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Binding and Incorporation of Lecithin-Cholesterol Vesicles to Lymphocytes: A Spin-Label Study

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Summary. When lecithin-cholesterol vesicles, containing the membrane-bound spin probe 3-doxyl-cholestane, were set in contact with mouse lymphocytes, the vesicles adsorbed to the cell and vesicle-membrane components were transferred to it. The spin probe was enzymatically reduced at the inside of the cell membrane. The spin-label method provided a means to determine quantitatively the extent of vesicles adsorption and vesicle-cell fusion by measuring the transfer of vesicles membrane material to the cell. This method, together with the reduction of spin label by the cell, allowed also a quantitative estimate of the extent of endocytosis during cell-liposome interaction.

Key words: lymphocyte-liposome interaction · vesicle-cell fusion · endocytosis · cellular vesicle adsorption · membrane-bound spin probe · cholestane spin label.

Introduction

The interaction between living cells and liposomes[1] has been the subject of numerous studies using a large variety of experimental procedures [13, 27, 28, 45, 47]. The necessity of using a large number of different techniques in these studies has emerged from the complexity of this interaction. Different phenomena such as liposome adsorption, fusion and endocytosis, can occur during this interaction. Commonly in these studies, liposomes labeled with radioactive or fluorescence markers have been used. Less commonly, spin-labeled liposomes have been used. Fusion between cell and liposome has deserved particular attention as it is of common occurrence during cell-liposome interaction. Two main criteria have been established as evidence of fusion: (1) the detection of transfer of liposome contents into the cell and (2) the detection of transfer of liposome membrane components to the cell membrane. Examples of the first group of studies are those of Weinstein et al. [5, 47], that used the water-soluble fluorescence label 6-carboxyfluorescein as a marker, and those of Dunham et al. [9], that used the calcium-sensitive dye arsenazo III as a marker. Examples of the second group of studies are those of Grant and McConnell [12], that used spin probes, and those of Owen [26], that used a fluorescent probe. However, as has been pointed out [27, 33], it may be difficult in a particular case to prove that these criteria have been met, either due to limitations established by the experimental procedure used or, as usually happens, by the simultaneous occurrence of several phenomena during cell-liposome interaction. Therefore the need for more than one type of measurement in these studies has been emphasized [27, 33]. And, for the same reason, the finding of additional methods that can discriminate between these different mechanisms of cell-liposome interaction is desirable.

We have been interested in studying the incorporation of lecithin vesicles containing cholesterol into lymphocytes, both from the viewpoint of the mechanisms involved in the interaction between cell and vesicles [8] and from the viewpoint of the effect of increased cholesterol content on cell activation [14]. This study is concerned with the first of these aspects. Spin probes have been used in an attempt to quantitatively assess different mechanisms of transfer of the components of the vesicle membrane to the cells. The paramagnetic resonance signal of these probes is sensitive to environment order, composition, and spin probe mobility and concentration. Our results show that the use of spin probes can provide a direct way to measure fusion between vesicle and cell membranes, but also to assess adsorption of vesicles to the cell surface and endocytosis. A detailed account on the preparation and properties of spin-labeled lecithin-cholesterol liposomes as

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[1] The terms vesicle and liposome are used as synonyms in the present work.

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those used in the present study has been published [7].

Materials and Methods

Lymphocytes were isolated from spleen of A.CA mice as described previously [14]. The preparation of LCC-liposomes[2] containing spin label I has been communicated [7]. All liposome preparations were subjected to phosphate and cholesterol analysis. Their spin-label concentration was also determined by double integration of their ESR spectra [7]. Cholesterol was determined by the method of Hanel and Dam [15] and the method of Zlatkis [48], according to Rudel and Morris [37]. Phosphate was determined with the method of Bartlett [4]. Membrane-bound dehydrogenase activity was measured according to Klingenberg [19]. ESR measurements and calculations were performed as referred to before [7].

I

A typical incubation experiment was performed as follows. Lymphocytes with a final concentration of 1×10^7 cell/ml were mixed with liposomes (final lipid concentration = 2.5 mM) in 0.15 M NaCl − 2.5 mM phosphate (pH 7.40) and left at 23 °C. Other conditions are indicated in the protocol of each experiment. At the end of the incubation period the cells were sedimented by centrifugation at $5{,}000 \times g$, for 10 min, washed, and the sediment, the supernatant, and the wash solutions kept. Cholesterol was determined on the cell pellet. ESR measurements were performed on the cell pellet, the supernatant, and the wash solutions. No ESR signal, phosphate, or cholesterol was detected in the wash solutions.

The total number of vesicles N_v in a volume of a suspension was calculated according to the following:

$$N_v = \frac{L_t}{L_v} \tag{1}$$

where L_t is the total number of lipid molecules that constitute vesicles present in the chosen volume of suspension and L_v is the total number of lipid molecules per vesicle. For a particular lipid component l, its mole fraction X_l in a volume of the suspension is given by:

$$X_l = \frac{L_l}{L_t} \tag{2}$$

where L_l is the total number of lipid molecules of l present in the volumen of suspension under consideration. The total number of lipid molecules per vesicle L_v is given by:

$$L_v = \frac{2A_v}{\sum x_i A_i} \tag{3}$$

where A_v is the area of an ideal curved surface lying between the two vesicle monolayers and $\sum x_i A_i$ is the average molecular surface area of a vesicle lipid molecule, obtained by summing the products of the mole fraction x_i of each lipid component i of the vesicle and its molecular surface area A_i.

If L_t from Eq. (2) and L_v from Eq. (3) are substituted into Eq. (1) one gets

$$N_v = \frac{L_t \sum x_i A_i}{X_l 2A_v}. \tag{4}$$

For 1 liter of suspension, where the molar concentration of l is c_l,

$$L_l = c_l N \tag{5}$$

where N denotes Avogadro's number. Substituting Eq. (5) in Eq. (4) yields

$$N_v = \frac{c_l N \sum x_i A_i}{X_l 2A_v}. \tag{6}$$

L_l and X_l where experimental values. A_i for lecithin was taken as 46.7 Å^2, which is the value found in egg lecithin bilayers containing 50% cholesterol [22]. A_i for cholesterol and 3-doxyl-cholestane was taken as 38 Å^2, which is the value experimentally obtained for fully packed cholesterol [6]. The diameter of a vesicle was assumed to be 350 Å [11] (and G. Dresdner and J. Prejza, *unpublished observations*) and the thickness of the vesicle bilayer 50 Å.

