labelled, low-molecular-weight PHB.

2. Materials and methods

2.1. Synthesis of dansyl labelled low-molecular-weight PHB (DPHB)

Hydroxy-terminated telechelic PHB (M_n = 2300) was obtained by degradative transesterification of the corresponding high-molecular-weight polymer with ethyleneglycol as described elsewhere [4]. The IUPAC name for this product is α, ω-dihydroxy-{oligo[(*R*)-3-hydroxybutyrate]-ethylene-oligo[(*R*)-3-hydroxybutyrate]}.

In a typical example, 6.020 g of the telechelic PHB were dissolved in 150 ml of 1,2-dichloroethane and the mixture heated to reflux in a Soxhlet extractor filled with molecular sieves. When the solvent in the Soxhlet showed a water content lower than 4 p.p.m. (as measured with the Karl Fischer apparatus), the mixture was cooled to room temperature and 1.205 g (6.88 mmoles) of BOC-glycine, 0.102 g (0.688 mmoles) of 4-pyrrolidino-pyridine (PPy) and 1.419 g (6.88 mmoles) and dicyclohexylcarbodiimide (DCC) were added. The mixture was stirred for 16 h at room temperature and then 4 h at 40 °C. After removal through filtration of the insoluble dicyclohexylurea, the product was precipitated in petroleum ether, washed with water and again with petroleum ether, and dried at 50 °C under reduced pressure.

1.5 g of the product were then dissolved in 3 ml methylene chloride. 0.75 ml of trifluoroacetic acid (TFA) were added to the solution and the reaction continued for 4 h. Both the solvent and TFA were removed by distillation under reduced pressure. The residue was dissolved in 10 ml of 1,2-dichloroethane and isolated as described.

0.36 g of the so obtained polymer were then dissolved in 1,2-dichloroethane and the water removed with the Soxhlet extractor as described. The solution was then cooled to 0 °C and 0.1 ml of triethylamine, 40 mg of pyridine, 100 mg (37 mmol) of dansyl (3,5-dimethylamminonaphtalensulphonyl) chloride were added. The solution was maintained for 20 h at room temperature and then the product isolated as described. The polymer was then washed with water until the water showed no more fluorescence.

The labelled polymer exhibited the typical fluorescence spectrum of a dansylsulphonamide fluorophore, with an absorption maximum at 335 nm and an emission maximum at 492 nm. No change in the DP of the polymers was observed after the various synthetic steps as determined from gel permeation chromatography (GPC), and vapour phase osmometry measurements. All reagents were commercial grade (Fluka, Switzerland) and were used without further purification except DCC that was distilled under reduced pressure (b.p. 103 °C at 0.2 mbar) and maintained under a nitrogen atmosphere, and PPy that was recrystallized from petroleum ether.

2.2. Preparation of particles of low-molecular-weight PHB (PHB-P) and dansyl-labelled PHB (DPHB-P)

The particles were obtained by precipitation in water of the polymers from a 1% (w/w) dioxane solution. They were irregularly shaped and had a diameter between 1 and 20 μm. The particles were washed in the culture medium before incubation with the cell cultures.

2.3. Cell cultures

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) supplemented with 10% fetal calf serum (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) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 μg/ml gentamicin.

2.4. DPHB-P phagocytosis

Macrophages 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 (40 pg DPHB-P/cell). After 2, 4, 24, and 96 h, cells were washed in phosphate buffer solution (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, 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).

2.5. Single cell analysis using the fluorescence activated cell sorter (FACS)

Cultures of macrophages were subcultured into 25 cm^2 culture flasks (NUNC, Roskilde, Denmark) at a density of 5×10^4 cells/cm^2. After an overnight period of attachment, the culture medium was exchanged against the same medium containing 2 μg/ml DPHB-P. After 4 h, 1 d, 4 d, and 8 d, cells were detached from the substrate using 0.25% trypsin and 0.02% EDTA in PBS. The cells were then fixed with 70% ethanol and were stored in this solution at 4 °C until analysis. The number of fluorescence labelled cells and the fluorescence intensity per cell were determined using FACS. For each test, 1×10^4 cells were analysed and three independent experiments were carried out in duplicates.

