International Journal of Pharmaceutics, 40 (1987) 23–31
Elsevier

IJP 01337

The effects of polyalkylcyanoacrylate nanoparticles on human normal and malignant mesenchymal cells in vitro

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(Received 17 December 1986)
(Modified version received 1 May 1987)
(Accepted 25 May 1987)

Key words: Polyalkylcyanoacrylate; Nanoparticle; Mesenchymal cell; Osteogenic sarcoma; Toxicity; Morphology

Summary

The interaction of a range of polyalkylcyanoacrylate nanoparticles with human mesenchymal malignant and normal cells was studied in vitro. The effects on cell viability and morphology (observed in transmission electron microscopy) were determined. Those nanoparticles based on polymers with shorter alkyl side chains were more toxic to the cells. Toxicity was also dependent on time, concentration, and the surfactant used in manufacture, but was independent of cell type. The adverse effects were reflected in TEM by loss of adhesion, followed by dilation of the rough endoplasmic reticulum. Perforation of the cell membrane occurred late in the sequence of damage.

Introduction

Solid particulate drug delivery systems or carriers for specific organ targeting should either increase the effect or decrease the side-effects of the drug carried. The interaction of these carriers with the target and non-target organs or cells contributes to the assessment of these systems. One form of carrier is polyalkylcyanoacrylate nanoparticles. These are polymeric colloidal particles with a diameter, normally, between 100 and 500 nm (Kreuter, 1983). Among those polymers used, have been: polymethylcyanoacrylate (pMCA), polyethylcyanoacrylate (pECA), polybutylcyanoacrylate (pBCA), polyisobutylcyanoacrylate (piBCA) and polyhexylcyanoacrylate (pHCA) (Couvreur et al., 1979; Kante et al., 1982; Kreuter et al., 1984).

In vitro, endocytosis has been reported as well as toxicity. Incubation of low concentrations of piBCA nanoparticles with mouse peritoneal macrophages appeared to result in endocytosis of the carrier (Guoit and Couvreur, 1983). However, treatment of the same cell type with higher doses of pMCA nanoparticles for 1 h resulted in perforation of the cell membrane as observed by scanning electron microscopy, while pBCA nanoparticles produced no such damage (Kante et al., 1982). Treatment of rat hepatocytes with pBCA

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nanoparticles indicated that cell membrane integrity, as measured by dye exclusion, and enzyme leakage were dependent on time and concentration (Kante et al., 1982). The latter property was confirmed by Kreuter et al. (1984), who also showed that pHCA nanoparticles were less toxic than the homologue with the shorter side chain, pBCA, against rat hepatocytes.

For the study presented here, the interaction of the various cell types and nanoparticles was studied in relation to the surfactant used in manufacture, length of the alkyl side chain and concentration of the nanoparticles, plus time of exposure and cell type. Due to the large number of parameters the effect of the nanoparticles on the viability of the cells was ascertained first. This allowed the levels to be chosen at which the morphology of the cells, in the presence of nanoparticles, was examined. The cells were selected as examples of possible targets (sarcoma cells), plus normal supportive cells (fibroblasts).

Materials and Methods

Nanoparticles

pMCA, pECA, pBCA and pHCA nanoparticles were produced by dispersing 1% of the monomer by mechanical stirring in an aqueous solution containing 0.05 M HCl (Siegfried, Switzerland), and 1% dextran 70 (Pharmacia, Sweden), and a surfactant, either 0.05% poloxamer 188 (BASF, Switzerland) or 0.2% polysorbate 20 (Atlas, U.S.A.). After polymerization was complete, the pH was adjusted to 7.0 and the suspension was made isotonic. When the suspension was not to be used directly, the nanoparticles were lyophilized.

Cell culture

Both normal and malignant mesenchymal cells derived from human tissue were used. These included 4 established cell lines: two osteogenic sarcomas (designated T and KN), a round cell (Ewing's) sarcoma (R), and a malignant fibrous histiocytoma (S). The origin and characteristics of the sarcoma cell lines have been been described previously (Hofmann et al., 1985). Human fetal lung fibroblasts (HLF) served as normal cells.