Results

After incubation with LCC-liposomes, the cell pellet exhibited a paramagnetic resonance signal (Fig. 1) and its cholesterol content increased (Figs. 2 and 3). That these results represented binding of liposomes to lymphocytes was deduced from the following observations. Lymphocytes and liposomes showed large differences in their sedimentation rates (*see* Materials and Methods). No sediment was observed on liposome suspensions centrifuged under the same conditions as those used for lymphocytes. The amplitude of the ESR signal and the cholesterol content of the cell pellet did not change after several successive washings. The wash solutions did not show any ESR signal nor detectable amounts of cholesterol. The ESR signal and the cholesterol content of the cell pellet increased with time of incubation (Fig. 3). The amplitude of the ESR signal of the cell pellet decayed at the postincubation period, whereas that of the supernatant or the original liposome suspension remained constant (Fig. 5*A*). The shape of the ESR signal of the cell pellet was different from that shown by the supernatant and the liposome suspension (Fig. 1).

It should be recalled here that no form of cholesterol or spin label other than that incorporated into liposomes was present in the suspension [7].

Although the characteristics and properties of all cell batches were like those to be described below,

[2] Abbreviations: ESR, electron spin resonance; doxyl, 2,2-dimethyl-N-oxyl-oxazolidine; LCC-liposomes, lecithin-cholesterol-(3-doxyl-cholestane)-liposomes; SL, spin label.

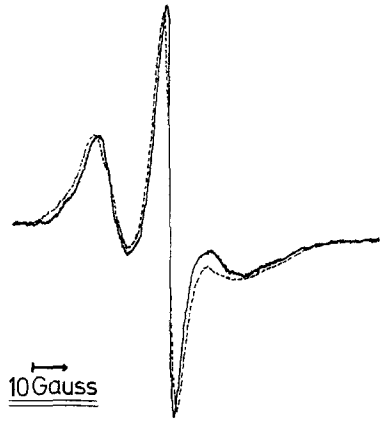

Fig. 1. ESR spectrum of lymphocytes after incubation with spin-labeled lecithin-cholesterol vesicles. Dotted line: ESR spectrum of vesicles present in the supernatant obtained by centrifugation of the incubation mixture. The vesicle composition was as follows: $X_{\text{lecithin}}=0.21$, $X_{\text{chol.}}=0.73$, $X_{\text{SL-chol.}}=0.061$. Incubation time $=15$ min

differences were, however, observed between batches. Parameters such as initial cholesterol concentration, maximum liposome binding capacity, time for achievement of maximum binding, rate of decay of the paramagnetic resonance signal, were characteristic of a particular cell batch but varied between batches.

Liposome-Concentration Dependence of Liposome Attachment[3] *to Lymphocytes*

The extent of attachment of liposomes to lymphocytes increased with liposome concentration during incubation (Fig. 2). There were two regions of linear increase with different slopes, suggesting that some saturable mechanism operated in the cellular uptake of liposomes, as found in other cases [5]. Both the cellular uptake of cholesterol and spin label occurred in a parallel fashion. In earlier studies a similar behavior for lecithin vesicles containing spin-labeled stearic acid was shown [8].

Time-Dependent Attachment of Liposomes to Lymphocytes

Attachment of liposomes to lymphocytes occurred already after 5 min of incubation (the shortest incubation time studied) as revealed by the cholsterol content and the paramagnetic resonance signal of the cell pellet (Fig. 3). Both showed a parallel increase and reached a plateau within 40–160 min, depending on the cell batch and the liposome-cell concentration ratio used. Binding and subsequent phenomena that followed incubation led to defined changes of the paramagnetic resonance spectrum of the cell-bound spin probe. These changes were monitored by means of the spectral parameters $2T'_{\parallel}$, δ, and H/D [7] (Fig. 4). H is the amplitude of the middle line of the spin-label ESR spectrum and D is the value of the double integral of the spectrum which is proportional to spin-label concentration. For a definition of $2T'_{\parallel}$ and δ see Fig. 4. The variation of δ and H/D can be followed with reference to Table 1. The observed changes indicated that the spin label was transferred from the liposomes to an environment where it reached a lower concentration, and support the view that it diffused laterally in the lymphocyte membrane. Such changes were not observed

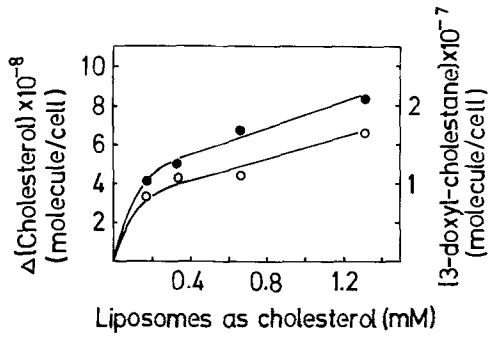

Fig. 2. Effect of LCC-liposome concentration during incubation on the incorporation of cholesterol and spin label into lymphocytes. o—o: cholesterol; ●—●: 3-doxyl-cholestane. The liposome composition was as follows: $X_{\text{lecithin}}=0.31$, $X_{\text{chol.}}=0.64$, $X_{\text{SL-chol.}}=0.055$. Incubation time $=5$ hr

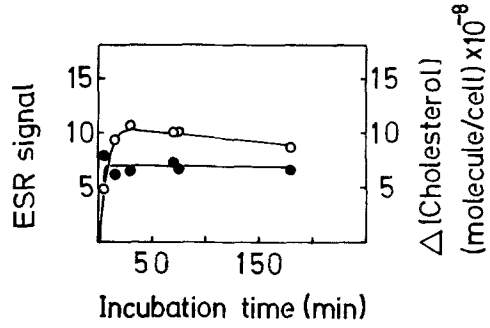

Fig. 3. Time-dependent incorporation of liposomes into lymphocytes. Spin-label incorporation was monitored by the amplitude of the middle resonance of the ESR signal of the cell pellet (o—o). ●—●: cholesterol. The liposome composition was the same as that indicated in Fig. 1

[3] Extent of attachment of vesicles to lymphocytes is used here to denote the number of vesicle-equivalents per lymphocyte after incubation of lymphocytes with a vesicle suspension, calculated on the basis of the cellular increment of cholesterol or spin label. It does not have any mechanistic significance.

