aluminum and total metal concentration of the mixtures in which hydrotalcite was found to form on aging.

The present monographs for these mixtures do not contain any tests which would detect the presence of hydrotalcite, nor do they require the inclusion of sorbitol in the mixtures. Thus, there is the possibility of the formation of hydrotalcite during the aging of official aluminum hydroxide gel and magnesium hydroxide gel mixtures.

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Toxicity of Polyalkylcyanoacrylate Nanoparticles I: Free Nanoparticles

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Received May 21, 1981 from the **Laboratoire de Pharmacie Galénique and the* ‡*Laboratoire de Chimie Médicale, Toxicologie, et Bromatologie, Université Catholique de Louvain, 1200 Bruxelles, Belgium;* the §*Laboratoire de Chimie Physiologique, Université Catholique de Louvain et International Institute of Cellular and Molecular Pathology, 1200 Bruxelles, Belgium;* and the ¶*School of Pharmacy, Federal Institute of Technology, Zürich, Switzerland.* Accepted for publication October 7, 1981.

Abstract □ The present report describes preliminary results concerning the acute toxicity of placebo polyalkylcyanoacrylate nanoparticles used as drug carrier. It was demonstrated that nanoparticles induced cellular damage only at relatively high concentrations in the cell culture medium. The absence of mutagenicity was shown for both nanoparticles and their degradation products, and the LD_{50} for polybutyl- and polyisobutylcyanoacrylate nanoparticles was also determined.

Keyphrases □ Polyalkylcyanoacrylate—toxicity of nanoparticles □ Nanoparticles—polyalkylcyanoacrylate toxicity □ Toxicity—polyalkylcyanoacrylate nanoparticles □ Drug carrier systems—nanoparticles, toxicity of polyalkylcyanoacrylate

Previous studies have demonstrated the interest of polyalkylcyanoacrylate nanoparticles as a drug carrier (1, 2). The main advantage of these particles is their degradability at a rate depending on the length of the alkyl chain (3). These ultrafine particles (diameter of ~0.2 μm) are able to efficiently adsorb a variety of drugs in a stable and reproducible way (4). It has been shown previously that the binding of cytostatic drugs to nanoparticles modifies their distribution pattern in rat tissues and generally increases the tissue capture of these drugs (5, 6).

Recently, preliminary results of experimental chemotherapy were published (7) using actinomycin D-loaded polymethylcyanoacrylate nanoparticles against the growth of a transplantable soft sarcoma tissue of the rat. The results indicated that the use of polymethylcyanoacrylate nanoparticles as a drug carrier increased the anticancer activity of actinomycin towards subcutaneous sarcoma. Furthermore, these particles could be of interest in the field of long-acting insulin therapy (8).

However, it was important to have information about the toxicity of the carrier to determine if these results did not prohibit the use of nanoparticles in human medicine. The present report describes preliminary results concerning the acute toxicity of nondrug-bound nanoparticles towards cells in culture and the determination of the LD_{50} of the carrier.

EXPERIMENTAL

Polyalkylcyanoacrylate Nanoparticles Preparation—Polymethyl-, polybutyl-, and polyisobutylcyanoacrylate nanoparticles were prepared following previous methods (1, 6), slightly modified. To an

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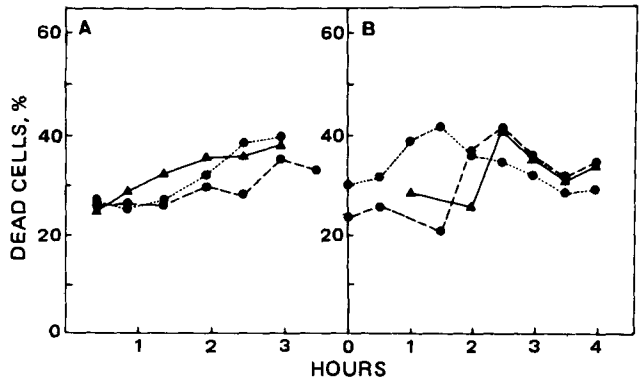

