The Role of Serum Complement on the Organ Distribution of Intravenously Administered Poly (methyl methacrylate) Nanoparticles: Effects of Pre-Coating with Plasma and with Serum Complement

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Received January 19, 1996; accepted April 10, 1996

Purpose. The organ distribution of radiolabeled poly (methyl methacrylate) (PMMA) nanoparticles coated with plasma proteins and serum complement in rats was studied in order to determine the effect of serum complement on the particle phagocytosis by the organs of the reticulo-endothelial system (RES).

Methods. PMMA-nanoparticles were coated overnight with plasma proteins or serum complement, and injected into Wistar rats. The body distribution of nanoparticles was measured by means of scintillation counting of organ samples. In addition, proteins adsorbed to the particle surface were inactivated by heat treatment prior to injection, and the particles's distribution was measured as described above.

Results. Whereas uncoated nanoparticles (control group) were mainly taken up by the Kupffer cells in the liver, incubation of the particles in plasma for 12 h followed by heat inactivation reduced the particle concentrations in the liver to merely 22% after 30 min. After 120 min, liver concentrations were still lower than the control group, and almost 30% of the administered dose of the heat-inactivated particle group was present in non-RES organs and tissues. Particles with non-inactivated complement were accumulated in the lung at concentrations of 29% after 30 min, which increased to 71% after 120 min, whereas those coated with inactivated complement reached lung concentrations above 70% already after 30 min.

Conclusions. Particles coated with plasma components are able to avoid uptake by the RES, especially after heat inactivation of the plasma components adsorbed. Adsorption and heat inactivation of complement proteins alone, however, does not have the same result as coating with plasma proteins followed by heat inactivation. Therefore, it is concluded that plasma components other than complement proteins take part in the process of RES activation and phagocytosis of injected nanoparticles.

KEY WORDS: nanoparticles; body distribution; plasma proteins; complement; reticulo-endothelial system; opsonization.

INTRODUCTION

Targeted drug delivery involving colloidal drug carriers such as nanoparticles faces the problem of recognition of the carriers' non-self character by the immune system after systemic administration. The rapid phagocytosis of the carriers by the organs comprising the reticulo-endothelial system (1) is triggered and enhanced by the adsorption of serum components, mostly proteins, to the particle surface (opsonization) (2). Inactivation of preadsorbed serum components was proposed as a method to decrease the RES concentrations of colloidal drug carriers (3). Several in vivo studies with particles differing in surface properties and size have suggested that the pattern of opsonins adsorbed to the particle surface is the determining factor in their organ distribution (4,5). Among these serum components, the complement proteins C3 and C5 were generally regarded as the major components involved in the opsonization and phagocytosis of colloidal drug carriers, such as liposomes (6). To investigate the composition of serum components adsorbed, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) analysis has successfully been employed (7,8). However, in contrast to earlier assumptions, in vitro examinations of adsorption patterns on polystyrene nanoparticles incubated in human citrate plasma using 2-D PAGE showed no significant amount of complement proteins on the particle surface (9). Another study involving poly (D,L lactic acid) nanoparticles incubated in human serum also showed the absence of complement proteins (10).

The aim of this study was to gain more insight into the role of serum complement as a component of total opsonin adsorption on the rate of particle phagocytosis by the organs of the RES. For this purpose, we studied the body distribution of systemically administered radiolabeled poly (methyl methacrylate) (PMMA) nanoparticles coated with plasma, or with complement proteins in rats. In addition, we examined the particle distribution after heat inactivation of the components adsorbed to the particle surface prior to injection.

MATERIAL AND METHOD

Nanoparticles

Methyl-2-^{14}C-methacrylate monomer was obtained as a 1% solution in phosphate buffered saline (PBS) from Amersham-Buchler (Braunschweig, Germany). Polymerization was performed after removal of polymerization inhibitors (11) by γ-irradiation (0.5 Mrad, 1–3 krad/min) at the Eidgenössische Versuchsanstalt Wädenswil, Switzerland. The suspension was freeze-dried and stored at −26°C until further use. The lyophilized powder contained 43.7% nanoparticles, with a specific activity of 4 mCi/g.

Size Measurements

The effective diameters of the particle groups (control, complement, inactivated complement) were determined by correlation spectroscopy (Goniometer, Brookhaven Instr. Co., Holtsville, N.Y., U.S.A.) in PBS and rat plasma, at a temperature of 25°C and a measuring angle of 90°. Rat plasma was obtained by centrifugation of heparinized blood collected from rats. The results of these measurements are shown in Table I.