The cells were mycoplasma-free as determined by transmission electron microscopy, TEM, or chemiluminescence (Bertoni et al., 1985).

In general, the cells were grown in Iscove's modified Dulbecco's medium, IMDM, (KC Biological, Lenexa, U.S.A.) supplemented with 10% fetal calf serum (Gibco, Basel, Switzerland). The cell line T was grown in RPMI 1640 (Amimed, Basel, Switzerland), with 20% fetal calf serum and 2% HEPES buffer (Sigma, St. Louis, U.S.A.). Antibiotics, giving a final dilution of penicillin 100 IU/ml and streptomycin 100 μg/ml (Inotech, Wohlen, Switzerland), and L-glutamine, 2 mM, (Flow Labs., Baar, Switzerland) were added to all media, the pH of which was adjusted with sodium bicarbonate (Merck, Darmstadt, F.R.G.). The cells were maintained as monolayers in flasks at 37 °C in 2.5% carbon dioxide with 90–95% relative humidity and subpassaged by recovery with trypsin 0.25% (Difco, Michigan, U.S.A.) in phosphate-buffered saline pH 7.4 (PBS) (Oxoid, Oxford, U.K.) after washing with the appropriate medium without serum.

The cells were counted after trypsinization, with a Coulter Counter, or with a haemocytometer by the Nigrosin dye (Fluka, Buchs, Switzerland) exclusion test. A third method, uptake of Neutral red dye, was used to determine the number of cells surviving the various treatments.

Determination of viable cell number by Neutral red dye uptake

The number of viable cells after treatment was determined through uptake of the vital stain, Neutral red. The dye was extracted from the cells and the UV absorbance was measured in a procedure adapted from Oku and associates (1982). Microtitre plates (96 flat-bottomed wells, Petra-Plastik, Switzerland) were seeded with cells in 100 μl of medium per well. The number of cells seeded was dependent on the time allowed for growth to a confluent monolayer, which varied between the different cell lines, usually 2–4 days.

Nanoparticles suspended in 50 or 100 μl of medium was incubated with the cells for the appropriate time. Each well was then rinsed with 100 μl PBS; 50 μl of 0.05% Neutral red (Merck, Darmstadt, West Germany) solution was added to

each well. This solution had been made from a stock solution of Neutral red 1% in 0.1 M sodium hydrogen phosphate (Fluka, Buchs, Switzerland) diluted with IMDM containing 2% fetal calf serum. The plate was incubated for 40 min. After rinsing with PBS, the dye was extracted with 200 μl of 0.05 M sodium hydrogen phosphate in 50% aqueous ethanol. After 20 min the absorbance was read at 550 nm by means of a fixed wavelength spectrophotometer (Virion, Rüschlikon, Switzerland). One row of cells which received fresh medium only served as treatment control and one row received only the alcoholic solution as apparatus control.

The mean $\pm$ S.D. of absorbance for each row of treated cells was calculated, and the LD_{50}, the level at which a 50% inhibition of viable cell number occurred, compared to untreated cells, was determined. In addition, the means of the untreated and treated rows were compared (Student's t-test, $P < 0.05$).

Verification of Neutral red uptake procedure

To confirm the linear relationship between Neutral red uptake and the number of viable cells, a geometric dilution series for each cell line was incubated until the most densely seeded wells had grown to a confluent monolayer. The number of viable cells was determined for one-half of the plate by the Neutral red uptake procedure, and for the other half, after trypsinization, by Nigrosin dye exclusion or with the Coulter Counter.

Effects of surfactants on cells

The effects of the surfactants, polysorbate 20 and poloxamer 188, used in manufacture were examined. The cells were treated for 1 h with a dilution series of 0.01–10% of the selected surfactant in medium.

Effects of nanoparticles with different surfactants on cells

Nanoparticles, made from pMCA, pECA, pBCA and pHCA in association with either polysorbate 20 or poloxamer 188, were used in a dilution series, 0.001–1%, calculated as the polymer, to treat the cells for 1 h. The concentration of polysorbate 20 was always one-half, and that of poloxamer 188 one-fifth, of the stated polymer concentration.