Figure 1—*Hepatocyte mortality after incubation with 0.5% of polybutylcyanoacrylate nanoparticles (- - - -) and 0.5% of nanoparticles polymerization medium (— • —) compared to controls (—). (A) Dye exclusion test (erythrosin B). (B) Lactic dehydrogenase leakage is expressed as a ratio of the lactic dehydrogenase activity in the medium compared to the total lactic dehydrogenase activity (medium and cells).*

aqueous solution (10 ml) of 0.01 *M* HCl containing 0.2% of a polysaccharide[1] and 0.8% of a polyoxyethylene–polyoxypropylene surfactant[2], butyl-[3], or isobutylcyanoacrylate[4] monomer (165 μl) was added under mechanical stirring. Polymethylcyanoacrylate nanoparticles were prepared in the same manner by adding the methylic monomer[3] (165 μl) to 10 ml of 0.01 *M* HCl containing 0.5% of polysorbate 20. After polymerization (at least 2 hr), each nanoparticle suspension was buffered at pH 7 using 100 μl of a molar solution of NaOH and 900 μl of a phosphate buffer[5]; NaCl (83 mg) was then added. No filtration was carried out before using, except in the case of the LD_{50} assays, where nanoparticles were filtered through a sintered glass filter (pore size 9–15 μm).

Scanning electron microscopy[6] showed mostly spherical particles with a diameter of ~0.2 μm for the polymethylcyanoacrylate preparations and of ~0.4 μm for the polyisobutyl- and polybutylcyanoacrylate preparations.

Cell Toxicity Procedures Using Placebo Nanoparticles—Hepatocytes were isolated using an *in situ* enzymatic perfusion method in the rat liver (9). Isolated cells were then incubated at 37° in a Waymouth medium supplemented with 10% newborn calf serum (10). Polybutylcyanoacrylate nanoparticles or their polymerization milieu were then added to obtain final concentrations of 0.5 and 1%, which corresponded,

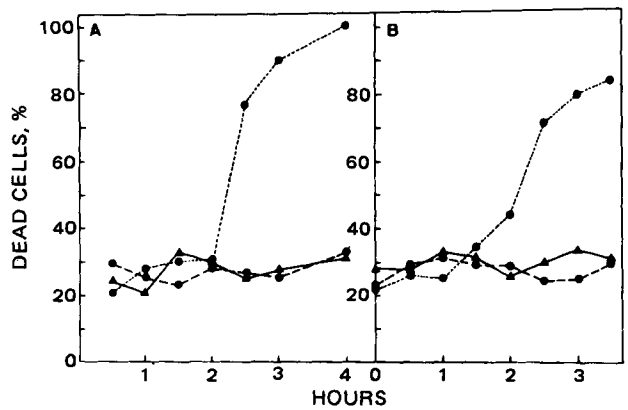

Figure 2—*Hepatocyte mortality after incubation with 1% of polybutylcyanoacrylate nanoparticles (- - - -) and 1% of nanoparticles polymerization medium (— • —) compared to controls (—). (A) Dye exclusion test (erythrosin B). (B) Lactic dehydrogenase leakage is expressed as a ratio of the lactic dehydrogenase activity in the medium compared to the total lactic dehydrogenase activity (medium and cells).*

[1] Dextran 70, Fison Laboratories, S.K.-RIT, Belgium.
[2] Pluronic L 63 Marles-Kuhlmann-Wyandotte, Paris, France.
[3] Loctite, Dublin, Ireland.
[4] Bucrylat Ethicon GmbH, Norderstedt, Germany.
[5] Phosphate buffer pH 7, USP XIX.
[6] Scanning Electron Microscope, Mini S.E.M. International Scientific Instruments, München, West Germany.

