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Cytotoxicity studies of Dynasan 114 solid lipid nanoparticles (SLN) on RAW 264.7 macrophages—impact of phagocytosis on viability and cytokine production

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Abstract

Solid lipid nanoparticles (SLN) based on Dynasan 114 (D114) were tested using RAW 264.7 cells. The influence of different surfactants on the cytotoxicity of this type of SLN was examined, expressed as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability and the production of cytokines such as interleukin 6 (IL-6), IL-12 and tumour necrosis factor-α (TNF-α). Results were compared with previously obtained data when peritoneal mouse macrophages were used. SLN produced with stabilizers/surfactants such as poloxamer 188, sodium cholate, Lipoid S75, Tween 80, Poloxamine 908 and sodium dodecylsulfate were shown to be nontoxic towards RAW 264.7 cells. Cytokine production was reduced and stimulation, expressed in elevated cytokine levels, could not be found. Using cetylpyridinium chloride (CPC) as stabilizing surfactant, SLN became cytotoxic in a concentration-dependent manner. Not only were the viabilities reduced but also cytokine production. Cytotoxic effects of CPC stabilized SLN could be antagonized using cytochalasin B to block phagocytosis. D114-SLN produced with pharmaceutically accepted surfactants for intravenous injection (poloxamer 188, Lipoid S75, sodium cholate, Tween 80) were very well tolerated by the cells. Even sodium dodecylsulfate-stabilized D114-SLN did not exert toxic effects. Comparison of the RAW 264.7 data with previously obtained data from toxicity studies of D114-SLN towards peritoneal mouse macrophages showed similar results. This offers the possibility of using the RAW 264.7 cell line for cytotoxicity studies of colloidal drug carrier systems, rather than using laboratory animals as source of macrophages for these kinds of studies.

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Introduction

Solid lipid nanoparticles (SLN) were developed as an alternative drug delivery system to emulsions, liposomes and polymeric nanoparticles (Müller & Lucks 1996; Müller et al 2000a). Similarly to emulsions and liposomes, SLN consist of ingredients that are well tolerated physiologically and offer the possibility of a controlled drug release (Schwarz et al 1993; Müller et al 1995; Müller & Lucks 1996). Equally to polymeric nanoparticles, their solid matrix protects active ingredients against chemical degradation and allows modulation of drug release profiles (Mehnert et al 1997; Zur Mühlen et al 1998). SLN can be produced without using toxic solvents and even large-scale production is possible (Müller et al 2000b). SLN are suitable for drug targeting to specific tissue sites (Yang et al 1999; Gessner et al 2001). For parenteral administration, information about the interaction of SLN with phagocytic cells is a prerequisite.

After intravenous injection of particulates such as solid lipid nanoparticles (SLN) their surface properties, mainly determined by the surfactants used, greatly impact their fate (Müller et al 1995). Surfactants have been identified leading to a decreased uptake of particulate delivery systems by monocytic phagocytes (Illum et al 1987; Rudt & Müller 1992; Müller & Olbrich 1999b). An increased uptake by phagocytic cells is needed when SLN are used as drug delivery systems for antibiotics against intracellular parasites and bacteria or as vaccine adjuvants for immunization by delivering specific

antigens to antigen presenting cells (APC), such as macrophages or dendritic cells (Olbrich et al 2000). Phagocytic cells – such as mononuclear phagocytes and granulocytes – are the first cells that interact with particles in the bloodstream and thereby represent the first line of defence of the immune system. Contact of SLN may lead to activated mononuclear phagocytes which play an important role in immune reactions by releasing NO, producing a respiratory burst and pro-inflammatory cytokines such as interleukins (IL-1, IL-6, IL-12) or tumour necrosis factor (TNF-α) (Claudio et al 1995; Cruz et al 1997; Kawai et al 2000). Secretion of these substances, however, may lead to adverse reactions, which cause severe damage to the host e.g. as described for lipopolysaccharide (LPS)-induced shock (Van Furth et al 1972; Baumann & Gauldie 1994).

On the other hand, cytokines such as IL-6 play an important role in the field of vaccination. Vaccine adjuvants e.g. MF59, a squalene–Tween 80/Span 85 emulsion, induce amongst others the formation of IL-6 by activation of macrophages and consequently enhance the immunogenicity of protein and subunit antigens (Harding et al 1991; Ott et al 1995). Recently, SLN were successfully tested as vaccine adjuvants (Müller et al 1999; Olbrich et al 2000). For this reason, a cell culture model allowing the determination of the induction of cytokines such as IL-6 and TNF-α being a stimulus for dendritic cells, promoting the antigen presentation could be useful. This is why IL-6, IL-12 and TNF-α were selected as indicators for the activation of macrophages.

