Synergistic Effect Between Size and Cholesterol Content in the Enhanced Hepatic Uptake Clearance of Liposomes Through Complement Activation in Rats

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Purpose. The effect of liposome size and cholesterol (CH) content on the pharmacokinetics of liposomes was investigated in rats.
Methods. The pharmacokinetics of liposomes was examined using 5(6)-carboxyfluorescein (CF) as an aqueous phase marker. The extent of complement activation (ECA) was also measured by the release of CF from liposomes in serum.
Results. Both the size and the CH content influenced the mean residence time, total body clearance, and the hepatic uptake clearance (CLh) of liposomes. The increase of the size of liposomes increased the CLh at each CH content. There was no CH dependency of CLh in small liposomes (200 nm in diameter), although the CLh increased with the increase in the CH content in large (800 nm) and medium (400 nm) liposomes. A significant interaction effect was observed between liposome size and the CH content on CLh according to the analysis of variance. The good correlation between CLh and ECA indicated the role of complements as opsonins in enhancing the hepatic uptake of liposomes. The interaction effect between the size and CH content on CLh was explained principally by the product of the size and CH content.
Conclusions. A synergistic effect was observed between the size and the CH content on CLh. An underlying hypothesis of the synergistic effect was postulated based on the size dependent recognition of liposomes by complement system.

KEY WORDS: liposome; complement; cholesterol; size.

INTRODUCTION

The size of liposomes is one of the principal factors influencing the uptake clearance of liposomes by the liver, which is the determinant organ of the blood clearance of liposomes (1–5). It was shown that entrapped 5(6)-carboxyfluoresceine (CF) was released from liposomes in rat plasma through the activation of an alternative complement pathway (6). The rate and extent of the release of CF from liposomes were also shown to depend on the size of liposomes (7) and there was a good correlation between the hepatic uptake clearance (CLh) of liposomes *in vivo* and the release of CF from liposomes (5). These results indicated that complement system contributes to both enhancement of CLh and disintegration of liposomes in blood circulation.

The effect of liposome size in the opsonization was examined under an isolated perfused liver system (8), an opsonic effect being seen for the large sizes (>400 nm) of liposomes. This size discrimination resulted from the opsonins not from the Kupffer cells. Since complements are considered to be opsonins—especially the fragments of the third component of the complement (C3b/iC3b)—it was suggested that the complement system recognized the size of liposomes. The putative binding (or recognition) site was suspected to be the hydroxyl group of the CH molecule, because although C3b covalently binds amino or hydroxyl groups (9), this composition of liposomes have no amino group. This consideration led us examine the effect of size and CH content on the CLh of liposomes.

In this study, we have systematically evaluated the effect of liposome size and CH content on the disposition of liposomes in *in vivo* experiments. The activation of the complement system was also evaluated in *in vitro* experiments. The CLh increased in proportion to the size of liposomes as expected, and the increase of CH content increased the CLh only for the large and medium sizes of liposomes. A significant interaction effect between the size and CH content was seen for CLh, thus a working hypothesis was postulated based on the size-dependent recognition of liposomes by the complement system.

MATERIALS AND METHODS

Materials

Hydrogenated egg phosphatidylcholine (HEPC) was donated by Nippon Fine Chemicals Co., Ltd. (Osaka, Japan). Dicetylphosphate (DCP) was purchased from Nacalai Tesque Inc. (Kyoto, Japan), CH from Wako Pure Chemicals Co., Ltd. (Osaka, Japan) and CF from Eastman Kodak Co. (New York, NY, USA). All other reagents were of commercially analytical grades.

