International Journal of Pharmaceutics, 84 (1992) 13–22

IJP 02712

Alkylcyanoacrylate drug carriers: II. Cytotoxicity of cyanoacrylate nanoparticles with different alkyl chain length

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(Received 2 August 1991)
(Modified version received 25 November 1991)
(Accepted 26 November 1991)

Key words: Alkylcyanoacrylate nanoparticles; Cytotoxicity; L929 fibroblast; Cell culture; Degradation

Summary

The cytotoxicity of four types of alkylcyanoacrylate particles was evaluated in L929 fibroblast cell cultures. The results revealed the ethyl- and isobutyl-derivatives to be the most toxic, the methyl-derivative to be of intermediate toxicity and the isohexyl-cyanoacrylate particles to have the lowest toxicity. The toxic effect was found to be correlated with the velocity of polymer degradation and the rate of release of the degradation products. The mechanism proposed to account for the observed cytotoxicity consists of the degradation of particles in the culture medium and/or of particles adhering to or in close proximity with the cell membrane. A contribution due to the internalization of particles by cells appeared to be negligible, if any, in the cytotoxicity of nanoparticles. Acute toxicity can be avoided by employing low doses of particles consisting of a slowly degrading cyanoacrylate polymer. This aspect is of interest in the development of a colloidal carrier for the chronic delivery of drugs. The use of such systems does not involve problems as a result of long-term toxicity during chronic treatments and the burden placed upon the body by overloading with polymeric drug carriers that undergo degradation more slowly, such as poly(hydroxybutyric acid) and poly(lactic acid).

Introduction

Polyalkylcyanoacrylate particles possess several properties making them of considerable interest as possible drug carriers for controlled drug delivery and drug targeting (Couvreur, 1988). Other polymers used for the production of particulate drug delivery systems such as poly(lactic acid) (PLA) and its co-polymers with glycolic acid (PLA/GA) degrade relatively slowly in vivo. For implants, degradation times ranging from 1 to 3 weeks for PLA/GA (50:50) and to 1 year for PLA (Tice and Cowsar, 1984) have been reported. For other polyesters like poly(hydroxybutyrate) (PHB), degradation in the body is unlikely to occur or is at least very slow (Koosha et al., 1989). The greater surface area of microspheres as compared to implants might accelerate this process to some extent. However, such slow degradation leads to the accumulation of non-de-

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graded polymer in the body during chronic treatment with multiple dosing.

In contrast, alkylcyanoacrylate polymers degrade relatively rapidly, depending on the alkyl chain length, within hours (methylcyanoacrylate) up to approx. 3 days (e.g., 80% of isobutylcyanoacrylate excreted within 3 days (Grislain et al., 1983)). The degradation of PLA, PLA/GA and PHB takes place by hydrolysis leading to the formation of lactic, glycolic and hydroxybutyric acids which are already present in the body. These acids are metabolic components and therefore as degradation products should be of low toxicity. The degradation of cyanoacrylates leads to the formation of alcohol together with poly(cyanoacrylic acid) (Lenaerts et al., 1984), compounds which could be more toxic than the acids mentioned above. However, in order to assess the relevance of the degradation products regarding possible toxic effects in humans, their rate of release during particle degradation and the maximum local concentrations must be considered.

Furthermore, it is essential to differentiate between systemic toxicity and toxicity at the cellular level. Implants of PLA and PLA/GA are well tolerated and of low systemic toxicity. However, at the cellular level, the death of macrophages after phagocytosis of PLA particles has been reported (Smith and Hunneyball, 1986). An excessively high cellular concentration of lactic acid creates cellular toxicity (e.g., shift in pH and metabolic disorders). Therefore, to assess the advantages and the drawbacks of cyanoacrylates as compared to polyesters, the release rates and cellular concentrations of the degradation products must be taken into account. Not only the systemic toxicity, but also that at the cellular level are relevant criteria.