For the evaluation of the in-vivo behaviour and toxicological acceptance of drug delivery systems, knowledge of what causes changes in the production of these pro-inflammatory cytokines is of great importance. Peritoneal mouse macrophages and J774 cells were often used to determine cytotoxic effects of nanoparticles on macrophages, expressed as viability in the MTT test (Peracchia et al 1998; Soma et al 2000). However, only a few studies were performed to evaluate the toxicity of colloidal drug carriers on peritoneal mouse macrophages in terms of cytokine production and activation of macrophages (native peritoneal mouse macrophages) (Schöler et al 1999, 2000a,b,c). Many experiments were performed to test activation and cytokine production of macrophages on exogenic stimuli using RAW 264.7 cells (Kubo et al 1998; Park et al 1999), demonstrating the comparability of the cells' behaviour with native peritoneal mouse macrophages. RAW 264.7 macrophages, a monocyte/macrophage-like cell line, was established from the ascites of a tumour induced in a male BALB/C mouse by the intraperitoneal injection of Abelson leukaemia virus (Ralph & Nakoinz 1977; Raschke et al 1978). RAW 264.7 cells are able to pinocytose neutral red, and phagocytose zymosan and latex beads. To evaluate the suitability of this cell line to be susceptible with respect to the IL-6, IL-12 and TNF-α production, as well as the MTT viability, on contact with solid lipid nanoparticles (SLN), Dynasan 114 (trimyristin) SLN were produced using different surfactants. Since the surfactant of SLN represents the particle surface and therefore affects their surface properties, it is of great interest to examine how SLN "coated" with different surfactants interact with mononuclear cells. Surface properties, e.g. hydrophobicity and surface charge, were found to have a major impact on phagocytic uptake of particulate drug delivery systems (Van Oss et al 1975, 1983). The surfactant also influences the amount and velocity of phagocytosis of SLN by human granulocytes (Müller et al 1997a). Sterically stabilizing surfactants such as block-copolymers, especially Poloxamer 407 and Poloxamine 908, led to a reduction of phagocytosis of SLN by freshly isolated human granulocytes (Müller et al 1997a) and by retinoic acid differentiated HL 60 cells (Müller et al 1997b). In addition macrophages failed to recognise polystyrene model drug carriers coated with Poloxamer 407 or Poloxamine 908 (Illum et al 1986, 1987). Studies on isolated granulocytes, retinoic acid differentiated HL 60 cells and by intravenous injection in mice in high doses demonstrated good tolerability of SLN (Müller & Olbrich 1999a, b). Recently immunomodulatory and cytotoxic effects of SLN on peritoneal mouse macrophages were investigated (Schöler et al 1999, 2000a,b,c). By varying the lipid matrix and using the same surfactant (Poloxamer 188) SLN did not cause an increase in the release of IL-6, IL-12, or TNF-α as an effect of macrophage activation. In contrast, levels of cytokines were reduced compared with the untreated control, which could be the result of observed concentration-dependent cytotoxic effects (Schöler et al 1999, 2000a,b,c). By comparing this data with data obtained for Dynasan 114 or Compritol (Glyceroltribehenate), both stabilized with the lecithin Lipoid S75, no cytotoxic effects on retinoic acid differentiated HL 60 cells (Müller et al 1997b) could be detected. No significant loss of viability of human granulocytes was observed after incubation with SLN consisting of cetylpalmitate or Compritol with Poloxamer 188 as surfactant (Müller et al 1997a).

To predict the cytotoxicity and/ or modulation of cytokine production of Dynasan 114-SLN produced with different surfactants, RAW 264.7 macrophages were used in this study. As a continuously growing cell line the cells were easily accessible, and thus animal saving. Results were compared with data previously obtained for peritoneal mouse macrophages and the influence of phagocytosis on cytotoxic effects on RAW 264.7 cells was studied using cytochalasin B as an inhibitor of phagocytosis (Zhou & Leaf 1994). For this reason we used a Dynasan 114 formulation with cetylpyridinium chloride, which was demonstrated to be cytotoxic.

Materials and Methods

Preparation and size measurement of SLN

D114-SLN were produced by hot high pressure homogenization. The lipid (10%, w/w) was melted at approximately 10°C over the melting point, poured into a hot aqueous surfactant solution (1%, w/w) and dispersed by high speed stirring to yield a pre-emulsion (Ultra turrax T 25, Janke and Kunkel, Staufen, Germany). This

was subsequently homogenized using an APV LAB 40 homogenizer (APV-Gaulin, Lübeck, Germany), applying three cycles of 500 bar (Müller et al 1995). Different SLN formulations were produced using different surfactants under aseptic conditions using a homogenizer placed in a laminar air-flow chamber. Particle size was determined by dynamic light scattering (photon correlation spectroscopy (PCS)) (Malvern Zetasizer IV, Malvern Instruments, Herrenberg, Germany), allowing particle size determination of colloids in the size range of 3 nm–3 μm. As a measure of the width of the particle size distribution the polydispersity index (PI) was determined. The PI of a particle distribution is in the range between 0 (monodisperse) and 1 (polydisperse). Parenteral fat emulsions typically possess PI values in the range of approximately 0.1–0.2.

