Interaction Between Polyalkylcyanoacrylate Nanoparticles and Peritoneal Macrophages: MTT Metabolism, NBT Reduction, and NO Production

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Purpose. The nature of interactions between macrophages and drug carriers is of primordial importance either in the design of more effective therapeutic strategies for macrophage-associated pathogenesis or in establishing new approaches for pharmacological action avoiding macrophages.

Methods. Polyalkylcyanoacrylate nanoparticles (PMCA, PECA, PBCA and PIBCA nanoparticles) were assayed for their toxicity on peritoneal resident and thioglycolate-elicited macrophages. Cellular viability was assessed by MTT tetrazolium salt assay, oxidative burst by NBT reduction and NO production by nitrite evaluation.

Results. The nanoparticles tested led to cellular morphological modifications and induced toxicity in both types of macrophages in culture. The polyalkylcyanoacrylate nanoparticles uptake by peritoneal macrophages caused an increase in respiratory burst, as assessed by the NBT reduction assay, and induced the release of soluble toxic factors to the culture medium. The association of LPS with the PMCA nanoparticles significantly stimulated the production of nitric oxide (NO) by resident macrophages. In contrast, the association of PBCA nanoparticles with LPS does not increase the nitrite production as compared with LPS alone, which may be due to a different physico-chemical interaction between LPS and the two types of polymers.

Conclusions. In cultured mice peritoneal macrophages, nanoparticles of PACA induce the production of oxygen reactive products, which cause changes in the cell metabolism of both resident and elicited macrophages. PMCA nanoparticles in association with LPS significantly increase the expression of the inducible isoform of nitric oxide synthase, leading to the release of large amount of NO, which may be highly cytotoxic to the cultured cells in the presence of peroxide generated from the oxidative burst.

KEY WORDS: nanoparticles; macrophages; cellular toxicity; NO production; NBT reduction.

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ABBREVIATIONS: FBS, fœtal bovine serum; LPS, lipopolysaccharide; MetArg, N^G-monomethyl-L-arginine; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium; NO, nitric oxide; np, nanoparticles; PACA, polyalkylcyanoacrylates; PBCA, polybutylcyanoacrylate; PECA, polyethylcyanoacrylate; PIBCA, polyisobutylcyanoacrylate; PMA, phorbol myristate acetate; PMCA, polymethylcyanoacrylate; ROI, reactive oxygen intermediates.

INTRODUCTION

The delivery of drugs to specific organs (*e.g.* antibiotics), or the decrease of drugs side effects (*e.g.* anthracyclines) was previously done by using polymeric carrier systems, such as nanoparticles (1). Particulate drug carriers are usually rapidly removed from the circulation by the macrophages of the mononuclear phagocyte system (2). However, the intracellular accumulation of drug carriers can lead to cytotoxic effects either due to the drug or to the polymeric carrier itself. At present, the mechanisms by which such toxic effects occur are not yet well understood.

Macrophages are phagocytic cells that secrete a variety of substances, whose actions range from induction of cell growth to cell death. These substances include signalling molecules such as nitric oxide (NO), cytokines and reactive oxygen intermediates (ROI), which are involved in host defence against pathogenic micro-organisms, parasites or tumour cells. However, NO and ROI trigger the activation of a number of pathways, some of which can exert an autotoxic effect on the macrophages (3,4).

In the last few years, studies in isolated cells have been useful in the elucidation of some of the biochemical events that are involved in cell death. One of the main contributions was a better identification of the mechanisms by which drug carrier systems may become toxic, but they are not yet completely understood. A better knowledge about the cellular and molecular events occurring during the interaction between macrophages and drug carriers will enhance the possibility of studying new therapeutic strategies for macrophage-associated pathogenesis and of designing new approaches for pharmacological action avoiding macrophages (design of so-called stealth drug carriers).

In the present work, polyalkylcyanoacrylate nanoparticles were assayed for their toxicity on the resident and thioglycolate-elicited peritoneal macrophages isolated from female BALB/c mice. We also evaluated whether the respiratory burst and the NO production occur when cellular toxicity is induced by polyalkylcyanoacrylate nanoparticles on the peritoneal macrophages in culture.