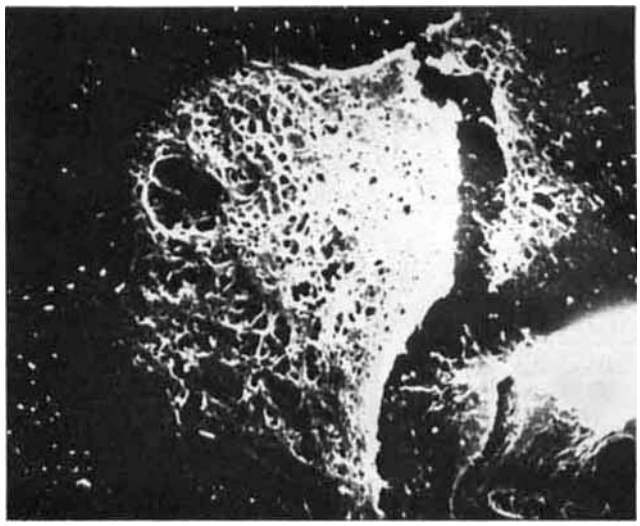

Figure 3—*Scanning electron microscopic appearance of a mouse peritoneal macrophage incubated 1 hr with polymethylcyanoacrylate nanoparticles at a concentration of 1% in the culture medium. The cell membrane appears to be completely perforated.*

respectively, to 7.5 and 15 mg of nanoparticles in 100 ml of the incubation medium, assuming the density of the polymer was equal to one.

Membrane integrity of the incubated cells was regularly controlled using both the 0.36% erythrosin B exclusion test and the leakage of lactic dehydrogenase, a cytosolic enzyme.

Macrophages were obtained after washing the peritoneal cavity of C 57 black mice three times with a medium[7] containing 0.1 mg/ml of streptomycin and 0.06 mg/ml of penicillin G. The liquid was incubated in plastic petri dishes containing a small glass coverslip. After 2 hr of incubation, the nonadhering cells were washed off with phosphate buffer solution, and the remaining macrophages were then further incubated at 37° in the culture medium[7] (containing 20% calf serum) in a 10% CO_2/90% air atmosphere incubator[8]. Nanoparticles were then added at a concentration of 1% in the culture medium. After 1 hr of incubation, nonadhering nanoparticles were washed off with phosphate buffer solution medium. Fresh medium was added to wash the macrophage monolayer culture. The macrophages were then fixed in glutaraldehyde (2.5% by volume in 0.1 *M* cacodylate buffer, pH 7.4) for 15 min at 37°, washed three times with cacodylate buffer, treated 1 hr at 4° with OsO_4 (1% w/v in 0.17 *M*

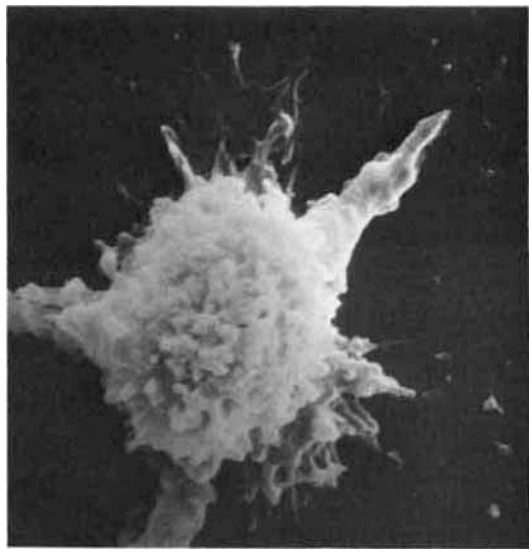

Figure 4—*Scanning electron microscopic appearance of a mouse peritoneal macrophage incubated 1 hr with polyisobutylcyanoacrylate nanoparticles at a concentration of 1% in the culture medium. The macrophage seems to be normal.*

[7] Eagle Dulbecco.
[8] Automatic CO_2 Controller type 3171, Forma Scientific, Marietta, Ohio.

Table I—Bacterial Toxicity Test

Salmonella typhimurium Strains	Bacterial Survival[a] × 10^7/Plate						
		Methyl Product,			Butyl Product,		
	Controls	150 μg	300 μg	1500 μg	150 μg	300 μg	1500 μg
TA 1530	129	116	112	3	150	140	135
TA 1535	101	84	74	0	111	75	67
TA 1538	90	105	70	0	107	78	68
TA 100	91	81	65	5	109	73	69
TA 98	83	77	68	3	104	73	72

[a] Number of survivals after incubation of the strains with polymethyl- and polybutylcyanoacrylate nanoparticles on nutrient agar plates.

