Activation of the Mononuclear Phagocyte System by Poloxamine 908: Its Implications for Targeted Drug Delivery

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Purpose. To investigate the effect of poloxamine 908 on the MPS activity and the importance of its mode of presentation to the immune system.

Methods. Solutions of endotoxin free poloxamine 908 were injected daily intravenously to rats, and the effect on the degree of sequestration by the liver of I^{125} labelled, poloxamine 908-coated 60 nm polystyrene particles was investigated by studying effect of dosing regimen(s) and assessment of opsonic activity.

Results. After 3 or 4 days repeated dosing with poloxamine 908 (0.7 mg) in solution, the poloxamine 908-coated polystyrene particles (60 nm) were rapidly cleared from the circulation. The increased sequestration of the particles by the liver lasted for more than 7 days after last dosing with the poloxamine 908 solution. In subsequent studies, it was found that a single dose of poloxamine 908 (0.7 mg) in solution was sufficient to activate the MPS 4 days after the injection. The increased uptake was found not be mediated by a serum component, nor was it due to proliferation of the Kupffer cells in the liver.

Conclusions. The results provide evidence that a solution of endotoxin-free poloxamine 908 activates the MPS so that 4 days after injection otherwise long-term circulating poloxamine 908-coated particles are sequestered by the liver. This finding has implications for use of such coated systems in therapeutic situations.

KEY WORDS: mononuclear phagocyte system; targeted drug delivery; nanoparticles; poloxamine 908.

INTRODUCTION

The main problem in achieving site specific drug delivery with colloidal carriers is the sequestration of such carriers by the mononuclearphagocyte system (MPS), especially the Kupffer cells of the liver. The coating of colloidal carriers with specific poloxamers and poloxamines has been shown to decrease considerably Kupffer cell uptake (1). One of the most effective polymers in this respect has been poloxamine 908 (1). This polymer, when adsorbed onto the surface of polystyrene particles (PS) of 60 nm in diameter produces a hydrophilic steric barrier that enable the particles (PS) of 60 nm in diameter produces a hydrophilic steric barrier that enable the particles to avoid Kupffer cell uptake, and if small enough, to remain in the circulation (2). It was also found that poloxamine 908-coated particles of larger sizes were filtered by the spleen after injection (3) and subsequently phagocytosed by the splenic macrophages (4). Interestingly, the same polymer, poloxamine 908, when adsorbed onto particles (PS-908) has also been found, after repeated injections, to enhance Kupffer cell uptake, resulting in the removal of the same long circulating nanoparticles (5). It was found after daily dosing with PS-908 (220 nm) particles for 4 days, that on the fifth day I^{125} radiolabelled PS-908 (220 nm) particles, which should have been essentially long circulating, were sequestered by the Kupffer cells of the liver. This response was found to be serum mediated and was explained by a possible increase in opsonic activity of the serum induced by the daily dosing with PS-908 (220 nm) particles. It was suggested that the massive accumulation of PS-908 (220 nm) particles in the spleen following 4 days dosing may elicit a humoral response responsible for the observed particle redirection. However, IgM levels, although elevated in the daily dosed animals, were still very low (6). Similarly, it has been found that daily dosing with smaller PS-908 (60 nm) particles also resulted in subsequent sequestration of PS-908 (60 nm) particles, whereas daily dosing with uncoated PS (60 and 220 nm) particles does not. Accordingly, it would appear that the presence of poloxamine 908 in the blood stream (bound or free) is crucial for the induction of an enhanced MPS activity. To investigate further the effect of poloxamine on the MPS activity and the importance of the way in which the polymer is presented to the immune system, solutions of poloxamine 908 were injected daily to rats and the effect on the degree of sequestration of poloxamine 908 coated particles investigated. The time for the response to return to normal and the role of opsonins in the sequestration process were also assessed.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma Chemicals (Poole, U.K.) unless otherwise stated. Poloxamine 908 was donated by BASF (Parsippany, USA). Polystyrene (PS) particles (60 nm ± 1.6 nm) were purchased from Park Scientific (Northampton, U.K.) Polymixin B Agarose was purchased from ICN Biomedicals Ltd. (High Wycombe, U.K.).

