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Preserved solid lipid nanoparticles (SLN) at low concentrations do cause neither direct nor indirect cytotoxic effects in peritoneal macrophages

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Abstract

In order to investigate the interaction of preserved solid lipid nanoparticles (SLN) with murine peritoneal macrophages (MØ), cytotoxicity and proinflammatory effects of two different solid lipid nanoparticles (SLN) preparations consisting of either compritol (CO) or cetyl palmitate (CP) preserved with thiomersal were analyzed. Concentration-dependent cytotoxic effects were observed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Secretion of interleukin-6 by MØ following incubation with CO and CP SLN did not differ from secretion by untreated cells; proinflammatory cytokines interleukin-12 and tumor-necrosis-factor-α as further indicators of immunomodulatory effects were not detectable. These findings paralleled our previous findings that unpreserved CO and CP SLN did not induce immunomodulatory effects but cytotoxicity at higher concentrations. There were no synergistic cytotoxic effects of preservative and SLN. Thus, preservation of SLN using thiomersal does not appear to cause increased cytotoxicity and immunomodulatory effects following incubation with MØ. © 2000 Elsevier Science B.V. All rights reserved.

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

Solid lipid nanoparticles (SLN) are appropriate for i.v. use due to their physiological lipid or lipid-like matrix and their low diameter of 100–1000 nm (Mueller and Lucks, 1991; Schwarz et al., 1993; Mueller et al., 1995). These preparations must be preserved in order to prevent microbial growth (Fassihi, 1991; Ascenzi, 1996). Preservatives do not only interact with the cytoplasmic membrane of microbes but have also been shown to interfere with the cell membrane of mammalian cells, and to cause dose-dependent cytotoxic effects (Takahashi, 1982; Withrow et al., 1989).

Mononuclear phagocytes are potent phagocytic cells that initiate cytokine-mediated immune re-

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sponses. While secretion of cytokines is crucial for cell-mediated immunity (Baumann and Gauldie, 1994; Trinchieri, 1994; Van Furth et al., 1997), uncontrolled secretion of proinflammatory cytokines can have detrimental effects for the host (Howard et al., 1993; Gazzinelli et al., 1996). Such cytokine secretion may initiate an immune cascade, which ultimately may result in shock.

Since the interaction of preserved SLN with peritoneal macrophages has not been studied yet, we investigated direct cytotoxicity on murine peritoneal macrophages (MØ) and their cytokine secretion following incubation with preserved SLN.

Glycerolbehenate (Compritol 888 ATO™, Gattefossé, Weil, Germany) (CO) or cetyl palmitate (Precifac ATO™, Gattefossé) (CP) SLN containing poloxamer 188 (Lutreol TM F68, BASF, Ludwigshafen, Germany) were prepared under sterile conditions (Schöler et al., in press) and preserved by thiomersal (Sigma, Deisenhofen, Germany). MØ were collected from peritoneal cavities of female BALB/c mice (bred in our own facility) 5 days after intraperitoneal injection of 0.5 ml sterile 3% Brewer's thioglycollate (Difco Laboratories, Detroit, USA), and seeded at a concentrations of 2×10^5 MØ per well. MØ were incubated for 1 h in the presence of thiomersal dilutions, SLN suspensions, or 0.0075% pansorbin (wt/vol) (Calbiochem, Bad Soden, Germany) after removal of non-adherent cells.

Concentrations of cytokines (IL-6, IL-12, TNF-α) were determined by ELISA in supernatants harvested after 20 h of incubation (Schöler et al., in press). Cytotoxicity of CO and CP SLN on MØ was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay on the same cell preparation used for cytokine detection (Mosmann, 1983). Statistical analysis was performed using the unpaired, two-tailed Student's *t*-test.

Direct cytotoxicity of preservative on MØ was examined using thiomersal dilutions of between 0.000075 to 0.0015%. A significantly lower cytotoxicity was observed following incubation with 0.000075 and 0.00015% compared to 0.00075 and 0.0015% thiomersal (Fig. 1). Whereas thiomersal is used in clinical practice at concentrations of 0.1 to 0.001% (Fassihi, 1991; Ascenzi, 1996), and 0.002% is recommended by the US Pharmacopeia (1990), these concentrations were found to be markedly cytotoxic for MØ. Observed cytotoxic effects of thiomersal at higher concentrations may be explained by unspecific interactions with macrophages which include membrane permeability changes, inhibition of enzyme functions, and loss of critical cellular processes (Fassihi, 1991).