2.6. Single cell analysis using an interactive laser cytometer

Macrophage cell lines were subcultured as described. Peritoneal macrophages were seeded after isolation from a rat peritonea into glass chamber slides at a density of 1×10^5 cells/well. After 24 h, the culture medium was exchanged with medium containing 2 μg DPHB-P/ml medium. After 4 h, 1 d, 4 d and 8 d, cells were fixed in 4% formaldehyde in PBS for 4 min at 4 °C, washed in PBS and were then taken for the single cell analysis using an interactive laser cytometer (ACAS 470). Two areas of 360 μm^2 were measured in each chamber. Three independent experiments were carried out for each time and in each experiment 24 areas were measured. The number of fluorescence-labelled cells and the fluorescence intensity per cell were determined.

2.7. Fluorescence spectroscopy measurements

Macrophages were incubated in 75 cm^2 flasks with 12 ml medium containing 60 μg DPHB-P. After an incubation time of 2 h, 4 h, 1 d, and 8 d, cells and medium were separated, the cells washed twice with 2 ml and once with 5 ml of a 1% water solution of Triton and once with water. The washing solutions were added to the culture medium. Ethanol was added to the cells and to the supernatant and the solvents evaporated under reduced pressure. The residue was then extracted with 4 ml dioxane for 5 h at room temperature and the solution filtrated. The culture medium was treated in the same way. Fluorescence of each solution was measured with a Spex Fluorolog 2 spectrofluorimeter equipped with a spectrometer Spex 1680 and a double spectrophotometer Spex 1681 with an excitation wavelength of 335 nm. For each experimental value, 4 different samples from independent experiments were measured.

2.8. High performance liquid chromatography

Macrophages were incubated with medium containing 2 μg/ml PHB-P for 8 d as described. After incubation and subsequent lysis of the cells, the supernatant was separated through centrifugation and extracted twice with diethylether. The ether was then dried overnight over $MgSO_4$ and then evaporated under reduced pressure. The residue was taken up with 1 ml water and analysed. Medium containing 2 μg/ml PHB-P and cells incubated with no PHB-P were treated in the same way and taken as negative controls.

The fractionation was performed by HPLC with a Gilson 302 spectrometer equipped with a 100 μl injection loop, a Gilson UV detector, and a Nucleosil 5 C18 reverse phase column 250 · 8 · 4 mm (Macherey-Nagel, Switzerland). 50 μl of the sample were injected, using an elution gradient starting with 30/70 solvent A/B, coming to 80% A in 20 min, then 5 min isocratically and ending in 3 min with 30% A, with 1 ml/min flow and UV detection at 210 nm. The composition of A was 90% acetonitrile, 10% water, 0.1% TFA, B was water containing 0.1% TFA.

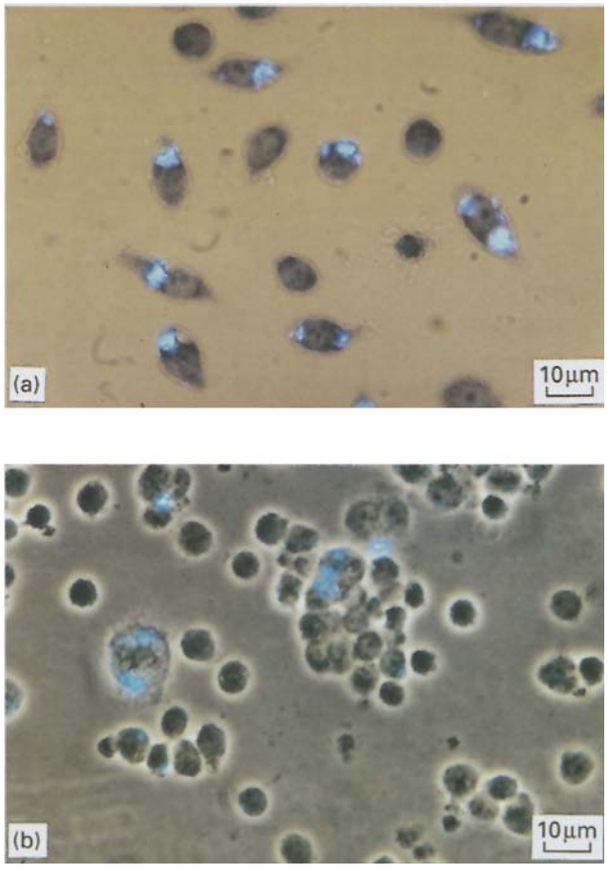

Figure 1 DPHB-P phagocytosis in macrophages. Fluorescence/phase contrast micrographs of cultured macrophages after 4 h (a) and 8 days (b) of incubation with DPHB-P (2 μg/ml).

