Specific interactions of liposomes with PMN leukocytes upon incorporating tuftsin in their bilayers*

Arun Singhal, Anu Bali, Rakesh K. Jain and C.M. Gupta+

Division of Biophysics, Central Drug Research Institute, Lucknow 226 001, India

Received 31 August 1984; revised version received 4 October 1984

Incorporation of tuftsin derivatives, Thr-Lys-Pro-Arg-NH-$C_{18}H_{37}$ or Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-$COC_{15}H_{31}$, into an egg phosphatidylcholine/cholesterol liposome bilayer led to significantly enhanced binding of the liposomes to PMN leukocytes at 37°C but not at 0°C. Under identical conditions, no such enhanced binding of the liposomes was observed with erythrocytes and lymphocytes. These results demonstrate that grafting of tuftsin on the liposome bilayer enables the liposome to recognize specifically the PMN leukocytes and to deliver its contents to these cells.

Drug targeting *Liposome* *Ligand-receptor interaction* *Tuftsin* *Leukocyte* *Endocytosis*

1. INTRODUCTION

Liposomes may serve as useful vehicles for site-specific delivery of drugs and macromolecules [1,2] provided that simple but efficient methods become available for homing them to selected cell types. For this reason, several investigators have attempted to enhance the interactions of liposomes with specific cells by covalently or noncovalently attaching the liposomes with such ligands for which specific receptor sites exist on certain types of cells. These ligands include cell-specific antibodies [3–6], heat-aggregated immunoglobulins [7,8], lectins [9] and glycolipids [10,11]. Here, we demonstrate that incorporation of hydrophobic derivatives of the tetrapeptide Thr-Lys-Pro-Arg (fig.1,I,II) in the liposomes bilayer enables them to recognize specifically the PMN leukocytes and to deliver their contents to these cells.

* This paper is dedicated to our teacher, friend and colleague, Dr Nitya Anand, on his 60th birthday

+ To whom correspondence should be addressed

Abbreviations: PC, phosphatidylcholine; CH, cholesterol; 6-CF, 6-carboxyfluorescein; TNBS, trinitrobenzenesulfonic acid; TBS (mM), Tris–HCl (10), NaCl (150), pH 7.4; HBSS (mM), NaCl (137), KCl (5.37), $CaCl_2 \cdot 2H_2O$ (1.00), $MgSO_4 \cdot 7H_2O$ (0.41), $MgCl_2 \cdot 6H_2O$ (0.50), KH_2PO_4 (0.44), $Na_2HPO_4 \cdot 2H_2O$ (0.34), glucose (5.55), $NaHCO_3$ (4.16), pH 7.4; SSTS (mM), Tris–HCl (10), NaCl (150), sucrose (44), EDTA (5), pH 7.4

2. MATERIALS AND METHODS

Egg PC and egg [*methyl*-^{14}C]PC (30 μCi/μmol) were prepared as in [12]. The tetrapeptides I and II were synthesized by conventional methods and characterized by FAB/CAD mass spectrometry (unpublished). 6-CF (Eastman-Kodak) was used after purification as in [13]. The radioactivity and 6-CF were measured as in [14].

2.1. *Liposomes*

Small unilamellar liposomes were prepared from 15 μmol egg PC, traces of egg [^{14}C]PC (about 10 μCi), 7.5 μmol CH and 6-CF (0.2 M) with or without I or II (7–8% by egg PC weight) in 0.8 ml TBS or SSTS by probe sonication [12], and fractionated by centrifugation in a Beckman L5-65B ultracentrifuge at 100×10^3 *g* (SW-50.1 rotor) for 1 h at 5°C. Only the liposomes found in the top 3/4 of the supernatant were used. Free and liposomal 6-CF were separated by gel filtration

Published by Elsevier Science Publishers B.V.

[14]. The outer diameter of the liposomes, as measured by electron microscopy, was about 35–55 nm (mean size, ~40 nm), which remained unaffected upon incorporating I (or II) in the liposomes bilayer. Also, the size distribution of egg PC/CH/I (or II) liposomes was similar to those of egg PC/CH liposomes, as was evident from their similar elution patterns from Bio-Gel A-50m column [15].