In the case of multiple dosing (chronic treatment), the use of rapidly degrading cyanoacrylate carriers could be more appropriate in order to avoid overloading the cells with slowly degrading polyesters. The acute cellular toxicity of cyanoacrylate particles might be less damaging in the long term than the chronic toxic effect due to the excessive burden placed upon the cells by carrier polymer. In particular, drug delivery to macrophages using slowly degrading particles can lead to the functioning of the RES being impaired. Cell culture studies were performed in order to obtain information permitting the selection of the most suitable alkyl cyanoacrylates for use as drug carriers and helping in the elucidation of the mechanism of the eventual cytotoxic effect. Particle-cell interactions are influenced by the physico-chemical properties of the particles such as the degree of hydrophobicity of the surface (Absolom, 1986) or surface charge (Wilkins and Myers, 1966). Consequently, the relevant parameters for cyanoacrylate nanoparticles were evaluated in the preceding paper (part I (Müller et al., 1992)) employing a range of methods for characterization developed using surface-modified polystyrene model drug carriers (Müller, 1991).

Materials and Methods

Materials

Methyl- and ethylcyanoacrylate were provided by Loctite Ltd (Ireland), isobutylcyanoacrylate was purchased from Sigma (U.S.A.) and isohexylcyanoacrylate was obtained from Sopar (Belgium). Poloxamine 908 and Poloxamer 407 were supplied by BASF (Wyandotte, U.S.A.).

Cell culture medium (MEM) and related chemicals were obtained from Flobio (Paris, France), and fetal calf serum (FCS) from IBF (Paris, France).

Methods

Polyalkylcyanoacrylate particles were polymerized as described before (Couvreur et al., 1979), and particle coating was performed by incubation with Poloxamine 908 and Poloxamer 407 solutions (Müller et al., 1992). Particle sizes were determined by photon correlation spectroscopy (PCS) (Müller, 1991) using a Malvern Spectrometer in connection with a 4-Bit Correlator (Malvern Instruments, Malvern, U.K.). Charge measurements were carried out by laser Doppler anemometry using a Zetasizer II (Malvern Instruments).

Toxicity was evaluated in L929 fibroblast cell cultures. The cells were cultured in 75 cm^3 flasks

at 37°C under a 5% CO_2/air atmosphere in a humidified incubator. MEM culture medium was supplemented with 5% FCS and antibiotics (200 IU/ml penicillin and 100 μg/ml streptomycin). Before incubation with particles, cells were removed from the incubation flasks by trypsinization, seeded into 35 mm petri dishes containing 2 ml medium (10^5 cells/dish) and cultured for 48 h. After renewal of the culture medium, cells were incubated with the test substance (particles or degradation products) for a period between 24 and 72 h. To determine the total number of cells and the percentage of viable cells, cells were trypsinized and counted in a Thoma chamber after the addition of trypan blue. In those studies where an increase in cell number occurred, the percentage of living cells was distinctly above 90%. The proportion of dead cells was similar in controls and in incubated populations, demonstrating that it was not the result of a cytotoxic effect. This indicates that growth inhibition took place. In the cases where a reduction in the number of living cells was observed, the total number of cells (living and dead cells) remained constant. This demonstrates that cell growth was inhibited and that a certain percentage of inhibited cells died during the incubation period. Therefore, the number of living cells is plotted vs time in the figures.

To evaluate the toxicity of the degradation products, degradation of nanoparticles was performed in NaOH. The solution of degraded nanoparticles was then neutralized with phosphoric acid immediately before addition to cell cultures. Untreated cells and those incubated with non-degraded nanoparticles were used as controls.

Results and Discussion

Cytotoxicity of alkylcyanoacrylate particles

Incubation of fibroblasts with increasing concentrations of rapidly degrading PECA particles over a period of 72 h did not lead to inhibition of cell growth at 1 μg/ml culture medium. A distinct reduction in cell multiplication was observed at 10 μg/ml. Concentrations above 25 μg/ml resulted in death of the majority of cells after 24 h (Fig. 1).