Dynasan 114 (glyceroltrimyristate) was obtained as a gift from Condea (Witten, Germany). The surfactants Poloxamer 188 (Pluronic F68) and Tween 80 (T80) were kindly provided by Uniquema (Eversberg, Belgium) and Poloxamine 908 from Ch. Erblöh (Düsseldorf, Germany). Cholic acid sodium salt (SC) and cetylpyridinium chloride (CPC) were purchased from Sigma Aldrich (Deisenhofen, Germany). Lipoid S 75 (soybean lecithin) was a gift from Lipoid KG (Ludwigshafen/Rhein, Germany) and Solutol HS 15 was kindly provided as a gift from BASF (Ludwigshafen, Germany). Glycerol for isotonization (2.25%, final concentration) was purchased from Merck (Darmstadt, Germany). Lipofundin MCT 10% was obtained from Braun Melsungen (Melsungen, Germany).

RAW 264.7 macrophages

RAW 264.7 macrophages (ATTC TIB 71) were obtained from Dr Kiderlen (The Robert Koch Institute, Berlin, Germany). They were cultured in a 5% carbon dioxide atmosphere at 37 °C in RPMI 1640 medium containing 10% heat inactivated foetal calf serum (FCS) and 2 mM L-glutamine, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin. After chilling for 2 h in the fridge, cells being spontaneously plastic adherent were harvested by soft scraping with a cell scraper. Viability was determined by trypan blue exclusion staining (Biochrom, Berlin, Germany). Cells were suspended in medium at concentrations of 1×10^6 cells mL^{-1} and seeded into 96-well flat bottom plates (Nunc, Roskilde, Denmark) (100 μL/well). Cell culture reagents were purchased from Sigma Aldrich (Deisenhofen, Germany). After a 3-h incubation (37°C, 5% CO_2) adherent cells were washed three times with culture medium. Subsequently, adherent cells were incubated in the presence of different concentrations of D114-SLN suspensions and Lipofundin MCT 10% (LIP) emulsions dispersed in cell culture medium or 75.0 μg mL^{-1} Pansorbin (Protein A of *Staphylococcus aureus*) (Calbiochem, Bad Soden, Germany). After a 1-h incubation, extracellular SLN were rinsed off and washed three times with cell culture medium. For cytokine detection, supernatants were harvested after a 20-h incubation and stored at −70°C until testing. Experiments were generally performed in triplicate and repeated at least once.

MTT assay

Cytotoxicity of SLN on RAW 264.7 macrophages was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). Cells were used for the MTT assay after removal of the supernatants for the cytokine detection and subsequent addition of cell culture medium. After 20 h, 50 μL MTT solution (Sigma-Aldrich) (2.5 mg mL^{-1} MTT in PBS) was added and incubated for 4 h at 37 °C in a 5% CO_2 incubator. MTT acts as a substrate for dehydrogenase enzymes and is modified to a coloured formazan product by living cells only. After solubilization of the formazan crystals by 25% sodium dodecylsulfate (SDS) in 0.075 M NaOH solution (Sigma Aldrich, Deisenhofen, Germany), absorbance that was directly proportional to the cellular metabolism was measured at 550 nm in an automated ELISA plate reader (Easy Reader, Gröding, Austria). Viability was expressed as percent compared with untreated cells. Experiments were performed in triplicate and repeated at least once. Surfactants/stabilizers were tested as controls in concentrations being present in the D114-SLN dispersions. Please note: the codes given in Figures 1B and 2 represent the concentrations of surfactant/stabilizer in the SLN formulation concerned.

Inhibition of phagocytosis

Phagocytosis was inhibited using cytochalasin B (10 μg mL^{-1}). Chloroquine (50 μmol) (Sigma Aldrich, Deisenhofen, Germany) was used to increase the intravesicular pH of endosomes (Zhou & Leaf 1994). Cytochalasin B was added to the cells after the adherence time of 4 h for an additional 4 h, then removed. Cells were incubated with SLN afterwards as described previously. Chloroquine was added after adherence of the cells but not removed before D114-SLN was added.