The extents of incorporations of I or II in the liposomes bilayer were also estimated. Briefly, to an aliquot (20 μl) of egg PC/CH/I (or II) liposomes were added 580 μl 10 mM phosphate buffer (pH 8.5), 20 μl TNBS (1.5% in 0.8 M $NaHCO_3$, pH 8.5) and 200 μl Triton X-100 (1% in 0.8 M $NaHCO_3$, pH 8.5) or buffer. It was mixed and then incubated in dark at 18–20°C for 1 h. After this period, the reaction was stopped by adding 400 μl of HCl (1.5 M). The absorbance for yellow color so obtained was read at 410 nm. Quantities of I or II incorporated in the bilayers were calculated from the standard curve, which was drawn by reacting varying amounts of I or II with TNBS under identical conditions. The absorbance at 410 nm was linear with concentration to at least 0.8 absorbance units. The amounts of I or II incorporated into the liposome bilayer were over 95%, and about 60% of this amount was localized in the outer monolayer.

2.2. *Cell isolation*

PMN leukocytes, erythrocytes and lymphocytes were isolated from freshly drawn rat blood using Ficoll-Paque gradient (Pharmacia). Removal of the gradient from PMN leukocytes and lymphocytes was affected by washing the cell fractions with HBSS at 4°C, while in case of erythrocytes the washing was done with SSTS. The erythrocytes so isolated were contaminated with only 2–3% PMN leukocytes, and the lymphocyte fraction contained 5–7% PMN leukocytes and monocytes. However, the PMN leukocyte fraction was heavily contaminated with lymphocytes and erythrocytes. Erythrocytes from this fraction were completely removed by lysing them with NH_4Cl [7], but no further attempts were made to remove the contamination of lymphocytes (28–30%) and monocytes (0–2%). The cells were suspended in HBSS or SSTS (36–40 $\times$ 10^6 cells/ml) and stored (0.5–1 h) at 0°C until used. The cell viability was determined by trypan blue exclusion. Over 95% cells were viable.

2.3. *Cell–liposome interaction*

Liposomes (0.13–2.5 μmol lipid P/ml) were mixed with PMN leukocytes (18–20 $\times$ 10^6 cells/ml) in HBSS. The mixture was incubated at 37°C for 1 h in a shaking water bath. After the incubation was complete, the cells were harvested by centrifugation. The cell pellet was repeatedly washed with HBSS till the supernatant was free from radioactivity. For incubation with lymphocytes and erythrocytes, only one concentration of liposomes (about 2 μmol lipid P/ml) was used. In addition, SSTS instead of HBSS was used as the incubation media for erythrocytes because incubation of these cells with egg PC/CH/II liposomes in HBSS led to clumping of the cells. The cell pellets, after washing, were suspended in HBSS. Recovery of cells was at least 90%. Over 95% cells were viable at this stage. The amounts of radioactivity and 6-CF in the cell pellets were assayed after disrupting the cells with Triton X-100 (1% final concentration). In case of erythrocytes, 6-CF could not be measured due to quenching of its fluorescence by hemoglobin. In one experiment, free 6-CF was also incubated with PMN leukocytes in identical conditions. About 0.01% of the dye remained bound to the cell pellet.

3. RESULTS AND DISCUSSION

Selection of the tetrapeptide Thr-Lys-Pro-Arg (tuftsin) as the recognition molecule for PMN leukocytes was based on studies which showed that specific receptor sites for tuftsin exist on PMN leukocytes and monocytes [16,17]. As binding of tuftsin to these receptors has been shown to be inhibited by chemical modifications of the side chains or the N-terminus of the tetrapeptide [16], the C-terminus of the peptide was modified to facilitate its incorporation in the liposomes bilayer. This was done by introducing sufficiently long hydrocarbon residues at the C-terminus (fig.1,I,II). These modifications should not influence the binding ability of tuftsin to the target cells because additions to the C-terminus have been known not to adversely affect the binding of tuftsin to these cells [18].

