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International Journal of Pharmaceutics 231 (2002) 167–176

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Effect of lipid matrix and size of solid lipid nanoparticles (SLN) on the viability and cytokine production of macrophages

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Received 31 July 2001; accepted 30 August 2001

Abstract

Solid lipid nanoparticles (SLN) interact with mononuclear cells following intravenous injection. Little is known about the interaction of SLN with these cells, including cytotoxic effects and a possible up-regulation of pro-inflammatory cytokines. Therefore, we investigated the influence of lipid matrix, concentration, and size of SLN on murine peritoneal macrophages (mϕ). mϕ were incubated with SLN consisting of different lipid matrices and coated with the same surfactant. Cytotoxicity as assessed by MTT test was found to be concentration-dependent and was dramatically influenced by the lipid matrix. Marked cytotoxic effects were observed when cells were incubated with SLN consisting of stearic acid (STE) or dimethyl-dioctadecylammonium bromide (DDA) at concentrations of 0.01%, whereas SLN consisting of triglycerides, cetylpalmitate or paraffin did not exert major cytotoxic effects at the same concentrations. Cytotoxic effects were most likely caused by products of enzymatic degradation including free stearic acid. Analysis of cytokine production by mϕ following incubation with SLN revealed concentration-dependent decreases in IL-6 production. These decreases seemed to be associated with cytotoxic effects. IL-12 and TNF-α production was neither detected in supernatants of mϕ treated with SLN at any concentration nor in those of untreated cells. The size of SLN did neither affect cytotoxicity of SLN nor resulted in induction or digression of cytokine production by mϕ. In conclusion, results of the present study revealed that the nature of the lipid matrix and the concentration of SLN dramatically impact cytotoxicity of SLN on mononuclear cells. Lipid matrices of SLN should therefore be carefully chosen and tested for later intravenous use.

Keywords: Solid lipid nanoparticles (SLN); Lipid matrix; Size; Peritoneal macrophages; Cytokines; Cytotoxicity

1. Introduction

Solid lipid nanoparticles (SLN) are an alternative drug delivery system to emulsions, liposomes, and polymeric nanoparticles. Similarly to emul-

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0378-5173/02/$ - see front matter
PII: S0378-5173(01)00882-1

sions and liposomes, they consist of physiologically well tolerated ingredients but allow controlled release of drug (Müller and Lucks, 1996; Müller et al., 1995; Schwarz et al., 1993). Equally to polymeric nanoparticles, their solid matrix protects against chemical degradation and allows modulation of drug release profiles (Mehnert et al., 1997; Zur Mühlen et al., 1998). SLN are good tools for targeting of drugs to specific tissue sites (Müller and Lucks, 1996). For parenteral administration, information about the interaction of SLN with phagocytic cells is prerequisite.

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. Activated mononuclear phagocytes play a potent role in immune reactions by releasing pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-12, or tumor necrosis factor (TNF)-α . Uncontrolled secretion of these molecules, however, may lead to a cascade of adverse reactions which cause severe damage to the host as is the case in lipopolysaccharide (LPS)-induced shock (Baumann and Gauldie, 1994; Van Furth et al., 1972). In order to evaluate the performance and toxicological acceptance of drug delivery systems, knowledge on what causes changes in the production of these pro-inflammatory cytokines is of utmost importance.

Immunomodulatory and cytotoxic effects of SLN on mononuclear phagocytes have recently been investigated by us (Schöler et al., 2000a,b, 1999). SLN consisting of either a lipid (Compritol) or a wax (cetylpalmitate) matrix, both coated with Poloxamer 188, did not cause an increase in the release of IL-6, IL-12, or TNF-α. However, concentration-dependent cytotoxicity of the same SLN systems was observed (Schöler et al., 2000a, 1999). Our group has reported that SLN consisting of Dynasan 114 or Compritol both stabilised by Lipoid S75 did not exert cytotoxic effects on HL 60 cells (Müller et al., 1997b). Furthermore, no significant loss of viability of human granulocytes was observed after incubation with SLN consisting of cetylpalmitate or Compritol coated with Poloxamer 188 (Müller et al., 1997a). With regard to the influence of the particle size on the interaction with phagocytic cells, increasing sizes of polyethylene particles decreased the number but increased the mass of particles internalised by human granulocytes (Rudt and Müller, 1992). However, polyethylene particles of different sizes did not exert cytotoxic effects on peritoneal macrophages (Green et al., 1998). The same authors also observed that particles of a 'critical size' induced IL-1β, IL-6, and TNF-α production in these cells.