Cytokine assay

IL-6, IL-2 and TNF-α were determined in supernatants by two-site enzyme-linked immunosorbent assays (ELISA) in Nunc-Immuno modules (Nunc, Roskilde, Denmark) according to standard procedures. Briefly, purified anti-cytokine antibodies (clones MP5-20F3, C15.6, MP6-XT22 for IL-6, IL-12, TNF-α, respectively) were used as primary antibodies, biotinylated rat anti-mouse cytokines (clones MP5-32C11, C17.8, MP6-XT3 for IL-6, IL-12, TNF-α, respectively) as the secondary antibodies and streptavidine-conjugated peroxidase as the developing reagent with 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate. All reagents used for ELISA were obtained from Pharmingen (San Diego, CA), except streptavidine horseradish peroxidase (Amersham, Little Chalfont, UK). TMB and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Cytokine concentrations were determined by referring to standard curves obtained with fixed amounts of mouse recombinant IL-6, IL-12 or TNF-α. Optical densities (OD) were determined using an automated ELISA plate reader (Tecan, Crailsheim,

Germany) at 405 nm using a 620 nm reference filter. Data from determinations in triplicate were expressed as ng mL^{-1} (mean ± s.d.). The sensitivity limits of the different assays were as follows: IL-6 0.20 ng mL^{-1}; IL-12 0.15 ng mL^{-1}; TNF-α 0.15 ng mL^{-1}.

Statistical analysis

Differences between two or more samples were statistically examined using the appropriate non-parametric test ($P < 0.05$ denoting significance).

Results and Discussion

The sizes of the D114-SLN were in the range 150–250 nm, with polydispersity index (PI) values of 0.15–0.210 (Table 1). Incubation of RAW 264.7 cells with D114-SLN coated with uncharged, sterically stabilizing surfactants (Poloxamer 188, Tween 80) at concentrations from 0.1% to 0.00001% did not result in major cytotoxic effects (Figure 1A). As a general feature viability of cells was reduced to approximately 80% compared with the untreated control, even when using the lowest concentration of nontoxic Lipofundin MCT. Additionally, D114-SLN produced with surfactants of the same chemical class as Poloxamer were tested (Poloxamine 908, Solutol HS 15, Poloxamer 407): they did not show any cytotoxic effects in this concentration range (data not shown). Also for D114-SLN produced with charged surfactants, no reduction in the viabilities could be detected (Figure 1A), even when using sodium dodecylsulfate as a stabilizer for the SLN. Cells were incubated with pure stabilizer solutions (controls), not showing any significant cytotoxicity. The reduced viabilities, being least for sodium dodecylsulfate solution, were not dose dependent (Figure 1B).

Contrary to these findings, a strong cytotoxic effect of cetylpyridinium chloride-coated D114-SLN could be detected (Figure 2). At a D114-SLN concentration of 0.1% cell viability was reduced to 3.8% (SD 0.87), and at 0.01% viability was 40.2% (SD 1.23). When we used D114-SLN stabilized with 0.0001% cetylpyridinium chloride the viability was only slightly reduced to 68.2% (SD 5%). These toxic effects of cetylpyridinium chloride could only be observed when administered as a stabilizer of SLN formulation. Application of cetylpyridinium chloride solution alone (i.e. in a concentration identical to the one present in the SLN dispersions) did not exert

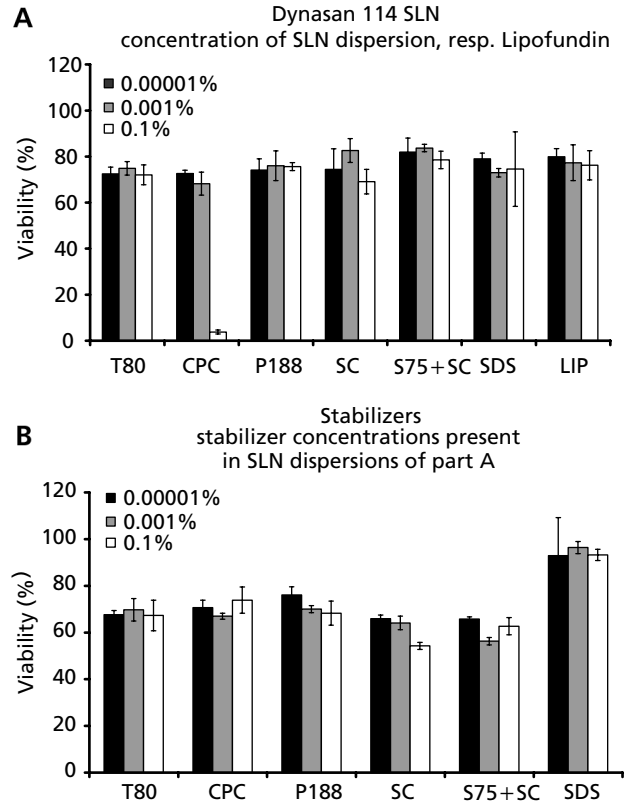

Figure 1 (A) Viability of RAW 264.7 macrophages after 20-h in-vitro incubation with different concentrations of Dynasan 114-SLN produced with different surfactants/stabilizers and incubation with Lipofundin MCT. (B) Viability of RAW 264.7 macrophages after 20-h in-vitro culture with the surfactants used as stabilizers for the SLN in part A. Viability of untreated RAW 264.7 cells was taken as 100% viability (n = 3). T80, Tween 80; CPC, cetylpyridinium chloride; P188, Poloxamer 188; SC, sodium cholate; S75+SC, Lipoid S75/sodium cholate 1:1; SDS, sodium dodecylsulfate; LIP, Lipofundin MCT 10%. The concentrations of surfactants were identical to their concentrations in the SLN dispersions of 0.00001%, 0.001% and 0.1%.

