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Calcium as a possible modulator of Kupffer cell phagocytic function by regulating liver-specific opsonic activity

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Recently we described some properties of organ-specific serum opsonins which differentiate between liver- and spleen-specific opsonic activities, and reported that, on dialysis of serum, its liver opsonic activity is enhanced by 2- to 3-fold, whereas spleen-specific activity is reduced by 20–30% of that of control serum (Moghimi, S.M. and Patel, H.M. (1989) Biochim. Biophys. Acta 984, 379–383). This observation suggests that serum contains dialysable factors which regulate liver- as well as spleen-specific opsonic activities. Our results from EGTA-treated serum suggest that dialysable factor(s) could be divalent cations such as Ca^{2+}, Mn^{2+}, Mg^{2+} or Co^{2+}, and among them, calcium may be the key regulatory factor for liver-specific opsonic activity. The regulatory mechanism of spleen-specific opsonic activity seems to be complex, since addition of dialysate or calcium or magnesium to the dialysed serum does not restore its activity; probably the removal of divalent cations has induced an irreversible conformational change in spleen-specific opsonin. In conclusion, we propose that the blood calcium concentration may play an important role in modulating hepatic phagocytic function by modifying liver-specific opsonic activity in serum. An increase in the physiological concentration of calcium will suppress and a decrease will enhance this opsonic activity.

Introduction

The clearance of colloidal particles from the circulation occurs mainly in the liver, spleen and bone marrow by phagocytes lining blood sinuses in these organs of the reticuloendothelial system. The relative distribution of the injected particles in these organs depends on various factors including the surface properties of the injected particles [1,2]. Surface properties are important in attracting serum opsonins or dysopsonins which determine the phagocytic fate of the particles [3,4].

Recently we have demonstrated that serum contains organ-specific opsonins which enhance phagocytosis of liposomes, depending on their lipid composition and surface properties, in Kupffer cells, spleen and bone marrow macrophages [3–6]. During the study of properties of organ-specific opsonins in serum, which differentiate their specificity for Kupffer cells and spleen macrophages, we observed [6] that, on dialysis of serum, the liver specific opsonic activity was enhanced by 2- to 3-fold and spleen specific opsonic activity was reduced by 20–30% of those in normal serum. When dialysate was added to the dialysed serum, the liver-specific activity was brought back to the level of normal serum, but this treatment did not reinstall the loss of activity of spleen-specific opsonin [4,6]. These observations led us to believe that organ-specific opsonic activities may be closely regulated by one or more dialysable small molecular weight substances present in serum. We believe that understanding of the regulatory mechanisms of organ-specific opsonic activities may provide an opportunity to target drug carriers like liposomes selectively to a specific organ of the reticuloendothelial system, and will also help us in further understanding of the pathogenesis of bacterial infections and immune complex diseases involving the reticuloendothelial system.

Hence, in this paper we have attempted to identify the nature of the dialysable factor which may be involved in regulating liver-specific opsonic activity in serum.

Materials and Methods

Egg phosphatidylcholine was purchased from Lipid Products (U.K.), cholesterol, dicetyl phosphate, and

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EGTA were purchased from Sigma (U.K.). ^{125}I-labelled iodinated poly(vinylpyrrolidone) (^{125}I-PVP), [^{3}H]cholesterol and [*carboxylic acid*-^{14}C]inulin were purchased from Amersham International (Amersham, U.K.).

Preparation of liposomes. Negatively charged multilamellar cholesterol-poor liposomes containing ^{125}I-PVP were prepared from a mixture of phosphatidylcholine/cholesterol/dicetyl phosphate in a molar ratio of 7 : 2 : 1 [7]. In some experiments [^{3}H]cholesterol-labelled liposomes containing an aqueous marker, [^{14}C]inulin, were prepared as described earlier [7].

Opsonisation of liposomes. Liposomes (15 mg) were opsonised by incubation in 50% fresh rat serum (diluted with saline) for 15 min at 37°C, followed by centrifugation at 200 000 × *g* for 30 min to pellet the opsonised liposomes. Washed opsonised liposomes were prepared by washing the pellet once with saline and centrifuging to obtain a liposomal pellet as described above.

Serum preparation. Male CFY rats (body weight 250 ± 25 g) were anaesthetised with diethyl ether and blood was collected by cardiac puncture and allowed to clot at room temperature for 30 min followed by centrifugation at 1500 × *g* for 20 min. Dialysed serum was prepared by dialysing fresh serum in 8/32 inch tubing against a large volume of deionised water overnight at 4°C.