Animals

Male Wistar BKW rats weighing 150–180 g were obtained from the Animal Unit, Medical School, The University of Nottingham, Nottingham, U.K.

Methods

Preparation of Endotoxin Free poloxamine 908 Solution.

In order to avoid any effect on MPS from endotoxins, the poloxamine 908 solutions were cleaned from endotoxins. A sterile glass wool plug was inserted into the barrel of a sterile 2.5 ml disposable syringe. 1 ml of sterilised Polymixin B Agarose gel was pipetted on top of the glass wool. 1 ml of a 5% w/v solution of poloxamine 908 prepared in water for injection (W.F.I.) was pipetted onto the column. The eluent was collected in a sterile vial and returned to the column a total of 10 times. This procedure has been shown to be sufficient to remove endotoxin from the sample (5). All vials, scissors, pipette tips, forceps, and glass wool used during this procedure were steri-

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lised by autoclaving. The endotoxin free poloxamine 908 solution was prepared on the day prior to the first injection and then stored in a steril closed vial for the duration of the study. Samples for injection were removed using a sterile needle and syringe.

Biodistribution Studies

Groups of 3 rats were used in each experiment. Following the desired dosing regimen(s) with either endotoxin free poloxamine 908 or W.F.I., each animal was injected via the right tail vein with 0.15 ml of a 4.67 mg/ml (0.7 mg) suspension of I^{125} radiolabelled PS-908 (60 nm) particles (containing 0.5 mg PS surface coated with 0.2 mg poloxamine 908). 20 µl blood samples were taken at 5, 15, 30 minutes, 1, 2, and 3 hours post i.v. injection. The rats were sacrificed at 3 hours post i.v. injection and the liver spleen, lungs, and one femur (left hind leg) were removed. The rats were sacrificed by intravenous injection of Pentobarbitone 60 mg/ml (0.3–0.4 ml) followed by cervical dislocation. The organs were weighed and their associated radioactivity was counted in a gamma counter (LKB 182 Compugamma CS, LKB Wallac, Finland). The results were expressed either as a % of the total injected dose for each organ, or as % of the total injected dose per gram of tissue. The total radioactivity in blood was determined by assuming that the total blood volume was 7.5% of the body weight (7). The carcass radioactivity was counted using a Model SD1 Scintillation Counter (Oakfield Instruments Ltd., Eynsham, U.K.). The preparation of I^{125} radiolabelled PS particles and testing of the stability of the label was performed as previously described (1). The coating of radiolabelled PS particles with poloxamine 908 and their cleaning by dialysis has been described previously (1,3,8).

Dosing regimen(s)

Determination of the Number of Daily Doses Required to Induce Sequestration of PS-908 (60 nm) Particles

The experimental rats were injected daily for 1, 2, 3, and 4 days with 0.15 ml of a 4.67 mg/ml (0.7 mg) endotoxin free poloxamine 908 solution via the left tail vein. The control rats were injected with 0.15 ml of W.F.I. (WFI) via the left tail vein over a similar time scale. Twenty four hours after the last poloxamine 908 injection, each rat was injected with 0.15 ml of a 4.67 mg/ml suspension of I^{125} radiolabelled PS-908 (60 nm) particles.

Determination of the Number of Days Required for Responses to Return to Normal

The experimental rats were injected daily for 4 days with 0.15 ml of a 4.67 mg/ml endotoxin free poloxamine 908 solution via the left tail vein. The control rats were injected with 0.15 ml of W.F.I. (WFI) via the left tail vein over a similar time scale. Three, 4, and 7 days after the last poloxamine 908 or W.F.I. injection, each rat was injected with 0.15 ml of a 4.67 mg/ml suspension of I^{125} radiolabelled PS-908 (60 nm) particles.