Since little is known about the influence of the individual components and of the size of SLN on their interaction with phagocytic cells, we performed a detailed analysis of the influence of the lipid matrix and size of SLN on cytokine release and viability of peritoneal mϕ.

2. Materials and methods

2.1. SLN preparation and size measurement

SLN were produced by hot high pressure homogenisation. Lipid (10% w/w) was melted, poured into a hot aqueous surfactant solution (1% w/w), dispersed by high speed stirring to yield a pre-emulsion (Ultra turrax T 25, Janke and Kunkel, Staufen, Germany), and subsequently homogenised using a Micron LAB 40 homogeniser (Dairy Engineering Company, Arvada, CO, USA) (Müller et al., 1995). Different particle sizes were obtained by varying the production parameters. Particles were produced under aseptic conditions using a laminar air flow chamber.

Particle size and width of distribution (polydispersity index) were determined by photon correlation spectroscopy (PCS) (Malvern Zetasizer IV, Malvern Instruments, Herrenberg, Germany) or laser diffractometry (Coulter LS 230, Coulter Electronics, Miami, FL, USA).

Lipid components Dynasan 114 (DYN 114), Dynasan 118 (DYN 118), and Imwitor (IMW) were kindly provided by Condea (Witten, Germany). Compritol 888 (COM) and cetylpalmitate (CET) were obtained from Gattefossé (Weil, Germany), stearic acid (STE) and paraffin (PAR) from Caesar und Loretz (Hilden, Germany), and

dimethyl-dioctadecylammonium bromide (DDA) from Gerbu Biotechnik (Heidelberg, Germany). The surfactant, Poloxamer 188, was purchased from BASF (Ludwigshafen, Germany), Tween 80 from ICI Surfactants (Eversberg, Belgium), and Lipoid S 75 (lecithin) from Lipoid KG (Ludwigshafen, Germany). Medium-chained triglycerides and soybean oil were obtained from Sigma–Aldrich (Deisenhofen, Germany). Glycerol for isotonisation was purchased from Merck (Darmstadt, Germany). Lipofundin MCT 10% was obtained from Braun Melsungen (Melsungen, Germany). Polystyrene particles (PSP) at different sizes (small: 250 nm, medium: 512 nm, large: 7.10 μm) were purchased from Polysciences Inc. (Warrington, PA, USA).

2.2. Murine peritoneal macrophages

Murine peritoneal cells were collected from peritoneal cavities of locally bred female BALB/c mice 5 days after intraperitoneal injection of 0.5 ml of sterile 3% Brewer's thioglycollate (Difco Laboratories, Detroit, MI, USA) (Schöler et al., 2000a). Donor mice were killed in a CO_2-atmosphere and cells were harvested using a 20-gauge needle by lavaging the peritoneal cavity twice with 5 ml sterile Dulbecco's phosphate-buffered saline (D-PBS) (Bio Whittaker, Walkersville, MD, USA), containing 2% heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany). Harvested cells were pooled and kept on ice. Cell counts were performed using a hemocytometer and viability was determined by trypan blue exclusion (Biochrom, Berlin, Germany). Differential counts were performed on fixed Pappenheim smears. Giemsa and May–Grünwald solutions were obtained from Merck (Darmstadt, Germany). Enumeration of harvested cells revealed $75.8 \pm 8.2\%$ mononuclear cells and $24.2 \pm 8.2\%$ lymphocytes and no neutrophils or other cells. Approximately $4–5 \times 10^6$ mϕ were harvested per mouse. Cells were suspended in RPMI 1640 in the presence of 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin at concentrations of 1×10^6 mϕ per ml, and seeded into 96-well flat bottom plates (Nunc, Roskilde, Denmark) (200 μl/well). All cell culture reagents were purchased from Biochrom (Berlin, Germany). After 3 h of incubation (37 °C, 5% CO_2), non-adherent cells were removed by washing three times with culture medium. Subsequently, adherent cells were incubated in the presence of SLN suspensions, polystyrene particles (PSP), Lipofundin MCT 10% (LIP) emulsions at different concentrations dispersed in cell medium, or 75.0 μg/ml Pansorbin (Protein A of *Staphylococcus aureus*) (Calbiochem, Bad Soden, Germany). After 1 h of incubation, extracellular SLN were rinsed off. For cytokine detection, supernatants were harvested at 20 h of incubation and stored at − 70 °C until testing. Condition of cultured cells was monitored microscopically and protocolled throughout the experiment. Experiments were generally performed in triplicate and repeated at least once.