Preparation of Liposomes

Multilamellar vesicles (MLV) were prepared according to the methods described previously (8). Three kinds of liposome composition were prepared to give the lipid molar ratio as follows: HEPC:CH:DCP = 4:4:1, 5:3:1, and 6:2:1. CF solutions of 25 mM were introduced as aqueous phase markers. Then the liposomes were extruded seven times through polycarbonate membrane filters (Nuclepore Co., CA, USA) with diameters at 800, 400 and 200 nm. The CF-encapsulated liposomes were dialysed in cellulose dialysing tubing against phosphate-buffered saline (PBS) for 7 days at room temperature with frequent changes of PBS. The diameters of MLV extruded at 800, 400 and 200 nm were measured with a Nicomp 370 HPL submicron particle analyzer (Particle Sizing Systems, Santa Barbara, CA, USA) as 786 ± 161, 392 ± 148 and 241 ± 71 nm, respectively.

Animal Experiments

Male Wistar rats weighing 200–300 g were used (Inoue Experimental Animal, Kumamoto, Japan). Rats were cannulated via the left femoral vein (PE-20, Natsume, Tokyo, Japan) and artery (PE-50) under light ether anesthesia. Each rat was kept in a Bollman's cage and left for at least 1 hr to recover from anesthesia. Liposome suspension was injected into a rat through the femoral vein cannula at a dose of 20 μmol lipid/kg. Blood was sampled

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at indicated times and plasma was obtained by centrifugation. At the end of each study, the rat was anesthetized with sodium pentobarbitone and a liver was perfused by PBS to remove blood. The whole liver was excised and weighed for determination of hepatic uptake amount of liposomes.

Assay

Plasma

One hundred μL of plasma was diluted with 4.9 mL of PBS. A 1 mL aliquot of this was shaken well with 1 mL of 5% Triton X-100, and again diluted by 2 mL distilled water. The remaining solution (4 mL) was assayed directly. The fluorescence intensity (FI) of CF was measured with a Hitachi 650-40 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at 520 nm (excited at 490 nm). The FI with or without Triton X-100 represent the total and free CF in plasma, respectively. The concentration of liposomally encapsulated CF (intact liposomes) was calculated by subtracting free from total concentration of CF and was expressed as %dose/mL.

Liver

One gram of liver was homogenized after addition of 4 mL of PBS. 50 μL of homogenate was incubated with 1.5 mL of 2N NaOH at 50°C overnight. The sample was mixed well with 1.5 mL of Triton X-100 and FI was measured as described above.

Release of CF from Liposomes in Serum

Blood was collected through the cannulated carotid artery under ether anesthesia and left at room temperature for 60 min. Serum was obtained by centrifugation at 3000 r.p.m. for 10 min at 4°C. One hundred μL (20 μmol lipid/mL) of liposome suspension was added to 900 μL normal serum and incubated at 37°C until 60 min. Ten μL of reaction mixture was sampled at 30, 45 and 60 min to obtain the maximum release of CF, and the FI of CF released from liposomes was determined as described above. Liposomes were also incubated with PBS as a control and this value was subtracted from % release value as baseline. Liposomes were also incubated with preheated serum at 56°C for 30 min, which inactivates the complement system. The percent release of CF in preheated serum indicates the complement-independent release.

Pharmacokinetic Analysis

The time courses of plasma concentration of liposomes were analyzed based on a one or two-compartment model, using MULTI (10). The Damping Gauss Newton method was chosen as an algorithm for the nonlinear least square's method and the inverse of plasma concentration was used as a weight. Pharmacokinetic parameters were calculated as explained in Table I.

Statistics

In order to test the effects of the size and the CH content, a two-factor analysis of variance (ANOVA) was applied. Regression analysis was also performed on CLh with size and CH content as independent variables.