Effect of Accumulated Dose

The experimental rats were injected with 0.15 ml of a 18.68 mg/ml (2.8 mg) endotoxin free poloxamine 908 solution via the left tail vein. The control rats were injected with 0.15 ml of W.F.I. (WFI) via the left tail vein. Thee hours, 24 hours, and 4 days after the poloxamine 908 injection, each rat was injected with 0.15 ml of a 4.67 mg/ml suspension of I^{125} radiolabelled PS-908 (60 nm) particles.

Assessment of Opsonic Activity

One group of rats was dosed for 4 days with 0.15 ml of a 4.67 mg/ml endotoxin free poloxamine 908 solution and another group of rats was dosed for 4 days with W.F.I. On the 5th day serum was prepared from each animal. 0.2 ml of a 4.67 mg/ml suspension of I^{125} radiolabelled PS-908 (60 nm) particles was incubated in 50% v/v serum from these animals for 15 minutes. 0.3 ml of the particle serum mixture was injected into the right tail vein of an untreated rat.

RESULTS AND DISCUSSION

0.7 mg of poloxamine 908 was chosen as the daily dose to determine the possible effect of free poloxamine 908 on the activation of MPS. The reasons for this choice are as follows. Firstly, 0.7 mg is equivalent to the weight of the daily dose of PS-908 particles previously used by Moghimi et al. (5). Secondly, since approximately 0.2 mg of poloxamine 908 is adsorbed onto 0.7 mg of PS (60nm) particles and 0.05 mg onto 0.7 mg of PS (220 nm) particles (9), 0.7 mg of poloxamine 908 (0.7 mg) is in excess of that previously present in the PS-908 systems, even assuming incomplete cleaning. Therefore, any response induced by the simple presence of poloxamine 908 in the PS-908 particle systems should also be induced by the poloxamine 908 solution.

Figure 1 shows the blood elimination profile of I^{125} radiolabelled PS-908 (60 nm) particles after I.V. injection into rats dosed with 0.7 mg of poloxamine 908 daily for 1, 2, 3, and 4 days. It is clearly shown that after 3 or 4 days repeated dosing

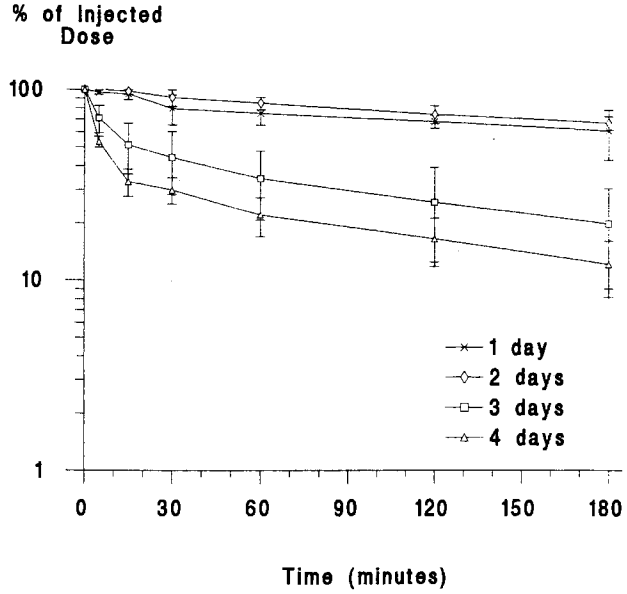

Fig. 1. Blood Elimination Profile of PS-908 (60 nm) Particles After 1, 2, 3 and 4 days dosing in rats with poloxamine 908. (±SD) X : 1 day dosing; ◇ : 2 days dosing; □: 3 days dosing; Δ : 4 days dosing.