2.3. Cytokine assay

IL-6, IL-12, and TNF-α in supernatants were determined by two-site enzyme-linked immunosorbent assays (ELISAs) 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 cytokine antibodies (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 tablets (TMB) as substrate. All reagents used for ELISA were obtained from Pharmingen (San Diego, CA, USA), except streptavidine horseradish peroxidase (Amersham, Little Chalfont, UK). TMB and bovine serum albumine (BSA) were purchased from Sigma–Aldrich. Cytokine concentrations were determined by referring to standard curves constructed with fixed amounts of mouse recombinant IL-6, IL-12, or TNF-α. Optical densities (OD) were measured using an automated ELISA plate reader (Tecan,

Crailsheim, Germany) at 405 nm using a 620 nm reference filter. Data from triplicate determinations were expressed as nanograms per ml (mean ± S.D.). The sensitivity limits for the different assays were as follows: IL-6, 0.20 ng/ml; IL-12, 0.15 ng/ml; TNF-α, 0.15 ng/ml.

2.4. Viability

Cytotoxicity of SLN on murine peritoneal mϕ was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Mosmann, 1983). Cells were incubated in parallel with cells used for cytokine detection by ELISA. Four hours prior to the 20 h time point, 50 μl MTT solution (Sigma–Aldrich) (2.5 mg/ml 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 live cells only. After solubilization of the formazan crystals by sodium dodecylsulfate (SDS) (Bio-Rad Laboratories, Munich, Germany), absorbance was measured at 550 nm in an automated ELISA plate reader (Tecan) and was directly proportional to the cellular metabolism. Viability was expressed in percent compared to untreated cells. Experiments were performed in triplicate and repeated at least once.

2.5. Statistical analysis

Statistical analysis was performed using the unpaired, two-tailed Student's *t*-test.

3. Results and discussion

Incubation of mϕ with SLN preparations (Table 1) consisting of four different lipids (COM, DYN 114, DYN 118, and IMW) coated with Poloxamer 188 at concentrations of 0.001% resulted in viabilities of minimum 85.7 ± 21.8% (Fig. 1a). Viability of cells incubated with SLN at concentrations lower than 0.001% did not differ from that observed at SLN concentrations of 0.001% (data not shown). Viability decreased markedly when DYN 118 SLN (0.1%) and IMW SLN (0.1%) were incubated with mϕ. Lipofundin MCT (LIP)—used for parenteral fat nutrition—at concentrations of 0.00–0.1% did not exert marked cytotoxic effects (Fig. 1a).

Incubation of SLN with mϕ did not result in increased cytokine production. mϕ maintained their capacity to produce pro-inflammatory cytokines as the production of IL-6 did not differ significantly between untreated cells and cells treated with COM, DYN 114, DYN 118, and IMW SLN, as well as LIP at concentrations of between 0.001 and 0.1% (Fig. 1b). TNF-α and IL-12 were undetectable in supernatants of untreated cells or supernatants of the same cells treated with any of the above mentioned SLN and control emulsion. Stimulation of cells with Pansorbin—performed to examine the capacity of cells to up-regulate cytokine production—resulted in significantly higher IL-6 concentrations (Fig. 1b). Following stimulation with Pansorbin, TNF-α (2.13 ± 1.13 ng/ml) and IL-12 (2.40 ± 0.03 ng/ml) were also detectable. These results indicate

Table 1
Diameters of SLN consisting of different lipid matrices as measured by photon correlation spectroscopy

Lipid	Abbreviation of SLN	Diameter (nm) ± S.D.	Polydispersity index ± S.D.
Compritol	COM	313.5 ± 3.1	0.38 ± 0.03
Dynasan 114	DYN 114	231.5 ± 1.4	0.13 ± 0.03
Dynasan 118	DYN 118	273.8 ± 2.9	0.10 ± 0.05
Imwitor	IMW	394.3 ± 4.4	0.32 ± 0.05
Cetylpalmitate	CET	359.9 ± 1.9	0.13 ± 0.03
Paraffin	PAR	233.5 ± 1.3	0.14 ± 0.01
Stearic acid	STE	359.9 ± 1.9	0.13 ± 0.03
Dimethyldistearyl-ammonium bromide	DDA	200.8 ± 0.7	0.28 ± 0.02