Small unilamellar liposomes were formed from

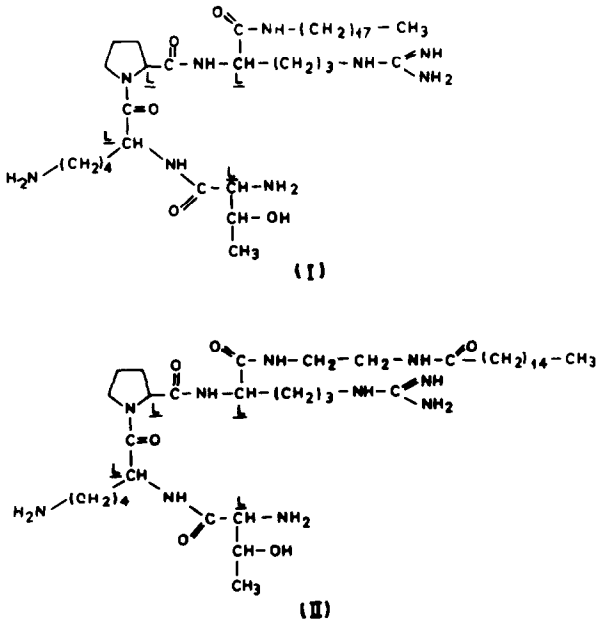

Fig.1. Hydrophobic derivatives of the tetrapeptide Thr-Lys-Pro-Arg (tuftsin).

egg PC and CH (50 mol%) in the presence as well as in the absence of I (or II) by sonication, and fractionated by centrifugation. Attempts to incorporate >10 mol% of I or II in the liposomes bilayer were not successful as the resulting lipid mixtures could not be dispersed even upon prolonged sonication. On the other hand, the liposomes containing <5 mol% of I or II were only poorly taken up by PMN leukocytes. Therefore, liposomes having 7–8 mol% I or II in their bilayers were used throughout. The leakage rates of 6-CF from egg PC/CH liposomes in buffer (pH 7.4) at 37°C were about 2–4%/h. The 6-CF leakage was dramatically enhanced upon incorporating I in the liposomes bilayer. This was probably due to binding of the dye [19] with positively charged Arg residue in I. Since this amino acid residue should be aligned just at the bilayer interface, the effect of its binding with 6-CF on the liposomes permeability must have been mediated through perturbation of the PC head-group packing [20] in the liposomes bilayer. This problem was, therefore, circumvented by introducing a spacer-arm in between the Arg residue and the hydrophobic anchor. Consequently, the 6-CF leakage from egg PC/CH/II liposomes became absolutely normal (2–5%/h).

The interactions between egg PC/CH/I liposomes and PMN leukocytes were studied using the liposomes that were formed in the absence of 6-CF. In this case, binding between liposomes and the cells was ascertained only by measuring ^{14}C in the cell pellet, while in case of egg PC/CH/II liposomes, the binding was assessed by measuring both the cell-associated ^{14}C and 6-CF. Parallel experiments were done using egg PC/CH liposomes as controls. Fig.2 shows that the binding/uptake of egg PC/CH/I liposomes increases with an increase in the liposome concentration, but virtually no such effect is observed in case of egg PC/CH liposomes. At 1.5 μmol lipid/ml concentration, the binding/uptake of egg PC/CH/I liposomes was at least 3-fold greater than that of the egg PC/CH liposomes. Similar results were obtained upon replacing I by II in the liposomes bilayer (fig.3A). As shown in fig.3A, the cell-associated ^{14}C to 6-CF ratio does not significantly differ from the liposomal ^{14}C to 6-CF ratio (before incubation). Therefore, it may be inferred that most of the egg PC/CH/II liposomes bind to the cells without losing their structural integrity.

These results indicate that incorporation of I or II in the liposomes bilayer leads to a significant increase in binding of the liposomes to PMN leukocytes. Since the amounts of cell-associated ^{14}C and 6-CF linearly increase with time at least up

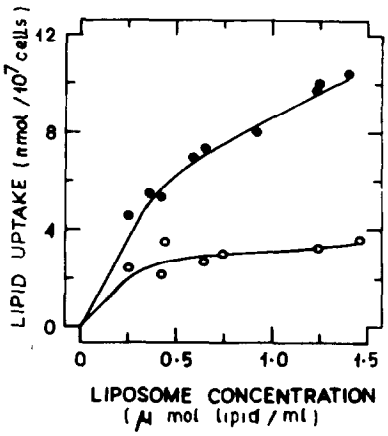

Fig.2. Effect of liposome concentration on binding/uptake of egg PC/CH/I (●), and egg PC/CH (○) liposomes by PMN leukocytes. Both the liposome concentration and lipid uptake were calculated from radioactivity, and expressed as μmol (nmol) PC.