Cell preparations and incubation with liposomes. 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 [5,6]. The hepatic cells (10^7 cells) were incubated in Ca^{2+}/Mg^{2+} free Hanks' balanced salt solution and splenic cells (8×10^7) were incubated in 10 mM oxygenated phosphate saline buffer (pH 7.4) with and without serum [6]. The serum opsonic activities were assessed by studying the uptake of liposomes in these cells in the presence of serum and the uptake of liposomes was monitored by measurement of the radioactivity of the entrapped ^{125}I-poly(vinylpyrrolidone) or [^{14}C]inulin in the cell at the end of incubation [5,6].

The data, expressed as the percentage uptake of radioactivity, are the means of three incubations ± S.D.

Results

Effect of EGTA on liver-specific opsonic activity

The effect of EGTA on the uptake of cholesterol-poor liposomes in liver cells in the presence and absence of serum was studied. The results in Fig. 1 show that 1.25 mM EGTA inhibits the uptake of liposomes in liver cells in the absence of serum but enhances it in the presence of serum, and this activity reaches the level of that found in the dialysed serum. Since EGTA is known to chelate divalent cations, particularly calcium, these results probably suggest that the dialysable serum factor may be cations which regulate liver-specific opsonic activity and thus influence phagocytosis of liposomes in hepatic macrophages.

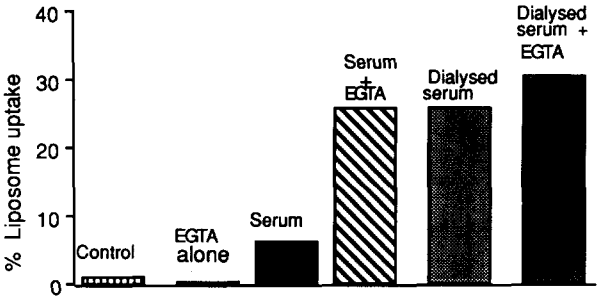

Fig. 1. Effect of 1.25 mM EGTA on liver-specific opsonic activity in dialysed and nondialysed serum.

Effect of divalent cations

Cations such as Ca^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+} are known to enhance phagocytosis in certain macrophages [8,9]. Hence, we studied the effect of these ions on the liver specific opsonic activity in dialysed serum. These cations at a concentration of 1.5 mM enhance the uptake of liposomes in liver cells in the absence of serum, but when incubated in the presence of dialysed serum they suppressed its liver specific opsonic activity (Table I) and the uptake of liposomes was greatly reduced. Mg2 + and Co2 + reduced the opsonic activity in the dialysed serum to 25–30%, which is still much more than that found in the non-dialysed (normal) serum (Table I). $MnCl_2$ and $CaCl_2$, on the other hand, caused maximum reduction in the opsonic activity of the dialysed serum. However, since this inhibitory effect of Co^{2+}, Mn^{2+} and Mg^{2+} is observed when non-physiological concentrations of these cations are used in the incubation, it probably suggests that they may not have any significant role in regulating liver opsonic activity in vivo.

Calcium is known to influence the process of phagocytosis [9–11] and the results in Fig. 2 show that when the calcium concentration, added as $CaCl_2$ in the incubation, is increased to 0.5 mM, the uptake of liposomes in liver cells is reduced by almost 60% and at 1

TABLE I

The effect of various divalent cations on uptake of negatively charged MLV liposomes by liver cells in absence and presence of dialysed serum

1.5 mM salt	Liposome uptake (% of initial ^{125}I-PVP)	
	control (no serum)	dialysed serum
None	1.4 ± 0.1	35.4 ± 2.9
$CaCl_2$	2.4 ± 0.1	1.6 ± 0.2
$MgCl_2$	2.0 ± 0.4	9.8 ± 0.9
$CoCl_2$	5.5 ± 1.0	11.3 ± 0.1
$MnCl_2$	3.0 ± 0.1	1.0 ± 0.3