RESULTS

In Vivo Disposition of Liposomes

Time courses of plasma concentration of liposomes are shown in Fig. 1. In each CH content, the larger liposomes cleared more rapidly than the smaller ones, however, this tendency seems to decrease for small CH contents. The size dependency in the elimination rate was remarkable in CH-rich

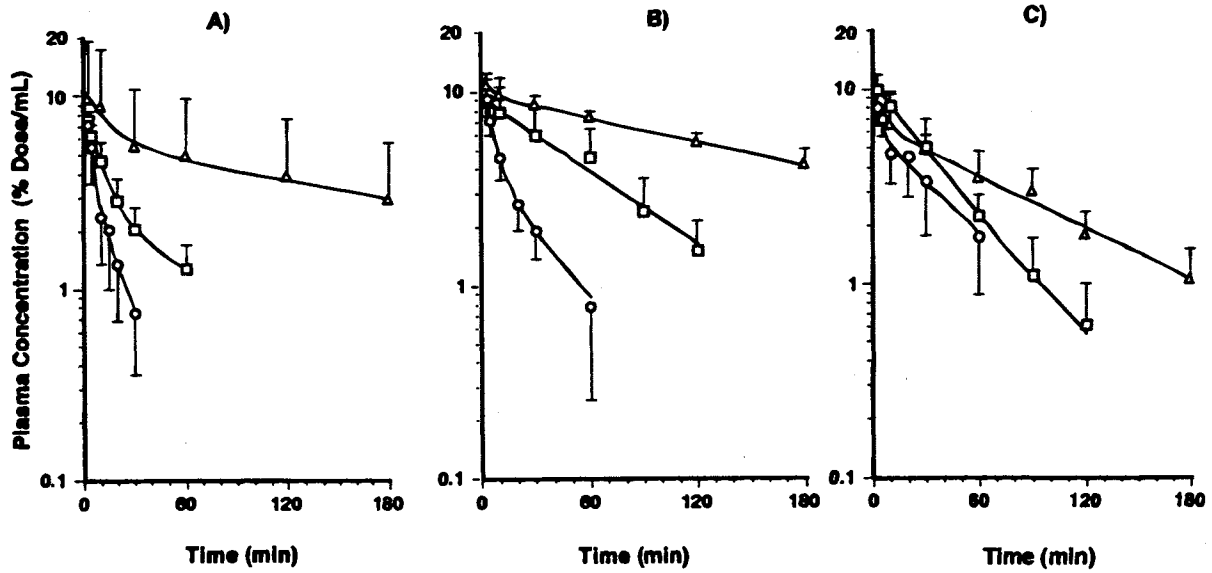

Fig. 1. Time courses of plasma concentration of intact liposomes in rats. Liposomes were administered intravenously at a dose of 20 μmol lipid/kg. Plasma concentration of liposomes is expressed as the percent dose per mL of plasma. Each data presents the mean ± SD of 4 to 6 rats. Three kinds of liposome composition were prepared as: HEPC:CH:DPC = 4:4:1 (A), 5:3:1 (B) and 6:2:1 (C). In each liposome composition, liposomes were sized by an extrusion method at 800, 400 and 200 nm: (○): 800 nm; (□): 400 nm; (△): 200 nm.

Table I. Pharmacokinetic Parameters of Liposomes

Lipid Composition (HEPC:CH:DCP)	Size (nm)	AUC[a] (% Dose · min/mL)	Vc[b] (mL)	CLtot[c] (mL/min)	CLh[d] (mL/min)	MRT[e] (min)
A) 4:4:1	800	120 ± 37	7.6 ± 2.1	0.904 ± 0.281	0.667 ± 0.273	27.7 ± 17.2
	400	255 ± 60	10.8 ± 2.3	0.417 ± 0.114	0.252 ± 0.09	52.6 ± 21.5
	200	1550 ± 435	9.5 ± 1.7	0.07 ± 0.024	0.046 ± 0.021	265 ± 113
B) 5:3:1	800	193 ± 41	8.8 ± 3.0	0.536 ± 0.100	0.271 ± 0.042	26.2 ± 7.1
	400	888 ± 504	11.3 ± 3.2	0.153 ± 0.101	0.085 ± 0.078	101 ± 40
	200	2180 ± 460	9.6 ± 2.1	0.048 ± 0.011	0.020 ± 0.010	231 ± 71
C) 6:2:1	800	327 ± 122	10.5 ± 3.4	0.340 ± 0.11	0.101 ± 0.064	47.3 ± 17.4
	400	617 ± 319	9.3 ± 1.6	0.193 ± 0.084	0.080 ± 0.051	60.9 ± 27.9
	200	676 ± 184	9.8 ± 1.0	0.157 ± 0.043	0.047 ± 0.011	112 ± 54