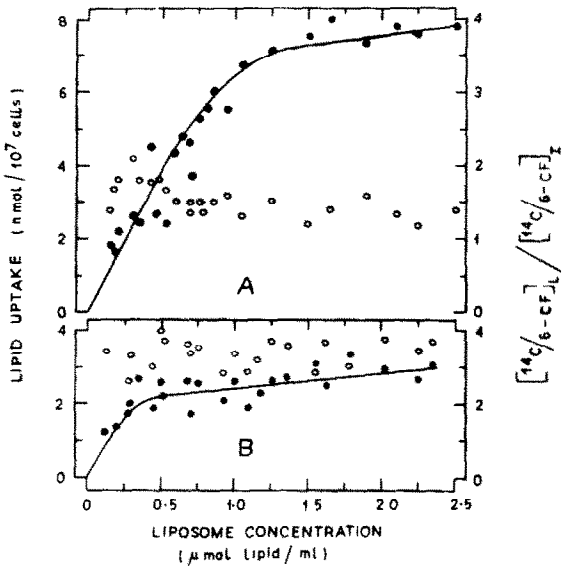

Fig.3. Effect of liposome concentration on binding/uptake of egg PC/CH/II (A) and egg PC/CH (B) liposomes by PMN leukocytes. $[^{14}C/6\text{-CF}]_L$ and $[^{14}C/6\text{-CF}]_I$ denote the ratios of ^{14}C to 6-CF in the cell pellet and liposomes (before incubation), respectively. Both the lipid uptake and liposome concentration were calculated from radioactivity, and expressed as nmol (μmol) PC. (●) Lipid uptake; (○) $[^{14}C/6\text{-CF}]_L/[^{14}C/6\text{-CF}]_I$.

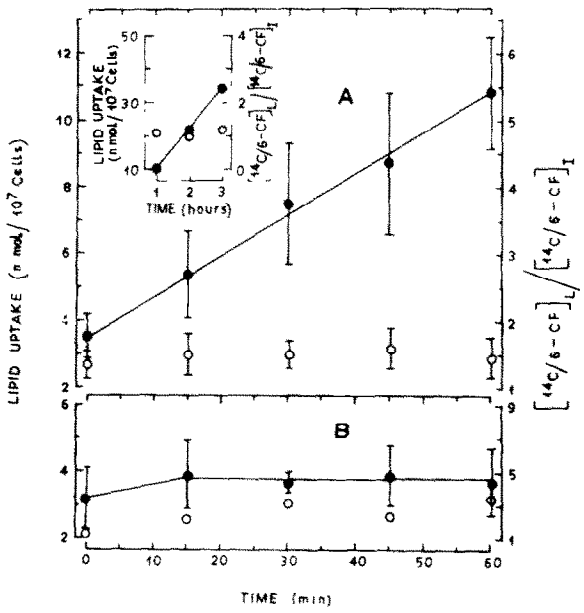

Fig.4. Kinetics of binding/uptake of egg PC/CH/II (A) and egg PC/CH (B) liposomes by PMN leukocytes. The liposomal concentration in these experiments was about 2 μmol PC/18–20 × 10^6 cells/ml. Incubations were done at 37°C. $[^{14}C/6\text{-CF}]_L$ and $[^{14}C/6\text{-CF}]_I$ denote the ratios of ^{14}C to 6-CF in the cell pellet and liposomes (before incubation), respectively. Lipid uptake was calculated from radioactivity, and expressed as nmol PC. Each point is the mean of 3 experiments. Bars, SD; (●) lipid uptake; (○) $[^{14}C/6\text{-CF}]_L/[^{14}C/6\text{-CF}]_I$. As egg PC/CH liposome binding/uptake was not influenced by the incubation time, the lipid uptake after 1–3 h incubation was measured for egg PC/CH/II liposomes only (inset). Values shown in the inset are the means of duplicate experiments. The maximum variation was ± 3 nmol. In another 2 experiments, the incubation temperature was lowered to 0°C. At this temperature, the mean values of lipid uptakes (nmol/10^7 cells) were as follows: egg PC/CH liposomes 3.7 (0 h), 3.6 (1 h), 3.7 (3 h); egg PC/CH/II liposomes, 5.1 (0 h), 6.3 (1 h), 6.6 (3 h). The maximum variation was ± 1.5 nmol.

to 3 h (fig.4), it is envisaged that this enhancement is probably due to tuftsin receptor-mediated endocytosis [21] of the liposomes by the leukocytes. That these liposomes are actively endocytosed by the cells is further suggested by our observation that lowering of the incubation temperature to 0°C inhibited the binding (fig.4).