($n = 3$) (concentration of SLN dispersions: 10.0% (w/w) lipid, 1.0% (w/w) Poloxamer 188)

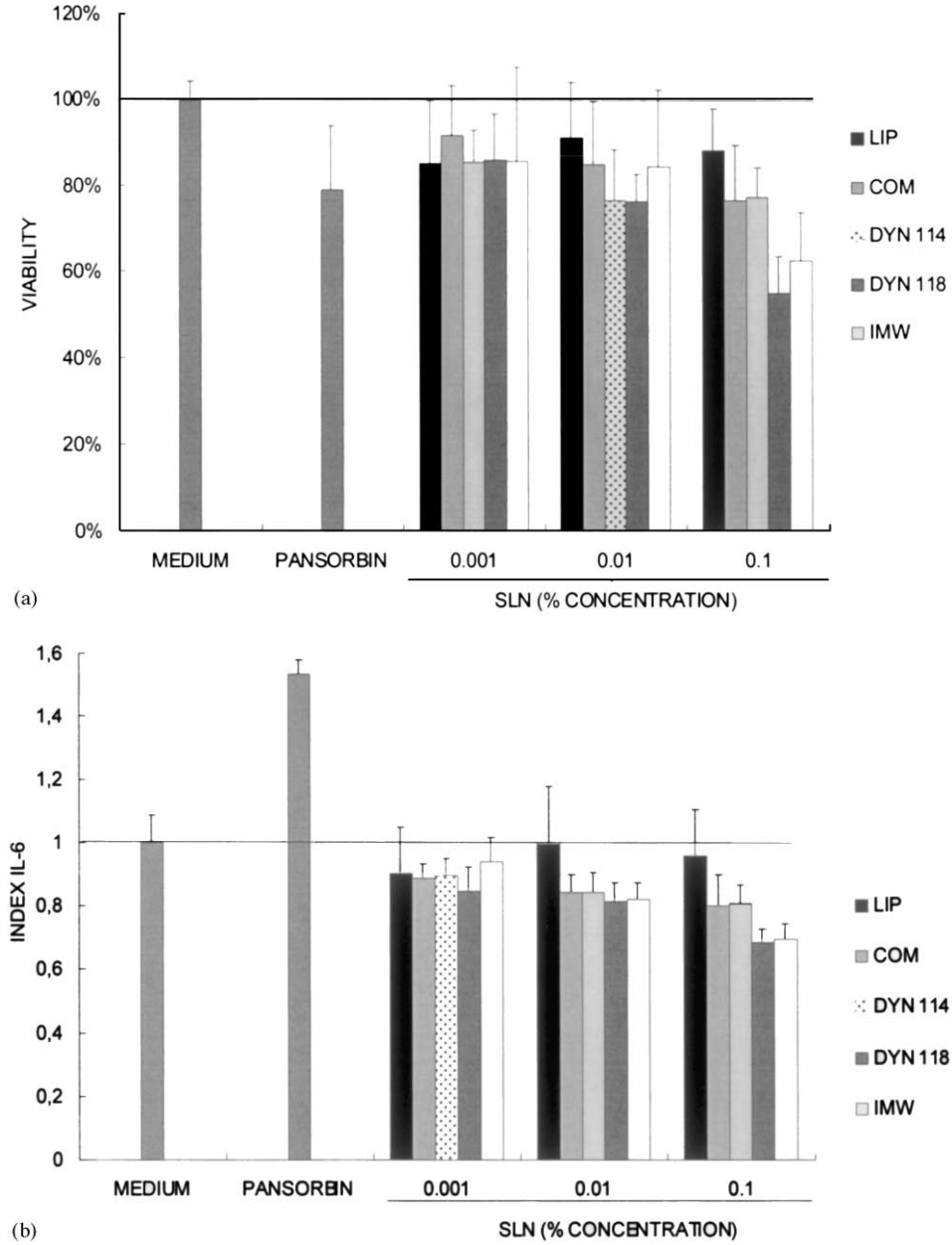

Fig. 1. (a) Viability of thioglycollate-elicited peritoneal macrophages after 20 h of in-vitro culture with different concentrations of SLN consisting of different lipid matrices coated with the same surfactant and with medium, Pansorbin, or Lipofundin MCT (LIP) ($n = 3$, $\pm$ S.D.). Viability of macrophages cultured in medium was considered 100%. (b) Secretion of IL-6 by ELISA in supernatants of the same cells after 20 h of in-vitro culture with same ingredients as tested in 1a. IL-6 levels are shown as mean index IL-6 $\pm$ standard deviation ($n = 3$).