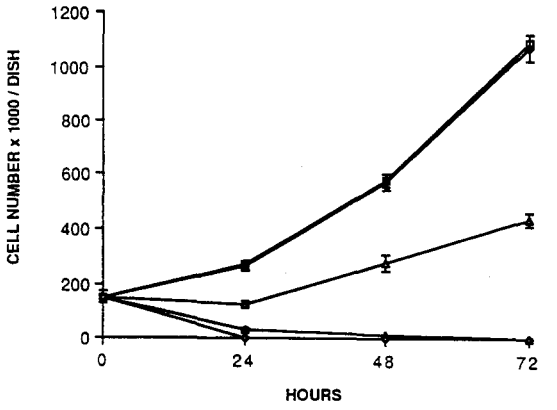

Fig. 1. Cytotoxicity of PECA nanoparticles on L929. Cell number as a function of time after addition of PECA at 1 (♦), 10 (△), 25 (▲), 100 (◇) μg/ml; (□) control.

Incubation with the slowly degrading PIHCA particles at concentrations up to 25 μg/ml did not significantly influence the increase in cell number; at a concentration of 100 μg/ml, they proved to be less cytotoxic than the ethyl derivative (Fig. 2).

Therefore, to compare the effects of all four types of particles, the concentration during incubation was chosen to be below 25 μg/ml in order to avoid 100% mortality of cells on incubation with the more cytotoxic PECA particles. At 20

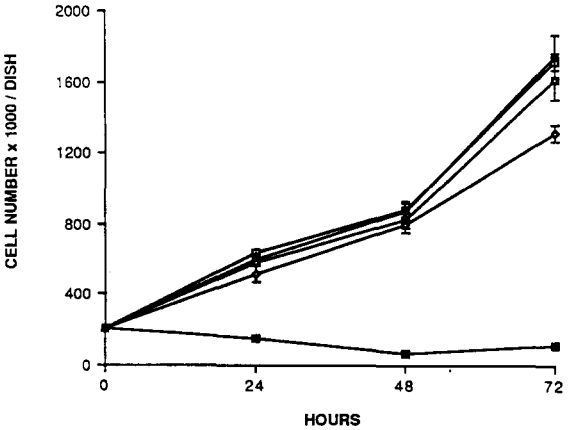

Fig. 2. Cytotoxicity of PIHCA nanoparticles on L929. Cell number as a function of time after addition of PIHCA at 1 (♦), 10 (□), 25 (◇), 100 (■) μg/ml; (⊡) control.

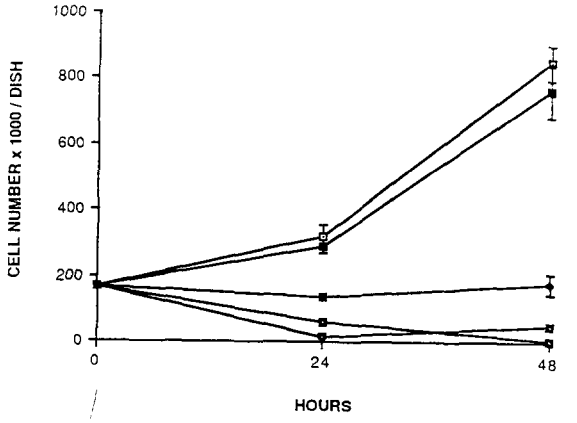

Fig. 3. Cytotoxicity of four different types of polyalkylcyanoacrylate particles on L929. Cell number over a period of 48 h incubation with each polymer at 20 μg/ml. (♦) PMCA, (□) PECA, (◇) PIBCA, (■) PIHCA; (⊡) control. Tween 20, used during the preparation of PMCA, was also added to the other at equivalent concentrations in order to compensate for any possible effect on cell growth by the surfactant.

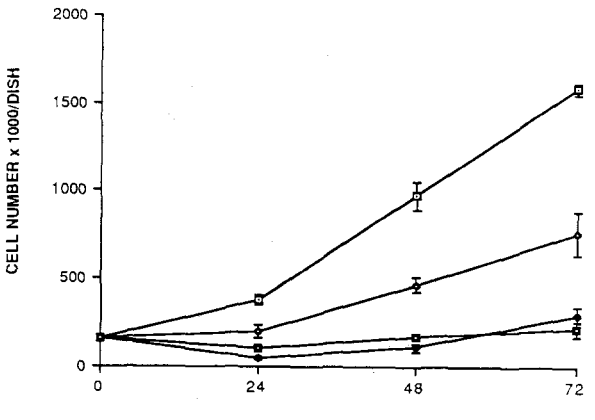

Fig. 4. Cytotoxicity of polyalkylcyanoacrylate particles on L929. Cell number over a period of 72 h incubation with 20 μg/ml polymer; incubation performed with dextran 70-stabilized (♦) PECA, (□) PIBCA and (◇) PIHCA particles; (⊡) control.