with poloxamine 908, the PS-908 (60 nm) particles were rapidly cleared from the circulation, whereas 1 and 2 days repeated dosing with poloxamine 908 had no significant effect on the elimination of the particles as compared to the control. In contrast the control animals dosed for 1 or 4 days with W.F.I. showed no significant clearance of the PS-908 particles within the 3 hours of the experiment. The effect of the repeated predosing with poloxamine 908 solution on the particle clearance is very similar to the effect found previously by us for predosing with PS-908 (220 nm) particles (5). It was shown in this work that the clearance of the PS-908 (60 nm) particles from the circulation was solely due to their increased sequestration by the liver, probably the Kupffer cells. Following daily dosing with poloxamine 908 solution it can be seen from Figure 2 that the clearance of PS-908 (60 nm) particles was due, not only to a significant ($p < 0.01$) increase in sequestration by the liver, but also to a significant increase ($p < 0.01$) in sequestration by the spleen. All other organ counts remained low. The liver cell sequestration accounted for approximately 55% of the injected dose of particles and the spleen 5%. When these data are normalised for the weight of each organ (Figure 3) it can be seen that the spleen was much more efficient at particle clearance than the liver and played a very significant role in the sequestration of PS-908 (60 nm) particles from the animals daily dosed with poloxamine 908.

The increased sequestration of essentially long circulating nanoparticles, lasted for more than 7 days after the last dosing with poloxamine 908 as seen in Figure 4. We previously found that when I^{125} radiolabelled PS-908 (60 nm) particles, which had been incubated in serum from an 'activated' rat (i.e. daily dosed for 4 days with PS-908 (220 nm) particles) were injected i.v. into a non-treated rat, the radiolabelled PS-908 (60 nm) particles were sequestered by the Kupffer cells of the liver and macrophages of the spleen, and the uptake of the PS-908 (60 nm) particles was found to be dependent upon the serum incubation concentration (5). It was concluded that a rapid increase in opsonic activity of serum was responsible for the redirection of the particles to the liver, and that this increase in opsonic activity was a direct result of the daily dosing regimen(s) with PS-908 (220 nm) particles. From a similar experiment in the present work, where the PS-908 (60 nm) particles were incubated in serum from rats which had been treated for 4 days with Poloxamer 908 solution, it appears that the response in this case was not mediated by a serum component, since a significant increase in PS-908 (60 nm) particle elimination was

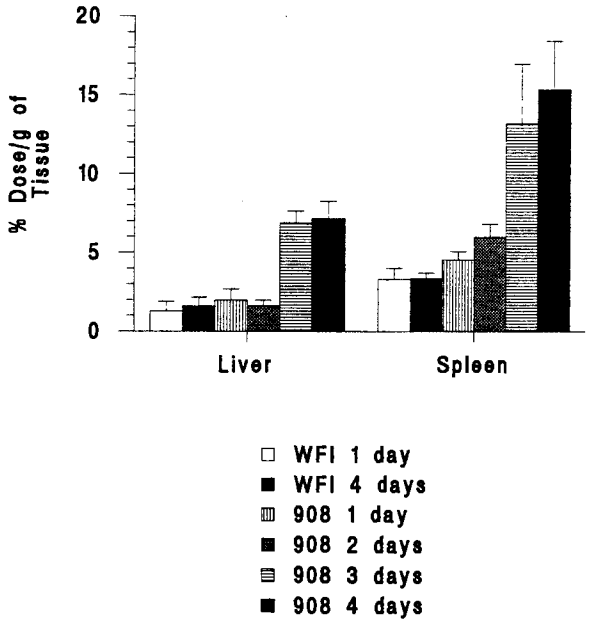

Fig. 3. Organ distribution of PS-908 (60 nm) Particles after 1, 2, 3 and 4 Days IV Dosing with poloxamine 908 or W.F.I. in rats (±SD).