Coating Procedure

Nanoparticles were suspended at a concentration of 10 mg/ml (40 μ Ci/ml) in either PBS (control), rat plasma (obtained from Wistar rats), or a physiological solution of whole rat serum

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0724-8741/96/0700-1055$09.50/0 © 1996 Plenum Publishing Corporation

Table I. Particle Sizes of Uncoated (Control) and Coated Nanoparticles. Effective Diameters (n = 5, mean ± S.D.) in PBS ($D_{eff.1}$) and Plasma ($D_{eff.2}$)

	PBS	Plasma
Coating material	$D_{eff.1}$ [nm]	$D_{eff.2}$ [nm]
Control (none)	8401 ± 1240	1299 ± 137
Plasma	258 ± 22	450 ± 31
Inactivated Plasma	265 ± 31	510 ± 23
Complement	206 ± 19	803 ± 33
Inactiv. Complement	257 ± 36	745 ± 41

complement in PBS (52 CH_{50} units/ml; obtained from Sigma, Deisenhofen, Germany), and incubated for 12 h at 37°C. Inactivation of proteins adsorbed to the particles was done by heat treatment of the suspensions at 56°C for 30 min (12).

Animal Studies

The animal studies protocol met the "Principles of Laboratory Animal Care," enforced by German regulations. After ultrasonication, the nanoparticle suspensions were injected via the tail veins of two male and two female Wistar rats (Savo-Ivanovas, Kisslegg, Germany) per group and time point (30 min and 2h after injection). During the i.v.-injection, the animals were anaesthetized with ether, the dose of injection (1 mL/300 grams body weight, injection rate 1 mL/min) varied between 0.6 and 0.7 mL (24–28 μCi/mL). After sacrificing, the animals were dissected and two samples of each organ, whole blood, muscle tissue, and bone marrow were taken.

Analytical Methodology

The organ samples were weighed into glass vials and dissolved in tissue solubilizer (BTS-450, Beckman Instr., München, Germany). The radioactivity of the samples was measured in a scintillation counter after addition of 10 mL scintillation cocktail (Beckman) and 70 μL of glacial acetic acid to each vial. Organ concentrations were determined as the percentage of the total dose administered. Statistical evaluation was done employing the Student's t-test.

RESULTS AND DISCUSSION

The fate of intravenously administered particulate carrier systems is mainly determined by the action of the reticuloendothelial system (RES). The function of this physiological defence system is the extraction of foreign and potentially antigenic material from the blood stream and its elimination by phagocytosis. The cells of the various organs and tissues of the RES are enabled to recognize and phagocytose foreign material after being marked by adsorption of specific serum proteins (opsonization). Although the liver is regarded as the most important organ in the process of phagocytosis, spleen, lung, the lymphatic system, and the bone marrow are also involved. Accordingly, in our experiments, uncoated nanoparticles (control group) were mainly taken up by the Kupffer cells in the liver (13,14; Tables II and III), and to smaller amounts by the lung and other RES organs and tissues (lymph, spleen, bone marrow). The concentrations of the uncoated particles in non-RES organs and tissues (muscles, kidneys, gut, heart, brain), and blood concentrations varied between 6 and 10%.

Plasma coated particles by 12 h incubation in serum prior to injection showed a body distribution significantly different from the control particles. After 30 min, more than 50% of the administered dose of plasma-coated and heat inactivated particles were present in non-RES tissues, about 10% were in systemic circulation. Heat inactivation of serum at 56°C for 30 min is a standard method to inactivate complement (12). Incubation of the particles in serum for 12 h followed by this heat inactivation process reduced the particle concentrations in the liver to merely 22%. After 120 min, liver concentrations were still lower than the control group, and almost 30% of the administered dose of heat-inactivated particle group was present in non-RES organs and tissues. Increased lung levels were found for both groups, especially after 120 min (Table III).

In contrast to these results, the coating of the particles with the commercially obtained complement preparation alone prior to injection resulted in high particle concentrations in the lungs. Particles with non-inactivated complement obviously underwent a significant redistribution process resulting in lung concentrations of 29% after 30 min (Table II) that increased to 71% after 120 min (Table III), whereas those coated with inactivated complement reached very high lung concentrations above 70% already after 30 min. The reason for the redistribution from liver to lung might possibly be a migration of nanoparticle-containing macrophages as already earlier observed by Adlersberg et al. (15). In the body distribution of both coated particle groups, other RES-organs played a minor, non-RES organs about the same role as in the control group.

There are a number of possible explanations for the high concentrations found in the lungs in the experiments described

Table II. Influence of Coating on Nanoparticle Concentrations 30 min After Injection. Values Are Given as Percentages of the Total Dose Administered (n = 4; mean ± S.D.)