3. Results

To evaluate the possibility that macrophages can phagocytize and degrade particles of short chain poly [(*R*)-3-hydroxybutyric acid], the mouse macrophage cell line J774 and murine primary macrophages were incubated with PHB-P and DPHB-P for several time intervals. The phagocytosis of polymer particles was investigated using light and fluorescence microscopy. Quantitative measurements of the time-dependence of phagocytosis were performed with FACS and laser cytometry. Possible macrophage-induced chemical modifications in the polymer morphology and molecular weight were investigated via fluorescence spectroscopy and HPLC analysis of the cell supernatants.

As shown by Saad *et al.* [6], concentration of PHB-P or DPHB-P lower than 10 μg/ml medium (200 pg PHB-P/cell) do not influence the cell functionality (as determined with MTT assay) neither activate macrophages, failing to stimulate production of NO and TNF-α. Therefore, concentrations of 2 μg/ml PHB-P or DPHB-P were used for the following experiments, designed to study PHB phagocytosis and biodegradability in cultured macrophages.

3.1. Time-dependence of phagocytosis as determined with fluorescence microscopy

Light and fluorescence microscopic analysis revealed phagocytized particles of DPHB-P present inside

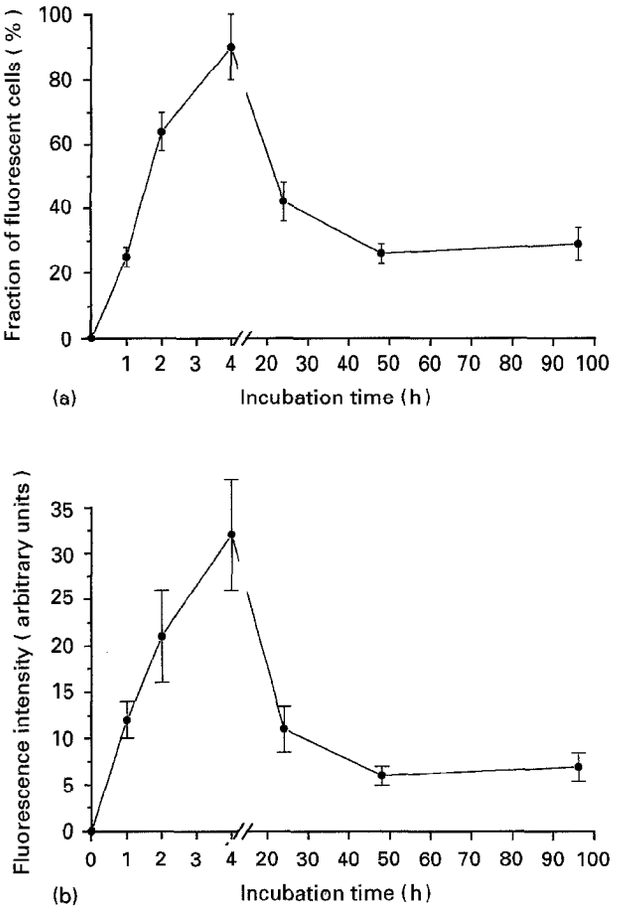

Figure 2 Time dependence of the PHB-P phagocytosis using FACS analysis. To determine the fraction of the fluorescent (DPHB-P positive) cells (a) and the fluorescence intensity/cell (b), 5×10^4 macrophages were incubated with non-toxic concentration of DPHB-P (2 μg/ml) for the indicated times. The plotted symbols represent the mean, the error bars indicate the estimated standard deviations.

macrophages (cell line and primary cultured peritoneal macrophages) already after 1 h of incubation. After 4 h, a large amount of fluorescent material was located in intracellular vacuoles as shown in Fig. 1a. The total fluorescence intensity of the cell population and the number of fluorescent cells gradually decreased with increasing incubation time. After eight days, only a few fluorescent particles were observed in the cells as shown in Fig. 1b. A small number of giant (polynuclear) cells was also observed after eight days.

3.2. Quantitative time-dependence of phagocytosis using FACS analysis

For the determination of the time dependence of the phagocytosis of DPHB-P particles in macrophages, the macrophage cell culture J774 was treated with non-toxic concentrations of the particles. The number of fluorescent cells increased during the first 4 h of incubation (about 90% of the total cell population) and then decreased to about 40% positive cells after 24 h (Fig. 2a). In parallel, the total fluorescence intensity per cell increased until 4 h and then decreased to about 30% of the maximum value at the fourth day (Fig. 2b). Therefore, 4 h was used as a reference value for the following experiments in order to better quantify the time dependence of phagocytosis.