Further experiments were done to examine the specificity of interactions between liposomes and other blood cells. Both egg PC/CH and egg PC/CH/II liposomes were separately incubated with rat erythrocytes and lymphocytes at 37°C for different periods of time. Results of these experiments revealed that there was virtually no interaction of both the types of liposomes with erythrocytes (1.5–2.5 nmol PC/10^7 cells, 3 h), though they appeared to have some binding (*n* mol PC/10^7 cells) with lymphocytes (egg PC/CH liposomes: 3.2–3.8 (0–3 h); egg PC/CH/II liposomes: 3.8–6.2 (0–3 h)). As this binding of egg PC/CH/II liposomes slightly increased with time, it may be concluded that the increase is primarily due to uptake of these liposomes by the PMN leukocytes/monocytes that were present as contamination in the lymphocyte preparation (see section 2).

This study clearly demonstrates that grafting of short sequence peptides like tuftsin, rather than cell-specific antibodies [3–6] or heat-aggregated immunoglobulins [7,8], on the liposomes bilayer may also help the liposome in selective delivery of its contents to specific cells. This approach of

liposome targeting is relatively simple and could possibly be extended to other cell types by appropriate selection of peptides which specifically interact with specific cells.

ACKNOWLEDGEMENTS

We thank ICMR and CSIR, New Delhi, for awarding research fellowships to A.S., A.B. and R.K.J. This is a communication (no.3601) from CDRI, Lucknow.

REFERENCES

[1] Finkelstein, M.C. and Weissmann, G. (1981) in: Liposomes – from Physical Structure to Therapeutic Applications (Knight, C.G. ed.) pp.443–464, Elsevier, Amsterdam, New York.
[2] Gregoriadis, G. (1983) Trends Pharmacol. Sci. 4, 304–307.
[3] Heath, T.D., Fraley, R.T. and Papahadjopoulos, D. (1980) Science 210, 539–541.
[4] Harsch, M., Walther, P. and Weder, H.G. (1981) Biochem. Biophys. Res. Commun. 103, 1069–1076.
[5] Leserman, L.D., Machy, P. and Barbet, J. (1981) Nature 293, 226–228.
[6] Heath, T.D., Montgomery, J.A., Piper, J.R. and Papahadjopoulos, D. (1983) Proc. Natl. Acad. Sci. USA 80, 1377–1381.
[7] Finkelstein, M.C., Kuhn, S.H., Schieren, H., Weissmann, G. and Hoffstein, S. (1981) Biochim. Biophys. Acta 673, 286–302.
[8] Kuhn, S.H., Gemperli, B., Shephard, E.G., Joubert, J.R., Weidemann, P.A.C., Weissmann, G. and Finkelstein, M.C. (1983) Biochim. Biophys. Acta 762, 119–127.
[9] Juliano, R.L. and Stamp, D. (1976) Nature 261, 235–238.
[10] Ghosh, P., Das, P.K. and Bachhawat, B.K. (1982) Arch. Biochem. Biophys. 213, 266–270.
[11] Spanjer, H.H. and Scherphof, G.L. (1983) Biochim. Biophys. Acta 734, 40–47.
[12] Gupta, C.M. and Bali, A. (1981) Biochim. Biophys. Acta 663, 506–515.
[13] Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137.
[14] Bali, A., Dhawan, S. and Gupta, C.M. (1983) FEBS Lett. 154, 373–377.
[15] Kumar, A. and Gupta, C.M. (1984) Biochim. Biophys. Acta 769, 419–428.
[16] Fridkin, M. and Gottlieb, P. (1981) Mol. Cell. Biochem. 41, 73–97.
[17] Gottlieb, P., Stabinsky, Y., Hiller, Y., Beretz, A., Hazum, E., Tzehoval, E., Feldman, M., Segal, S., Zakuth, V., Spirer, Z. and Fridkin, M. (1983) Ann. NY Acad. Sci. 419, 93–106.
[18] Gottlieb, P., Beretz, A. and Fridkin, M. (1982) Eur. J. Biochem. 125, 631–638.
[19] Oku, N., Kendall, D.A. and MacDonald, R.C. (1982) Biochim. Biophys. Acta 691, 332–340.
[20] Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Biochim. Biophys. Acta 650, 21–52.
[21] Amoscato, A.A., Davies, P.J.A., Babcock, G.F. and Nishioka, K. (1983) Ann. NY Acad. Sci. 419, 114–134.