MATERIALS AND METHODS

Macrophages Isolation

Peritoneal macrophages were harvested from female BALB/c mice (8–10 weeks) as described (5). Briefly, resident macrophages were removed from mice peritoneal exudate with PBS 0.15 M, pH 7.2. The cells were centrifuged at 112 g, during 10 minutes, and resuspended in culture medium (RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomicyn, 1 mM sodium pyruvate, 10 mM Hepes, pH 7.4). Then, the cell suspension was plated and incubated at 37°C for 2 hours, in a 5% CO_2 atmosphere, allowing the cells to adhere. Non adherent cells were removed by washing, and the adherent cells were cultured. Elicited peritoneal macrophages were obtained from peritoneal exudate of mice, that were injected i.p. with 1 ml of sodium thioglycolate (3%), 48 hours before cells harvest. The cell viability was 97–99% as assessed by trypan blue exclusion.

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The research adhered to the "Principles of Laboratory Animal Care".

Morphological and Cytochemical Characterization of Mouse Peritoneal Macrophages

Resident and thioglycolate-elicited macrophages (2×10^5 cells/well) were incubated with RPMI-1640 in 24-well microplates, at 37°C for 24 hours, in a 5% CO_2 atmosphere. The macrophages in culture medium were examined by an inverted microscope. In some experiments, the adherent cells were air dried, fixed in 100% methanol and were stained by the May-Grunwald Giemsa technique. In other experiments, macrophages (1×10^6 cells/well) were stained with the Sigma KIT (ref. *n*° CP-1) for α-naphtyl non-specific esterase and for acid phosphatase activity, according to the manufacturer's instructions and subsequently examined by light microscope. The immunostaining for macrophages was performed using the monoclonal mouse antibody anti-human macrophage (Dakopatts *n*° M718). Macrophages (7×10^5 cells/well) were incubated with supplemented RPMI-1640, in a 5% CO_2 atmosphere. After 4 hours in culture, the supernatant medium was removed and the cells were fixed with 37% formaldehyde and 25% glutaraldehyde, in 0.15 M PBS, pH 7.2. Then, the cells were incubated with the recommended concentration of the monoclonal mouse antibody anti-human macrophage in Tris-HCl 0.05 M, pH 7.6, during 2 hours. A second incubation was performed with monoclonal rabbit anti-mouse antibody, during 30 minutes. The avidin-biotin complex was added and incubated during 30 minutes, followed by the addition of diaminobenzidine solution. The cells were counterstained with Herlich hematoxillin.

Nanoparticles Preparation and Physico-Chemical Characterization

Polyalkylcyanoacrylate nanoparticles were prepared by a standard procedure. Container materials and monomer were sterilised by dry-heat; polymerisation medium sterilised by filtration through a 0,22 μm sterile filtration apparatus. Preparation procedure until obtaining final freeze-dried product was performed in a sterile room. The cyanoacrylic monomer (12 μl/ml) was added to a polymerisation medium containing 5% glucose, 1% dextran and citric acid 0.001 M, at pH = 3 and polymerised under magnetic stirring for different time according to the monomer used (6). After polymerisation and appropriate physico-chemical characterisation, colloidal suspensions were freeze-dried in a LyoFreeze III GT15 (Leybold-Heraeus, Germany). In the freeze-drying process nanoparticles were submitted to a freezing temperature of −42°C, followed by drying at 4×10^{-2} mbar, as previously optimised (7). Nanoparticles were stored in sterile, closed, freeze-dried form at −30°C until use. Before use redispersion was performed in sterile water and nanoparticles suspension diluted in cell culture medium adequate for the *in vitro* assays. All polymers were obtained at the same time and processed through the freeze-drying process in simultaneous to avoid any discrepancies arising from the preparation procedure. After nanoparticles were redispersed, their size and surface properties (electrophoretic mobility and zeta potential) were assayed by Photon correlation spectroscopy (AutoSizer II) and Doppler Electrophoretic Light Scattering Analysis (Coulter® DELSA 440). Nanoparticles were also controlled for absence of pyrogens (LAL method).