μg/ml and over a period of 48 h, the rapidly degrading PMCA inhibited increase in cell number, but did not lead to a reduction in the number of viable cells as observed for PECA (Fig. 3). To eliminate a possible effect of Tween 20 (Gipps et al., 1987) used during the preparation of PMCA particles, Tween 20 was added in equivalent concentrations to PECA, PIBCA and PIHCA particles. Both PECA and PIBCA reduced the number of living cells to a similar extent whereas the most slowly degrading PIHCA had no effect on multiplication of cells.

Incubation of cells with dextran 70-stabilized PECA particles over a period of 72 h revealed an initial decrease followed by a slight increase in cell number (Fig. 4). This effect was observed repeatedly. It is believed that the degradation products of the rapidly degrading PECA were metabolized, and that the cells recovered, starting to divide again. The same effect has been described elsewhere for human fetal lung fibroblasts (Gipps et al., 1987).

From these results, PECA and PIBCA were found to be of similar cytotoxicity and PMCA to be slightly less toxic, whilst PIHCA showed no cytotoxic effects at the concentration investigated. A cytotoxicity index was calculated by using the data from four independent experiments. In each experiment, the four types of particles were placed in order of decreasing cytotoxicity. To indicate the degree of toxicity, a numerical value was assigned to each particle type on a scale ranging from 4 (most cytotoxic) to 1 (least cytotoxic). Summation of the values led to an index providing a measure of the relative cytotoxicity between the four particle types (Fig. 5).

These results were found to be consistent with the ID_{50} values (dose required for 50% inhibition of growth of viable cells) determined for *n*-al-

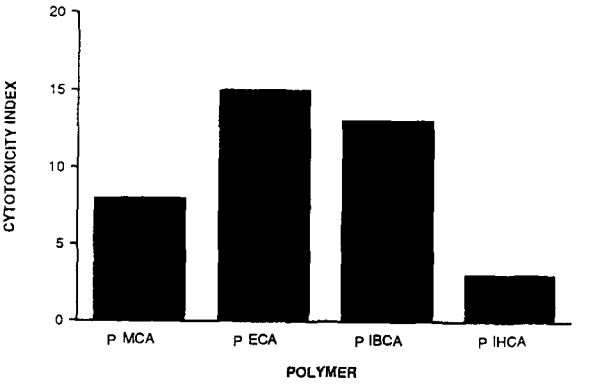

Fig. 5. Comparison of cytotoxicity index of PMCA, PECA, PIBCA and PIHCA nanoparticles as determined in four independent experiments (calculation as given in the text).

kylcyanoacrylate polymers on testing with human normal and malignant cell lines (Gipps et al., 1987). However, the latter studies were only performed over periods of 1–24 h. The greatest decrease in number of viable cells was observed for PECA and polybutylcyanoacrylate nanoparticles (PBCA). PMCA showed a smaller extent of reduction and the lowest degree of reduction was found for polyhexylcyanoacrylate particles (PHCA). The extent of inhibition of viable cells was correlated with the individual cell line, indicating the different sensitivities of the cells. In another cytotoxicity study with rat hepatocytes, PBCA was reported to have an LD_{50} value of 400 μg/ml culture medium (after 1 h incubation) and was found to be much more toxic than PHCA (Kreuter et al., 1984). Kante et al. (1982) determined cell mortality of 80–100% for PBCA at 150 μg/ml culture medium (hepatocytes, 4 h incubation). The measurement of acute toxicity after 1–4 h required higher polymer concentrations in order to elicit a cytotoxic effect in comparison with the present experiments for periods of up to 72 h.