that cultivated mϕ retained their potential to up-regulate cytokine production in our assay system. The in-vitro system used in the present study allows the parallel analysis of cytokine production and of cytotoxic effects of SLN and their components with high sensitivity, since a viability of

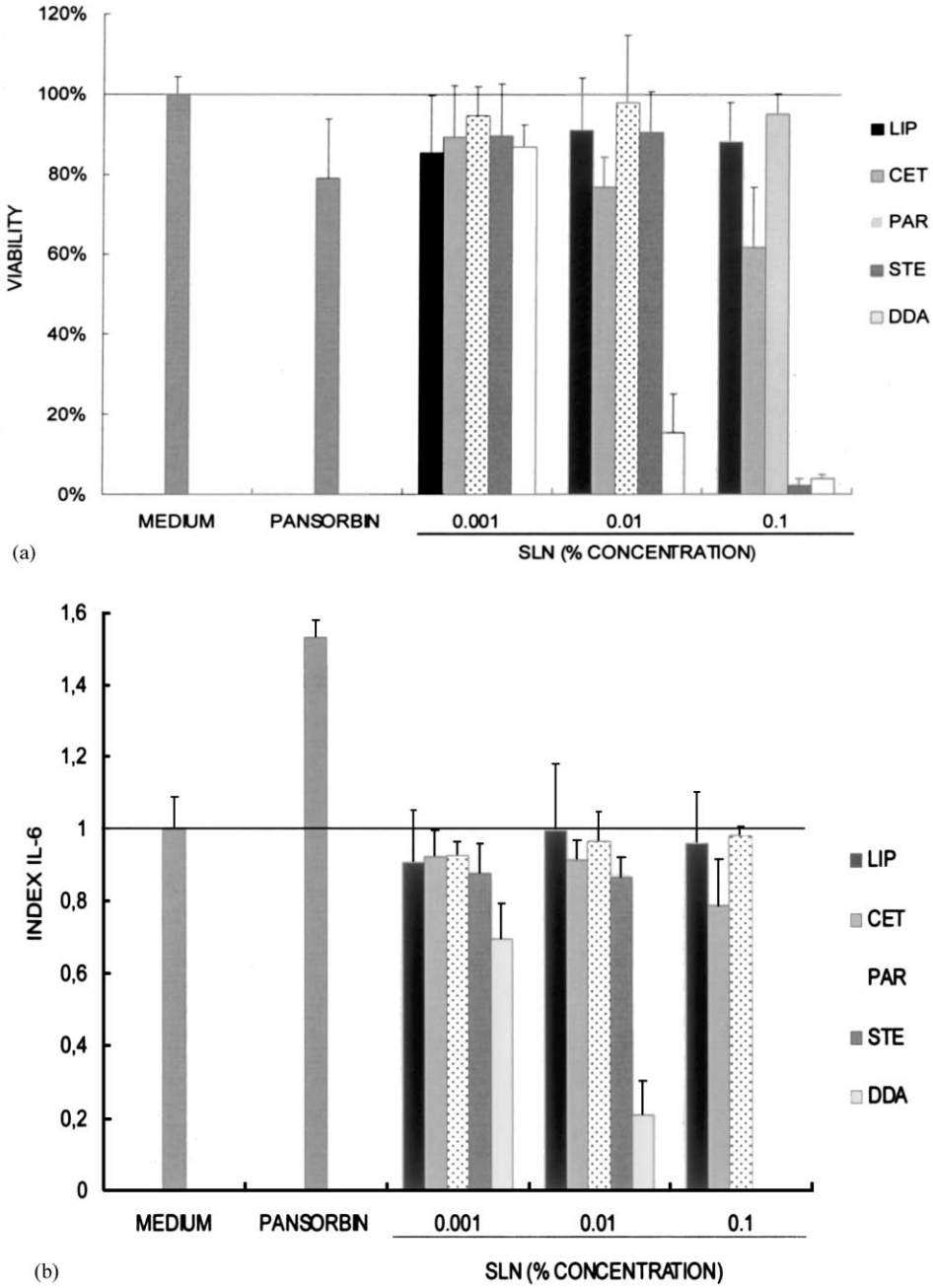

Fig. 2. (a) Viability of thioglycollate-elicited peritoneal macrophages after 20 h of in-vitro culture with different concentrations of SLN consisting of different lipid matrices coated with the same surfactant and with medium, Pansorbin, or Lipofundin MCT (LIP) ($n = 3$, $\pm$ S.D.). Viability of macrophages cultured in medium was considered 100%. (b) Secretion of IL-6 by ELISA in supernatants of the same cells after 20 h of in-vitro cultures with the same ingredients as tested in 2a. IL-6 level are shown as mean index IL-6 $\pm$ standard deviation ($n = 3$).

Table 2
Diameters of SLN and control emulsion consisting of 10.0% (w/w) lipid coated with 1.0% (w/w) surfactant as measured by laser diffractometry ($n = 3$)

Composition	Abbreviation	Diameter (μm) ± S.D.	
Stearic acid/ Tween 80	STE 80	Small	0.325 ± 0.430
		Medium	0.535 ± 0.461
		Large	7.257 ± 6.298
MCT/ Soyaoil 1:1/ Lipoid S75	MCT	Small	0.232 ± 0.124
		Medium	0.530 ± 0.429
		Large	8.808 ± 5.842

9.1 ± 5.5% was observed following incubation of mϕ with LIP at concentrations of 10%—commonly used concentration used for parenteral nutrition.

When mϕ were incubated with SLN consisting of CET, PAR, STE, and DDA SLN (Table 1) at low concentrations no cytotoxic effects were seen (Fig. 2a). Viability of cells incubated with SLN at concentrations lower than 0.001% did not differ from that observed at SLN concentrations of 0.001% (data not shown). In contrast, treatment of mϕ with DDA SLN at concentrations of 0.01% resulted in markedly decreased viability (15.3 ± 9.9%) (Fig. 2a). At higher concentrations of 0.1%, CET and STE SLN, too, exerted