Table II—Ames Mutagenicity Test with Nanoparticles Without Metabolic Activation

Salmonella typhimurium Strains	Histidine Revertants[a]/Plate						
		Methyl Product,			Butyl Product,		
	Spontaneous	30 μg	60 μg	150 μg	30 μg	60 μg	150 μg
TA 1530	23	19	13	19	17	17	12
TA 1535	6	10	9	12	9	14	12
TA 1538	25	14	37	15	17	9	16
TA 100	122	171	151	127	113	158	160
TA 98	44	10	14	22	23	24	23

[a] Number of revertants after incubation of the strains with polymethyl- and polybutylcyanoacrylate nanoparticles.

Table III—Ames Mutagenicity Test Involving Metabolic Activation with Nanoparticles

Salmonella typhimurium Strains	Histidine Revertants[a]/Plate						
		Methyl Product,			Butyl Product,		
	Spontaneous	15 μg	30 μg	150 μg	15 μg	30 μg	150 μg
TA 1530	9	8	9	5	5	11	9
TA 1535	11	7	8	10	7	9	11
TA 1538	15	8	11	8	9	11	9
TA 100	180	125	135	123	149	174	169
TA 98	14	12	13	10	13		19

[a] Number of revertants after metabolic activation and incubation with polymethyl- and polybutylcyanoacrylate nanoparticles.

Table IV—Ames Mutagenicity Test with the Degradation Products of Nanoparticles Without Metabolic Activation

Salmonella typhimurium Strains	Histidine Revertants[a]/Plate						
		Methyl Product,			Butyl Product,		
	Spontaneous	15 μg	30 μg	150 μg	15 μg	30 μg	150 μg
TA 1530	14	22	14	16	17	17	20
TA 1535	15	10	7	12	11	12	11
TA 1538	13	12	7	8	9	5	20
TA 100	145	122	137	131	168	141	138
TA 98	13	24	22	16	10	19	28

[a] Number of revertants after incubation of the strains with polymethyl- and polybutylcyanoacrylate nanoparticle degradation products.

barbital acetate buffer, pH 7.4) and washed with barbital acetate buffer.

Finally, the macrophages were washed five times with double distilled water, frozen in liquid nitrogen, and dried by lyophilization for 3 hr. The glass coverslips with the dried macrophages were then attached to metal disks and coated with a thin film of gold in a vacuum evaporator[9]. The specimens were then stored at room temperature until examination under a scanning electron microscope.

Mutagenicity Tests—Mutagenicity tests were performed on polybutyl- and polymethylcyanoacrylate nanoparticles using the *Salmonella typhimurium* method (11). This test was performed with and without metabolic activation[10] on both intact and degraded nanoparticles. A preliminary toxicity test was carried out to determine the maximum noninhibitory doses of nanoparticles.

Determination of Nanoparticle Histotoxicity—A total of 14 male mice[11] (20 g) were injected subcutaneously with 0.2 ml of a polyisobutylcyanoacrylate suspension containing 3 mg of nanoparticles. After 24 hr, six mice were sacrificed under carbon dioxide atmosphere, the skin was turned inside out and macroscopically examined according to a previously described method (12).

Three days later, the remaining animals were rechallenged with the same dose of nanoparticles and were divided into two groups of four mice. The first group was sacrificed 48 hr after the second injection, while the second group received a third injection and was sacrificed 2 days later. All these mice were treated as mentioned previously. Control mice, which received no injection, were treated in the same manner.

Determination of Nanoparticle LD_{50}—Six groups of 10 male NMRI mice (20 g) were treated with nanoparticles by injection in the tail vein. Each group received one of the following doses in a single injection, respectively: 12.5, 17.5, 22.5, 27.5, 32.5, and 40 ml/kg of polybutyl- or polyisobutylcyanoacrylate nanoparticles suspension. Both suspensions contained 9.2 mg of nanoparticles/ml. The same volumes of the polymerization milieu were injected into different groups of mice and the LD_{50} of this milieu was then determined.

RESULTS AND DISCUSSION

Toxicity of Polybutylcyanoacrylate Nanoparticles on Isolated Hepatocytes—At a concentration of 0.5% in the culture medium, neither polybutylcyanoacrylate nanoparticles nor nanoparticle polymerization medium modified the cellular integrity of the hepatocytes; in fact the lactic dehydrogenase leakage and the dye exclusion capacity of the cells remained comparable to the untreated hepatocytes (Fig. 1). In the case of the 1% final concentration, the polybutylcyanoacrylate nanoparticles greatly affected the integrity of the hepatocytes; the polymerization medium, on the other hand, exerted no effect (Fig. 2).