Cytotoxicity of degradation products

At 25 μg/ml, the degradation products of PIBCA nanoparticles had no effect on cell growth whereas an equal concentration of the non-degraded PIBCA particles showed a distinct cytotoxic effect, reducing the number of living cells (Fig. 6). This might be explained by differences in cellular distribution of soluble degradation products and nanoparticles in suspension. The soluble degradation products become evenly distributed throughout the cell culture medium. In contrast, nanoparticles adhere partially to cell membranes. In addition, internalization may take place to some extent. Degradation of nanoparticles adhering to cells would lead to high local concentrations of degradation products on the cell membrane, which could damage the membrane, leading to inhibition of cell growth or death. Using the same procedure, incubation with formaldehyde and isobutanol, which are possibly formed via degradation of alkylcyanoacrylate particles (Lenaerts et al., 1984), led to no acute cytotoxic effect. This could lead one to suppose that the

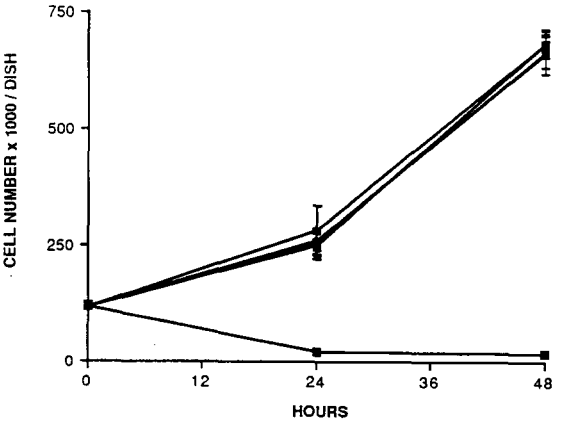

Fig. 6. Cytotoxicity of degradation products of PIBCA on L929. Cell number over a period of 48 h after incubation with each of the following at 25 μg/ml: (■) non-degraded PIBCA particles, (□) degraded PIBCA nanoparticles, (◇) isobutanol and (◆) formaldehyde; (⊡) control.

cells are capable of dealing with these compounds by metabolization (Fig. 6). Formaldehyde was found to be of similarly low cytotoxicity in hepatocyte cultures and it was concluded that the cytotoxicity of cyanoacrylate is not solely attributable to the formation of formaldehyde (Kreuter et al., 1984). However, high local concentrations of formaldehyde due to degradation of membrane-adherent nanoparticles could explain the higher cytotoxicity of particle suspensions as compared to formaldehyde solutions at equivalent concentration.

Incubation of the fibroblasts with a relatively high concentration of degraded nanoparticles (50 μg/ml) resulted in an identical toxic effect to that observed for non-degraded particles (Fig. 7). This suggested that the local concentration of degradation products leading to cell damage was around 50 μg/ml.

Mechanism of cytotoxicity

Damage to the cells could be due to external particles undergoing degradation in the culture medium and/or adhering externally to the cell membranes as well as to cell-internalized particles. To determine whether the external or internal mechanism contributes most to the observed cytotoxic effects, nanoparticles were coated with

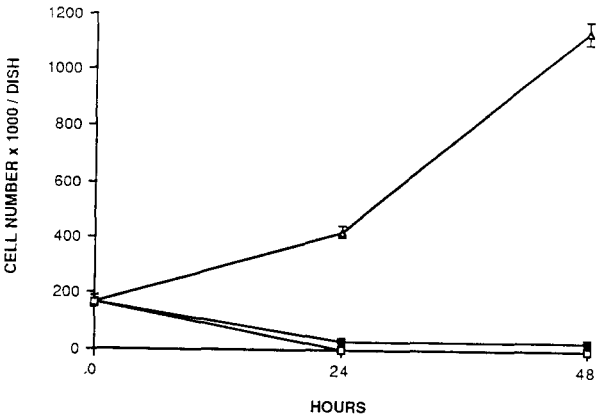

Fig. 7. Cytotoxicity of degradation products of PIBCA on L929. Cell number over a period of 48 h after incubation with (■) non-degraded PIBCA particles and (□) degraded PIBCA nanoparticles (both at 50 μg/ml). As control, untreated cells and (△) cells that had been treated with degradation medium were used. The increase in cell number for both controls was identical (only one growth curve shown).