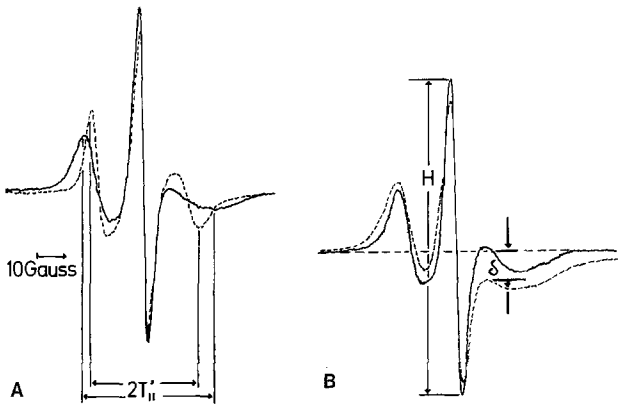

Fig. 4. ESR parameters used for monitoring the interaction between lymphocytes and LCC-liposomes. The spectra are the same as those shown in Figs. 6A and 7A, reference [7]. They are ESR spectra from LCC-liposomes with different mole fractions of cholesterol (*A*) and spin label (*B*). *A*. Hyperfine splitting $2T'_{\parallel}$. *B*. Parameter δ. δ values are positive above the base line and negative below it. Before calculating δ, the ESR spectra of all samples were normalized by assigning to the double integral of their spectra the same arbitrary value

in the ESR spectra of vesicles that remained in the supernatant after incubation. Variations of the hyperfine splitting $2T'_{\parallel}$ of the cell-bound spin label with time of incubation were observed only in cases where the vesicles had a low concentration of spin label. Compared with the liposomes, those lymphocytes that were incubated with liposomes whose 3-doxyl-cholestane mole fraction was lower than 0.05, showed a value for $2T'_{\parallel}$ that was $90.4 \pm 4.9\,\%$ of that of the liposomes. Instead, lymphocytes incubated with liposomes whose 3-doxyl-cholestane mole fraction was higher than 0.065 showed a $2T'_{\parallel}$ value that was $99.8 \pm 4.3\,\%$ of that of the liposomes. These values are the average and standard deviation of two groups each of 15 and 18 experiments, respectively. This result can be explained by the opposed effects that cholesterol and spin-label concentration had on $2T'_{\parallel}$ [7] and to the relatively moderate expected change of the cholesterol-lecithin molar ratio of the lymphocyte membrane as a consequence of liposome uptake and incorporation[4]. The observed change in $2T'_{\parallel}$ was also in agreement with the view that 3-doxyl-cholestane was transferred during incubation from the liposome membrane to the lymphocyte membrane.

[4] In fact, assuming: (a) a cholesterol-lecithin molar ratio of the lymphocyte membrane of 1.0 [1, 10, 21, 40] and of the liposome membrane of 2.0 [7], (b) a cholesterol content of the lymphocyte membrane corresponding to 6×10^8 molecules/cell [14] and of the liposome of 9×10^8 molecules/10^5 vesicles [7], and (c) a lecithin content of the lymphocyte membrane corresponding to 6×10^8 molecules/cell [1, 10, 21, 40] and of the liposome of 4.5×10^8 molecules/10^5 vesicles [7], a maximal increase of the cholesterol-lecithin molar ratio of the lymphocyte-liposome complex to 1.4 was expected by the attachment of 1×10^5 vesicles per cell. The mole fraction of cholesterol would increase under the same circumstances from 0.50 to 0.58.

Table 1. Course of the incubation of lymphocytes with LCC-liposomes monitored by the ESR parameters H/D and δ[a]

Incubation time (min)	H/D		δ	
	Cell pellet	Supernatant	Cell pellet	Supernatant
5	7.8	4.8	−0.30	−0.50
15	7.1	4.8	−0.29	−0.55
70	7.1	5.1	−0.31	−0.58
180	7.2	5.1	−0.30	−0.58

[a] Lymphocytes (10^7 cell/ml) were incubated with LCC-liposomes (final concentration = 1.45 μmol lipid/ml) with the following composition: $X_{\text{lecithin}} = 0.21$, $X_{\text{chol.}} = 0.73$, $X_{\text{SL-chol.}} = 0.061$

Spin-Label Reduction at the Lymphocyte Membrane

Earlier investigations have shown that spin labels are reduced at the plasma membranes of living cells [3, 12, 18, 36, 42]. This capacity to reduce spin labels has also been observed in human lymphocytes [18].

The amplitude of the paramagnetic signal of 3-doxyl-cholestane, measured by the amplitude of the center line of the spectrum, decayed, after spin-label incorporation into the lymphocyte, by reduction at the cell membrane (Fig. 5*A*). The decay followed a first-order kinetics and it was often preceded by an initial period where no change was detected or even a slight increase in the amplitude of the paramagnetic resonance signal was observed (Fig. 6*B*). An explanation for this apparent initial lack of reduction of 3-doxyl-cholestane is given below where post-incubation phenomena are described. The rate of decay of the ESR signal was temperature-dependent [8]. It was independent of the time of incubation. It was also observed on spin-labeled stearic acid incorporated in lymphocytes that had been incubated with lecithin liposomes containing that spin label [8]. These changes were not reversed by addition to the cells of potassium ferricyanide (final concentration = 2 mM) at the postincubation period as it has been reported for human lymphocytes [18]. Nor by adding this substance during incubation or pretreating the cells 10 min with it before incubation. The same result was obtained by adding to the cells, in the fashion described for potassium ferricyanide, the membrane-reductase inhibitors [23] sodium azide (final