Table 1 Mean diameter and polydispersity indices (PI) of SLN formulations determined by photon correlation spectroscopy (PCS) (n = 3). The lipid content was 10% Dynasan 114 in each formulation and the surfactant concentration was 1%, except for Lipoid S 75/sodium cholate (0.5%/0.5%)

Surfactant	Code of formulation	PCS mean diameter (nm) (s.d.)	Polydispersity index (s.d.)
Poloxamine 908	P908	253 (1)	0.140 (0.03)
Poloxamer 188	P188	252 (4)	0.210 (0.02)
Tween 80	T80	146 (1)	0.210 (0.01)
Sodium cholate	SC	254 (2)	0.194 (0.03)
Lipoid S 75/sodium cholate	S75/SC	192 (2)	0.120 (0.03)
Sodium dodecylsulfate	SDS	167 (3)	0.190 (0.01)
Cetylpyridinium chloride	CPC	160 (1)	0.190 (0.01)

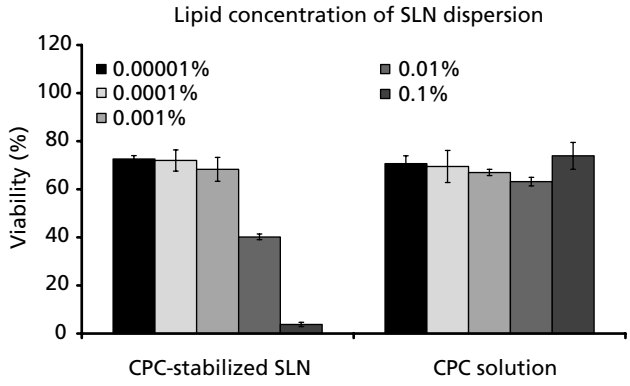

Figure 2 Viability of RAW macrophages after incubation with of cetylpyridinium chloride (CPC) solution and CPC-stabilized SLN formulation. Viability of untreated RAW 264.7 cells was taken as 100% viability (n = 3). The concentration of CPC in the CPC-solutions was equivalent to its concentration in the corresponding SLN formulation. The percentages of SLN dispersions are given (i.e. in a 0.1% SLN dispersion was 0.01% CPC).

any cytotoxic effects in the MTT assay (Figure 2). To evaluate the influence of phagocytosis and intracellular processing on the toxicity of cetylpyridinium chloride stabilized D114-SLN, cytochalasine B and chloroquine were used. At a concentration of 0.01% SLN, significant differences in the viabilities could be observed. Pre-treatment with cytochalasin B led to viabilities compared with untreated cells (Figure 3), whereas addition of chloroquine resulted in a reduced viability compared with Figure 2. When using higher concentrations (0.1 and 1%) the viability was reduced to almost zero.

In addition to direct cytotoxic effects, the production of pro-inflammatory cytokines IL-6, IL-12 and TNF-α was examined. IL-12 was not detectable after incubation of cells with all D114-SLN formulations and even stimulation with pansorbin did not lead to detectable concentrations. Pansorbin did upregulate the production of IL-6 and TNF-α in RAW 264.7 cells, proving the ability of the cells to react on suitable stimulants in producing pro-inflammatory cytokines. IL-6 production by RAW 264.7 cells following incubation with the afore mentioned SLN formulations was reduced at SLN concentrations of 0.00001 to 0.1% in a concentration-dependent manner for some formulations only (Figure 4). For D114-SLN stabilized with Poloxamer 188, sodium cholate/Lipoid S75 and sodium dodecylsulfate, a decrease of IL-6 production was detected. The levels of IL-6, even with non-toxic SLN-concentrations, were reduced to 80–90% compared with the untreated control. For the cholic acid sodium salt stabilized-D114-SLN, a slight increase in IL-6 production with increasing SLN concentration seemed to be present, but was not significant. No changes in IL-6 production were found with Tween 80-stabilized D114-SLN. A significant dose-dependent decrease in IL-6 levels was found for cetylpyridinium chloride-stabilized D114-SLN (Figure 4).