Assessment of Cellular Toxicity

Cellular toxicity was evaluated according to previously reported methods (8). We used MTT tetrazolium salt assay to assess metabolic active cells. For the MTT assay, macrophages were isolated (1×10^5 cells/well) and incubated during 18 hours, in 96-well microplates with culture medium. Different concentrations of polyalkylcyanoacrylate nanoparticles (PMCA, PECA, PBCA and PIBCA) were added to cells in a final volume of 200 μl (from 10 to 1000 μg polymer/ml). Then, the cells were incubated with 10 μl MTT (5 mg/ml) and the extent of MTT reduction was quantified by an ELISA reader at 570 nm. The absorbance of control cells (incubated without polyalkylcyanoacrylate nanoparticles) was defined as 100% viability. In additional experiments, the cell supernatants obtained from macrophages (1×10^5 cells/well), previously incubated with nanoparticles (250 μg/ml) were used in a second set of resident or elicited peritoneal macrophages. After incubation, the MTT assay was performed as described above and the results were expressed as percentage of metabolic intact macrophages, compared to control cells incubated with fresh culture medium (100% viability).

NBT Reduction Assay

The NBT reduction assay was performed according to the method described by Pick (9). Macrophages (1×10^5 cells/well) were incubated in 96-wells microplates at 37°C for 18 hours in a 5% CO_2 atmosphere, with 100 μl RPMI-1640. Polyalkylcyanoacrylate nanoparticles (100 μg/ml) and 100 μl of NBT solution (1 mg/ml) were added to cells in a final volume of 200 μl. The supernatants were removed and the cells were solubilized in 100 μl of acid isopropanol (0.04 N HCl in isopropanol). The extent of NBT reduction was quantified by an ELISA reader at 570 nm. As a positive control, macrophages were incubated with 100 μl of phorbol myristate acetate (500 ng/ml) in a final volume of 200 μl. Results were corrected for blanks in which cells were preincubated with iodoacetamide (10 mM) at 37°C for 10 minutes.

Measurement of Nitrite Formation

NO production was evaluated quantifying nitrite accumulation in cell culture supernatants by the Griess reaction (10).

Macrophages (5×10^5 cells/well) were incubated, at 37°C for 18 hours, in a 5% CO_2 atmosphere, with RPMI-1640 containing 500 ng/ml LPS (extracted from *Escherichia coli*, serotype 026:26). Different concentrations of PMCA or PBCA nanoparticles (25 and 100 μg/ml) with or without LPS (500 ng/ml) plus N^G-monomethyl-L-arginine (1 mM), was added to cells in a final volume of 1 ml. Then, 1 ml of Griess reagent (1% w/v sulphanilamide and 0.1% w/v naphtylethylenediamide in 5% v/v phosphoric acid) was added to 1 ml of sample culture medium, and the absorbance was measured at 546 nm. Nitrite concentrations in the medium were determined by using standard solutions of sodium nitrite (0.125–10 nmol/ml). In control experiments, macrophages were incubated in the presence of N^G-monomethyl-L-arginine (1 mM), LPS (500 ng/ml) and PMCA or PBCA nanoparticles (100 μg/ml).

RESULTS

Nanoparticles Characterization

Nanoparticles were characterized for their size and surface properties according to two techniques (see Methods). Results (Table I) show minor differences between the two techniques (using Coulter® DELSA 440 or AutoSizer II) for size evaluation, which is related to different detection angles used, as previously shown in separate report (11).

Morphological and Cytochemical Characterization of Mouse Peritoneal Macrophages

The morphological examination of peritoneal macrophages was performed using both the macrophages in culture medium (Fig. 1A) and the macrophages fixed and stained by May-Grünwald Giemsa technique (Fig. 1B). After 24 hours in culture, adherent peritoneal macrophages exhibit two basic types of morphology: they are either extended and stellate, or they are rounded (Fig. 1A and 1B). However, the stellate forms were predominantly detected in elicited macrophages, which also exhibit greater cytoplasmic vacuolisation, as compared with resident macrophages. After treatment with polyalkylcyanoacrylate nanoparticles the cells started to become rounder (data not shown). Although the mechanisms underlying this morphological alteration are unclear, they are probably related to cell response to the stimulation during the phagocytic process.

To confirm whether the cells in culture were macrophages, the monolayers were characterised by cytochemical and immunocytochemical staining (Fig. 1C), and the percentage of positive cells was estimated according to standard method. Considering these criteria, 99% of the adherent cells were macrophages. The cytochemical characterisation of the adherent macrophages was performed by the reaction of two enzymes, the α-naftil acetate esterase and acid phosphatase (data not shown). In both reactions, we observed that the cytochemical staining was stronger in elicited macrophages as compared with the resident cells, which indicates that those enzymes are expressed in higher levels in elicited macrophages. In Fig. 1C is illustrated a representative result of the immunocytochemical study using the monoclonal mouse antibody anti-human macrophage (anti-MAC). The microscopic examination of the positive cells labelled with cytoplasmic dark-brown granulations confirmed the percentage (> 99%) of macrophages on the monolayer. Negative controls with omission of the primary antiserum (anti-MAC) were consistently negative.