cytotoxic effects on mϕ resulting in viabilities of 61.8 ± 15.2 and 2.2 ± 1.8%, respectively. Neither CET, PAR, STE, and DDA SLN nor LIP increased the IL-6 secretion by mϕ (Fig. 2b). In contrast, all SLN that exerted cytotoxic effects also decreased the IL-6 production by mϕ in a concentration-dependent manner (Fig. 2b). As shown above for COM, DYN 114, DYN 118, and IMW SLN, decreased IL-6 production by mϕ following treatment with STE and DDA SLN can be attributed to a concentration-dependent cytotoxicity. These results are in line with our previous findings that COM and CET SLN exert cytotoxic and cytokine-depressing effects at concentrations of between 0.0075 and 0.75% (Schöler et al., 2000a).

In addition, Poloxamer 188 solutions at concentrations equivalent to the concentrations present in SLN suspensions did not exert cytotoxic effects on mϕ and did not alter IL-6 production by these cells (data not shown).

These results clearly indicate that cytotoxicity of SLN is dramatically influenced by the lipid matrix. Comparison of SLN consisting of free stearic acid (STE SLN) with SLN consisting of triglycerides of stearic acid (DYN 118 and IMW SLN) revealed that STE SLN at concentrations of 0.1% exerted significantly higher cytotoxic effects than DYN 118 and IMW SLN at equal concentrations. Slightly reduced viabilities of mϕ treated with DYN 118 and IMW SLN at concentrations of 0.1% may be attributed to stearic acid which is released after enzymatic degradation of these SLN. Thus, free stearic acid most likely is the cytotoxic component of STE SLN. Lipid matrices of SLN were found to be decomposed by enzymatic degradation resulting in the release of free fatty acids (Müller et al., 1996; Olbrich et al., 1997; Olbrich and Müller, 1999). Slight cytotoxic effects of SLN on human granulocytes have previously been described to be caused by free fatty acids following intracellular degradation of SLN (Müller et al., 1997a).