A cytotoxic effect appeared between 0.5 and 1% and seemed to proceed

[9] P-S1 Diode Sputter Coater, International Scientific Instruments, München, West Germany.

[10] Addition of cofactors supplemented liver "S_9" from Arochlor 1254 pretreated rats (11): 50 μl of "S_9" per plate.

[11] NMRI mice, Animal House of U.C.L., Louvain-en-Woluwe, Belgium.

Table V—Ames Mutagenicity Test Involving Metabolic Activation with the Degradation Products of Nanoparticles

Salmonella typhimurium Strains	Histidine Revertants[a]/Plate						
		Methyl Product,			Butyl Product,		
	Spontaneous	15 μg	30 μg	150 μg	15 μg	30 μg	150 μg
TA 1530	10	11	17	9	9	11	16
TA 1535	10	12	12	13	15	7	12
TA 1538	28	36		29	27		29
TA 100	178	137		120	170		133
TA 98	46	47	39	40	39	35	44

[a] Number of revertants after metabolic activation and incubation with polymethyl- and polybutylcyanoacrylate nanoparticle degradation products.

from the polymer itself rather than from the surfactants present in the nanoparticle polymerization medium. However, the 1% dose corresponded to a high ratio ($\sim 2 \times 10^4$) of particles per hepatocyte.

Scanning Electron Microscopy Studies with Macrophages—This microscopic study was performed to determine the exact morphological cell changes resulting from nanoparticle ingestion. Macrophages were used because of their high phagocytic capacity. It is known that toxicity of the polymer seems to be dependent on the biodegradation half-life which is prolonged with increasing alkylchain length (13). For this reason, it was decided to test polymethyl- and polyisobutylcyanoacrylate nanoparticles.

Scanning electron micrographs revealed marked morphological membrane modifications after incubation of the macrophages for 1 hr in the presence of polymethylcyanoacrylate nanoparticles at a 1% concentration in the culture medium (Fig. 3). The cell membrane appeared to be completely perforated. After incubation of the macrophages with polyisobutylcyanoacrylate nanoparticles under the same experimental conditions (1 hr at a concentration of 1%), no signs of toxicity were observed, and the morphological appearance of the macrophages under the scanning electron microscope seemed to be normal as compared with control (Fig. 4).

Mutagenicity Tests—Table I shows the bacterial survival of the *S. typhimurium* strains after incubation with nanoparticles on nutrient agar plates seeded with $2-7 \times 10^7$ viable cells. Polymethylcyanoacrylate induced an appreciable toxic effect when it was used at the dose of 1500 μg/plate. A slight inhibitory effect was observed with polybutyl- and polymethylcyanoacrylate nanoparticles at a concentration of 300 μg/plate. No toxicity was noted at the 150-μg dose. This dose was then considered the maximum noninhibitory level for the two types of polymer. The results of the Ames test, performed with different doses of nanoparticles and degradation products, are presented in Tables II–V. The assays were made either with or without metabolic activation. No mutagenic effect was observed using both nanoparticles and their degradation products. In all tests carried out, the number of induced revertants as compared with spontaneous revertants was not significantly increased. Furthermore, no dose–response effect was obtained. Although the Ames test was performed only with the polymethyl- and the polybutyl product, from the data obtained, it can be inferred that polyethyl- and polyisobutylcyanoacrylate nanoparticles are unlikely to be mutagenic. Indeed, in a mutagenic assay, the observed effect is closely dependent upon the chemical nature of the product tested, and polyalkylcyanoacrylates are very similar in their chemical structure.

In the Ames test, as in any mutagenicity assay, the observed mutations are related to only a very small part of the genome. Therefore, a negative result does not necessarily mean that the product under study is not mutagenic for humans (14); however, the Ames method is reliable in detecting carcinogens (15, 16). Moreover, cyanoacrylate polymers have been used in surgery for a long time, and until now, no carcinogenic action has been observed with these products (17, 18).