Time scheduling experiments

pMCA, pECA, pBCA and pHCA nanoparticles, manufactured with poloxamer 188, were incubated for various times with the cell monolayers. The dilution series was contracted to 0.01% to 1% polymer (with one-fifth of the surfactant). The treatments lasted one, two and 24 h.

Effect of glycine on toxicity of polyhexylcyanoacrylate nanoparticles

Glycine, present in normal IMDM medium (30 μg/ml), is a non-essential amino acid which would react with excess aldehyde groups. Additional and increasing amounts of glycine (finally, 60, 100, 300 and 1000 μg/ml) were added to the medium with 3 different concentrations of pHCA nanoparticles (0.01, 0.1 and 0.5%) for 1 and 24 h.

Morphology

After determination of the concentrations at which toxicity was produced, the morphology of the cell damage was investigated by transmission electron microscopy (TEM). The cell line S was incubated with pMCA and pECA for periods between 1 and 24 h. The cell lines R, T, and HLF were incubated with pBCA and pHCA nanoparticles for between 1 and 48 h. The concentration most commonly used was 0.01%, but 0.1% was also used. After treatment had finished, the cell monolayer was fixed with 2.5% glutaraldehyde and 0.8% paraformaldehyde in 0.05 M cacodylate buffer (310 mOsm/ml, pH 7.4) for 30 min at 4° C. Postfixation was performed using aqueous osmium tetroxide 1% and potassium ferrohexacyanide 1.5%. The cells were scratched from the plastic with a rubber spatula, washed with cacodylate buffer, and transferred into a 2.5% solution of agar in 0.05 M cacodylate buffer. After dehydration in a progressive alcoholic series, the specimens were embedded into Epon. Ultrathin sections (50 nm) were contrasted with uranyl acetate 4% and lead citrate (Reynold's solution), and studied with a TEM 420 (Philips).

Results and Discussion

Cell culture

The number of cells, as determined with the

Coulter Cytometer or Nigrosin dye exclusion correlated with the amount of Neutral red dye taken up by the cells. The correlation coefficients are shown in Table 1. The staining of viable cells by this method allowed quantitation of the effects of the nanoparticles. For presentation of the results, the choice of 50% inhibition of viable cells to compare results was arbitrary but allowed examination of trends.

After the cells had been treated for 1 h with surfactant, a dose-related decrease in the number of viable cells was seen. As shown in Table 2, treatment with polysorbate 20 produced a more marked toxicity than poloxamer 188. In both cases, the number of viable cells decreased with increasing surfactant concentration. The cell line T was most resistant to both surfactants, while the fibroblasts, HLF, were the most sensitive. Thus, for the individual cell lines, such as HLF, which were most sensitive to polysorbate 20, the presence of the surfactant may influence the toxicity in conjunction with the nanoparticles. Poloxamer 188 appeared to be the more suitable surface active agent under the circumstances.

The concentrations of nanoparticles at which the viable cell number was halved as compared to untreated controls (LD_{50}), are shown in Tables 3 and 4, for polysorbate 20 and poloxamer 188, respectively. As stated previously, these nanoparticles had not been separated from the polymerization milieu. As was to be expected those nanoparticles made in association with polysorbate 20 elicited greater toxicity, due to the surfactant.

This toxicity was related to the length of the alkyl side chain. The hydrolytic degradation of these polymers has been shown to decrease with increasing side chain length (Leonard et al., 1966).

A linear relationship was, however, not found. In general, treatment with pECA and pBCA produced the greatest decrease in viable cell number, while pHCA nanoparticles elicited the least reaction. This reflects the finding by Lehman et al. (1966) that, of a range of these polymers, the pECA derivative was the most toxic against *Staphylococcus aureus* and *Escherichia coli*. These authors suggested that the toxicity could be due to an alkyl acetate, in addition to the alcohol. Since the toxicity of alcohols decreases with increasing chain length (Albert, 1979), the rate of breakdown of the polymer may be the determinant of the rate of toxicity.