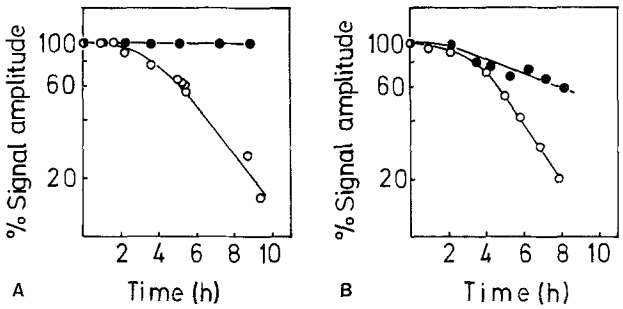

Fig. 5. Reduction of spin label incorporated into lymphocytes after incubation with LCC-liposomes. Reduction was monitored by the amplitude of the middle resonance of the ESR signal of the cell-bound spin label. The composition of the liposomes was as follows: $X_{\text{lecithin}}=0.31$, $X_{\text{chol.}}=0.64$, $X_{\text{SL-chol.}}=0.048$. *A*. Comparison between the cell pellet (○—○) and the supernatant (vesicles) (●—●). *B*. Comparison between undiluted and diluted pellet. (*a*) ○—○: cell pellet of a single sample controlled by successive measurements. (*b*) ●—●: each point was obtained from a sample containing the same number of cells as that in (*a*) that were resuspended, after incubation and separation from the supernatant, to the same concentration used during incubation, and left in that way until, just before measurement of the ESR spectrum, was centrifuged again

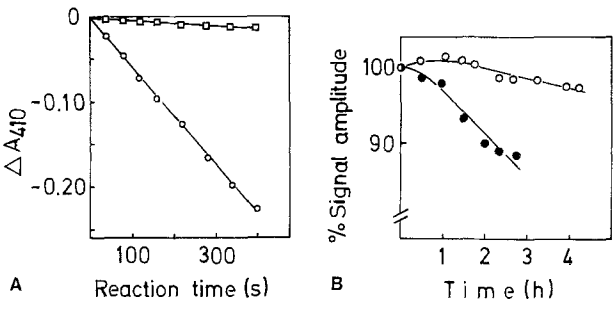

Fig. 6. Reductase activity of the lymphocyte membrane. In each case a 1-ml sample containing 1×10^6 cells was used. *A*. External ferricyanide-reductase activity. ○—○: intact lymphocytes. □—□: lymphocytes broken down by means of 15 passes in a Potter-Elvehjem homogenizer. *B*. Nitroxide-reductase activity. ○—○: intact lymphocytes. ●—●: pellet of lymphocytes previously homogenized as in *A*

concentration: 0.10–0.17 M) or *p*-hydroxi-mercuribenzoate (final concentration 9×10^{-6} to 3×10^{-4} M). Dilution of the cell pellet to the cell levels present during incubation decreased the rate of the reaction to about one-fourth of the value obtained with the undiluted pellet (Fig. 5*B*).

Reductase activity of intact lymphocytes was investigated with the ferricyanide method [19]. Ferricyanide does not diffuse through the lymphocyte cell membrane [18, 19]. Its reduction by intact cells is thus a measure of the reducing activity located at the external lymphocyte surface. It was found that lymphocytes had a high ferricyanide-reducing activity (Fig. 6*A*). This activity was almost abolished by rupturing the cells by mild homogenization (Fig. 6*A*). That the exernal reductase activity was different from that observed with cell-incorporated spin labels was supported by the following facts. While breakdown of the lymphocytes leads to an almost complete disappearance of the ferricyanide-reductase activity, it increased instead the nitroxide-reductase activity, responsible for the decay of the paramagnetic resonance signal of the lymphocyte (Fig. 6*B*). Also, as mentioned before, the external addition of potassium ferricyanide did not modify the reduction rate of cell-bound spin label. These results also support the view that the enzyme (or enzymes) responsible for the ferricyanide-reductase activity was located on the outside membrane surface of the lymphocyte, whereas that responsible for the nitroxide-reductase activity was located at the inside of the membrane. The last must have also been in close contact with the membrane lipids since the spin label itself is located in the lipid phase of the membrane [39]. This has also been shown to be the case of other lipid-soluble spin probes [42].

Extent of Vesicle Attachment to Lymphocytes

The extent of vesicle attachment to lymphocytes during incubation was estimated both from spin-label incorporation data and cholesterol incorporation data (Tables 2 and 3). In order to give a correct estimate of the total amount of vesicles incorporated into the cells within a certain period of time, spin-label incorporation data were corrected for spin-label reduction within that period of time. Details of the correction procedure are given below in the section titled *Rate of Vesicle Attachment to Lymphocytes*. The rate of spin-label reduction was in turn corrected for cell concentration, as ESR measurements were performed on the cell pellet. The amounts of vesicles attached to a lymphocyte, calculated on the basis of these corrected spin-label incorporation data, are shown in Table 4. If comparison is made between the data of Table 2 and Table 3, it can be seen that there is a good agreement between the extent of vesicle attachment to lymphocytes calculated from cholesterol-increment data and spin-label incorporation data. Comparison between the data of Table 2 and Table 4 shows that the difference between the extent of vesicle attachment of lymphocytes calculated from corrected spin-label incorporation data and cholesterol-increment data is given by the amount of vesicles that were modified by some kind of cellular mechanism, in this case enzymatic reduction of the vesicle component 3-doxyl-

Table 2. Number of vesicles attached to lymphocytes, at 23 °C at an incubation ratio of 4×10^6 vesicles/cell, calculated on the basis of the increment of cell cholesterol after incubation

Incubation time (hr)	Attached No. vesicles per cell[a]	Average rate of vesicles attachment (vesicle/cell/hr)
3	65,000 ± 12,000(2)	22,000
6	67,000 ± 4,000(4)	11,000
22	103,000 ± 40,000(2)	5,000

[a] Mean ± standard error. Number of experiments is in parentheses

Table 3. Number of vesicles attached to lymphocytes, at 23 °C at an incubation ratio of 4×10^6 vesicles/cell, calculated on the basis of the basis of the amount of spin label 3-doxyl-cholestane present in the cell after incubation

Incubation time (hr)	Attached No. vesicles per cell[a]	Average rate of vesicle attachment (vesicle/cell/hr)
3	41,000 (1)	14,000
4	72,000 ± 4,000(2)	18,000
6	65,000 ± 29,000(4)	11,000
8	94,000 ± 13,000(2)	12,000
12	99,000 ± 15,000(2)	8,000
14	98,000 (1)	7,000
16	102,000 ± 25,000(2)	6,000

[a] Mean ± standard error. Number of experiments in parentheses.

cholestane. These data sustain the view therefore that it is not only the 3-doxyl-cholestane moiety which was modified after the attachment of vesicles to the cell, but also cholesterol was modified, presumably part of it decomposed. This suggested, therefore, that part of the vesicles might have been broken down by the cell as whole units after attachment.