The degradation profile of SLN was found to be influenced by their lipid matrix (Müller et al., 1996; Olbrich et al., 1997; Olbrich and Müller, 1999). The velocity of degradation increases with decreasing the length of fatty acid chains when triglycerides are used as lipid matrices (Müller et al., 1996; Olbrich and Müller, 1999). SLN consisting of wax matrices (e.g. cetylpalmitate) degrade faster compared to SLN consisting of triglyceride matrices (Müller et al., 1996). In the present study, cytotoxic effects of SLN consisting of a triglyceride of stearic acid (DYN 118 SLN) and SLN consisting of glyceryl monostearate (IMW SLN) did not differ significantly. Therefore, intracellular degradation most likely is completed after 20 h of incubation and the velocity of degradation does not influence cytotoxic effects of SLN in-vitro. SLN consisting of mono-, di-, and triglycerides of the long-chained behenic acid (COM SLN) and SLN consisting of triglycerides of the short-chained myristic acid (DYN 114 SLN) were both well tolerated. Similar results were obtained when SLN consisting of Compritol or Dynasan at

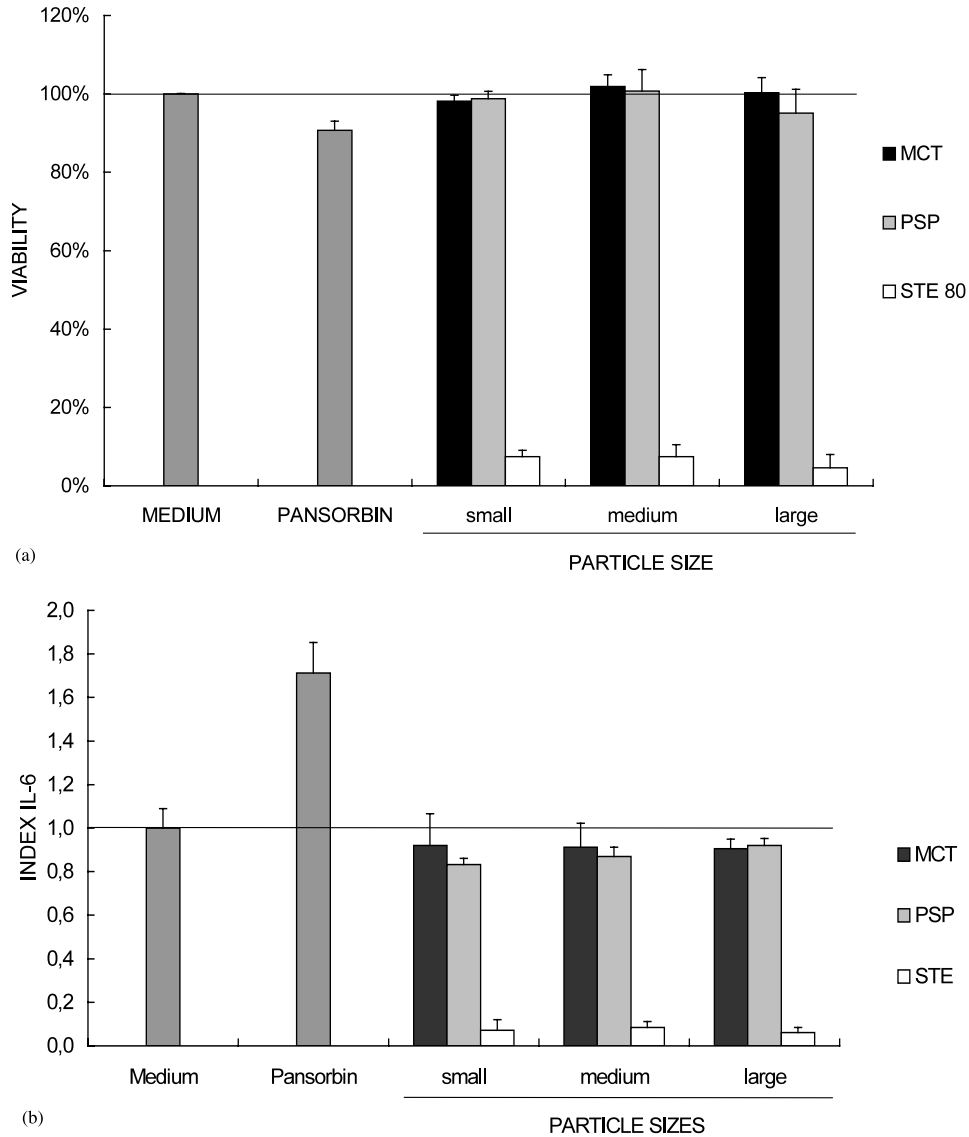

Fig. 3. (a) Viability of thioglycollate-elicited peritoneal macrophages after 20 h of in-vitro culture with medium, controls, and small (325 nm)-, medium (535 nm)-, and large (7.257 μm)- sized SLN—consisting of stearic acid coated with Tween 80—at a concentration of 0.1% ($n = 3, \pm$ standard deviation). Viability of macrophages cultured in medium was considered 100%. (b) Secretion of IL-6 by ELISA in supernatants of the same cells after 20 h of in-vitro cultures with the same ingredients as tested in 3a. IL-6 level are shown as mean index IL-6 $\pm$ S.D. ($n = 3$).