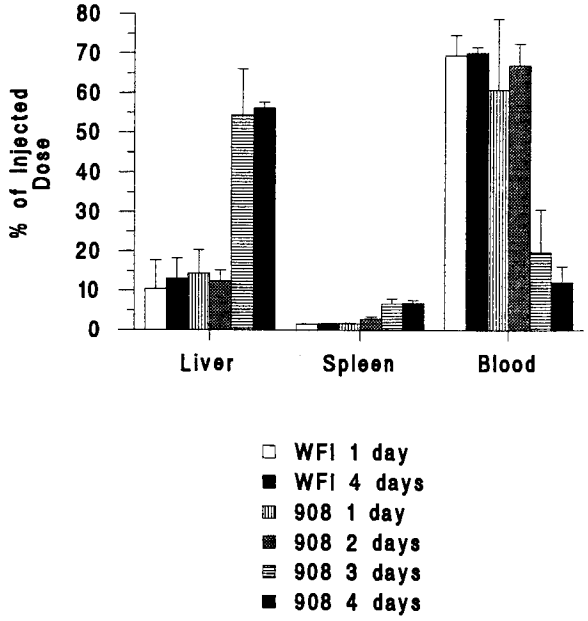

Fig. 2. Organ distribution of PS-908 (60 nm) Particles After 1, 2, 3 and 4 Days IV Dosing with poloxamine 908 or W.F.I. in rats (±SD).

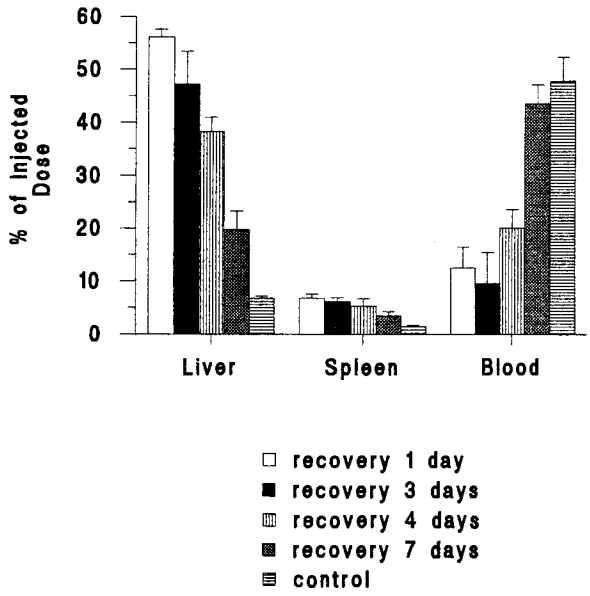

Fig. 4. Organ distribution of PS-908 (60 nm) Particles in rats after 1, 3, 4 and 7 Days Recovery after Daily Dosing with poloxamine 908 for Four Days (±SD).

not seen (Figure 5). It is possible that the opsonic component may have adsorbed on to the particle surface upon incubation *in vitro*, but was subsequently displaced upon introduction into the rat. However, since it is unlikely that an opsonic component would have such a low affinity, we tentatively conclude that the elimination response in the present study was not mediated by a serum component.

Since daily dosing with poloxamine 908 did not result in the proliferation of the Kupffer cells, as indicated by no change in liver weight between experimental groups and that sequestration of PS-908 particles does not appear to be serum mediated, it is likely that enhanced phagocytic activity of the Kupffer cells was responsible for the sequestration of the PS-908 particles. However, the mechanism of activation is unknown.