The ability of RAW 264.7 cells to produce TNF-α on selected D114-SLN was tested. Incubation of cells with SLN did not lead to an increase but to a reduction of TNF-α levels in the cell supernatants (Figure 5). For all D114-SLN the TNF-α levels were lower than the untreated control. Incubation with Lipofundin MCT influenced only a little TNF-α secretion, whereas the effect of cetylpyridinium chloride D114-SLN was very drastic. Even at an SLN concentration as low as 0.00001%, the TNF-α secretion was reduced to 50% of the untreated control. Increasing the concentration to 0.01%, TNF-α levels were reduced to 15%. After the incubation with 0.1% D114-SLN, no TNF-α could be detected in the supernatant. With regard to Tween 80 D114-SLN, a dose-dependent decrease of TNF-α was

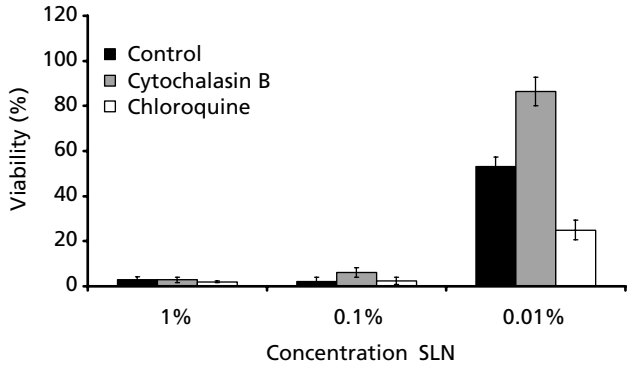

Figure 3 The influence of pre-incubation with cytochalasin B and chloroquine. Viability of RAW 264.7 macrophages after 20-h in-vitro incubation with different concentrations of Dynasan 114-SLN produced with cetylpyridinium chloride. The cells were pre-incubated with cytochalasin B and chloroquine. Viability of untreated RAW 264.7 cells was taken as 100% viability (control) (n = 3).

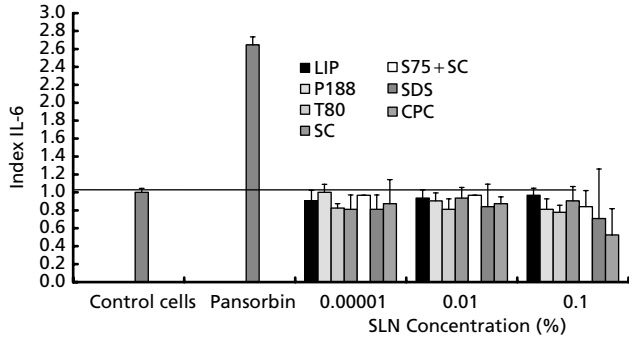

Figure 4 Secretion of IL-6 in supernatants of RAW 264.7 cells after 20-h incubation with SLN at increasing concentrations produced with different surfactants from Table 1. Detection was performed using an ELISA assay. IL-6 levels are shown as index IL-6 with standard deviation. LIP, Lipofundin MCT 10%; P188, Poloxamer 188; T80, Tween 80; SC, sodium cholate; S75+SC, Lipoid S75/sodium cholate 1:1; SDS, sodium dodecylsulfate; CPC, cetylpyridinium chloride. As a control, IL-6 levels were determined in the medium of untreated cells and after Pansorbin incubation.

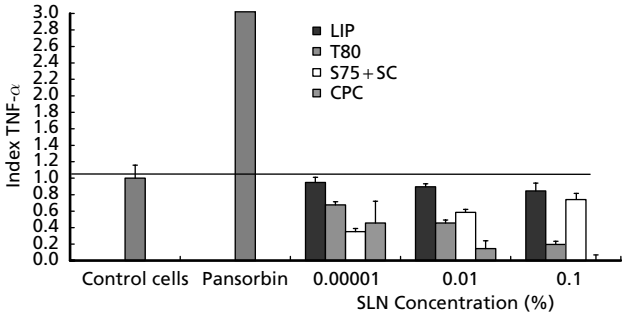

Figure 5 Secretion of TNF-α in supernatants of RAW 264.7 cells after 20-h incubation with SLN at increasing concentrations produced with different surfactants from Table 1. Detection was performed using an ELISA assay. TNF-α levels are shown as index IL-6 with standard deviation. LIP, Lipofundin MCT 10%; T80, Tween 80; S75+SC, Lipoid S75/sodium cholate 1:1; CPC, cetylpyridinium chloride. As a control, IL-6 levels were determined in the medium of untreated cells and after Pansorbin incubation.

observed. A contrary result was obtained using sodium cholate/Lipoid S75 stabilized SLN. Here, a dose-dependent increase in TNF-α levels could be observed (Figure 5). This effect could not be observed for sodium cholate-stabilized D114-SLN (data not shown).

D114-SLN produced using different surfactants did not show cytotoxic effects on RAW 264.7 cells, except that the cetylpyridinium chloride-stabilized formulation was cytotoxic in a concentration-dependent manner. Sodium cholate is a substance that causes membrane damage in various test systems e.g. expressed as haemolysis (Martin et al 1992). Haemolytic effects occurred at concentrations from 6 mM. Within the formulations tested in this study the highest concentration was 0.25 mM. Cytotoxic effects could arise when higher concentrations of sodium cholate were used. To achieve a sufficient physical stability of D114-SLN dispersions, the use of higher amounts of the bile salt is not necessary. Therefore the sodium cholate formulation is supposed to be nontoxic towards RAW 264.7 cells.

