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Differential properties of organ-specific serum opsonins for liver and spleen macrophages

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Earlier we reported that serum contains organ-specific opsonins which selectively enhance recognition of liposomes by macrophages in the specific organs of the reticuloendothelial system (Moghimi, S.M. and Patel, H.M. (1988) FEBS Lett. 233, 143–147). The results presented here describe the properties of these organ-specific opsonins which differentiate between liver-specific and spleen-specific opsonins responsible for the enhancement of phagocytosis of liposomes by Kupffer cells and spleen macrophage, respectively. Liver-specific opsonin is a heat-stable macromolecule which on heating or on freezing and thawing exhibits enhanced opsonic activity. Serum also contains a dialysable factor which inhibits its opsonic activity. On the other hand, the spleen-specific opsonin is a heat-labile macromolecule which is sensitive to freezing and thawing and requires a dialysable serum co-factor for its optimum opsonic activity on spleen macrophages. Removal of this factor from serum brings about an irreversible conformational change in the opsonin. Evidence suggests that the spleen-specific opsonin may be composed of more than one different opsonin molecule. It is suggested that the serum factor(s) that inhibits liver-specific opsonic activity and enhances the spleen-specific activity may not be the same molecule, but in both the cases the factor(s) may mediate its function by modifying the process of the opsonisation of liposomes or by influencing the interaction of the opsonised liposomes with the respective cells. We propose that purification of the organ-specific opsonins may provide an opportunity to target drug carriers selectively to a specific organ of the reticuloendothelial system, and help us to evaluate their role in the altered opsonin states known to exist in certain diseases.

Introduction

Intravenously injected colloidal particles such as drug carriers, liposomes, nanoparticles, microspheres, etc., are cleared from the circulation in liver, spleen and bone marrow by phagocytes lining blood sinuses, and of these sites, hepatic clearance by Kupffer cells predominates [1,2]. The site of clearance of the particles by these organs of the reticuloendothelial system (RES) is influenced by blood flow, local tissue damage, the presence of opsonins including antibodies and probably other factors. In recent years, several investigators have studied the phagocytosis of the drug carrier, liposomes, in vivo and have shown that the relative distribution of the injected liposomes in the RES organs depends on their size [3,4], charge [5] and other surface properties such as hydrophobicity, fluidity [6], etc. A variety of serum components such as albumin, blood coagulation factors, globulins, fibronectins, and lipoproteins are absorbed on to the surface of liposomes depending on the surface properties of liposomes [8–10], which may influence their tissue distribution. However, there is a lack of information regarding the identity of serum components that are responsible for the phagocytosis of liposome and their distribution in various organs of the reticuloendothelial system.

Recently we demonstrated [11,12] that the intravenously injected cholesterol-free and cholesterol-rich liposomes are handled differently by liver and spleen. Liver takes up cholesterol-free more than cholesterol-rich liposomes, whereas spleen preferes cholesterol-rich to cholesterol-poor liposomes. These findings are further supported by our in vitro studies [13,14] which have led us to believe that serum contains organ-specific opsonins which determine the distribution of the injected particles in the various organs of the RES. The degree of distribution of the injected particles in the two main organs of the RES, liver and spleen, depends on their ability to attract either liver- or spleen-specific

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opsonins and this depends on the surface properties of the particles [14].

Our observation [13] of the presence of the organ-specific opsonins in serum may be important in explaining the mechanism of distribution of the intravenously injected drug carriers in the organs of the reticuloendothelial system. Hence it is worthwhile to investigate the properties of liver- and spleen-specific opsonins and we report here that these properties are distinctly different for both opsonins.

Materials and Methods

Egg phosphatidylcholine was purchased from Lipid Products (U.K.), cholesterol, dicetyl phosphate were purchased from the Sigma Chemical Co. Ltd. (U.K.), gelatin-Sepharose beads from Pharmacia (LKB) Ltd. and ^{125}I-labelled iodinate polyvinylpyrrolidone (^{125}I-PVP) were purchased from Amersham International plc.

Preparation of liposomes

Negatively charged multilamellar liposomes with entrapped ^{125}I-polyvinylpyrrolidone were prepared as described earlier [11,12] from a mixture of phosphatidylcholine/cholesterol/dicetyl phosphate in a molar ratio of 7:2:1, respectively, for cholesterol-poor liposomes and 7:7:1 for cholesterol-rich liposomes.