Since thiomersal was found to have low cytotoxicity on MØ at concentrations of less than

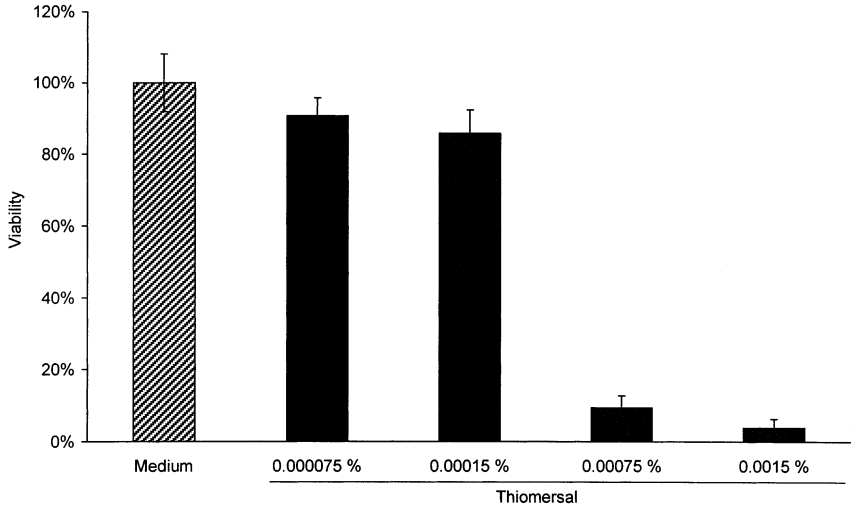

Fig. 1. Viability of MØ after 20 h of incubation with different concentrations of thiomersal shown as mean viability in percent.

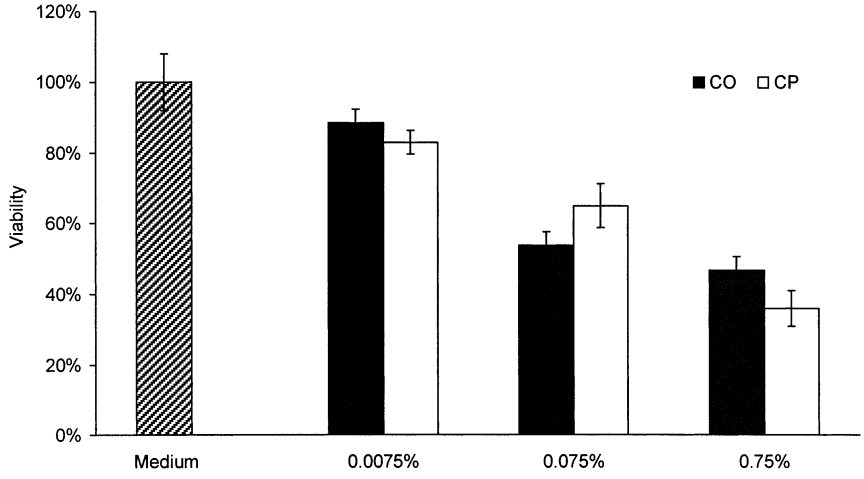

Fig. 2. Viability of MØ after 20 h of incubation with preserved CO and CP SLN at concentrations of 0.0075, 0.075, and 0.75% shown as mean viability in percent. Thiomersal concentrations were 0.0000015, 0.000015, and 0.00015%, respectively.

0.00015%, cytotoxic effects of preserved CO and CP SLN on MØ were subsequently investigated. CO and CP SLN were used at concentrations of 0.0075, 0.075, and 0.75% with final concentrations of thiomersal of 0.0000015, 0.000015, and 0.00015%, respectively. Treatment of cells with CO and CP SLN at low concentrations revealed high viability. A concentration-dependent increase in cytotoxicity was observed at higher concentrations; there were no differences in cytotoxicity between CO and CP SLN (Fig. 2). These findings parallel our previous findings which had revealed concentration-dependent cytotoxicity for unpreserved CO and CP SLN at the same concentrations (Schöler et al., in press). These results suggest that preservative, and SLN do not have synergistic cytotoxic effects on MØ.

Following incubation of MØ with thiomersal at concentrations of 0.000075, and 0.00015%, IL-6 secretion by MØ did not differ significantly from baseline concentrations in supernatants of untreated cells ($p = 0.1132$ and $p = 0.0483$, respectively), but declined steeply following treatment with thiomersal at higher concentrations (Fig. 3). The decrease in IL-6 secretion by MØ following thiomersal treatment at higher concentrations was paralleled by a decrease in viability of the same cells as shown above (Fig. 1). The capability of MØ to increase cytokine secretion was checked by treatment with Pansorbin–Protein A of *Staphylococcus aureus* which resulted in significantly increased secretion of IL-6 as compared to unstimulated MØ (41.33 ± 1.08 versus $24.38 + 2.90$ ng/ml). Thus, the steep decrease in IL-6 levels is likely to have been caused by the low viability of cells. TNF-α and IL-12 could only be detected in supernatants of Pansorbin-treated MØ, but not in supernatants of untreated and thiomersal-treated cells (data not shown).