TABLE 1

Correlation coefficients (r) of cell numbers determined by Neutral red dye uptake compared to other procedures (Nigrosin dye exclusion or Coulter Cytometer)

Cell line	*r*
T	0.9971
KN	0.9965
R	0.9965
S	0.9861
HLF	0.8385

TABLE 2

Concentration (%) of the surfactant at which the LD_{50} was found after treatment of the cells for 1 h

Cell line	Poloxamer 188	Polysorbate 20
T	*	1
KN	>1	0.1
R	*	0.2
S	*	1
HLF	>1	0.05

>1, an LD_{50} was not found but a significant decrease in viable cell number occurred.

*, neither LD_{50} nor significant decrease in cell number were found (Student's *t*-test, $\alpha = 0.05$).

TABLE 3

Concentration (%) of polyalkylcyanoacrylate nanoparticles with associated polysorbate 20, at which LD_{50} was found after treatment of cells for 1 h

Cell line	pMCA	pECA	pBCA	pHCA
T	0.2	0.2	0.2	*
KN	1	1	0.2	*
R	1	*	0.5	1
S	>1	>1	>1	>1
HLF	0.02	0.2	*	1

>1, an LD_{50} was not found but a significant decrease in viable cell number occurred.

* neither LD_{50} nor significant decrease in viable cell number were found (Student's *t*-test, $\alpha = 0.05$).

TABLE 4

Concentration (%) of polyalkylcyanoacrylate nanoparticles with associated poloxamer 188, at which LD_{50} was found after treatment of the cells for 1 h

Cell line	pMCA	pECA	pBCA	pHCA
T	*	1	1	>1
KN	>1	0.02	>1	*
R	>1	*	*	*
S	>1	>1	>1	*
HLF	>1	>1	*	*

>1, an LD_{50} was not found but a significant decrease in viable cell number occurred.
* neither LD_{50} nor significant decrease in viable cell number were found (Student's *t*-test, $\alpha = 0.05$).

TABLE 5

Concentration (%) of polyalkylcyanoacrylate nanoparticles with associated poloxamer 188, at which LD_{50} was found after treatment of the cells for stated times

Nanoparticle	Cell line	1 h	2 h	1 Day
pMCA	T	>1	0.5	0.5
	KN	>1	>1	1
	R	>1	>1	1
	S	>1	>1	0.05
	HLF	0.5	>1	>1
pECA	T	0.5	0.1	0.1
	KN	>1	0.1	0.5
	R	1	*	*
	S	*	*	0.1
	HLF	0.05	0.05	>1
pBCA	T	>1	0.01	0.1
	KN	*	>1	1
	R	>1	>1	>1
	S	>1	>1	0.01
	HLF	0.1	0.01	>1
pHCA	T	>1	>1	1
	KN	*	*	1
	R	>1	>1	>1
	S	>1	>1	0.05
	HLF	0.1	0.05	>1

>1, an LD_{50} was not found but a significant decrease in viable cell number occurred.
* Neither LD_{50} nor significant decrease in viable cell number were found (Student's *t*-test, $\alpha = 0.05$).

Decrease in cell number was also related to individual cell line. For those nanoparticles manufactured in association with polysorbate 20, the S cells were most resistant while the T and HLF cells were most sensitive. In contrast, for nanoparticles made with poloxamer 188, the R cells were most resistant and the KN cells were most sensitive.

The results of the time scheduling experiments are listed in Table 5. In general, with prolonged times, there was a slight tendency for a decrease in the number of viable cells found. The sensitivity of the cell lines to the polyalkylcyanoacrylate nanoparticles varied. R remained the most resistant to the adverse effects. In contrast, at 1 and 2 h, HLF were the most sensitive. However, after 24 h the number of viable HLF cells determined was relatively high, indicating an apparent resurgence of viability.

The additional amounts of glycine in the media had no effect on the interaction of the cells with the nanoparticles, as determined by viable cell number.

Morphology

When examined by TEM all cell lines showed a similar morphological pattern of damage after treatment with the nanoparticles. Increasing concentration and shorter alkyl side chain raised the number of dying cells.

The morphological alterations observed after treatment with pBCA nanoparticles are illustrated in Figs. 1–3 on the human round cell sarcoma line, R. In this context it is appropriate to mention that neither single nor aggregated nanoparticles were detectable in the fixed and embedded specimens with TEM.