Nanoparticle Histotoxicity and LD_{50}—Twenty four hours after subcutaneous injection of 3 mg of polyisobutylcyanoacrylate nanoparticles, neither necrosis nor tissue irritation was visible. Furthermore, 48 hr after multiple treatments (2 or 3 injections) no hypersensitivity reaction nor granulomatous response was observed. Figure 5 shows the cumulative mortality of the mice after intravenous administration of different nanoparticle suspension volumes (milliliters per kilogram). Brought again to dry polymer weight per kilogram, the LD_{50} was determined to be 196 and 230 mg/kg for polyisobutylcyanoacrylate and polybutylcyanoacrylate nanoparticles, respectively. It should be noted, however, that the polymerization medium alone is not free of toxicity (LD_{50} = 33.4 ml/kg).

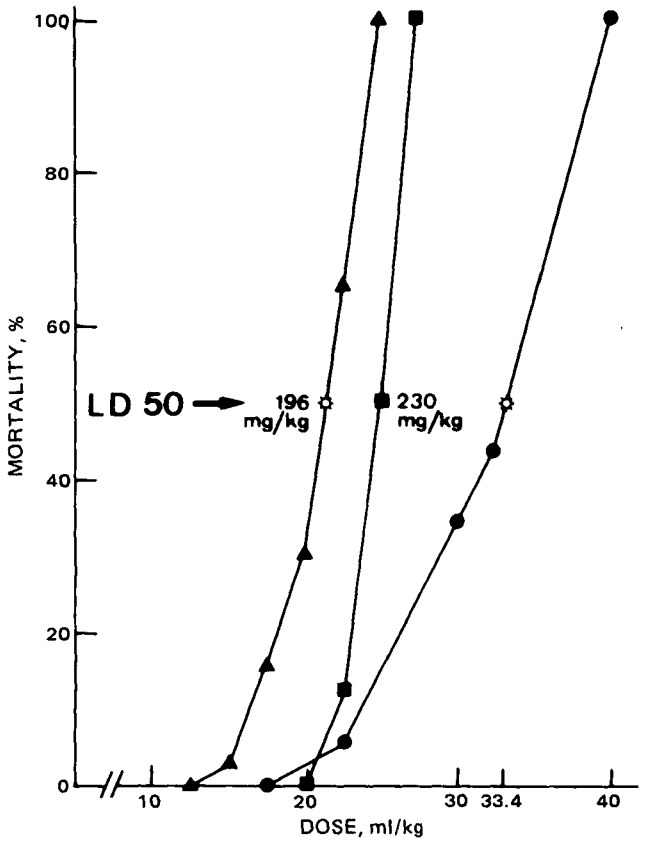

Figure 5—*Cumulative mortality of mice after intravenous administration of various nanoparticle suspension volumes. Key: (▲) polyisobutylcyanoacrylate nanoparticle; (■) polybutylcyanoacrylate nanoparticle; (●) nanoparticles polymerization medium.*

CONCLUSIONS

These first toxicological data obtained at the cellular and whole body animal levels did not demonstrate any acute toxicity susceptible to hinder the use of polyalkylcyanoacrylate nanoparticles in human medicine. Indeed, they induced cellular damage only at a relatively high concentration in the culture medium (1%). This toxicity is probably due to the presence of nanoparticle degradation products in the cytoplasm following their phagocytosis. This consideration could explain the lesser toxicity of the isobutyl product, which is more slowly biodegradable than the methyl derivative. Moreover, LD_{50} values are reasonable, and because of the presence of surfactants in the polymerization medium, it could be possible to reduce considerably the acute toxicity of the particles by washing off the surfactants and eventual remaining monomers. Finally, the absence of histotoxicity and mutagenicity for both nanoparticles and their degradation products are encouraging. These results will be completed by subacute and chronic toxicological studies and we will examine the possibility of decreasing the toxicity of an anticancer agent by its adsorption on polyisobutylcyanoacrylate nanoparticles.

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Toxicity of Polyalkylcyanoacrylate Nanoparticles II: Doxorubicin-Loaded Nanoparticles

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Received May 21, 1981, from the * *Laboratoire de Pharmacie Galénique, Université Catholique de Louvain, 1200 Bruxelles, Belgium* and the ‡ *School of Pharmacy, Federal Institute of Technology, Zürich, Switzerland.* Accepted for publication October 7, 1981.