Treatment of MØ with CO and CP SLN resulted in a pronounced concentration-dependent decrease in IL-6 secretion (Fig. 4). IL-6 secretion by MØ treated with CO and CP SLN did not differ (Fig. 4). Whereas at concentrations of 0.0075% of both SLN, IL-6 secretion was near baseline ($p = 0.3747$ for CO, $p = 0.1073$ for CP), levels of IL-6 were significantly lower at higher concentrations of CO and CP SLN ($p = 0.0022$ for all concentrations) (Fig. 4). Furthermore, TNF-α and IL-12 could not be detected in SLN-treated MØ in either SLN concentration.

Since a high viability was found for cells treated with CO and CP SLN at concentrations of 0.0075% (Fig. 2), the near baseline secretion of IL-6 and the lack of detection of other proinflammatory cytokines indicate that macrophages were not activated by interaction with CO and CP SLN. At higher concentrations, direct cytotoxic

effects on MØ were observed which resulted in decreased cytokine secretion. These findings parallel our previous findings that unpreserved CO and CP SLN at low concentrations did not induce cytokine secretion by MØ (Schöler et al., in press).

The findings presented above reveal that preserved SLN did neither cause cytotoxic effects on MØ nor led to secretion of proinflammatory cytokines by these cells. The results suggest that preservative, and SLN do not have synergistic cytotoxic effects on MØ. Thus, thiomersal may be suitable for preservation of SLN preparations.

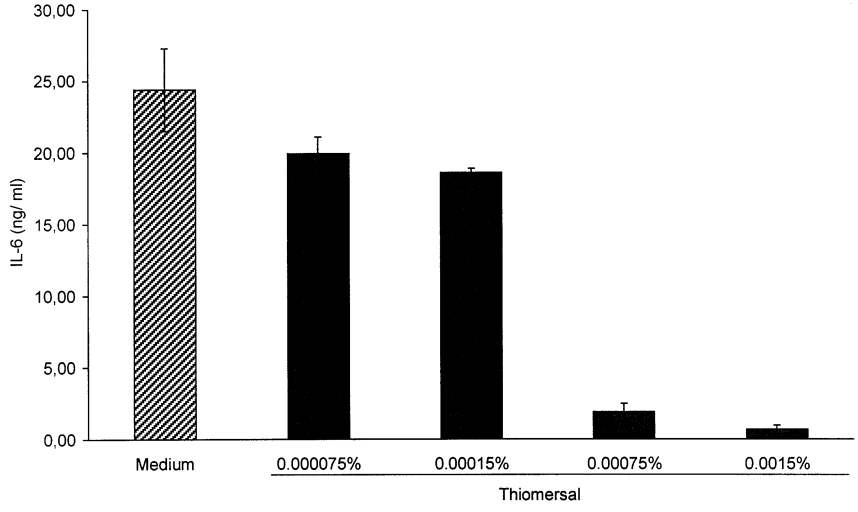

Fig. 3. Secretion of IL-6 in supernatants of MØ after 20 h of incubation with different concentrations of thiomersal. IL-6 concentrations shown are mean concentrations of triplicate cultures.

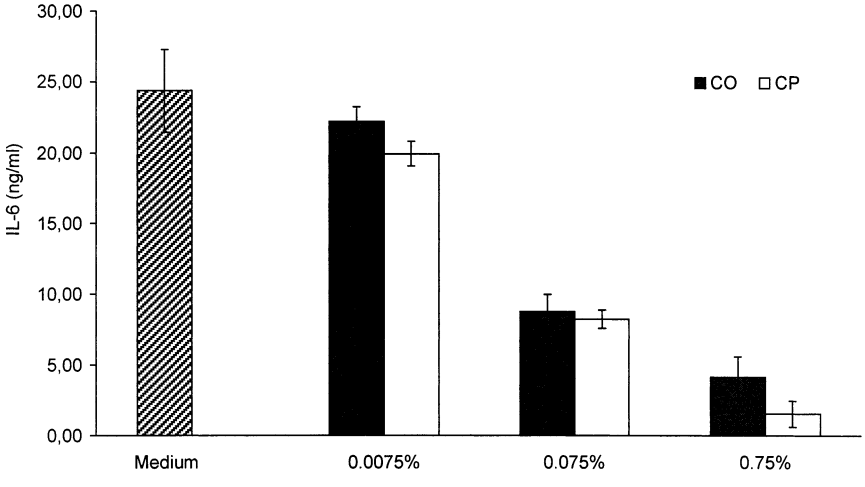

Fig. 4. Secretion of IL-6 in supernatants of MØ after 20 h of incubation with preserved CO and CP SLN at concentrations of 0.0075, 0.075, and 0.75%. Thiomersal concentrations were 0.0000015, 0.000015, and 0.00015%, respectively. IL-6 concentrations shown are mean concentrations of triplicate culture.

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