Note: Liposomes were administered intravenously at a dose of 20 μmol/kg. Time courses of plasma concentration of intact liposomes are shown in Figure 1 and fitted by following equation: $Cb = Ae^{-\alpha t} + Be^{-\beta t}$, where α and β represent the apparent rate constants. A and B represent the corresponding zero-time intercepts and t represents time.

[a] AUC represents the area under the plasma concentration-time curve and calculated as: $AUC = A/\alpha + B/\beta$.

[b] Vc represents the volume of distribution for the central compartment and calculated as: $Vc = Dose/(A + B)$.

[c] CLtot represents the total body clearance of liposomes and calculated as: CLtot = Dose/AUC.

[d] CLh represents the hepatic uptake clearance and calculated as: CLh = X(t)/AUC(0-t), where X(t) and AUC(0-t) represent the hepatic uptake amount of liposomes at time t and AUC from time 0 to t, respectively (11).

[e] MRT represents the mean residence time of liposomes and calculated as: $MRT = (A/\alpha^2 + B/\beta^2)/(A/\alpha + B/\beta)$.

liposomes. The pharmacokinetic parameters of liposome disposition are summarized in Table I. The Vc ranged around 10 mL and this value corresponds to the plasma space of rats (~7 mL/250 g, unpublished data). The AUC varied remarkably depending on the size and the CH content. The larger the size, the smaller the AUC in each CH content.

The effects of the size and the CH content on CLtot, CLh and CLtot-CLh are shown in Fig. 2. The size effect was observed for each parameter. The increase of CH content increased the CLtot in large liposomes, while decreased in small liposomes. As for CLh, the increase of CH content increased the CLh in large and medium liposomes, while there was no change in small liposomes. There was no significant effect of CH content in CLtot-CLh. In general, CLh contributed to 30 ~ 70% of CLtot and the contribution of CLh was higher in CH-rich liposomes.

Statistical Analysis

ANOVA

The ANOVA was applied to each parameter and there was a significant effect of liposome size on MRT ($p < 0.001$), CLtot ($p < 0.001$), CLh ($p < 0.001$), CLtot-CLh ($p < 0.001$). There was also a significant effect of CH content on MRT ($p < 0.05$), CLtot ($p < 0.001$), CLh ($p < 0.001$). A significant interaction effect between size and CH content was observed in MRT ($p < 0.005$), CLtot ($p < 0.001$) and CLh ($p < 0.001$).

Regression Analysis

Since there was a significant interaction effect between the size and the CH content, the CLh was regressed as a function of [size] and/or [CH] as follows.

$$a[\text{size}] + b \quad (1a)$$

$$a[\text{CH}] + b \quad (1b)$$

$$a[\text{size}] + b[\text{CH}] + c \quad (1c)$$

$$a[\text{size}][\text{CH}] + b \quad (1d)$$

where a, b and c represent the constants. The coefficient of determination (r^2) in each regression (1 – a, b, c and d) was 44.1 ($p < 0.1$), 26.9 ($p > 0.1$), 71.0 ($p < 0.05$) and 84.9% ($p < 0.001$), respectively. As shown in Fig. 3, the product of [size] and [CH] can explain the variation of CLh better than the [size] or [CH]. These results indicate the importance of the synergistic effect between size and CH content of liposomes in determining the CLh of liposomes.

Release of CF from Liposomes in Serum

The complement-dependent release was calculated by subtracting the release in pretreated serum from that in normal serum. The larger the size, the higher the extent of CF release. There was little release of CF from small liposomes (200 nm). The effect of CH on the release of CF from liposomes was prominent in larger liposomes. Treatment of serum at 56°C for 30 min abolished the release of CF, except for large liposomes with small CH content, which suggested the complement-mediated release of CF from liposomes.