Serum preparation and treatment

Male CFY rats, body weight 250 ± 25 g were anaesthetized with ether and blood was collected by cardiac puncture and allowed to clot at room temperature for 30 min followed by centrifugation at $1500 \times g$ for 20 min. Serum was used fresh or stored at $-20\,^{\circ}C$ for period of up to 6 months. For certain experiments, serum was heat-treated by heating it at a temperature from 55 °C to 65 °C for a specified time. Fibronectin-depleted serum was prepared by treating it with gelatin-Sepharose beads as described by Bevidacqua et al. [15]. Serum (10 ml) was mixed with 2.5 ml of gelatin-Sepharose beads and incubated in ice for 30 min. The beads were then separated from the serum and the procedure was repeated twice more. Dilution of serum caused by this treatment was taken into account in the volume of the serum used in the incubation with the cells. Dialysed serum was prepared by dialysing fresh serum in 8/32″ tubing (from Medicell International Ltd., London) against a large volume of deionised water overnight at 4 °C. Dialysate was prepared by freeze drying the bulk of the dialysate collected after dialysing serum and resuspending it in saline to the original volume of the serum.

Cell preparation and incubation with cells

Hepatic non-parenchymal cells containing predominantly endothelial and Kupffer cells and splenic white cells were prepared from male CFY rats of body weight 250 ± 25 g as previously described [13]. The cells were incubated at 37 °C in polythene vials with liposomes in the presence or absence of 25% serum as described earlier [13]. The hepatic cells (10^7 cells) were incubated in Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (Gibco) and the splenic cells ($8 \cdot 10^7$) were incubated in 10 mM oxygenated phosphate-saline buffer, pH 7.4 with and without serum. 25% serum used in the incubation does not cause aggregation of both liver and spleen cells and we have shown earlier [13] that the results obtained in the presence of 25% serum in vitro are comparable with those obtained in vivo. Furthermore, the uptake of liposomes in the presence of serum was inhibited by 50–65% by substances known to inhibit endocytosis.

The uptake of liposomes by cells was monitored by measurement of the radioactivity of the entrapped ^{125}I-labelled polyvinylpyrrolidone in the cells at the end of the incubation. Serum from Lewis and CFY male and female rats exhibited liver- and spleen-specific opsonic activity but no activity was observed in serum collected from WAG rats. Furthermore, the opsonic activities varied from day to day and animal to animal and may be influenced by the possible day-to-day variation in the cell preparations. We also suspect seasonal variation in these activities. These problems slow down the progress of this research and to assure the reliability of the results, serum and cell preparations used in each experiment were generally obtained from the same animals. Each experiment was designed to be small with the appropriate controls and used a maximum of two or three rats in order to achieve the best results. Most of the incubations were done in triplicate but in some experiments duplicate incubations were done due to the design of the experiment and shortage of serum. However, each experiment was carried out at least three times and the results of a typical experiment are presented here. The data, expressed as the percentage uptake of ^{125}I radioactivity, are the means of two or three incubations ± S.D. whenever possible [13].

Results

Heat-treatment of serum

The results in Table I show that serum opsonic activity on the interaction of liposomes with liver cells is modified when the serum is preheated at different temperatures for 10 min. The activity of the fresh serum is enhanced by about 35% at 55 °C but at a temperature of 60 °C it is reduced by 25% and abolished completely when it is preheated at 65 °C for 10 min. The results in Fig. 1 show the effect of prolonged heating of serum at 55 °C on the interaction of liposomes with both liver and spleen cells. The liver-specific opsonic activity is increased initially and reaches a maximum when the

TABLE I

Opsonic effect of pre-heated rat serum on the interaction of negatively charged cholesterol-poor liposomes with rat liver non-parenchymal cells

For experimental details see Materials and Methods.

Treatment	% of initial liposomal ^{125}I-PVP
Control	2.2 ± 0.1
Serum	12.4 ± 0.1
Heated serum:	
55 °C/10 min	17.5 ± 1.3
60 °C/10 min	9.2 ± 0.1
65 °C/10 min	< 0.1

serum is preheated at 55 °C for 10 min, and thereafter further heating of serum caused a gradual decrease in the activity. However, the opsonic activity, even after prolonged heating of serum for 60 min, is still elevated and enhances the interaction of liposomes with liver cells as compared to that observed with the control serum.

The spleen-specific opsonic activity of serum, on the other hand, responded differently to the heat treatment. The activity in serum is gradually reduced and eventually lost after 30 min of heating prior to incubation with the spleen cells (Fig. 1).

Effect of freezing-thawing (cold storage) of serum

The results in Table II show that the serum stored at −20 °C for 6 months not only retains its liver-specific opsonin activity but has 24% more activity compared to that of the fresh serum. The effect of freezing on serum activity is very similar to the effect of dialysis and heating of the serum. Spleen-specific activity of the serum, on the other hand, is reduced by 20% on storage

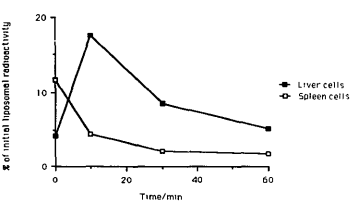

Fig. 1. Effect of heated serum at 55 °C on its opsonic activity specific for liver and spleen phagocytes. Negatively charged cholesterol-poor liposomes entrapped ^{125}I-PVP were incubated with the liver and spleen cells in the presence and absence of pre-heated rat serum for 1 h. The cell associated liposomal radioactivity was measured at the end of incubation. Each point represents the mean of duplicate incubations and the % associated radioactivity with cells in each incubation at the end of the experiment was within the 5% of the mean value. For experimental details see Materials and Methods.