Poloxamine 908 and Poloxamer 407 to avoid particle internalisation by the cells (Müller, 1991). Incubation of fibroblasts with uncoated and coated PIBCA nanoparticles resulted in identical cytotoxic effects (Fig. 8). This supports the proposal that the mechanism of cytotoxicity most probably involves the degradation of particles outside the cells rather than the internalization of nanoparticles. This is consistent with findings reporting that internalization of particles by non-phagocytotic cells does not occur to a significant extent (e.g., hepatocytes (Kante et al., 1982; Johnson et al., 1986)).

Bioadhesion of nanoparticles on cell membranes could lead – for relatively slowly biodegradable nanoparticles (e.g., PIBCA) – to a high local concentration of cytotoxic compounds. Very rapidly degrading alkylcyanoacrylate particles (e.g., PMCA) possess a half-life of less than 0.5 min in culture medium (Müller et al., 1992). For such particles, the observed cytotoxicity should mainly be due to the concentration of degradation products in the culture medium, with only a minor contribution being made by particles adhering to membranes. These particles probably undergo degradation before substantial adherence or even phagocytosis can take place. Adhesion and phagocytosis are time-dependent processes, e.g., phagocytosis attains maximum effect in cell cultures after approx. 1 h (Roberts and Quastel, 1963; Muller and Schuber, 1986). Finally, slowly biodegradable PIHCA was found to be the least toxic since the release of degradation products was slower, thereby preventing high local concentrations even in close proximity to the cell membrane. Therefore, the observed cytotoxicity could be considered as a superposition of the toxic effects exerted by degradation products in the culture medium and by particles adhering to cell membranes. Morphological examination of dead cells under SEM revealed completely perforated membranes (Kante et al., 1982). The presence of holes in the membranes supports the contention that particle attachment is an additional mechanism of cytotoxicity. The contribution of each effect depends on the degradation velocity of the nanoparticles leading to the observed relative cytotoxicities of the four alkylcyanoacrylates (Fig. 5). Quantification of the contribution of each effect was difficult to perform, however, a model illustrating the possible contributions is given in Fig. 9.

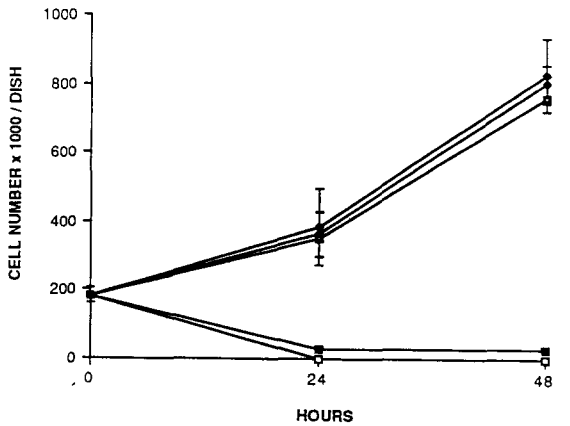

Fig. 8. Cell number over a period of 48 h after incubation with PIBCA particles (25 μg/ml) coated with Poloxamine 908 (□) and particles coated with Poloxamer 407 (■). The coating prevents internalization of the particles by the macrophages and the measured toxic effect is caused by particles degrading outside the cells. Controls: cells incubated with culture medium containing Poloxamine 908 (◆) and Poloxamer 407 (■); Control (◇).

Influence of particle size on cytotoxicity

To study the effect of nanoparticle size, PIHCA of 50 and 200 nm particle sizes was prepared by variation of the surfactant concentration (2.0 and 0.1% Poloxamer 188, respectively (Müller et al., 1992)).

In repeated experiments ($n = 3$) the smaller particles proved to be more toxic than the larger type (Fig. 10). These findings provide support for the consideration that the toxic effect was caused by particles degrading outside the cells. Indeed, cyanoacrylate particle degradation takes place via a surface degradation process (Lherm, 1990). Due to their larger surface area the smaller particles degraded much faster, leading to higher concentrations of degradation products in the cell culture medium. In the case of particle internalization, larger particles should have been more toxic because the total polymer mass taken up generally increases with increasing particle size (Johnson et al., 1986).