Correlation Between CLh and CF Release in Serum

As shown in Fig. 4, there is a good correlation between CLh and CF release in serum ($r^2 = 0.759$, $p < 0.0025$). The regression between CLh and the complement dependent CF release shows better regression ($r^2 = 0.877$, $p < 0.0005$).

DISCUSSION

The present study has shown that both the size and the CH content are important factors in determining the disposition of liposomes. The CLh is the principal parameter to explain the variation of CLtot, since the CLh explains 30 to 70% of

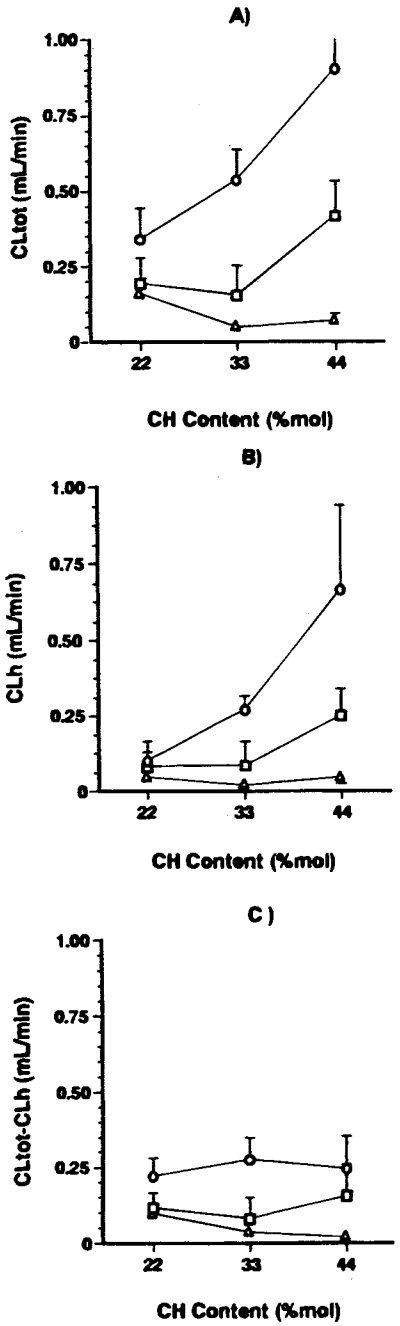

Fig. 2. Effects of the size and CH content on the clearance of liposomes. A) CLtot, B) CLh, and C) CLtot-CLh. Circles represent the clearance of large liposomes (800 nm). Squares represent the medium liposomes (400 nm). Triangles represent the small (200 nm) liposomes.

CLtot. According to ANOVA, the size and the CH content were shown to be important in determining the CLh and a remarkable interaction effect was shown.

The liposomes used in this study have been shown to release CF in rat plasma through the activation of alternative complement pathway (6). Since the release of CF from all liposomes except the large (800 nm) liposomes with low CH content (22%) were inhibited by heat treatment, the release of CF was considered to occur through the activation of the complement system. The good correlation between complement-dependent release of CF and CLh (Fig. 3) suggested that the variation in CLh due to the different size and CH content resulted from the variation of the extent of complement activation. The possibility of saturation of complement activation in small liposomes due to the increased number of liposomes was excluded by the experiments both in isolated perfused liver system (8) and CF release in rat serum (unpublished data).

The size dependency in the activation of the complement system has been reported by other groups (12, 13). Devine et al. showed the effect of liposome size in the consumption of complement and a remarkable difference was observed between 100 and 200 nm (13). The importance of the inclusion of CH into liposome membrane was also reported in the activation of complement system in human (14, 15) guinea pig (16) and rat (13) serum. Uncharacterized serum factor in the CH-dependent complement activation has been reported in human serum (14) and rat plasma (17). These factors may bind directly to the CH molecule or require the membrane fluidity induced by CH to initiate complement activation on the surface of liposomes.