Assessment of Cellular Toxicity of the Nanoparticles

Metabolic active cells have the capacity to transform MTT tetrazolium salt into MTT formazan and a good correlation has been achieved between the MTT metabolism and cell viability (8).

In this work, the toxicity induced by polyalkylcyanoacrylate nanoparticles on peritoneal macrophages in culture was assessed by MTT tetrazolium salt assay. Cells were exposed to increasing concentrations of different cyanoacrylate polymers: PMCA, PECA, PBCA and PIBCA (Fig. 2). We observed a decrease in cell viability both in resident and in thioglycolate-elicited macrophages, as the concentrations of nanoparticles increase. However, the highest degree of toxicity for the resident macrophages (55 ± 5% viability) was observed with the nanoparticles concentrations of 100 μg/ml (Fig. 2A). For the elicited macrophages, the viability decreased until the concentration of 500 μg/ml of nanoparticles and thereafter a plateau was reached in cell survival (30 ± 5% viability) (Fig. 2B). The results of this study also indicated that the mouse peritoneal macrophages undergo identical cellular damage when incubated with the different polymers (PMCA, PECA, PBCA and PIBCA nanoparticles).

Another aspect to be considered was to evaluate whether the interaction between the nanoparticles and macrophages in culture causes the production and release of soluble toxic factors. Resident (Fig. 3A) and elicited (Fig. 3B) macrophages were incubated with the supernatant solutions obtained from macrophages previously cultured in the presence of 250 μg/ml of polyalkylcyanoacrylate nanoparticles. The results indicate a reduction in the survival of macrophages, as compared to the control (Fig. 3). In the following section we studied if this toxicity was due to the release of reactive oxygen intermediates.

Effect of Polyalkylcyanoacrylate Nanoparticles on the Reactive Oxygen Intermediates Production by Resident and Elicited Macrophages

As indicated in Fig. 4, the reduction of NBT in basal conditions (macrophages cultured in RPMI alone) was very low (0.49 ± 0.2 mOD/1 × 10^5 cells). A significant increase in the reduction of NBT (more than two times) was observed, when polyalkylcyanoacrylate nanoparticles were incubated either with resident (Fig. 4A) or with elicited (Fig. 4B) macrophages. These results indicate that the polymers induce an oxidative burst in peritoneal macrophages.

Effect of Polyalkylcyanoacrylate Nanoparticles on the Nitrite Production by Resident and Elicited Macrophages

Experiments were carried out to assess the effect of the PMCA and PBCA nanoparticles on the nitrite production by peritoneal macrophages. The results indicate that the nanoparticles alone had no effect (0.01 ± 0.02 nmol nitrite/ml) on nitrite production. The addition of LPS alone to the culture medium induced an increase in nitrite production (0.50 ± 0.05 nmol nitrite/ml). The effect of combining LPS with different concentrations of PMCA or PBCA nanoparticles (25 and 100 μg/ml) was assessed. In the group where 100 μg PMCA/ml were incubated with LPS (500 ng/ml) a significant increase in nitrite production was observed when compared to LPS alone. In contrast, the combination of LPS with the same PBCA concentrations does not increase the nitrite production as compared with LPS alone (Table II). The NO synthase inhibitor, N^G-monomethyl-L-arginine, inhibited the nitrite production, as compared with test experiments (Table II). This indicates that nitrite formation was specifically associated with NO synthase-catalyzed oxidation of L-arginine (10).