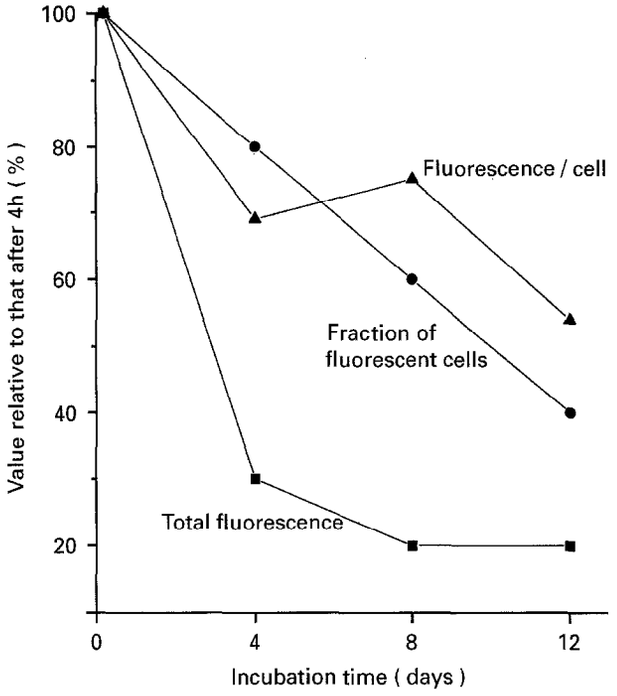

Figure 3 Time dependence of the PHB-P phagocytosis as determined with laser cytometry. 5×10^4 macrophages were incubated with non-toxic concentration of DPHB-P (2 μg/ml) for the indicated times. To determine the total fluorescence intensity of the cell population, the fluorescent intensity/cell, and the fraction of fluorescent (DPHB-P positive) cells, cells were fixed with formaldehyde and examined in the laser cytometer. The values given represent the means from three independent experiments carried out in duplicates.

3.3. Quantitative time-dependence of phagocytosis using the laser cytometer

Both, peritoneal macrophages and the cell line J774, were treated with non-toxic concentrations of DPHB-P (2 μg/ml). After 4 h, 4 d, 8 d, and 12 d, the fluorescence intensity/cell, the number of fluorescent cells and the total fluorescence intensity were measured in single-cell analysis using laser cytometry.

The maximal fluorescence intensity/cell and the largest number of labelled cells (70%) were found after 4 h of incubation, confirming what had been observed with FACS. The number of DPHB-P positive cells decreased at longer incubation time. If the maximum value (after 4 h) is taken as 100%, only 80%, 60%, and 40% of all cells were still fluorescent after 4, 8, and 12 days, respectively (Fig. 3). The fluorescence intensity/cell decreased after 4 d to about 70% of the value after 4 h, barely significantly increased again to about 80% (8 d), and then decreased to 55% after 12 days of incubation. The cell size of fluorescent cells (taken as a measure for the appearance of giant cells), increased from day four to day eight (data not shown), indicating that the increase in the fluorescence intensity/cell at day eight is most likely associated with the appearance of giant cells.

The total fluorescence intensity (calculated by multiplying the fluorescence intensity per cell by the number of DPHB-P positive cells), decreased after 4 d to 30% of the 4 h-value and to 20% at day 8 and 12 (Fig. 3).

These results were verified using primary cultured peritoneal macrophages, that showed similar patterns