In certain pathological and experimental conditions the macrophages of the MPS can display augmented phagocytic behaviour (5,10–14). The enhanced behaviour of the MPS is normally a response to an inflammatory stimulus and is often accompanied by an increased Kupffer cell number and function. Interestingly, Moghimi et al. (5) demonstrated that a zymosan stimulated liver was able to sequester PS-908 (220 nm) particles and to a lesser extent PS-908 (60 nm) particles. This ability was the result of enhanced proliferation and phagocytic response of the Kupffer cells, and was not related to any specific opsonization process. The mechanism of zymosan stimulation of the Kupffer cell activity is not well understood but may arise from changes in cell hydrophobicity as well as changes in the mobility of certain plasmalemma receptors. It should be noted that endotoxin administration has been shown to result in Kupffer cell proliferation (15) and enhanced phagocytosis *in vivo* (16). Endotoxin is a cell wall component (lipopolysaccharide or LPS) of gram negative bacteria that induces the release of interleukins 1 and 6 (17) which results in general activation of the MPS. To exclude this possibility, endotoxin was removed from all the poloxamine 908 solutions that were used for dosing.

Since in the present work at day 4 the repeated dosing of 0.7 mg of poloxamine 908 was found to induce a response that resulted in an increased sequestration of long circulating PS-908 (60 nm) particles, it was important to clarify whether such a daily dosing regimen was needed for the induction of this response, or whether the same response could be achieved by a bolus injection of poloxamine 908 equivalent to four doses

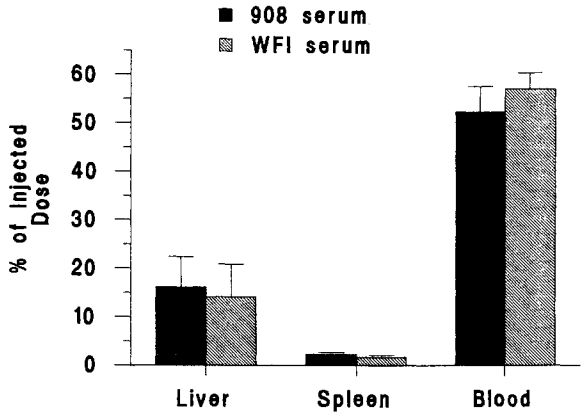

Fig. 5. Organ distribution in rats of IV injected PS-908 (60 nm) Particles incubated in serum from rats daily dosed with poloxamine 908 or W.F.I. (±SD).

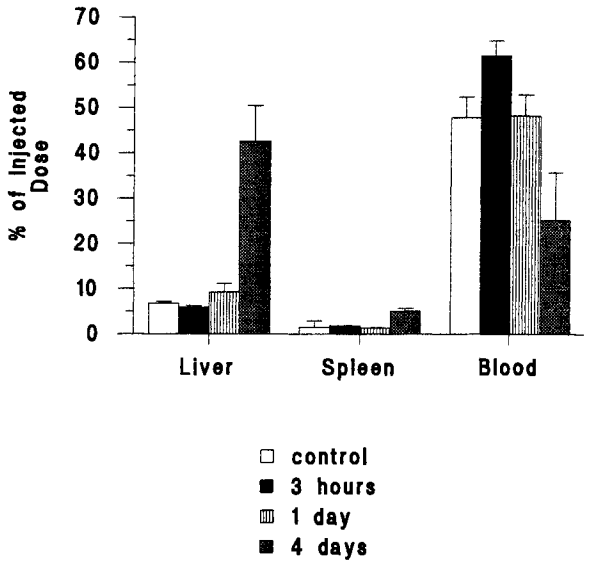

Fig. 6. Organ distribution of IV injected PS-908 (60 nm) in rats treated with a Single Predose of 2.8 mg of poloxamine 908 (±SD).

in the system. From recent studies by Yamaoka et al. (18) regarding the distribution of different molecular weight PEOs following i.v. administration, it would be expected that poloxamine 908 would be cleared from the circulation through the kidneys quite rapidly and so would not accumulate. It is clear from the results in Figure 6 that a single dose of 2.8 mg of poloxamine 908 in solution was sufficient to induce activation of the MPS. However, the effect was not immediate, as one might expect if it was triggered by the accumulation of a defined amount of polymer. Rather, the activation did not become effective until 4 days after the single bolus injection. In a subsequent study, where decreasing amounts of poloxamine 908 were given as bolus injections, it was found that a single i.v. dose of 0.7 mg of poloxamine 908 was sufficient to activate the MPS 4 days later. This implies that the polymer given on days 2, 3 and 4 of the chosen dosing regimen(s) were not required to give the observed effect.