DISCUSSION

The phagocytic cells of the mononuclear phagocyte system are able to remove circulating particulate matter, including polyalkylcyanoacrylate nanoparticles (1,2). Although macrophages constitute an important natural target for drug delivery systems,

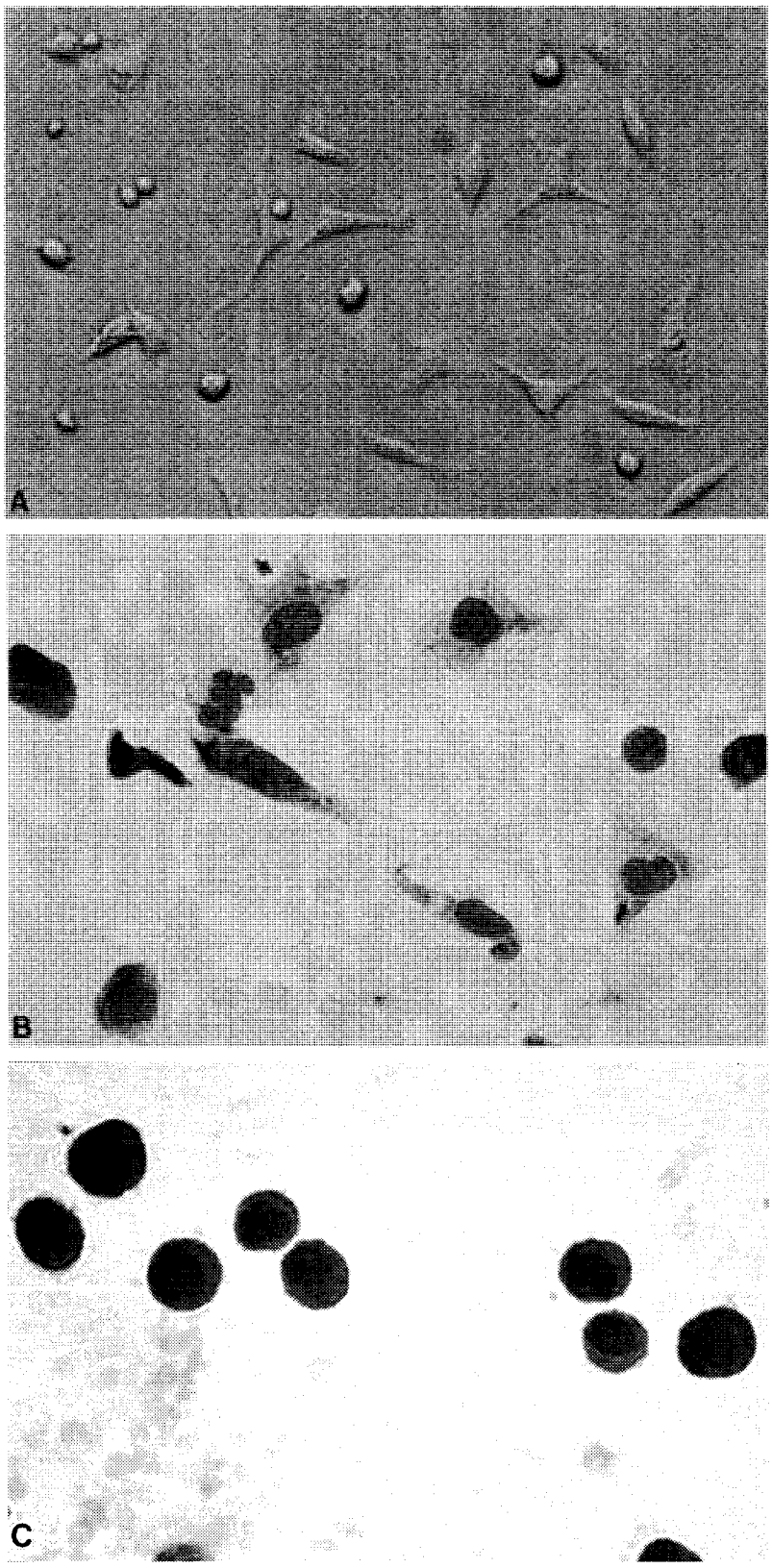

Fig. 1. Morphological and cytochemical characterization of resident peritoneal macrophages in culture. Resident peritoneal macrophages (2×10^5 cells/well) were incubated in RPMI-1640, at 37°C, for 24 hours, in a 5% CO_2 atmosphere, and the cells were examined by inverted microscope (magnification ×400) (A). In some experiments, the cells were fixed and stained by May-Grünwald Giemsa technique (magnification ×1000) (B). Immunostaining for macrophages was performed with the monoclonal antibody anti-human macrophage (anti-MAC) (C), as described in Materials and Methods (magnification ×1000).