Untreated R cells grew in a densely packed monolayer (Fig. 1). The individual cell had an elongated nucleus with single or multiple distinct nucleoli. In the cytoplasm, a few ovoid mitochondria, short profiles of rough endoplasmic reticulum (rER) and large accumulations of glycogen granules were observed. The first stages of cell damage are demonstrated in Fig. 2. The cells started to become rounder, resulting in dilation of the extracellular space. The extent of rounding and detachment varied between the cell lines. Within the next stages of toxic damage, the cells

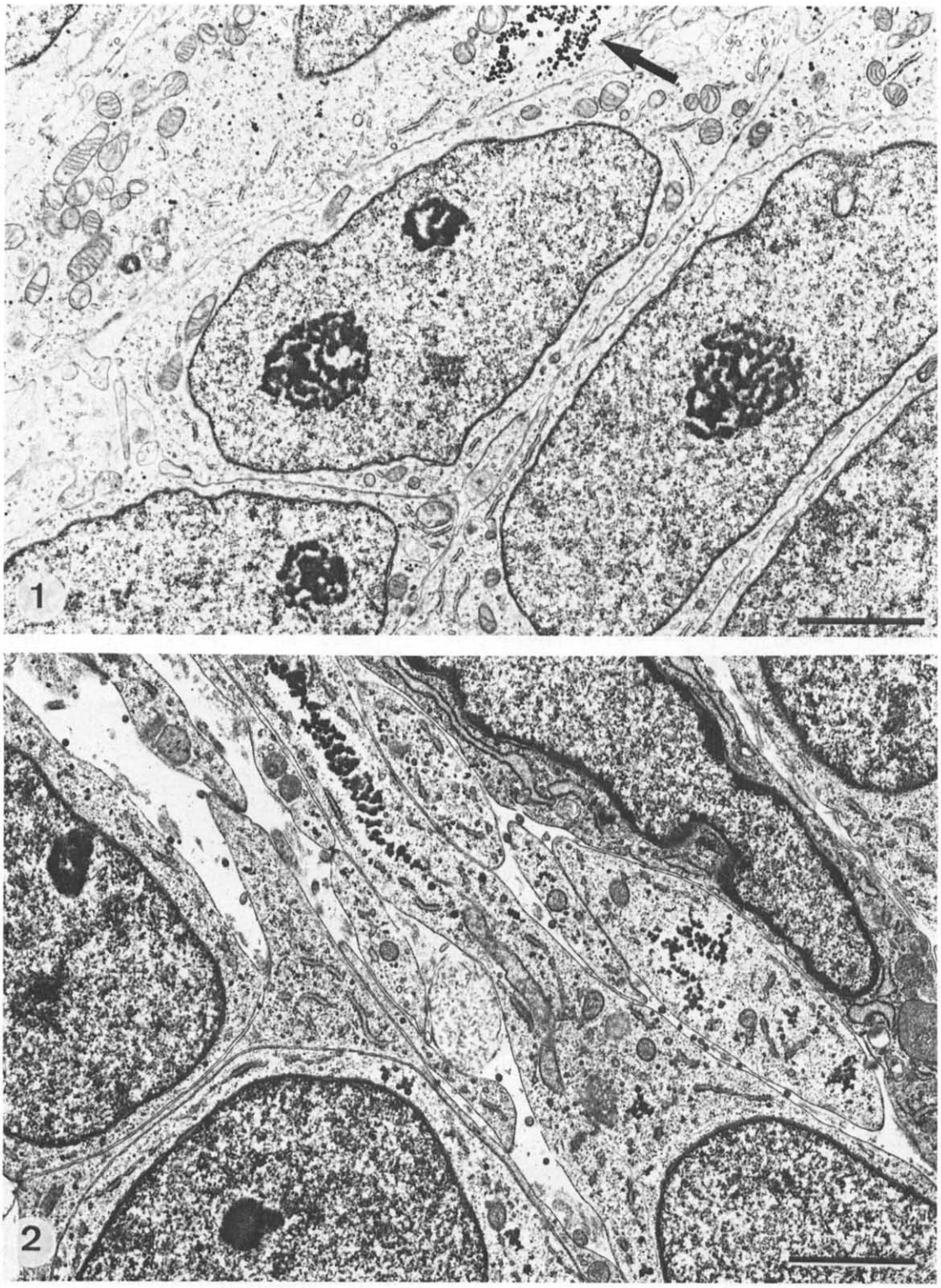

Fig. 1. Untreated R cells. Arrow indicates glycogen field. Bar = 5.4 μm.