30 Min	Control	Plasma	Inactivated Plasma	Complement	Inactivated Complement
Lung	1.92 ± 1.46	21.57 ± 19.5	14.49 ± 20.27	29.18 ± 2.27	74.68 ± 5.48
Liver	70.35 ± 18.16	31.78 ± 16.53	22.33 ± 21.46	59.30 ± 3.30	16.37 ± 3.97
Other RES[a]	3.99 ± 2.17	1.20 ± 0.58	1.21 ± 0.99	1.98 ± 0.49	0.77 ± 0.23
Blood	0.96 ± 0.84	2.56 ± 3.62	10.21 ± 10.42	0.60 ± 0.13	1.05 ± 0.44
Non RES[b]	17.86 ± 13.58	36.16 ± 25.86	52.06 ± 33.07	9.07 ± 4.01	7.53 ± 4.39

[a] Other RES: bone marrow, lymph nodes, spleen.
[b] Non RES: brain, GI-tract, gonades, heart, kidneys, muscles.

Table III. Influence of Coating on Nanoparticle Concentrations 120 min After Injection. Values Are Given as Percentages of the Total Dose Administered (n = 4; Mean ± S.D.)

120 min	Control	Plasma	Inactivated Plasma	Complement	Inactivated Complement
Lung	8.05 ± 9.81	34.02 ± 4.90	19.27 ± 13.50	71.42 ± 3.80	65.87 ± 6.77
Liver	75.48 ± 6.43	53.22 ± 5.36	50.50 ± 17.67	23.99 ± 2.92	28.34 ± 6.22
Other RES[a]	3.33 ± 1.54	2.06 ± 0.76	2.45 ± 1.39	1.09 ± 0.29	0.77 ± 0.23
Blood	1.05 ± 0.87	0.92 ± 0.58	1.85 ± 1.13	0.28 ± 0.11	1.05 ± 0.44
Non RES[b]	10.47 ± 6.39	10.06 ± 7.47	28.94 ± 15.38	3.85 ± 1.54	4.04 ± 1.82

[a] Other RES: bone marrow, lymph nodes, spleen.
[b] Non RES: brain, GI-tract, gonades, heart, kidneys, muscles.

above. One possibility is an agglomeration of the particles that could have occurred after injection. This, in turn would have led to a blockade of the lung vessels. In fact, agglomeration of injected particles was generally observed after fast (1 ml/5 sec), but not after slow injection (1 ml/min) of nanoparticle suspensions (16). For this reason, the injection rate in the experiments described here was kept at a slow rate of 1 mL/min. Another reason for agglomeration could have been a size increase of the particles through the coating with serum and complement components. However, size measurements did not show any tendency of particle aggregation neither in PBS nor in plasma (Table I). In fact, although the effective diameters of the uncoated control particles were much bigger, their concentration in the lung was very low. It is possible, that size measurements in vitro are not supplying enough information about the aggregation tendency in vivo.

The most likely explanation of the preferential retention of nanoparticles coated with complement in the lung, especially after inactivation of the adsorbed proteins, is the occurrence of specific interactions of the particles with the macrophages present in pulmonary capillaries (17). A prerequisite for such a specific interaction could be the high potency of the protein coatings to selectively trigger the phagocytotic activity of the pulmonary macrophages. It has to be taken into account that the lung endothelium represents the first portion of the RES that the particles encounter after i.v.-injection and that the lung is the only RES organ through which the blood is recirculated after each body passage. The relatively slow increase in lung accumulation with the non-inactivated complement coated particles shows that this process was not a first pass effect but was dependent on the increase in lung macrophage or endothelium interaction with the particles. In contrast, this interaction was strong and rapid with the inactivated complement. This difference in body distribution behaviour of the particles shows that slight changes in structure of coating opsonins such as complement proteins that for instance occur as a result of the heat treatment can have very significant consequences.

This investigation clearly demonstrated a difference in body distribution between nanoparticles incubated in plasma from heparinized blood and those incubated in the commercially obtained complement preparation used in the present study. This complement is from coagulated blood and also contains other serum components. It has to be taken into consideration that, as a result of this, the complement in the different types of nanoparticle preparations may be present on the nanoparticle surface in different structural conformations. Consequently, the blood interaction and recognition by the blood cells, especially the macrophages, may be different and may lead to the observed differences in body distribution. Moreover, it is possible that complement does not play such a significant role in cell internalization as believed earlier (3,6,12). Leroux et al. (10) found no evidence of serum complement components on the surface of nanoparticles that were readily phagocytosed by monocytes. It is possible that other plasma components that are equally heat sensitive as complement are responsible at least in part for triggering the uptake by the different RES-tissues.

In summary, particles coated with plasma components are able to avoid uptake by the RES, especially after heat inactivation of the plasma components adsorbed. On the other hand, adsorption and heat inactivation of complement proteins alone does not have the same result. Therefore, it seems to be very likely that plasma components other than complement proteins take part in the process of RES activation and phagocytosis of injected nanoparticles.

ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG), grant Kr 867/1-1.

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