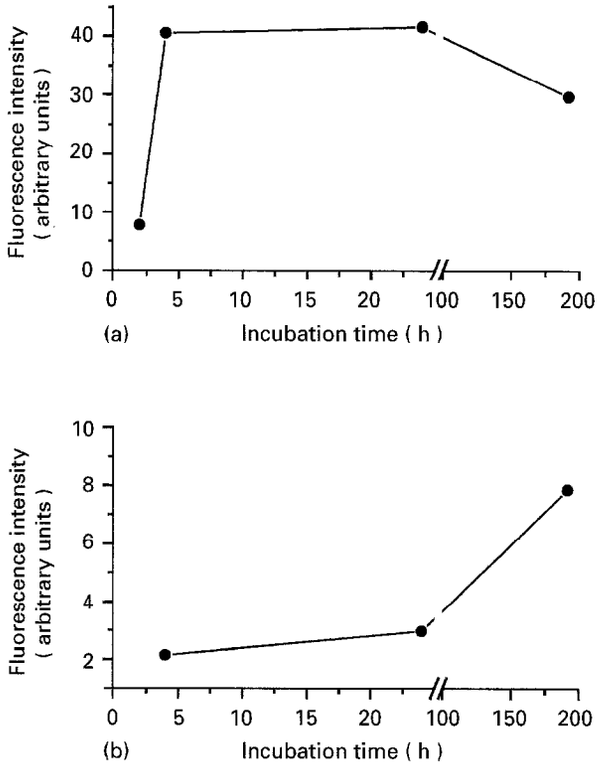

Figure 4 Tracing of dansyl labelled-compounds in cells and cell culture supernatant through fluorescence spectroscopy. Macrophages were incubated with non-toxic concentration of DPHB-P (5 µg/ml) for the indicated times. Cells and supernatant were dried and then the residue extracted with dioxane. The fluorescence of the solutions was then measured by an excitation of 335 nm in a spectrofluorimeter. The given values are measured at the emission maximum of 492 nm. For each experimental value 4 different samples from independent experiments were measured.

as the cell line J774 concerning all end points measured. The decrease in fluorescence intensity/cell, the number of positive cells, and the total fluorescence intensity can be interpreted as an exocytosis of the fluorescent material. From this data it is, however, not possible to deduce in which form the labelled PHB leave the cells. For this reason, fluorescence spectroscopy measurements and HPLC analysis were performed to gain better understanding.

3.4. Fluorescence-spectroscopy measurements of cells and medium extracts

The macrophage cell line J774 was treated with non-toxic concentrations of DPHB-P (2 µg/ml) for 4 h, 1 d, and 8 d. For each time, cells and medium were separated, the cell lysed, and both extracted with dioxane, after evaporation of water. The fluorescence of the dioxane solutions was then measured in a spectrofluorimeter using an excitation wavelength of 335 nm and the fluorescence intensity at 492 nm registered with increasing incubation time.

The fluorescence decreased from 4 h to 8 d in cell extracts in qualitative accordance (taking into account the different sensitivity of the techniques) with the results of the FACS and laser cytometry as shown in Fig. 4a. In medium extracts, the fluorescence increased significantly from 4 h to 8 d of incubation, showing the presence of a fluorescent and probably water-soluble material in cell supernatants and suggesting a possible biodegradation of DPHB-P after incubation (Fig. 4b).

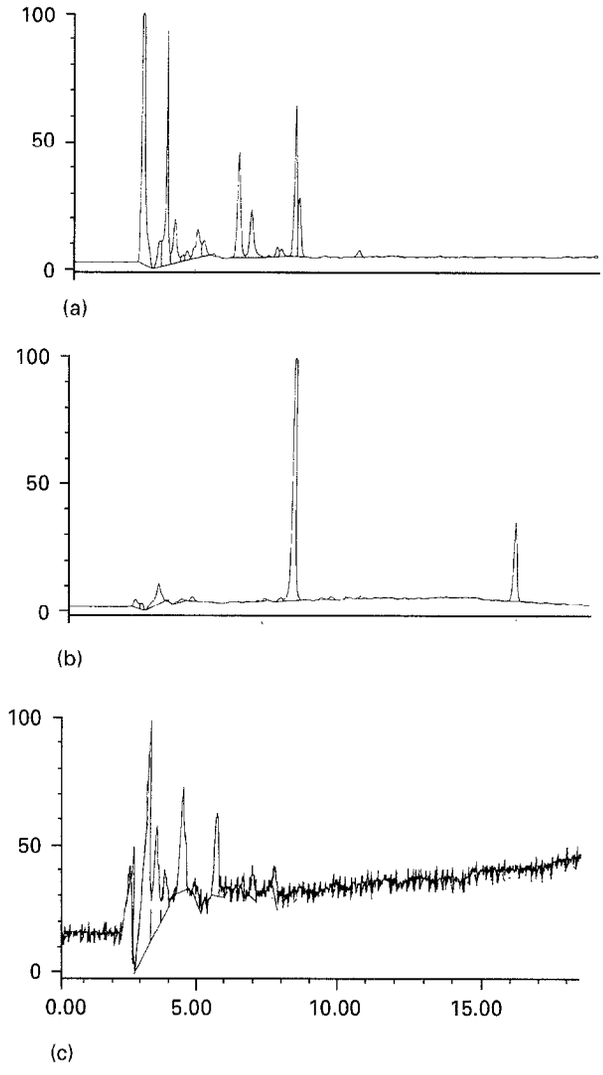

Figure 5 HPLC chromatograms of products extracted with diethylether from acidified (pH = 3) Dulbecco's culture medium containing 2 µg/ml PHB-P and used for incubation with macrophages for 8 days (a), containing 2 µg/ml PHB-P (b), and containing no PHB-P and used for incubation with macrophages (c). Chromatogram C is represented with a 20 times higher sensitivity.