Fig. 2. R cells after treatment for 1 h with 0.01% pBCA nanoparticles. Note expanded extracellular space. Bar = 4.4 μm.

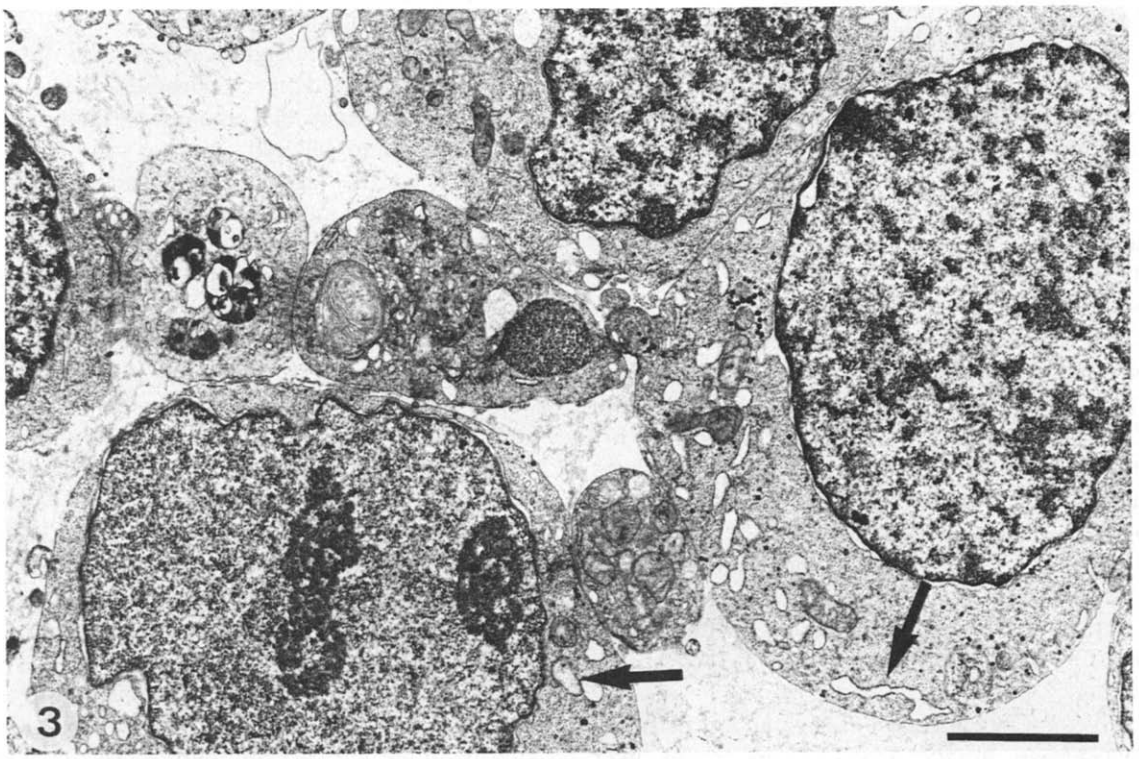

Fig. 3. R cells after treatment for 2 h with 0.01% pBCA nanoparticles. Arrows indicate dilated ER profiles. Bar = 3.44 μm.

began to swell (Fig. 3). The nucleus was characterised by disorganization of the chromatin structure. In the cytoplasm the rER profiles appeared dilated. This may be due to direct damage to the endoplasmic reticulum, or possibly the cells attempt to produce enough enzymes to cope with the environment produced by the nanoparticles. The cells would finally exhaust their capacity to synthesise the enzymes. These enzymes, particularly those for endocytosis are produced in the rER (Cohn and Fedorko, 1969). The mitochondria showed an electron-dense matrix and the glycogen fields were loosened. However the cell membrane remained intact.