Adsorption of Vesicles to Lymphocytes

The magnitudes of H/D and δ depend on the local concentration of spin label [7], both at the lymphocyte and at the liposome membrane. Since, after fusion, the spin label diffuses in the lymphocyte membrane and therefore its concentration decreases, the value of these parameters increases. The value of these parameters gives therefore a measure of the ratio between fused and adsorbed intact vesicles. It is thus possible, form the measured ESR parameters, a parameter-spin-label concentration plot [7], and the amount of spin label present, calculated by double integration of the ESR spectrum, to estimate the

Table 4. Number of vesicles attached to lymphocytes at 23 °C calculated on the basis of the amount of spin label 3-doxyl-cholestane present in the cell after incubation[a]

Experiment No.	vesicle/cell at incubation $\times 10^{-6}$	Incubation time (hr)	No. attached vesicles/cell	
			Uncorrected	Corrected
1	3.8	4	67,000	77,000
		8	99,000	150,000
		12	105,000	209,000
		16	109,000	216,000
2	4.6	4	95,000	106,000
		8	108,000	146,000
		12	111,000	180,000
		16	116,000	216,000

[a] Both uncorrected data and data corrected for spin-label reduction after vesicle attachment to the cell are shown.

relative and absolute amounts of fused and nonfused (adsorbed) vesicles.

Assuming, when a fraction of vesicles fuses with the lymphocyte cell membrane, that the spin label remains in the lipid phase of the membranes only, and that it diffuses rapidly, the values of H/D and δ will vary linearly with spin-label concentration. Taking δ as an example, its value at the cell pellet after incubation will be given by

$$\delta_{Lv} = a\,\delta_v + (1-a)\,\delta_f \tag{7}$$

where Lv refers to the lymphocyte-vesicle complex of the cell pellet, v to vesicles, f to spin label that has diffused laterally after fusion, and a to the fraction of vesicles adsorbed to the lymphocyte external surface. From Eq. (7) it follows that

$$a = \frac{\delta_{Lv} - \delta_f}{\delta_v - \delta_f}. \tag{8}$$

A homologous Equation such as (8) can be derived for H/D. In Eq. (8), δ_{Lv} and δ_v are obtained experimentally, δ_f can be calculated from dilution of the spin label using values of cell uptake of spin label and a set of parameters related to lymphocyte membrane composition. Since the value of δ_f depends on the fraction of vesicles $(1-a)$ that has fused with the lymphocyte membrane, a solution for δ_f and a is in fact obtained simultaneously. For further details *see* the Appendix.

A calculation, made using the procedure just described, is shown in Table 5. The range of the values in H/D found in the experiments whose results are shown in Table 5 were as follows: (5.3, 9.1) for the cell pellet and (4.6, 5.4) for the vesicles. The ranges of the δ values were: $(-0.62, -0.32)$ for the cell pel-

Table 5. Per cent of attached lecithin-cholesterol vesicles adsorbed to lymphocytes after various times of incubation[a]

Incubation conditions		% Adsorbed vesicles[b]	
Time (min)	$\frac{\text{No. vesicles}}{\text{cell}} \times 10^{-6}$	H/D	δ
5	2.9	58	73
15	2.9	88	69
70	2.9	100	86
180	2.9	75	78
240	3.5	99	71
300	1.1	83	89
300	8.9	53	82

[a] The procedure used for these calculations is indicated in the Appendix

[b] The headings of columns 3 and 4 indicates the ESR parameter that was used for the calculation of the percentage of vesicles adsorbed to the cell.

let and (−0.68, −0.62) for the vesicles. In this calculation a lymphocyte 8 μm in diameter with a cell membrane containing a 50-Å-thick lipid bilayer, 57% protein, 30% lipid [1, 10, 21, 40] (*see also* footnote 3), and a cell membrane cholesterol-lecithin molar ratio close to 1 has been considered. The last assumption is justified since the cells have a membrane cholesterol-lecithin molar ratio of 0.9–1.0 [1, 10, 21, 40] and were incubated with vesicles with a ratio larger than 1.5, in most cases larger than 2.

In all experiments the per cent of vesicles adsorbed to the lymphocyte surface exceeded 50% of the total number of attached vesicles. The values shown in Table 4 were not corrected for spin-label reduction in the cell. A correction for spin-label reduction would decrease those values in amounts that depend on the incubation time. For a 4- to 5-hr incubation experiment a maximal decrease of 5% would be expected.

Rate of Vesicle Attachment to Lymphocytes

Average rates of attachment of vesicles to lymphocytes, calculated both from spin-label data and cholesterol data, are shown in Table 2 and Table 3. These results are comparable to each other if due consideration is paid to the variability observed owing to the use of different cell batches in these experiments. The average rate of vesicle attachment decreases with time of incubation on account of the lower contribution that the large initial rates of attachment make time of incubation. These rates of attachment are comparable with those obtained by Weinstein et al. [47] for the incorporation of lecithin vesicles into human lymphocytes.

More accurate values for the rates of attachment of vesicles to lymphocytes can be calculated from spin-label incorporation data assuming the attainment of a steady state at the plateau region of Fig. 3. The assumption made is that, after the onset of incubation, as the amount of vesicles attached to the cell rises, the rate of disappearance (measured in this case by the rate of loss of the paramagnetic resonance signal of the cell-bound spin label) progressively increases, until a steady state is reached, where the rate of vesicle attachment is equal to the rate of disappearance.

The change with time of the total amount of vesicle-equivalents per cell v is given by

$$v = v_a - v_d \tag{9}$$

where v_a is the rate of attachment of vesicle-equivalents per cell and v_d is the rate of disappearance of vesicle-equivalents per cell due to reduction of spin label. At the plateau region (Fig. 3), the number of vesicle-equivalents per cell is constant, thus $v=0$. From Eq. (9) it follows that

$$v_a = v_d = \text{constant}. \tag{10}$$

Since the rate of disappearance of vesicle-equivalents per cell v_d follows a first-order kinetics,

$$v_a = v_d = k c_p \tag{11}$$

where c_p denotes the measurable number of vesicle-equivalents per cell at the plateau region and k is a first order kinetic constant. Therefore

$$\Delta c = k c_p \Delta t \tag{12}$$

gives the correction for the number of vesicle-equivalents per cell that disappearance in the interval Δt. For any time interval preceding the plateau region, c_p in Eq. (12) can be replaced by the average number of vesicle-equivalents per cell in that interval. The error introduced by this approximation is quite small. The value of k in Eq. (12) can be obtained from measurements on the zone of constant rate of decay of the cell paramagnetic resonance signal at the postincubation period (Fig. 5). This value must be corrected for the cell concentration of the pellet (Fig. 5*B*).