The cytotoxicity of cetylpyridinium chloride-stabilized D114-SLN was due to phagocytosed particles, as shown by the co-administration of cytochalasin B. Previous experiments with colloidal drug carrier systems demonstrated that their cytotoxicity might be caused by different mechanisms. Polycyanoacrylate nanoparticles were reported to be cytotoxic due to adherence to the cell membrane, the subsequent degradation of particles and release of toxic degradation products leading to holes in the cell membrane (Lherm et al 1991; Müller et al 1996). Muller et al (1997a) discussed this mechanism of cytotoxicity for SLN. Cetylpyridinium chloride is commonly used as a cationic antiseptic. The antimicrobial activity is correlated with bacterial membrane functions. Therefore cytotoxic effects on RAW 264.7 macrophages could possibly be explained by adherence to the cell membrane. However, the results obtained with cytochalasin B pretreatment revealed the contribution of phagocytosis on cytotoxicity (Figure 3). No cytotoxic effects were observed when incubating RAW 264.7 cells with cetylpyridinium chloride solutions at concentrations equivalent to those used in cetylpyridinium chloride D114-SLN. The cetylpyridinium chloride molecules in the solution were dissolved in cell culture medium and were adsorbed on the cell surface as well. Cetylpyridinium chloride-stabilized D114-SLN adhering to the cell surface resulted in high local concentrations of cetylpyridinium chloride at those points of adsorption which could lead to membrane damage. However, from the results obtained with the phagocytosis inhibiting substance cytochalasin B it could be concluded that the cytotoxic effects referred mainly to intracellular events when the positively charged D114-SLN were taken up by phagocytosis. The positive charge on the particle surface is a phagocytosis promoting factor (van Oss 1975), and so it can be assumed that many of the cetylpyridiniumchloride-stabilized D114-SLN had been taken up by the RAW 264.7 macrophages. The positive charge promoted adherence to the negatively charged cell membranes as well. Adherence to the membrane is the first step of internalization, thus the positive charge further promotes phagocytosis. Due to the small particle size of the formulation an essential amount of the particles will have been taken up by pinocytosis. Pinocytosis is not inhibited using cytochalasin B. This is the reason for observing the cytochalasin B effect only at the 0.01% SLN concentration. At higher D114-SLN concentrations (0.1% or 1%) more particles may be taken up by pinocytosis leading to cytotoxic effects. Rudt & Müller (1992) showed that particle internalization increased with increasing particle concentration in the cell suspension. The increased D114-SLN concentration led to the uptake of an amount of pinocytosed particles sufficient to cause distinct cytotoxicity. Therefore, to avoid cytotoxic effects by preventing uptake of particles in a larger size range cytochalasin B is not effective.

Toxic effects of positively charged substances after ingestion in cells are described for cationic lipids, used as liposomes as cell transfection agents e.g. DOTAP (Wattiaux et al 1997). Cytotoxic effects of these substances were reported to be caused by destabilization or by disruption of the lysosomal or the endosomal membrane. The effect of chloroquine was to disable the degradative function of the lysosomes or to prevent the fusion of endosomes with the lysosomes (Fraley et al 1981). In general, the fusion of the endosomes with the lysosomes leads to a detoxification and the presence of lysosomal enzymes may lead to a decrease in the cytotoxicity of the cetylpyridinium chloride D114-SLN. If cationic liposomes for the delivery of DNA are used, pretreatment with chloroquine results in enhanced transfection activity. For DNA–cationic liposome complexes it could be shown that chloroquine leads to an escape of the complexes from the endocytic vacuoles into the cytoplasm (Zhou & Leaf 1994). If this chloroquine effect is valuable for the cetylpyridinium chloride D114-SLN as well, the presence of the stabilizer cetylpyridinium chloride in the cytoplasm will be toxic for the cell. In fact pretreatment of cells with chloroquine enhanced the cytotoxicity of cetylpyridinium chloride-stabilized D114-SLN. This is an example of

a toxification of a substance when applied with SLN. As an example of detoxification the cytotoxic drug hexadecylphosphocholine has been employed as a surfactant in an emulsion formulation resulting in a reduction of haemolytic effects at 1/1000 (Müller & Olbrich 1999b).