These results support the suggestion that increased sequestration of PS-908 particles was due to enhanced phagocytic activity of the Kupffer cells. The mechanism for this enhanced activity is presently unknown but may be similar to that documented by Johnston et al. (19) who suggested the occurrence of an acute phase response to explain elevated white blood cell levels in rats which had received 4 days intraperitoneal doses of 1.0 g/kg Poloxamer 407. The acute phase response (20) is a systemic reaction to inflammatory stimuli such as infection or trauma and is induced by factors (mainly cytokines) generated at the site of infection or trauma. The time required for significant increases in acute phase proteins is dependent on the protein, e.g. mannose binding protein (MBP) 1–9 days (21) and CRP 2–3 days after surgery (22). It is likely that a single dose of poloxamine 908 could induce potentially an acute phase response. The response may take 3–4 days before the Kupffer cells are primed to sequester the PS-908 (60 nm) particles. It is noteworthy that a similar response, over a similar time scale has been reported by Aramaki et al. (23) after a single dose of empty liposomes. The authors suggested the involvement of a

soluble serum factor secreted from liposome modified B lymphocytes.

Our previous findings (3–5) in combination with this present work have great implications upon the use of poloxamine coated particulate carriers for drug delivery in that a single i.v. dose of poloxamine 908 induced an as yet unknown response that led to the enhanced phagocytic activity of the Kupffer cells. It obviously has the possibility of limiting the dosing regimen(s) that can be offered to potential patients, and furthermore, it casts doubt on the effectiveness of colloidal carriers in the treatment of disease states that possess Kupffer cell hyperactivity.

The potential of colloidal targeting in diseased states remains largely unaddressed. Undoubtedly, the mononuclear phagocyte function and the state of 'responsiveness' of the host defence system at various stages of disease will be a determining factor in the exploitation and success of colloidal carriers. Kupffer cell hyperactivity (e.g. diabetes, hypervitaminosis A, and non-Hodgkin's lymphoma, etc.) and hypoactivity (e.g. chronic alcohol consumption, sepsis, traumatic shock, and neoplastic disease, etc.) disease states are well documented and have previously been discussed by us (24). Splenic congestion associated with haemolytic anaemia has been shown to reduce the splenic filtration capacity of colloidal systems (25). Serum levels of certain key proteins involved in the removal of colloidal systems by the MPS are known to be influenced by disease conditions and so will affect particle clearance. Fibronectin levels have been shown to increase in post-myocardial infarction (26) and were also elevated in patients with Werner Syndrome (27). Increased levels of IgG fragments were present in patients with cystic fibrosis (28), whilst reduced levels of tuftsin have been identified in patients presenting loss of spleen function due to infarction or infection (29). Deficiencies of individual complement components are associated with immune complex disease (30). Thus, in general, colloidal targeting in disease states requires much greater attention, for ultimately these will be the conditions in which the colloid must function.

CONCLUSIONS

It was found that a single dose injection of poloxamine 908 was sufficient to induce a response that resulted in the rapid clearance of PS-908 (60 nm) particles from the circulation. The rapid clearance of essentially long circulating nanoparticles was due to increased sequestration by the Kupffer cells of the liver and macrophages of the spleen. The sequestration of PS-908 (60 nm) particles occurred 3–4 days after the poloxamine 908 predose and continued for approximately 7 days. The majority of the particles were sequestered by the Kupffer cells of the liver, but when the results are given as per gram of tissue, the spleen was found to be a much more active organ. The induced response was not a result of the proliferation of Kupffer cells or spleen macrophages and sequestration of PS-908 particles did not appear to be serum mediated. It was concluded that enhanced phagocytic activity of the Kupffer cells may be responsible although the mechanism of activation is unknown.

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