Vesicle attachment data calculated in the way just described are shown in Fig. 7.

Postincubation Phenomena

After separation of the lymphocytes from the vesicles, at the end of the incubation period, other

changes of the ESR spectrum of the cell-bound spin label than decay of amplitude of the paramagnetic resonance signal were also observed. The course of these changes can be followed with reference to Fig. 8*A*. Parameter H/D increased and reached a maximum at about 2 hr of postincubation and then decreased progressively. The high initial values of parameter δ remained constant for about 2 hr and then decreased simultaneously with H/D. The initial increase in H/D explains why the decay of the ESR signal of the cell-bound spin label was often observed only after 2 hr of postincubation and even a small increase of its amplitude detected within this period (Fig. 5, Fig. 6*B*). This increase in H/D was not always observed. But in all experiments there was present a high initial H/D value followed by a decrease, in a similar fashion to that shown for δ in Fig. 8*A*. Changes in $2T'_{\parallel}$ were less defined among different experiments as those observed for H/D and δ, due parhaps to the simultaneous diffusion of both spin label and cholesterol into the lymphocyte following fusion. Within a particular experiment there was nevertheless a defined pattern of change of $2T'_{\parallel}$. Both an initial increase in $2T'_{\parallel}$ followed by a decrease and the reverse pattern were observed.

After 6 to 8 hr postincubation the paramagnetic resonance spectra of the cell pellet, the supernatant (containing the liposomes), and the liposome suspension were the same. The ESR parameters of the cell pellet mentioned above reached, therefore, identical values with those of the supernatant and the liposome suspension. However the ESR signal of the cell pellet continued to decay until it vanished completely. The ESR signal amplitude and ESR parameters of the supernatant and the original liposome suspension remained instead unchanged for significantly longer periods of time.

The changes in H/D and δ, observed after the supply of vesicles from the medium to the lymphocyte was stopped, indicated continued diffusion of spin label at the lymphocyte membrane, following fusion with the cell of vesicles adsorbed on the cell surface. As the amount of spin label that diffused into the lymphocyte membrane decreased by reduction, the contribution that the spin label present in adsorbed vesicles made to the ESR spectrum was larger and the ESR spectrum of the lymphocyte-vesicle complex became more similar to that of the vesicles. A calculation of the relative and absolute amounts of adsorbed vesicles during the postincubation period is shown in Fig. 8*B*. It should be recalled here that during the postincubation period the spin label was continuously reduced without resupply of vesicles from the medium, which explains the exponential decrease of vesicle-equivalents calculated on this basis. In particular is noticed here, for later discussion, the fraction of vesicles that remained unmodified attached to the cell surface (arrows in Fig. 8) and incorporated as such into the cell.

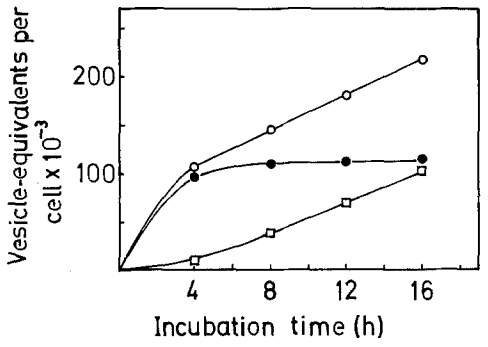

Fig. 7. Attachment of LCC-liposomes to lymphocytes, calculated from spin-label uptake data. ●—●: calculated from ESR measurements performed on the cell pellet, without correction for spin-label reduction. □—□: calculated from spin-label reduction in the cell pellet, and corrected for cell concentration of the pellet. ○—○: total number of vesicles attached. The liposome composition was as in Fig. 5

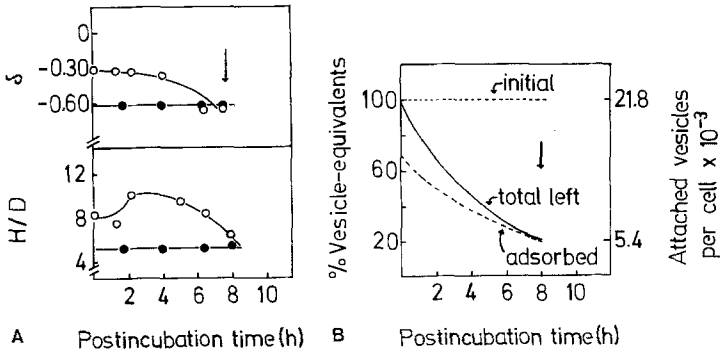

Fig. 8. Postincubation phenomena on lymphocytes incubated with spin-labeled lecithin-cholesterol vesicles. The vesicle composition was as follows: $X_{\text{lecithin}} = 0.21$, $X_{\text{chol.}} = 0.73$, $X_{\text{SL-chol.}} = 0.061$. *A*. Variation of ESR parameters H/D and δ. ○—○: cell pellet; ●—●: supernatant (vesicles). *B*. Number and per cent of equivalents of total and adsorbed vesicles in lymphocytes, in the same experiment shown in *A*

Discussion

When lymphocytes were incubated with spin-labeled lecithin-cholesterol vesicles, binding of the vesicles to the cell was rapidly followed by incorporation of vesicle membrane components into the cell. The predominant hydrophobicity of the spin probe used, the abundant evidence available concerning its location in lipid bilayers [39], the changes of the paramagnetic resonance spectrum that indicated lateral diffusion of the probe in a membrane-like environment, plus its reduction after attachment to the cell, indicated that the probe became attached to the lymphocyte membrane and fusion between liposomes and cells occurred. Additional evidence was provided by previous findings that showed that lecithin vesicles containing spin-labeled stearic acid were incorporated in these cells in a similar fashion [8] and that the binding of lecithin-cholesterol vesicles decreased the reactivity of the cells towards *B* and *T* cell activators [14], which are known to act at the cell membrane. These results are in agreement with those of Weinstein et al. [5, 45], who studied the interaction between human peripheral blood lymphocytes and dioleoyl lecithin-unilamellar vesicles containing 6-carboxylfluorescein in their internal aqueous compartment and those of Huang et al. [16] who studied the interaction between mouse thymocytes and, among others, egg lecithin-unilamellar vesicles containing [^{3}H]-inulin in the internal aqueous compartment. A major difference between the present investigation and those of the forementioned authors is that in our studies the transfer of vesicle membrane components to the lymphocyte membrane instead of the transfer of vesicle contents has been monitored.