concentrations of between 0.015 and 1.5% were incubated with HL 60 cells (Müller et al., 1997b). In addition, SLN consisting of a fast biodegraded wax (CET SLN) showed a slightly increased cytotoxicity on mϕ with increasing concentrations, whereas in human granulocytes, the same SLN formulations did not exert cytotoxic effects even at concentrations of 2.5% (Müller et al., 1997a). These results further underline the high sensitivity of our assay system compared to other in-vitro culture systems including human granulocytes and HL60 cells. SLN consisting of degradation-inert

paraffin (PAR SLN) did not show cytotoxic effects, whereas SLN consisting of a hydrophilic quarternary amine (DDA SLN) are highly cytotoxic at concentrations of 0.1%. Since DDA is used in emulsions in association with liposomes for parenteral administration, this finding is remarkable.

The results presented above reveal dramatic differences in cytotoxic effects of different lipid matrices on mϕ. Our results also point towards significant differences in sensitivity of different cell culture systems to cytotoxic effects of SLN. These findings should be considered when SLN are developed and tested for later in-vivo use.

In addition to the effect of lipid matrices, we were also interested in the effect of SLN size on the viability and the cytokine production of mϕ. Small-(325 nm), medium-(535 nm), and large-(7.257 μm) sized SLN consisting of stearic acid coated with Tween 80 were produced (Table 2). Fig. 3a shows that cytotoxic effects did not change with increasing sizes of SLN. STE SLN coated with Tween 80 at 0.1% exerted cytotoxic effects on mϕ—as shown for STE SLN coated with Poloxamer 188 at the same concentration. In addition, control emulsions (MCT)—consisting of medium-chained triglycerides and soybean oil (1:1) coated with Lipoid S 75—as well as non-degradable polystyrene particles (PSP) did not exert cytotoxic effects on mϕ at any size similar to the sizes of SLN (Fig. 3a). IL-6 production by mϕ incubated with STE 80 SLN at 0.1% was decreased and paralleled cytotoxic effects at all sizes tested as shown above for other SLN systems (Fig. 3b). IL-12 and TNF-α were not detectable. These results indicate: (i) that the size of SLN does not influence cytotoxic effects of SLN; and (ii) that production of cytokines by mϕ is not altered. In contrast to our findings, the size of polyethylene particles has been reported to be crucial for the up-regulation of IL-1β, IL-6, and TNF-α production by peritoneal mϕ (Green et al., 1998). Increased cytokine levels were measured in supernatants of peritoneal mϕ after incubating cells with particles sized between 0.49 and 4.3 μm, whereas no differences in viability were observed after incubation with 88 μm particles (Green et al., 1998). In a size range of between 200 nm and 88 μm no cytotoxic effects of polyethylene particles on mϕ were found (Green et al., 1998). Furthermore, using Al_2O_3 particles, cytotoxic effects on the J774 mϕ cell line increased with particle sizes of greater than 2 μm, whereas up-regulation of IL-6 or TNF-α production was not observed (Catelas et al., 1998).

Our studies regarding the influence of the size of SLN on viability and cytokine production by mϕ suggest that the size of SLN is not a major factor that attributes to cytotoxicity or activation or decrease of cytokine production by mϕ.

In conclusion, results of the present study indicate that the change in lipid matrix, concentration, and size of SLN does not result in activation of mϕ. In contrast, the nature of the lipid matrix and concentration of SLN but not the size influenced cytotoxic effects of SLN on mϕ. Cytotoxicity of SLN was most likely caused by products of enzymatic degradation of SLN including free fatty acid. Further studies will have to determine the influence of surfactants of SLN on viability and cytokine production by mϕ.

Acknowledgements

This work was supported by a Deutsche Forschungsgemeinschaft grant, Mu708/9, E, to H. Hahn, O. Liesenfeld, R.H. Müller.

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