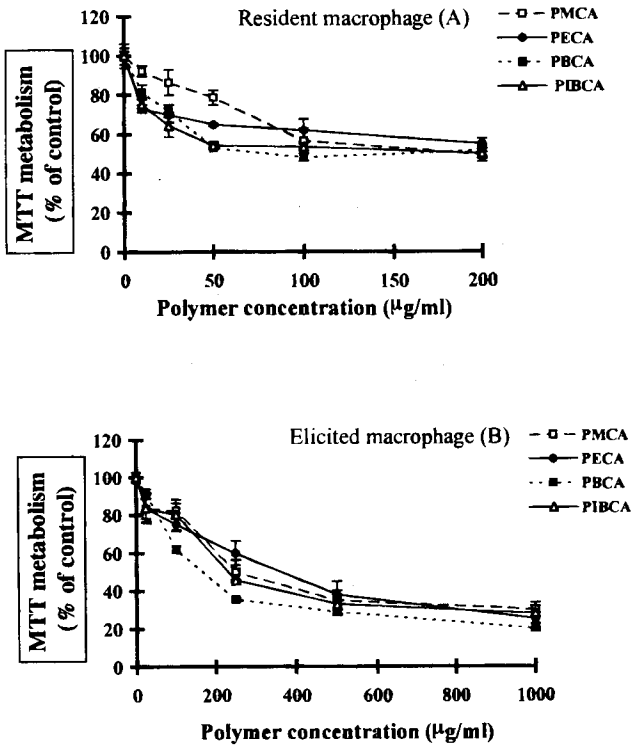

Fig. 2. Assessment of toxicity induced by polyalkylcyanoacrylate nanoparticles on resident and elicited peritoneal macrophages in culture. Resident and elicited peritoneal macrophages (1×10^5 cells/well) were incubated, at 37°C, for 18 hours, in a 5% CO_2 atmosphere, with concentrations ranging fom 0 to 1000 µg/ml of polyalkylcyanoacrylate nanoparticles (PMCA, PECA, PBCA and PIBCA) in a final volume of 200 µl. The cellular toxicity was assessed by the MTT metabolism, as described in Materials and Methods. Values are means ± S.E.M. for three different experiments, each done in triplicate.

it is now clear that they are susceptible to suffer toxic effects because of the intracellular accumulation of drug carriers.

It was previously demonstrated that polyalkylcyanoacrylate nanoparticles with short alkyl side chain are much more rapidly biodegradable, and produced more toxic degradation products, when compared to polymers with longer side chain length (12,13). No difference in the ability to metabolise MTT to formazan was observed when macrophages were incubated with different cyanoacrylate polymers (PMCA, PECA, PBCA and PIBCA). Our results appear to indicate that the toxicity of polyalkylcyanoacrylate nanoparticles on resident and elicited macrophages is not only dependent on the length of the alkyl chain (Fig. 2).

The possible explanation for this apparent discrepancy is that peritoneal macrophages have efficient phagocytic receptors (they are "professional phagocytes"), whereas the cells (fibroblasts and mesenchymal cells) used by other authors are not phagocytes. Other experimental evidences indicate that phagocytosis plays a major role in the polyalkylcyanoacrylate nanoparticles uptake by macrophages (14). Furthermore, resident macrophages are significantly less phagocytic than elicited macrophages (15). Thus, elicited macrophages can accumulate a higher intracellular level of the polyalkylcyanoacrylate nanoparticles than resident macrophages, which leads to a greater reduction in the cell viability of elicited cells, as it is indicated in Fig. 2. Indeed, Nemati et al. noted that cytotoxicity of several cyanoacrylates in P388 cells was dependent on incubation time and number of particles, taken up by these cells (16). It is reasonable to assume that degradation products, resulting from the bioerosion of nanoparticles, are the major factor in cellular toxicity when non-phagocytic cells are used, but in macrophages the phagocytic uptake of nanoparticles must be taken into account.

It was previously suggested that the presence of monomer within the inner structure of the nanoparticles could account for intracellular toxicity, but the data obtained by HPLC-GPC indicated that the presence of monomer, when occurring, was very residual (17). Another aspect of which some attention was given relates with the toxicity induced by formaldehyde production from nanoparticles degradation. However, this aspect is questionable and different groups demonstrated that the chemical degradation pathway producing formaldehyde seems to play a marginal role in the overall degradation of nanoparticles in physiological conditions (18,19).