Our results suggest that in the system we have studied endocytosis plays a larger role than that exhibited by other systems [5, 16, 47]. Results of the postincubation period show that at least 20% of the vesicles that remained attached to the cell after separation of the cells from the bulk of the vesicle suspension were incorporated as intact vesicles (Fig. 8*B*). But this fraction must have been much larger, since such form of incorporation must have been operating during the whole postincubation period. This form of incorporation must have also been operating during the whole incubation period.

An analysis of the possibility that the observed reduction of spin label of adsorbed vesicles could be due to reductase activity of the outer lymphocyte surface shows that most of the evidence is against this possibility. First, the addition of external potassium ferricyanide at any stage of cell-liposome interaction does not affect the rate of spin-label reduction, nor the addition of the reductase inhibitors such as sodium azide of *p*-hydroxy-mercuribenzoate. In the second place, the nitroxide-reducing activity increases by cell disruption, which supports the view that there are intracellular enzymes that play a more direct or indirect role on the rate of this reducing activity. In the third place, the external ferricyanide-reductase activity of the cell decreases by mild cell disruption. The two last results show that both reductase activities must be associated, if not to different membrane proteins, at least to parts of a protein moiety accessible from different sides of the lymphocyte membrane. In the fourth place, it is difficult to explain how the whole amount of spin label of adsorbed vesicles could be completely reduced, at the rate observed, by an enzyme located on the lymphocyte outer surface. Particularly difficult would be to explain a complete reduction of the spin label located at the inner vesicle monolayer. Kornberger and McConnell [20] have shown that complete fast oxidation of spin-labeled phospholipid located on the outside vesicle monolayer preserves the integrity of the spin label located at the inside monolayer, and that the decay of the asymmetry in spin-label distribution between the two monolayers of the vesicle has a half-time of 6.5 hr at 30 °C. The half-time of the transbilayer movement of cholesterol in phospholipid vesicles has been found to be larger [34] and lower [2] than this. No information of this type has been found in the literature for 3-doxyl-cholestane. In the fifth place, the vanishing of the paramagnetic resonance signal of the adsorbed vesicles in the cell pellet could not explain the concomitant vanishing of cholesterol in the same proportion as the vanishing of the spin-label signal by reduction, even assuming that, after reduction, the vesicles would be released from the cell surface. A concomitant vanishing of components of the vesicle membrane could be explained if, for instance, the liposome wall would be broken down enzymatically within a small closed volume, allowing spin-label molecules to get in close contact with the reductase. These conditions could be met in a small endocytic vesicle that originated at the cell membrane [25, 43]. In the sixth place, dilution of the cell pellet leads to a decrease of the rate of the nitroxide reducing reaction. This effect could be partly explained by a decrease on the proximity of vesicles adsorbed to the surface of neighboring cells, but it must be recalled also that cell dilution decreases the rate of endocytosis [17, 41].

Another argument that supports the view that endocytosis plays a significant role in vesicle incorporation in our experiments is the comparison between the extent of vesicle incorporation calculated

from cholesterol data and that calculated from spin-label data. It follows from this comparison that the cellular concentration of spin-label and cholesterol increase with time of incubation to about the same extent (Tables 2–4). But since a large part of spin label has been degraded by reduction, cholesterol must also have been degraded at about the same rate. However, the normal rate of cholesterol breakdown of the lymphocyte [30–31] is much lower than the rate estimated here from spin-label reduction. This suggests therefore that some common faster mechanism for the breakdown of 3-doxyl-cholestane and cholesterol must have operated in the cell.

Another argument that supports a larger role of endocytosis in our experiments is the following. If the results obtained from experiments on cell-cell fusion [32] and liposome-liposome fusion [29] are valid also for cell-liposome fusion, for a lymphocyte 8 μm in diameter and a cell membrane containing 57% protein [1] and lipid in the form of a bilayer 50-Å thick, to incorporate let us say 5×10^4 lecithin-cholesterol vesicles by fusion, would cause an increase in cell diameter of about 75%, assuming that the thickness of the bilayer has remained constant, i.e., an increase in diameter up to about 14 μm. If it would incorporate 25×10^4 vesicles by fusion then the diameter should increase to about 21 μm. But no increase in cell diameter at all has been observed in our experiments, even after incubating the cells 22 hr. Therefore, a large part of vesicle membrane material must have been transferred into the cell.

Our results show therefore that even when fusion is a quantitatively important mechanism of liposome incorporation in lymphocytes, endocytosis plays a significant role. These results agree with results recently published by Owen [26] using a vesicle membrane-bound fluorescent probe.

Studying cell-liposome interaction by means of the use of spin probes thus represents an effective way to assess liposome adsorption to the cell surface; fusion, by measuring the transfer of vesicle-membrane components to the cell; and provides complementary information for the assessment of endocytosis.

Appendix

Calculation of the Mole Fraction at the Lymphocyte Cell Membrane of Spin Label Transferred to the Membrane After Cell-Vesicle Fusion

If during the interaction between lymphocytes and spin-labeled lecithin-cholesterol vesicles, a fraction of the vesicles adsorbs to the cell surface and another fraction fuses with the cell membrane, the spin label will be present in two environments with different composition that will affect the motional freedom and the collision frequency of the label molecules. The paramagnetic resonance signals of the spin label, that originate at these two environments, will also be different. If, at a certain time during the incubation of lymphocytes and vesicles, parameter δ is used to characterize and measure these signals (the same reasoning applies to parameter H/D), there will be a value of δ peculiar to the spin label present in the adsorbed vesicles δ_v and a value of δ peculiat to the spin label that has diffused laterally in the lymphocyte membrane after fusion δ_f. Experimentally, however, after the incubation of cells and vesicles, it will only be possible to measure δ_v and a value δ_{Lv} of the cell pellet that arises from the lymphocyte-vesicle complex, to which both vesicle-bound and laterally diffused spin label will contribute.