TABLE II

Effect of cold-stored serum on interaction of negatively charged cholesterol-poor liposomes with liver non-parenchymal and splenic white cells

Serum was stored at −20 °C for a period of 6 months and thawed only once at room temperature on the day of experiment.

Treatment	Change in opsonic activity (% of serum) [a]	
	liver cells	spleen cells
Fresh serum	100	100
Fresh serum heated at 55 °C/10 min	141 ± 2	not done [b]
Cold-stored serum for 6 months at −20 °C	124 ± 9	81 ± 3
Cold-stored serum heated at 55 °C/10 min	131 ± 2	not done [b]

[a] Mean of three incubations ± S.D.

[b] Not done since spleen-specific opsonin is heat labile (see Fig. 1).

at −20 °C for 6 months in comparison to the fresh serum activity.

Effect of dialysis of serum

The results in Table III show the effect of the dialysed serum on the interaction of cholesterol-poor liposomes with liver and spleen cells. Upon dialysis, the liver-specific opsonic activity of serum is enhanced by about 2-fold, whereas the spleen-specific activity is reduced by about 50% of the control serum. This effect of the dialysed serum was much more pronounced on the interaction of cholesterol-rich liposomes with spleen cells, where it was completely abolished (data not shown). The dialysate by itself has no effect on the interaction of liposomes by liver and spleen cells, but when the dialysate together with the dialysed serum is added to the incubation media, the enhanced liver-specific opsonic activity of the dialysed serum observed earlier is suppressed to the level of that of the control serum and this effect of the dialysate was not reversed

TABLE III

Opsonic activity of dialysed rat serum on interaction of negatively charged cholesterol-poor liposomes with liver non-parenchymal and splenic white cells

For experimental details see Materials and Methods.

Treatment	% of initial liposomal ^{125}I-PVP	
	liver cells	spleen cells
Control	2.3 ± 0.6	2.8 ± 0.2
Serum	13.4 ± 0.6	7.9 ± 0.2
Dialysed serum	28.6 ± 2.0	4.4 ± 0.5
Serum dialysate	2.1 ± 0.2	3.0 ± 0.1
Dialysed serum + serum dialysate	13.6 ± 0.1	3.1 ± 0.4
Dialysed serum + boiled serum dialysate	10.1 ± 0.1	not done

TABLE IV

Effect of fibronectin depleted serum and addition of heparin to normal serum on its opsonic activity specific for liver and spleen phagocytes

Cholesterol-poor PC liposomes were used for the experiments. For experimental details see Materials and Methods.

Treatment	% of initial liposomal ^{125}I-PVP	
	liver cells	spleen cells
Control	1.5±0.1	3.4±0.3
Serum	12.3±0.6	6.8±1.4
Serum (fibronectin depleted)	11.8±0.5	6.0±0.7
Heparin (5 U) + buffer	1.3±0.1	not done
Heparin (5 U) + serum	13.5±3.2	not done

when it is preboiled, whereas the dialysate, when added to the dialysed serum, fails to reverse the lost opsonic effect of the dialysed serum specific for the spleen cells. On the contrary, it further suppresses the interaction of liposomes with the cells.

Effect of heparin

Heparin is known to influence the phagocytic activity of macrophages and enhances the opsonic function of fibronectin [16,17]. Hence the effect of heparin on liver-specific opsonin was investigated. The results in Table IV show that heparin does not have any significant effect on opsonin activity of the fresh and dialysed serum in stimulating the interaction of liposomes with liver cells. Heparin in larger doses caused aggregation of cells and thus reduced liposome–liver cell interaction. It was not possible to assess the effect of heparin on the spleen-specific opsonic activity since heparin caused aggregation and lysis of spleen cells.

The results in Table IV also show fibronectin-depleted serum has no significant effect on the interaction of liposomes with liver or spleen cells. These results are very similar to those obtained with the control serum.