In this work, we used MTT tetrazolium salt assay that indicates the integrity of mitochondrial enzymes activity, whereas in other previous works the cell viability was measured by the ability of cells to take up trypan blue, which indicates plasma membrane damage (13). In some additional experiments (data not shown), we observed that loss of cell viability indicated

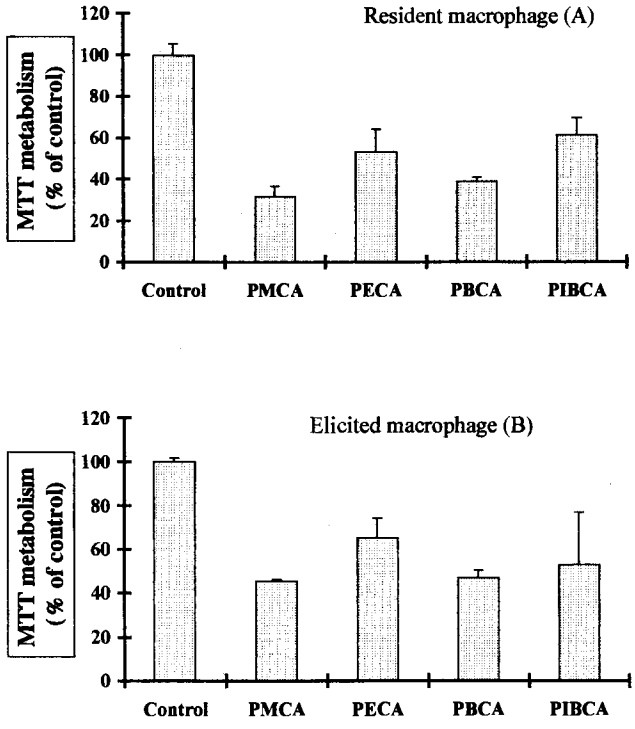

Fig. 3. Assessment of toxicity induced on resident and elicited macrophages in culture by the supernatants obtained from previous cultured macrophages with 250 µg/ml of polyalkylcyanoacrylate nanoparticles. Cell supernants of the cell cultures of resident and elicited macrophages with 250 µg/ml of the nanoparticles were incubated with a second set of resident and elicited peritoneal macrophages, respectively. The cellular toxicity was assessed by the MTT metabolism as described in Materials and Methods. Values are means ± S.E.M. for three different experiments, each done in triplicate.

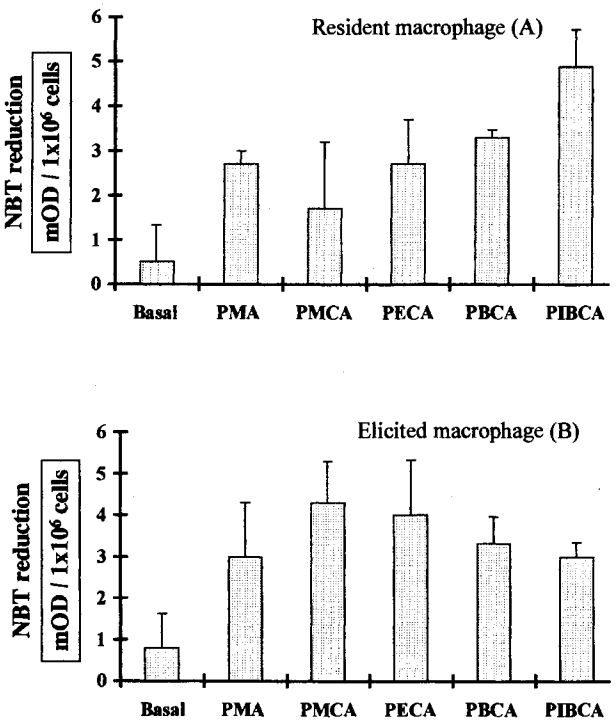

Fig. 4. Intracellular reduction of NBT by resident and elicited peritoneal macrophages incubated with polyalkylcyanoacrylate nanoparticles. Resident and elicited peritoneal macrophages (1×10^5 cells/well) were incubated in RPMI-1640 containing 100 μg/ml polyalkylcyanoacrylate nanoparticles before the assay of NBT reduction, as described in Materials and Methods. In positive control, cells were incubated with phorbol myristate acetate. Basal level of NBT reduction was determined with NBT. Values are mean ± S.E.M. for three different experiments, each done in triplicate. [mAbs-milliabsorbance; PMA-phorbol myristate acetate].

by the trypan blue assay was less pronounced then that displayed in the MTT assay, which is probably due to cell fragmentation. Moreover, results from our physico-chemical characterisation of nanoparticles and MTT metabolism assessment show that differences in zeta potential and size do not influence the cellular toxicity (Fig. 2 and Table I). Charge and size, at least for the observed values, seemed to have no influence on nanoparticles toxicity in macrophages.