If δ varies linearly with spin-label concentration, then the following relationship will hold:

$$\delta_{Lv} = a\,\delta_v + (1-a)\,\delta_f \tag{A.1}$$

where a represents the fraction of spin label present in the adsorbed vesicles. Thus

$$a = \frac{\delta_{Lv} - \delta_f}{\delta_v - \delta_f}. \tag{A.2}$$

An approximate solution of Eq. (A.2) can be obtained under the following assumptions:

1) After cell-vesicle fusion, the vesicle lipids diffuse laterally in the lymphocyte cell membrane until homogeneous distribution of the lipid molecules that have diffused is achieved in the lipid phase of the cell membrane, and within a time interval which is much shorter than the time necessary for measuring the spin-label concentration at the lymphocyte membrane [38, 44];

2) the spin label distributes only in the lipid phase of the lymphocyte membrane and does not interact with other membrane components;

3) the lipid phase of the lymphocyte cell membrane is constituted mostly by phospholipid and cholesterol [1, 21, 40];

4) the cholesterol molar fraction of the lymphocyte membrane is about 0.5 [1, 21, 37] and remains around 0.5–0.6 (*see* footnote 4);

5) almost all the cellular cholesterol of the lymphocyte is present in the cell membrane (cholesterol is in fact assumed to be present only in the cell membrane and it is used as a marker to estimate the extent of contamination of plasma cell membrane preparations with membranes from other cell organelles [1, 24, 36, 46]);

6) the variation of δ with variations of spin-label concentration that will occur at the lymphocyte-vesicle complex as a consequence of fusion will be linear, which is a good approximation for $X_{\text{SL-cholestane}} \lesssim 0.065$ (Figs. 5*B* and 7*C* in ref. [7]).

In the following paragraphs, the number of phospholipid, cholesterol, and spin-label molecules will be designated by P, C, and C^o, respectively. The subscripts L and v will indicate whether they are present at the lymphocyte membrane or at the vesicle membrane, respectively.

Before cell-vesicle fusion, the total number of lipid molecules L_v of a spin-labeled vesicle composed of phospholipid and cholesterol, and the homologous number for the lymphocyte membrane L_L, are given, respectively, by

$$L_v = P_v + C_v + C_v^o \tag{A.3}$$

and

$$L_L = P_L + C_L. \tag{A.4}$$

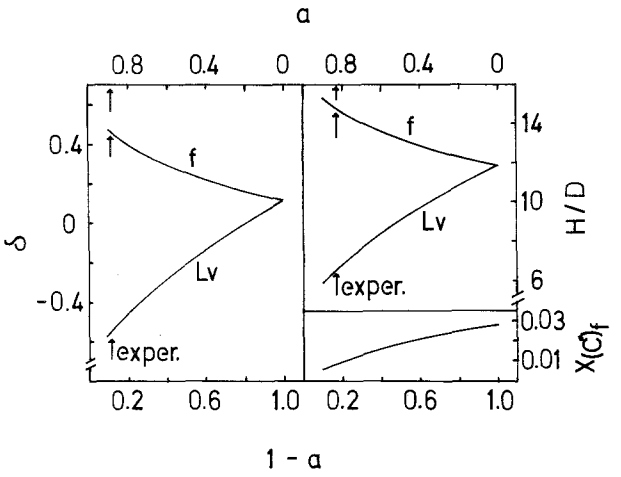

Fig. 9. Plot of calculated values of δ and H/D against the fraction $(1-a)$ of spin-labeled lecithin-cholesterol vesicles assumed to have fused with a lymphocyte; f refers to spin label that would diffuse laterally in the plane of the membrane after fusion; Lv refers to the lymphocyte-vesicle complex present in the cell pellet obtained by centrifugation after incubating lymphocytes with vesicles. These calculations were made from an experiment in which, after 5-hr incubation, 5.2×10^4 vesicles were attached per cell, calculated on the basis of the increment of cell cholesterol. The vesicle composition was as given in Fig. 2. Before incubation, the lymphocytes had 3.3×10^8 cholesterol molecules per cell. Other experimental values were: $\delta_v = -0.68$, $\delta_{Lv} = -0.63$, $(H/D)_v = 4.9$, and $(H/D)_{Lv} = 6.6$. By calculation $(X_{C^o})_f = 0.006$ and $(X_C)_f = 0.52$ was obtained. The corresponding value of δ_f was 0.46 and that of $(H/D)_f$ 14.8

After the fusion of n vesicles with a lymphocyte, the total number of lipid molecules of the lymphocyte membrane $(L_L)_f$ will be given by

$$(L_L)_f = P_L + C_L + n(P_v + C_v + C_v^o) \tag{A.5}$$

and the mole fraction of spin label at the lymphocyte membrane after fusion is given by

$$(X_{C^o})_f = \frac{n C_v^o}{(L_L)_f}. \tag{A.6}$$

The mole fractions of cholesterol and phospholipid at the lymphocyte membrane after fusion can be calculated by relationships homologous to Eq. (A.6) above.

Assume now that, after incubation of lymphocytes with spin-labeled lecithin-cholesterol vesicles, it is determined that n vesicles became attached to the cell, either adsorbed to it or fused with it. Calculate now the amount of spin label that can laterally diffuse in the lymphocyte membrane by assigning arbitrary increments to the fraction of fused vesicles $(1-a)$ in Eq. (A.1) above, for various values of a. Calculate the corresponding molar fractions of spin label at the lymphocyte membrane after each increment of $(1-a)$ by means of Eq. (A.6) above. From these values and the plots of Fig. 7*C* (Fig. 5*B* for H/D) of the accompanying paper, calculate the corresponding values of δ_f. Calculate now a series of δ_{Lv} values by means of: Eq. (A.1) above, the obtained δ_f values, their corresponding a values, and the experimentally obtained δ_v values. A plot like that shown in Fig. 9 can now be made and the calculated δ_{Lv} value that matches the experimental one is chosen for determining a.

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