Discussion

Our earlier evidence suggests that serum contains opsonins which are specific for macrophages of specific organs of the reticuloendothelial system, and the results presented here describe their properties which differentiate between liver-specific and spleen-specific opsonins responsible for the enhancement of phagocytosis of liposomes by Kupffer cells and spleen macrophages, respectively [13]. Liver(Kupffer cells)-specific opsonin is heat-stable and its opsonic activity is elevated either on heating at 55°C or on freezing and thawing of serum. Thus this opsonin probably undergoes conformational changes on heating or freezing and thawing, which enhance its opsonic activity as monitored by an increase in the interaction of liposomes with Kupffer cells. The spleen-specific opsonin, on the other hand, is heat-labile since most of its opsonic activity is lost on heating of serum at 55°C. However, freezing and thawing of serum causes partial loss of the activity. These treatments perhaps bring conformational changes in the opsonic molecule which have a reverse effect on its opsonic function to that observed with the liver-specific opsonin.

The second difference in the properties of these two opsonins is the effect of dialysis of the serum on their activity. Upon dialysis, liver-specific opsonic activity in serum is enhanced, which suggests that liver-specific opsonin is a macromolecule and its activity is partially inhibited by small molecular weight factor(s), which is heat-stable, even at the boiling temperature, whereas the reduction in the spleen-specific opsonic activity in the dialysed serum suggests that the spleen-specific opsonin is either a dialysable small molecular weight substance(s) or it is a macromolecule which requires a dialysable co-factor for its optimum activity. Since the dialysate by itself has no opsonic activity on the uptake of the spleen cells (see Table III), the spleen-specific opsonin must be a macromolecule(s) which requires a dialysable co-factor for its optimum opsonic activity. As in the case of the spleen cells, the dialysate by itself has no effect on the interaction of liposomes with the liver cells and this suggests that the dialysable factor does not cause the inhibitory effect on the interaction of liposomes by acting directly on the liver cells. Hence its effect must be mediated by its interaction with the liver-specific opsonin molecule and thus it either interferes with the process of opsonisation of liposomes or with the interaction of the opsonised liposomes with the cells. This argument is further supported by the observation that when the dialysate is added to the dialysed serum in order to replace the inhibitory factor removed by the dialysis of the serum (Table III) the elevated opsonin activity of the dialysed serum is reduced to the level of that of the control serum. The dialysis of serum brings about irreversible changes in the spleen-specific opsonin, since the addition of the dialysate to the dialysed serum does not bring back its lost opsonic activity (see Table III). This probably suggests that this co-factor for the spleen-specific opsonin, when removed by dialysis, brings about irreversible conformational changes in the opsonin molecule which causes partial loss of its activity.

Fibronectin, a serum heat-labile opsonin, is known to interact with liposomes and enhance their phagocytosis in the peritoneal macrophages [20], but since the properties of fibronectin [18] do not match with those of the liver-specific opsonin, fibronectin may not be our liver-specific opsonin. This conclusion is further supported by the evidence that the depletion of fibronectin from the serum does not alter its liver-specific opsonic activity. Heparin, which is known to enhance the function of fibronectin [16,17], has no significant effect on the opsonic activity when added to the serum. On the other

hand, some properties of fibronectin are similar to those of spleen-specific opsonin. Both of these opsonins are heat-labile but unlike fibronectin, the spleen-specific opsonin, on cold storage, loses only part of its activity. Moreover, the fibronectin-depleted serum does not show significant reduction in the uptake of liposomes. This discrepancy apparently rules out the possibility of fibronectin as our spleen-specific opsonin, however, at the same time the discrepancy can be explained if we follow our earlier suggestion [13] that there are more than one spleen-specific opsonins so that the depletion or inactivation of one of the serum opsonins, which may be fibronectin, will produce only partial loss in the opsoninc function of the serum. Whereas the total loss of the serum activity on heating at 55°C suggests that the other spleen-specific opsonin may also be heat-labile. The direct, independent role of fibronectin as an opsonic molecule is still not established [21], but its complementary role in the enhancement of the complement factor C3b-mediated endocytosis of erythrocytes is well known [22]. Complement factor C3b and fibronectin are both heat-labile and hence it is tempting to suggest that spleen-specific opsonin may include both fibronectin and complement components for its optimum activity. However, studies are in progress in this laboratory to identify the nature of these opsonins.. During our attempts to purify the liver-specific opsonin we have established that this opsonin appears to be a proteinaceous macromolecule and the dialysable heat-stable factor which inhibits its opsonin activity appears to be Ca^{2+} (manuscript in preparation).

The results discussed here demonstrate that the properties of liver-specific and spleen-specific opsonins are different, and suggest they are different macromolecules and their opsonic functions are regulated by the small molecular weight serum factors. Purification and characterisation of these organ-specific opsonins may not only provide an opportunity to target drug carriers selectively to the particular organ of the reticuloendothelial system, but also help us to evaluate their role in infection and other pathological conditions. In our next manuscript (in preparation), we hope to elucidate further the properties of these opsonins and those specific for other organs of the reticulendothelial system.

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