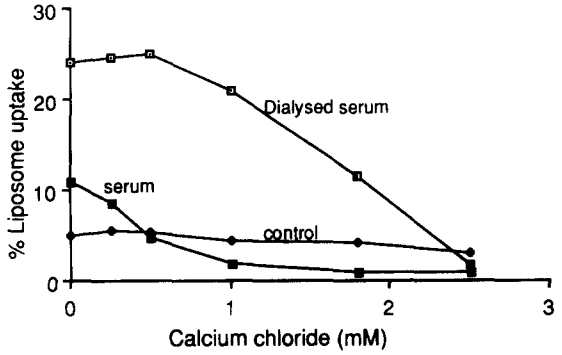

Fig. 2. The effect of calcium chloride on liver-specific activity in dialysed and nondialysed serum.

mM concentration the uptake is almost abolished, whereas up to 0.5 mM of $CaCl_2$ added to the dialysed serum has no significant effect on the uptake of liposomes, and a further increase in its concentration causes rapid suppression in liposome uptake in these cells and uptake is completely inhibited when $CaCl_2$ concentration reaches 2.5 mM. This effect of $CaCl_2$ is not due to the change in ionic strength in the incubation media, since replacing $CaCl_2$ with NaCl in a concentration to give a similar ionic strength showed no significant effect on the opsonic activity.

Possible site(s) of action of calcium

To investigate the mechanism of action of calcium on liver-specific opsonin, liposomes were opsonised by incubating with fresh serum (see Materials and Methods) and their uptake by liver cells in the presence and absence of 1.5 mM $CaCl_2$ was studied. The results in Fig. 3 show that the pre-opsonised liposomes, as shown earlier [5], are taken up by these cells much more than unopsonised liposomes in the absence of $CaCl_2$, but when $CaCl_2$ is present in the incubation the uptake of pre-opsonised liposomes is suppressed to the level of that of unopsonised liposomes, and the results are comparable with those obtained when prewashed opsonised lipsomes are used. Since washing of opsonised liposomes removes liver-specific opsonin bound on the liposomal surface [5], these results suggest that calcium is either interfering with the process of opsonisation of liposomes or with the interaction of the opsonised liposomes with cell surfaces.

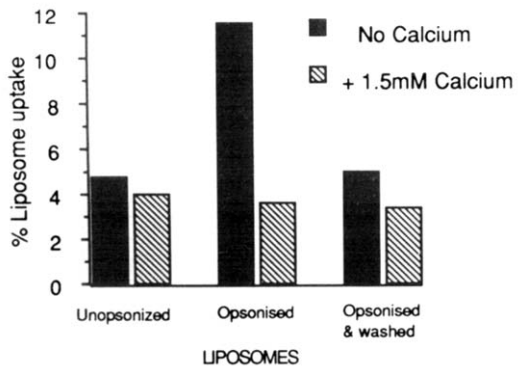

Fig. 3. Effect of 1.5 mM calcium chloride on uptake of opsonised, washed opsonised and unopsonised liposomes by liver cells.

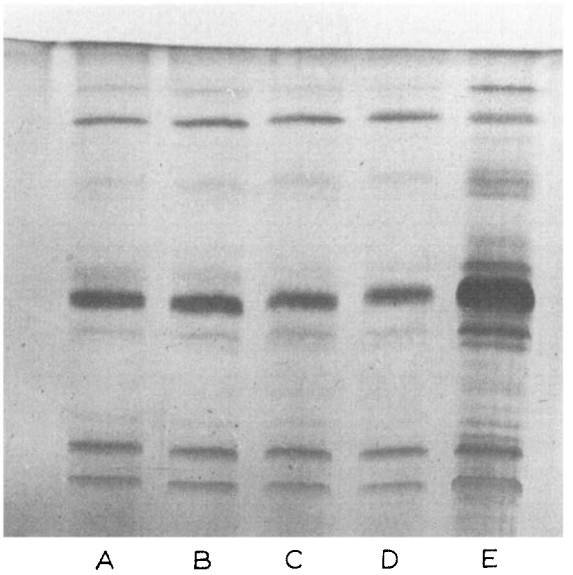

Fig. 4. Electrophoresis of proteins associated with liposomes opsonised in the presence of $CaCl_2$. SDS-PAGE electrophoresis of opsonised cholesterol-poor liposomes was done with a Pharmacia Phast electrophoresis system on a 10–15% gradient gel. (A) liposomes opsonised in absence of $CaCl_2$, (B, C and D) liposomes opsonised in presence of 2.0, 5.0 and 10.0 mM $CaCl_2$, respectively; (E) serum.