Abstract □ The possibility of significantly reducing toxicity of an anticancer drug such as doxorubicin by fixing it on polyisobutylcyanoacrylate nanoparticles was studied. It was shown that, when the drug was adsorbed on nanoparticles, significant reduction of both mortality and weight loss of mice were recorded under various administration schedules. Furthermore, cardiotoxicity was decreased due to the poor uptake by the myocardium.

Keyphrases □ Polyalkylcyanoacrylate—toxicity of doxorubicin-loaded nanoparticles □ Doxorubicin—toxicity of polyalkylcyanoacrylate nanoparticles □ Toxicity—doxorubicin-loaded polyalkylcyanoacrylate nanoparticles □ Drug carrier systems—toxicity of doxorubicin-loaded polyalkylcyanoacrylate nanoparticles

The first toxicological data concerning the polyalkylcyanoacrylate nanoparticles did not demonstrate any distinct toxicity susceptible to hinder their use in human medicine (1). The aim of the present investigation was to reduce considerably the toxicity of an anticancer drug such as doxorubicin by fixing it on nanoparticles. The idea of using doxorubicin in association with a macromolecular carrier such as DNA, to minimize the detrimental effect of this anticancer drug on normal cells, has been previously investigated (2, 3).

Doxorubicin, an anthracycline antibiotic, has produced encouraging results in the treatment of neoplastic diseases (4). However, it is toxic, with its most severe side effects involving the heart (acute and chronic cardiomyopathy), bone marrow, and intestine (5, 6). For these reasons, and because doxorubicin is highly adsorbed on nanoparticles, this cytostatic drug was chosen as the experimental model.

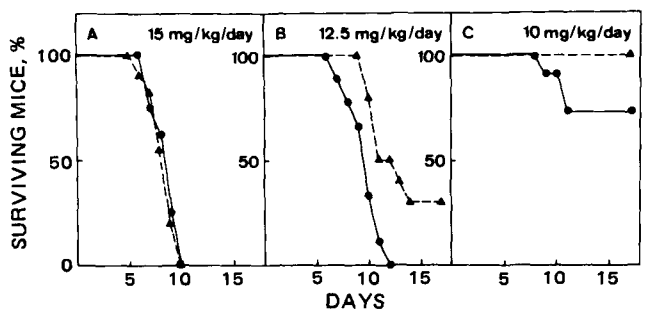

Figure 1—*Percent of surviving mice after intravenous administration on 3 consecutive days of various doses of free (●) and nanoparticle-bound doxorubicin (▲).*

EXPERIMENTAL

Polyalkylcyanoacrylate Nanoparticle Preparation—After dissolution of doxorubicin[1] (10 mg) in 10 ml of aqueous solution containing 100 mg of a polysaccharide[2], 50 mg of citric acid, and 1 mg of calcium chloride, 100 μl of isobutylcyanoacrylate monomer was dispersed under mechanical stirring.

After polymerization (3 hr), the resulting milky suspension was brought to isotonicity with 72 mg of sodium chloride. The size of the particles obtained was then estimated by measuring light scattering, arising from a laser source[3].

Measurement of Doxorubicin Linked to Nanoparticles—A 10-ml nanoparticle suspension was centrifuged[4] at 20,000 rpm for 1 hr. Sediment was then separated and dissolved in 10 ml of dioxane containing 20% water.

The content of doxorubicin was determined in both supernate and sediment by fluorimetric[5] dosage. For this purpose, 200-μl samples were diluted to 10 ml by water (for the supernate) or by dioxane (for the sediment) and measurements were performed using reference solutions of doxorubicin.

[1] Adriablastina, Montedison Farmaceutica Benelux, Bruxelles, Belgium.
[2] Dextran 70, Fison Laboratories, S.K.-RIT, Belgium.
[3] Nano-Sizer, Coulter Electronics, Harpenden, England.
[4] Beckman Centrifuge, model J-21C, Beckman Instruments, Palo Alto, Calif.
[5] Vitatron Fluorimeter, type U.F.D., Vitatron, Holland.

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