Cell death was associated with distinct shrinkage and total disorganisation of the cytoplasm and nucleus. The nuclear membrane was destroyed. Perforation of the cell membrane, detected by Kante et al. (1982) occurred late in the sequence of damage.

The pattern of cell damage was similar to that reported by Hegyeli (1973), after incubation of various polyalkylcyanoacrylates with embryonic chicken liver. In general the cells tested within the present study demonstrated no convincing evidence of phagocytosis. In only one cell line, the round cell sarcoma, R, membrane-bound vacuoles with clear content were found in the cytoplasm of some cells (not shown), but the possibility of this being caused by the dextran in the polymerization milieu cannot be excluded.

This lack of interaction was also demonstrated by Illum et al. (1983), using another osteosarcoma cell line. They however showed that binding of nanoparticles to the cells occurred when specific monoclonal antibodies were used. The endocytosis reported by Guoit and Couvreur (1983) was achieved with cells of epithelial, rather than mesenchymal origin with a high phagocytic capacity.

Conclusions

The results show that incubation of polyalkylcyanoacrylate nanoparticles with a range of normal and malignant mesenchymal cells resulted in toxicity. This was related to the surfactant used in manufacture, the length of the alkyl side chain, concentration and time. It was independent of cell type, whether the cells were normal or malignant, and also varied between related tumors.

The cause of the adverse effects is likely to be the hydrolytic or metabolic degradation products. If the polyalkylcyanoacrylate nanoparticles are not to be endocytosed, they would be subject to hydrolytic degradation to an alcohol and a more water-soluble polymer, which in turn may degrade producing formaldehyde. Diffusion of the alcohol into the cells may provide the active toxin, since the larger more water-soluble polymer is unlikely to diffuse into the cell, and the formaldehyde should be inactivated by the excess protein in the medium.

In vivo, a large portion of the nanoparticles are phagocytosed by the Kupffer cells in the liver (Lenaerts et al., 1984b). In an acidic environment, as in the lysosome, production of both the alcohol (Grislain et al., 1983) and formaldehyde (Leonard et al., 1966) is slowed. The alcohol is sufficiently small to pass through the lysosomal membrane, and in vivo could then be metabolized by hepatic microsomes. The backbone of the polymer may be susceptible to degradation by lysozyme (Wood et al., 1985).

Until the polymer has been degraded to one or two subunits of cyanoacetate, it would not diffuse out of the lysosome (Lucy, 1969). The direct susceptibility of polyalkylcyanoacrylate nanoparticles to microsomal degradation as reported by Lenaerts et al. (1984a) is likely to be of decreased importance.

The lack of direct interaction with tumor cells observed in our experiments suggests that the increased efficiency of D-actinomycin-pMCA nanoparticles against the sarcoma S 250 in the rat (Brasseur et al., 1980) and of 5-fluorouracil-pBCA nanoparticles against Crocker sarcoma S 180 in the mouse (Kreuter and Hartmann, 1983) may not be due to a specific interaction between the nanoparticles and the tumor cells.

In evaluating the significance of the observed toxicity for drug delivery purposes, it has to be kept in mind that the amount of nanoparticles administered to the cell cultures was very high: the dose at the highest nanoparticle concentrations added to one well was equivalent to what normally is administered to one mouse. In addition, under in vivo conditions the degradation products are eliminated from the nanoparticle location, thus decreasing the contact time with individual cells. In an in vivo study in which several organs were investigated after i.v. injection into mice using autoradiography no signs for cell damage were observed even at locations where a significant accumulation of nanoparticles occurred (Waser et al., manuscript in preparation). After visual inspection of pBCA and pHCA nanoparticles also no signs for adverse effects were detectable even after multiple doses (unpublished results). Therefore our in vitro cell toxicity study does not discourage further interest in these drug carriers. Nevertheless the toxicity demonstrates that these particles are not inert, especially at high concentrations. In particular the toxicity of these nanoparticles to the Kupffer cells should be examined.

Acknowledgements

The authors wish to express their thanks to Marina Balzer for ther skillful technical assistance, and Rolf Bues for photographic processing. The study was supported by the Swiss Federal Institute Zürich Research Fund, the Kantonal Zürcherische Krebsliga and the Herzog Egli Stiftung.

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