Next, the effect of calcium on the binding of serum proteins on liposomes surfaces was studied. Liposomes were incubated with fresh serum in the absence and presence of 2.0, 5.0 and 10 mM $CaCl_2$. Fig. 4 shows the electrophoretic pattern of serum proteins associated with liposomes. The electrophoretic patterns of detectable proteins associated with liposomes were very similar in both cases of the opsonisation of liposomes in the absence and presence of $CaCl_2$. Thus $CaCl_2$ does not appear to interfere significantly in the binding of serum proteins on liposomal surfaces.

Lastly, to investigate the possibility of calcium interfering with the interaction of lipsomes with liver cells, we examined the effect of $CaCl_2$ on the uptake of double labelled liposomes in the presence of dialysed serum. As reported earlier, 1.5 mM $CaCl_2$ added in the beginning of incubation with liver cells caused a significant reduction in the opsonic activity in dialysed serum (see Table II) as judged by the decrease in uptake of both the markers, [^{14}C]inulin and [^{3}H]cholesterol. $CaCl_2$ also altered the ratio of lipid marker, [^{3}H]cholesterol, and aqueous marker, [^{14}C]inulin. This suggests [12,13] that $CaCl_2$ causes an alteration in the mode of interaction of liposomes with the cells. When liposomes are incubated with cells for 30 min, the ^{3}H and ^{14}C radioactivities associated with cells are 8.8% and 5.8%, respec-

TABLE II

Effect of calcium on interaction of negatively charged MLV liposomes with liver non-parenchymal cells

Treatment	% of initial radioactivity		
	[^{3}H]cholesterol	[^{14}C]inulin	$^3H/^{14}C$
A Dialysed serum (incubated 60 min)	10.2±0.1	7.2±0.2	1.5
B Dialysed serum + 1.5 mM calcium (incubated 60 min)	2.5±0.1	0.6±0.1	4.1
C Dialysed serum (incubated 30 min)	8.8±0.3	5.8±0.3	1.5
D Dialysed serum incubated 30 min followed by addition of 1.5 mM calcium and incubated for further 30 min	5.7±0.2	3.3±0.2	1.7

tively. When 1.5 mM $CaCl_2$ is added at the end of 30 min incubation, which is then prolonged for 30 min, the two radioactivities associated with liposomes are 5.7% and 3.3%, respectively. However, the ratios of ^{3}H and ^{14}C radioactivity in both cases are very similar. This suggests that $CaCl_2$ has not only prevented further binding of liposomes to the cells but also effectively removed some intact liposomes (about 40%) associated with the cells when incubated for 30 min prior to addition of $CaCl_2$. This perhaps indicates that $CaCl_2$ interferes with binding and eventually with the process of internalisation of liposomes in the cells. However, it is not clear how calcium mediates its action: whether by modifying the receptor on the cell or the opsonin, which may make a bridge between liposomes and cells.

The effect of Ca^{2+} on spleen-specific opsonic activity

Unlike its effect on liver-specific opsonin, dialysis of serum causes suppression in its spleen-specific opsonic activity [3]. Furthermore, the results in Table III show that EGTA also suppresses the spleen-specific opsonin activity in serum. This raises the possibility that calcium may also be involved in regulation of spleen-specific opsonic activity but having the reverse effect to that observed with liver-specific activity. Hence to investigate this possibility, the effect of 1.0 mM $CaCl_2$ on the uptake of liposomes by spleen cells in the presence of dialysed serum was studied. The results in Table III show that calcium does not reinstall the opsonic activity that has been suppressed during dialysis of serum. Similarly, 1.0 mM $MgCl_2$ has no significant effect on the activity in dialysed serum.

TABLE III

The effect of diavalent cations on the uptake of negatively charged cholesterol-poor egg PC liposomes by spleen cells in the absence and presence of dialysed serum

Liposomes were labelled with membrane marker [^{3}H]cholesterol and aqueous marker, [^{14}C]inulin, and 1.25 mM EGTA and 1.0 mM $CaCl_2$ and $MgCl_2$ were used.