Toxicity of cyanoacrylates compared to polyesters

Incubation of the fibroblasts with polyhydroxybutyrate (PHB) particles did not influence cell growth (Lherm et al., 1989). Degradation of PHB particles was so slow that the amounts of degradation products released, if any, were too small to cause any growth inhibition. In contrast, incubation of the cells with more rapidly degrading PLA/GA at a concentration of 250 μg/ml led to inhibition of cell growth (Lherm et al., 1989). This was probably due to the release of degradation products, since no toxic effect was observed with non-degraded PHB particles. The toxic effect of polyesters is even more distinct when the particles are internalized by macrophages. Smith and Hunneyball (1986) observed 100% mortality in cultures of peritoneal macrophages at a PLA microsphere concentration of 75 μg/ml culture medium. The lower cytotoxicity of polyester compared to cyanoacrylate particles is therefore partially due to the slow release of degradation products. This should be considered when comparing the polymers.

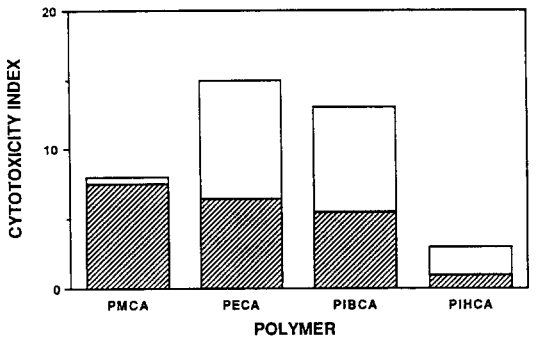

Fig. 9. Possible contributions (model) to the observed cytotoxicity: cytotoxic effect caused by nanoparticle degradation products in the cell culture (hatched bars), cytotoxic effect caused by degrading nanoparticles adhering to cell membranes (open bars). The fastest degrading PMCA exerts little toxicity due to particle adherence; particle adherence is the dominant factor for the slowest degrading nanoparticles (PIHCA). The toxic effect due to particle adherence (open bars) diminishes with increasing degradation half-life of the particles from PECA and PIBCA to PIHCA.

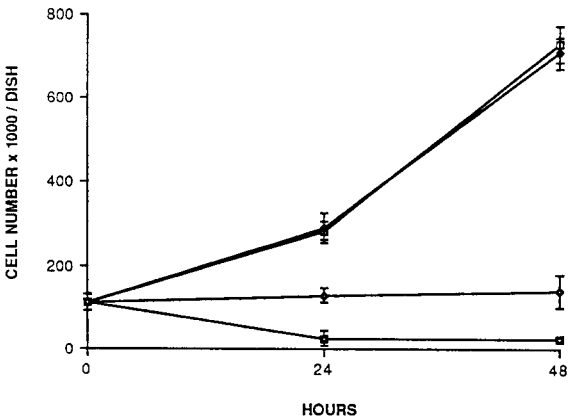

Fig. 10. Cell number over a period of 48 h after incubation with 50 nm (■) and 200 nm (◇) PIBCA particles (20 μg/ml). Untreated cells (□) and cells treated with the polymerisation medium (♦) were used as controls.

Conclusions

Among the different alkylcyanoacrylate particles, the ethyl and isobutyl derivatives comprised the highest relative cytotoxicity, the methyl type being less toxic and the isohexyl form the least toxic.

The toxicity of the methyl and ethyl derivatives can be explained by their rapid degradation lead-

ing to the burst release of toxic degradation products. The hydrolytic degradation of alkylcyanoacrylate polymers (Leonard et al., 1966) and nanoparticles (Müller et al., 1992) increases in extent with decreasing alkyl-chain length. There is some indication that the cells recover after metabolization of the degradation products. This capacity for metabolization and recovery are of interest for the chronic administration of cyanoacrylate particles and could explain the discrepancy between in vitro cytotoxicity and in vivo toxicity assays (Brasseur et al., 1983).