The synergistic effect between the size and the CH content in CLh is considered to be an important characteristic in the recognition of liposomes by the complement system. In our previous paper, we hypothesized that the size dependent difference in the density of recognition sites can exist due to the different curvature in the local area on the surface of liposomes, because the complement activation results from a chain reaction of several components of the complement system (5). If we assume that the complement system requires a certain local area, which is represented by a disc with a radius γc and depth d as shown in Fig. 5, for the recognition of foreign particles and for the activation of the complement cascade, the surface area of liposomes recognized by the complement system (SRC) can be calculated by the radius of liposomes (r) and d as follows:

$$\mathrm{SRC} = 2\pi \int_{r-d}^{r} \sqrt{\{r^2-(r-d)^2\}}\sqrt{[1+(r-d)^2/\{r^2-(r-d)^2\}]}\, dx$$

$$= 2\pi\, dr \qquad (2)$$

Thus, the SRC is proportional to the diameter of liposomes and not to the square of the diameter. Since the number of recognition sites in the local area is proportional to both the SRC and the density of recognition sites on the liposome surface, the total number of recognition sites in the local area can be described as:

$$\mathrm{NRS} = \epsilon\pi d[2r][\mathrm{CH}] \qquad (3)$$

where ϵ represents a constant. The minimum radius for complement activation (γm) seemed to exist around 200 nm in this study. This size dependency of complement activation is consistent with the observation of the other group as described above (13). They suggested that the more curved surfaces of smaller liposomes can not achieve the proper geometric configuration for efficient complement activation because complement activation requires the assembly and activation of complement proteins. These considerations led us to assume the minimum number of recognition sites (NRSmin) for the extent of complement activation (ECA) as follows:

$$\mathrm{ECA} = \epsilon\pi d[2r][\mathrm{CH}] - \mathrm{NRSmin} \qquad (r > \gamma m) \qquad (4)$$

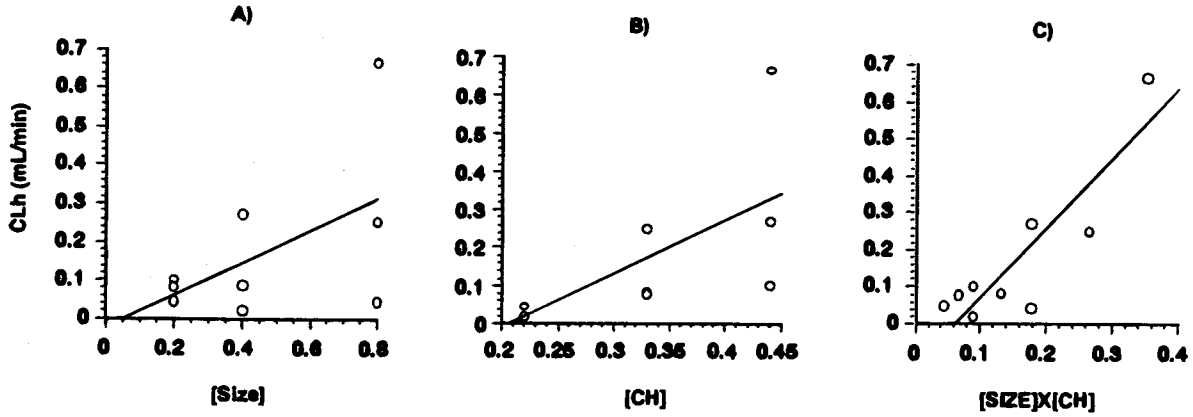

Fig. 3. Regression of hepatic uptake clearance of liposomes as functions of size and/or CH content. CLh was regressed by the size and/or CH content according to the Eqs-(1-*a*, *b*, and *d*). A) Regression of CLh as a function of [size]. $a = 0.515$, $b = -0.066$, $r^2 = 0.441$. B) Regression of CLh as a function of [CH]. $a = 1.117$, $b = -0.194$, $r^2 = 0.269$. C) Regression of CLh as a function of [size] × [CH]. $a = 1.876$, $b = -0.115$, $r^2 = 0.849$.