3.5. HPLC analysis of organic extracts of cell supernatants

The macrophage cell line J774 was treated with non-toxic concentrations of PHB-P (2 µg/ml) for 8 d. After lysis of the cells and centrifugation of the culture, the supernatant was acidified to pH 3 and then extracted with diethylether. After evaporation of the solvent, the residue was redissolved in water and eluted with a gradient of water and CH_3CN in a RP C18 chromatographic column.

As shown in Fig. 5a several peaks were identified with an UV detector at 210 nm. The chromatogram showed significant differences from the control systems, (PHB-P and medium without cells; cells without PHB-P) that are shown in Fig. 5b and c, respectively. This observation suggests that water soluble degradation products of PHB-P are present in the cell supernatants after eight days of incubation.

4. Discussion

To our knowledge, only high-molecular-weight PHB was used in most of the available literature concerning the degradation of this material while nothing is

known about the degradability of low-molecular-weight PHB in a cellular environment. The published results are often contradictory concerning the degradation potential of high molecular weight poly [(*R*)-3-hydroxybutyric acid]. A study of *in vitro* hydrolysis reported no PHB degradation after 6 months [10]. Moreover, an investigation of PHB monofilament reported little evidence for *in vitro* or *in vivo* degradation after 180 days of subcutaneous implantation in rats, except when the polymer was pretreated with 10 Mrd of γ-irradiation [11]. Two *in vivo* studies report, however, degradation of PHB following 30 weeks of implantation in mice [2]. In addition, PHB degradation has been reported *in vivo* [12], although at a much slower rate than the polylactides: the molecular weight at the end of 6 months of implantation was reduced to 85% of its initial value.

In our experiments, both primary cultured peritoneal macrophages and cell line J774, showed signs of PHB-P degradation. The FACS and laser-cytometry analysis clearly support the observations coming from fluorescence microscopy that PHB-P do not remain inside macrophages for a long time after phagocytosis. At non-toxic concentrations, the number of PHB-P per cell was decreasing already after 4 d of incubation. This may be due to

1. cell damage and cell death — this possibility can be excluded since the MTT assay showed that the cell viability was not affected at the PHB-P or DPHB-P concentrations employed [6],
2. cell division and mitotic activity of the cells — this possibility can also be excluded since the decrease in the PHB-P number per cell would have resulted in an increase in the number of PHB-P positive cells, contrary to our observations;
3. terminal and selective cleavage of the dansyl group or quenching of fluorescence in the macrophages for some unknown reason — these possibilities seem quite improbable, however, because the fluorescence intensity per PHB-P particle remained unchanged during incubation times as measured in laser cytometry.

The finding of dansyl-fluorescent molecules in the culture supernatants at eight days of incubation suggests that a degradation process takes place in the macrophages. In fact, an exocytosis process could only produce water-insoluble molecules that would not be traced in the supernatants after centrifugation. An explanation of these observations with a selective cleavage of the dansyl group is also quite improbable because it was possible to trace water soluble materials with HPLC even in supernatants of cells incubated with non-fluorescent PHB-P. Although these products were not fully characterized, preliminary mass spectrometry analysis of the peaks indicated that the substances have a significantly lower molecular weight (200–300), than the starting material (PHB with $M_n = 2300$) [13], giving support to the hypothesis that macrophages can indeed degrade PHB.

5. Conclusions

The results obtained here clearly indicate that macrophages are able to take up PHB low-molecular-weight particles (2–20 μm). The decrease in the amount of intracellular particles with incubation time, as shown by the FACS analysis and laser cytometry, can be explained by either an exocytosis process or an active intracellular biodegradation of the polymeric material. The latter hypothesis is supported by the identification of possible water-soluble degradation products in cell supernatants after eight days of incubation by fluorescence spectroscopy and HPLC analysis. Further work is in progress to identify and characterize at least some of these products through spectroscopic techniques.

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