Table I. Results for Polyalkylcyanoacrylate Nanoparticles Related to Their Size and Surface Properties Analysis Using Photon Correlation Spectroscopy (Autosizer II) and Doppler Electrophoretic Light Scattering Analysis (Coulter® DELSA 440)

	DELSA			Autosizer II	
	Size (nm)	Zeta potential (mV)	Electrophoretic mobility (μm.cm/V.s)	Size (nm)	Polydispersity index
PMCA	461.1	−17.88	−1.380	562.8	0.657
PECA	394.3	−7.95	−0.624	319.0	0.047
PBCA	290.4	−7.43	−0.586	186.1	0.015
PIBCA	317.9	−7.48	−0.587	186.8	0.072

Previous reports suggest that polystyrene nanoparticles may activate complement system by the classical and/or alternative pathways (20), which could lead to a deposition of the C3b and $C3b_i$ proteins on nanoparticles surfaces. These complement proteins may stimulate an oxygen burst in neutrophils and monocytes and promote phagocytosis. It was also observed the production of an oxidative burst with anion superoxide formation in a murine macrophage-like cell line (J774) after the phagocytic uptake of polyalkylcyanoacrylate nanoparticles (6). In this work, we observed that the polyalkylcyanoacrylate nanoparticles phagocytosis by peritoneal macrophages was associated with an increase in the oxidative burst (Fig. 4) and with the release of soluble toxic factors, which directly affected the viability of resident and elicited macrophages (Fig. 3).

In macrophages, the expression of the inducible NO synthase isozyme is activated by LPS in association with growth factors (10). We observed that simultaneous treatment of resident macrophages with LPS and PMCA nanoparticles potentiate the NO synthase activity (increases of nitrites in culture medium) as compared to that of macrophages incubated with LPS alone (Table II). These findings suggest that PMCA nanoparticles adsorb LPS on their surface and as a consequence a greater number of LPS molecules are internalised during the phagocytosis of these nanoparticles. On the contrary, the simultaneous treatment of resident macrophages with LPS and PBCA nanoparticles (25 and 100 μg/ml) produces the same nitrite concentration as LPS alone, which indicates a different physicochemical interaction between LPS and both polymers.

In conclusion, we present evidence that the phagocytosis of polyalkylcyanoacrylate nanoparticles is associated with the release of soluble toxic factors and that the toxicity of these drug carriers is not only a function of the alkyl chain length. In addition, we demonstrated that the association of PMCA nanoparticles with LPS significantly stimulates the expression of the inducible isoform of the nitric oxide synthase on resident peritoneal macrophages. Further studies are needed to increase our knowledge about nanoparticle-macrophage interactions and as a result modern therapeutic strategies might be designed in order to enhance drug specificity when considering macrophage-targeted drug delivery.

Table II. Nitrite Formation by Resident Peritoneal Macrophages Incubated with LPS and PMCA or PBCA Nanoparticles

Additions	Nitrite production (nmol/ml)	
	np-PMCA	np-PBCA
LPS	0.50 ± 0.05	0.52 ± 0.03
LPS + np (25 μg/ml)	0.50 ± 0.1	0.50 ± 0.05
LPS + np (100 μg/ml)	0.90 ± 0.15	0.50 ± 0.04
LPS + np + MetArg	0.21 ± 0.001	0.2 ± 0.001
LPS + MetArg	0.20 ± 0.001	0.22 ± 0.001
np (100 μg/ml)	0.01 ± 0.02	0.012 ± 0.02

Note: Resident macrophages (5×10^5 cells/well) were incubated in RPMI 1640 containing LPS (500 ng/ml) and various PMCA or PBCA nanoparticles concentrations (25 and 100 μg/ml) before analysis of nitrite production as described in Materials and Methods. Control experiments were performed with nanoparticles alone, or with MetArg (1 mM) added simultaneously with LPS and nanoparticles. Values are mean ± S.E.M. for three different experiments, each done in triplicate [MetArg- N^G-monomethyl-L-arginine].

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