Isobutylcyanoacrylate was found to be comparable to the ethyl derivative in toxic effect, despite the markedly slower degradation velocity (Müller et al., 1992). A possible explanation is that the particles could adhere to the fibroblasts and become degraded close to the cell membrane. The degradation velocity was still fast enough to create high local concentrations of degradation products leading to cell membrane damage. The contribution of internalized particles appears to be minor as could be concluded from the results obtained with particles protected against phagocytosis.

Isohexylcyanoacrylate possessed the lowest toxicity due to slow degradation which allowed time for the cells to metabolize the products.

Physico-chemical characterization of the particles revealed similar properties with regard to charge, interaction with charged serum components and surface hydrophobicity whereby the isohexyl derivative seemed to be slightly more hydrophobic (Müller et al., 1992). From the results it was concluded that the influence of these parameters on toxicity was not large. However, size influenced cytotoxicity, since it is one of the factors determining the release of degradation products. During the degradation of cyanoacrylate particles a process of surface erosion takes place as shown by previous PCS measurements (Müller et al., 1992). The specific surface area increases with decreasing particle size, resulting in the faster release of degradation products and greater cytotoxicity for smaller particles.

The kinetics of release of the degradation products were clearly found to be the determining factor for cytotoxicity. The low toxicity of polyesters such as PHB and PLA is most probably due to their slow degradation. In cell culture studies PHB can be regarded as an inert material such as non-biodegradable polystyrene. In the case of more rapid degradation (e.g., PLA/GA), cytotoxic effects were also observed. Cytotoxic effects in macrophage cultures have also been described previously for PLA (Smith and Hunneyball, 1986).

Drug delivery devices made from polyesters such as poly(lactic acid) (PLA) are registered by the regulatory authorities (Zoladex®, ICI, in the U.K. and Germany). However, the degradation times of these implant devices are relatively long. Nanoparticles made from polyester polymers possess a larger surface area than implants which should result in increased degradation velocity on hydrolysis. However, in vivo degradation data are sparsely available to date. This would support considerations of preferring rapidly degrading cyanoacrylate particles with higher acute cytotoxicity for chronic treatment to slowly degrading polyesters which could lead to accumulation of polymer in the body (e.g., blockade of the macrophage system). They might solve the likely problems of long-term toxicity caused by overloading of the body with slowly degrading polymeric particles.

These investigations suggest that introduction of cyanoacrylate particles into therapy (at least for cancer treatment) might be possible by optimization of the polymer dose and release kinetics of the degradation products in order to avoid acute toxicity. Alkylcyanoacrylate particles should degrade slowly enough:

(1) to prevent high local concentrations of degradation products occurring; and

(2) to release degradation products at a rate at which they can be metabolized without causing acute toxic effects. For PIHCA nanoparticles, no cytotoxic effects were observed at a concentration of 25 μg/ml cell culture medium. Assuming 10^{12} nanoparticles per ml (Kante et al., 1982), this corresponds to 50000 nanoparticles/cell in the dish. Injecting a nanoparticle dose of 20 mg/kg in vivo (PIBCA and hexylcyanoacrylate, Wistar rats), no toxic effects could be attributed to the particles after histopathological examination

(Brasseur et al., 1983). Assuming 10^8 hepatocytes, 42×10^6 endothelial cells and 12×10^6 Kupffer cells per rat liver (3-month-old rats, 140–170 g) (Knook and Sleyster, 1980) and 60% accumulation of the particles in this organ (Grislain et al., 1983), this dose is equivalent to approx. 1600 nanoparticles per liver cell (assuming even distribution throughout the liver) or 20000 nanoparticles per Kupffer cell (assuming maximum concentration by 100% localization in Kupffer cells). These numbers are in the range of or below the number of non-toxic particles/cell in the culture but sufficient for administration of a drug dose in chemotherapeutic treatment (e.g., with doxorubicin; Chiannilkulchai et al., 1990). The polymer dose of 20 mg/kg is well below the LD_{50} for PIHCA as observed, e.g., in mice (> 500 mg polymer/kg; Brasseur et al., 1983). Slowly degrading cyanoacrylate particles can therefore be administered in vivo in such a dose that acute toxicity can be avoided.

Acknowledgement

This research was supported by the European Community within the framework of the program 'Biotechnology', for which we would like to express our sincere thanks.

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