rum	% of initial radioactivity	
	[^{3}H]cholesterol	[^{14}C]inulin
Experiment I		
Control	7.4±0.1	1.6±0.1
Control + EGTA	6.3±0.3	1.5±0.1
Serum	17.1±0.6	20.3±0.6
Serum + EGTA	13.8±0.4	14.9±0.6
Experiment II		
Control	10.6±0.2	2.9±0.2
Control + $CaCl_2$	10.3±0.3	3.3±0.2
Control + $MgCl_2$	9.3±0.3	2.6±0.1
Serum	19.2±0.9	20.3±0.8
Dialysed serum (DS)	8.0±0.7	6.4±1.0
DS + $CaCl_2$	8.3±0.6	6.2±0.8
DS + $MgCl_2$	9.3±0.2	8.0±0.5

Discussion

Enhancement in the apparent uptake of liposomes in the presence of dialysed serum is due mainly to binding of liposomes on the surface of the liver cells [14], and the results presented here suggest that the enhanced liver-specific opsonic activity observed in the dialysed serum [6] may be due to removal of divalent cations on dialysis of serum. Cations such as Co^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} at a concentration of 1–1.5 mM stimulates phagocytosis of opsonised albumin particles in leukocytes and rabbit alveolar macrophages [8], but we found that these cations suppressed the uptake of liposomes by Kupffer cells in the presence of dialysed serum (Table I). Thus, they have an inhibitory effect on liver-specific opsonic activity. However, the amount of these cations, particularly Co^{2+}, Mn^{2+} and Mg^{2+}, used in our experiments is much greater than physiological concentrations in serum, and hence their role in influencing liver-specific opsonic activity in vivo may not be significant. Calcium, on the other hand, at low concentrations is a prerequisite for the process of phagocytosis of certain particulate material [9–11], but at a high concentration is known to inhibit the process in some cell types [8]. The concentration of calcium in blood is controlled closely in man and it remains almost constant except in certain diseases such as cancer [15,16]. The calcium level in our rat serum, measured by atomic

absorption, indicated that dialysis removes about 1.5 to 1.8 mM calcium from the total of an average 2.6 mM in normal serum. Our results in Fig. 2 show that the enhanced opsonic activity in the dialysed serum is reduced on addition of $CaCl_2$ in the incubation. When the concentration of $CaCl_2$ reaches about 1.8 mM, the calcium level in the incubation containing dialysed serum is now very similar to that in the control (serum) incubation. At this concentration the opsonic activity in the dialysed serum is almost back to the level of that of control serum. These results are comparable with those reported earlier [6], where it was demonstrated that when dialysate is added back to the dialysed serum, its liver-specific opsonic activity is brought back to the level of control serum. Thus these results suggest that the blood calcium concentration may play an important role in regulating the liver-specific opsonic activity. A small change in calcium level may cause a fluctuation in its activity. An increase in calcium will suppress and a decrease will stimulate the liver-specific opsonic activity.

To test this hypothesis, we have studied the opsonic activity in hypercalcaemic blood of tumour-bearing animals. The results of this study (unpublished) demonstrate that, in hypercalcaemic serum, the opsonic activity is suppressed and its treatment with EGTA restores its activity to normal levels. However, all organ-specific opsonic activities are not influenced by calcium. For example, our results (Table III) indicate that spleen-specific opsonic activity, which is suppressed on dialysis of serum is not affected by calcium, but it is possible that removal of calcium by dialysis of serum might have caused an irreversible change in spleen-specific opsonin [6] and hence replacing calcium in dialysed serum fails to restore its lost activity.

If calcium is a key regulatory factor, then the question that is to be addressed is, how does it regulate liver-specific opsonic activity? Calcium is known to influence many membrane-associated and intracellular events [17,18] and there are several possible ways in which it can modulate the opsonic activity. Firstly, calcium may bring about conformational changes in the opsonic molecule which may prevent opsonisation of liposomes, or these changes may prevent the interaction of opsonised lipsomes with cells. A second possibility is that calcium may modify the receptor on Kupffer cells [19] and as a result the opsonised liposomes find it difficult to interact with the cells and thus fail to internalise. The third possibility is that calcium may facilitate the binding of dysopsonins [4] or other serum proteins which may mask the recognition regions of the liver-specific opsonin, and as a result suppression of their phagocytosis may occur. Experiments (see results in Figs. 3 and 4 and Table II) designed to understand the mode of action of calcium failed to give a clear-cut answer. On the contrary, the results suggest that the regulation of liver-specific opsonic activity by calcium may involve more than one site. Further study with purified liver-specific opsonin may perhaps help us to understand the involvement of calcium in regulating its activity.

Acknowledgements

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