Comparing our results with the results of former studies with other cell types, differences were apparent. Using retinoic acid differentiated granulocyte-like HL 60 cells it could be found that stabilizing surfactants influenced the extent of cytotoxic effects of SLN (Müller et al 1997b). SLN consisting of Compritol (glyceroltribehenate) coated with Poloxamer 407 did not lead to reduced viabilities of cells at concentrations of up to 10%, whereas a distinct reduction was observed when using SLN consisting of the same matrix stabilized with Tween 80 at concentrations of $>0.1\%$ (Müller et al 1997b). In contrast to findings in RAW 264.7 and macrophage cultures, the viability of HL 60 cells incubated with solutions of Tween 80 alone at concentrations present in SLN of 0.0001% was reduced to 50%. Besides that finding, a decrease in viability linked with a decrease in the molecular weight of the Poloxamers could be detected (Müller et al 1997b). Using SLN stabilized with Poloxamer 407, Poloxamer 184, Poloxamer 188, Poloxamer 235 or Poloxamer 335 at SLN concentrations $>0.01\%$, a decrease in viability was observed, which was attributed to the decreasing sterically-stabilizing layer of the SLN being responsible for higher rates of phagocytosis of nanoparticles (Müller 1991).

Cytotoxicity was most pronounced for the low molecular weight Poloxamer 188, which can be attributed to highest uptake. In addition, SDS-stabilized SLN were well tolerated at concentrations $<0.01\%$, whereas a solution of SDS (at concentrations present in 0.00001% SLN) exerted distinct cytotoxic effects (Müller et al 1997b). For RAW 264.7 macrophages no cytotoxic effects could be seen, on the contrary less reduced viability of the cells in SDS solutions was observed (Figure 1B). Using freshly isolated human granulocytes, similar results to the results with differentiated HL 60 cells were observed when granulocytes were incubated with the same SLN (Müller et al 1997b).

Other researchers obtained different results concerning the behaviour of the cells with respect to the production of cytokines when using different particulate carriers. Green et al (1998) reported the size-dependent upregulation of IL-6 and TNF-α production by peritoneal macrophages when polyethylene particles were used. The highest concentration of cytokines was measured in the cell supernatants after incubation of macrophages with polyethylene particles in a phagocytosable size range (0.3–10 μm). This effect was correlated with the nature of the particles because no differences in cell viability were observed after incubation of macrophages with polystyrene particles in the range of 0.2 to 88 μm (Green et al 1998). Additionally, cytotoxic effects of AL_2O_3 particles on the J774 macrophage cell line increased with particle size greater than 2 μm but no IL-6 and TNF-α upregulating effect could be observed (Catelas et al 1998). Using RAW 264.7 cells, Claudio (1995) found macrophage activation with increasing levels of TNF-α secretion when silica particles were used. The lack of effect or limited effect of most SLN on IL and TNF-α secretion proved the good tolerability of this drug carrier system.

By comparing the SLN data for RAW 264.7 cells with data obtained for peritoneal mouse macrophages, Schöler et al (2000a,b,c) found the following similarities and differences. The viability of the cells treated with the nontoxic Lipofundin or surfactant solutions was approximately 90% for the peritoneal mouse macrophages, whereas for the RAW 264.7 macrophages viabilities were shown to be in the range of only 75–80% (Figure 1B). A remarkable cytotoxic effect was exerted on macrophages by the positively charged cetylpyridinium chloride D114-SLN at 0.01 and 0.1%. In parallel to these findings, IL-6 production was reduced in a concentration-dependent manner following incubation with cetylpyridinium chloride-stabilized D114-SLN. Solutions of surfactants described above as components of SLN formulations were tested alone at equivalent concentrations to those used in SLN. As well as found for the RAW 264.7 cells, these solutions did not result in reduced viabilities of peritoneal mouse macrophages. Incubation of macrophages or RAW 264.7 cells with D114-SLN did not result in upregulation of cytokine production. Down-regulatory effects were associated with cytotoxicity of D114-SLN or could be due to inhibition of proliferation in RAW 264.7 cells. For macrophages, concentration-dependent changes in viability and reduction of cytokine production for increasing D114-SLN concentration could be demonstrated, though they were not very pronounced except for the cetylpyridinium SLN, where they were significant.

Conclusion

The results showed the good tolerability of Dynasan 114 solid lipid nanoparticles by the RAW 264.7 macrophage cell line when using pharmaceutically accepted surfactants. D114-SLN were well tolerated in terms of MTT reduction and with respect to IL-6 and TNF-α secretion, except for the positively charged cetylpyridinium chloride formulation. Neither SLN nor the pure surfactant solutions caused any increase in cytokine production but did cause a decrease. For cetylpyridinium chloride it could be shown that the internalization of the particles seemed to be mainly responsible for the toxic effects. When particles internalized by cells are released into the cytoplasm – as occurs by a co-incubation with chloroquine – they become even more cytotoxic. When solutions of surfactants (as described above) used as components of D114-SLN were tested alone at equivalent concentrations to those used in the D114-SLN formulations, they did not exert cytotoxic effects on RAW 264.7 cells. When comparing these data with data obtained previously from peritoneal mouse macrophages, the same tendencies where shown, making the cell line interesting for an alternative use in the testing of colloidal drug carriers. With respect to interactions of D114-SLN with RAW 264.7 cells, further studies are necessary to evaluate their ability to induce the formation of NO or to give a phagocytic burst.

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