This model can explain the size-and CH content-dependent CLh and complement-dependent release of CF from liposomes well, and also correspond to Eq-(1-d). As shown in Fig. 5, the surface area recognized by the complement system is large in large liposomes due to their low curvature compared to small liposomes. In this study, small liposomes with a diameter of 200 nm did not activate the complement system as shown in CF release and CLh (8). Thus the number of recognition sites on the small liposomes might not be enough to activate the complement system, although SRC of small liposomes was not zero. Therefore, a threshold was introduced in the activation of complement system as shown in Eq-(4). This model is also supported by our recent observation where the threshold of bound C3 fragments was suggested from the relationship between CF release and bound C3 fragments depending on the size of liposomes in human serum (18). The precise molecular mechanism of this synergism observed in this study is not clear

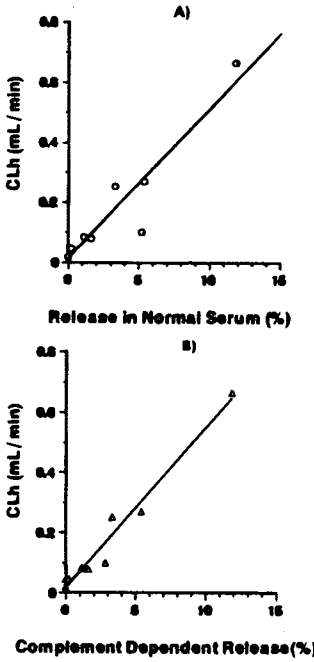

Fig. 4. Correlation between hepatic uptake clearance and serum induced release of CF from liposomes. A) Correlation between CLh and the release of CF in normal serum. The coefficient of determination (r^2) was 0.759 ($p < 0.0025$). B) Correlation between CLh and the complement dependent release of CF, which was calculated by subtracting the release of CF in heat treated serum from the release of CF in normal serum. The coefficient of determination was 0.877 ($p < 0.0005$).

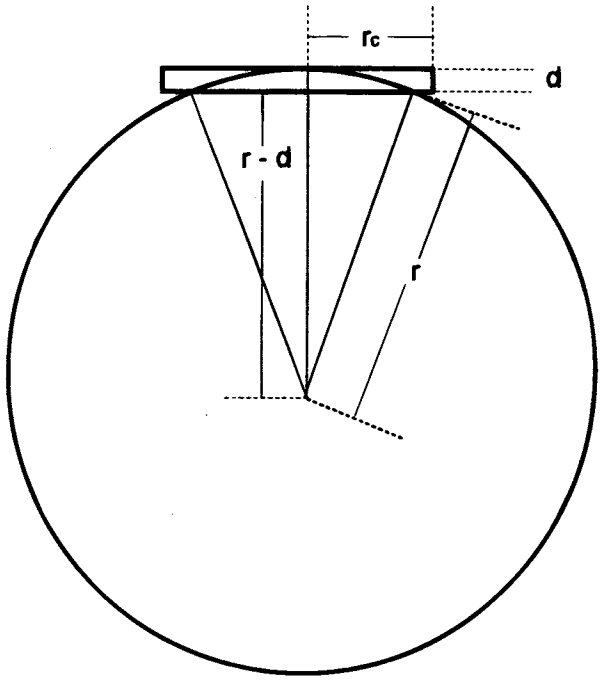

Fig. 5. Hypothesis on the size and CH dependent complement activation by liposomes. The circle represents the surface of a liposome with the radius *r*. The square represents the local area required for complement recognition described by the radius γc and the depth *d*. The surface area (SRC) of a liposome recognized by complement system in this area can be calculated as a function of *r* and *d*: SRC = $2\pi dr$. See Discussion in detail.

at this moment and assumptions used in this hypothesis to explain this synergism in the complement activation by liposomes should be tested experimentally in a further study.

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