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Covalent binding of antibodies to liposomes using a novel lipid derivative

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N-[3-(2-Pyridyldithio) propionyl] stearylamine (PDP-SA) was synthesized from a reaction between stearylamine and the heterobifunctional reagent *N*-succinimidyl-3-(2-pyridyldithio) propionate. Use of this PDP-SA to covalently couple antibodies to liposomes was investigated. The binding efficiency was found to be 24–32%. The antibodies bound to liposomes were shown to retain the specific antibody activity. This new procedure of coupling antibodies to lipsosomes could be an efficient means to deliver drugs to selected target organs, especially in cancer chemotherapy.

Several methods to couple antibodies to liposomes via a stable covalent linkage have been reported (Barbet et al 1981; Endoh et al 1981; Jansons & Mallett 1981; Martin et al 1981; Shen et al 1982; Goldmacher 1983). Martin et al (1981) have described a method which has a higher efficiency of binding than achieved by the other methods and which avoids dialysis in the presence of a detergent. However, because of the use of an unsaturated phosphatidylethanolamine derivative, the long-term stability of the product might be questionable due to the risk of oxidation. We report here the successful linkage of a rabbit anti-BSA IgG to liposomes using a stearylamine derivative of *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). This method has a high efficiency of binding without substantially interfering with the antibody activity of the bound IgG and the product does not have the risk of undergoing oxidation.

Methods

Synthesis of N-[3-(2-pyridyldithio) propionyl] stearylamine (PDP-SA). Stearylamine (SA) (260 mg, 965 μmol) and *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (200 mg, 640 μmol) were dissolved separately in 12 ml portions of absolute methanol. The solution of SPDP was added dropwise to the stearylamine solution and the mixture was stirred at room temperature (20 °C) for 30 min. Methanol was then removed under reduced pressure. The crude product was purified by column chromatography on silica gel; eluting with ethyl acetate–light petroleum (40:60) (282 mg, 94%): mp 61 °C; ir (CH_2Cl_2): 3450 (–NH–), 2950 [$(CH_2)_{18}$], 1690 (Amide I) and 1525 cm^{-1} (Amide II); [^{13}C]-nmr ($CDCl_3$): 175, 170 (*cis* and *trans* CO), 160–120 (5, pyridyl), 40–27 (several, CH_2), 23 ppm (CH_3). Anal.-Calc. for $C_{26}H_{46}N_2S_2O$: C, 66·90; H, 9·93; N, 6·00; S, 13·74. Found C, 67·28: H, 9·89; N, 5·81; S, 13·75.

Preparation of liposomes. Preparations containing large unilamellar and oligolamellar liposomes were obtained by the reverse-phase evaporation method described by Szoka & Papahadjopoulos (1978) using DL-α-dipalmitoyl phosphatidyl choline (DPPC), cholesterol, SA, and PDP-SA in a molar ratio of 4:4:1:1. Specifically, DPPC (80 mg, 109 μmol), cholesterol (42 mg, 109 μmol), SA (7·5 mg, 27·8 μmol) and PDP-SA (12·7 mg, 27·3 μmol) were dissolved in a mixture of chloroform (4·5 ml) and diethyl ether (8 ml). Next, 4·2 ml of 0·5% $NaHCO_3$ (pH 8) was added, after which, the lipid solution was sonicated at 0–5 °C for 5 min in a bath-type sonicator. Organic solvents were removed from the resulting emulsion-like dispersion by a rotary vacuum evaporator. The aqueous dispersion was then shaken at 55 °C for half an hour to obtain liposomes.

To incorporate methotrexate (MTX) into the liposomes a 20 mM solution of MTX in 0·5% $NaHCO_3$ was used for entrapment. Crude liposomes thus obtained were washed twice with 0·5% $NaHCO_3$ by centrifugation at 22 000*g* for 25 min. The washed pellet, which consisted of MTX liposomes free from unentrapped MTX, was finally dispersed in a volume of 0·5% $NaHCO_3$. The leakage of entrapped MTX during antibody-liposome coupling was calculated as the difference between liposomal MTX content (determined spectrophotometrically at 303 nm in methanol) before and after attachment of thiolated normal rabbit IgG to the liposomes.

Preparation of PDP-IgG. Rabbit anti-BSA IgG was fractionated with 33% saturated ammonium sulphate from immune sera and its antibody activity was assayed by radial immunodiffusion as described by Kulkarni et al (1981). Pyridyl disulphide moieties were introduced into IgG, using the heterobifunctional reagent SPDP by the method of Carlsson et al (1978). Ten moles of SPDP was reacted with 1 mol of IgG in 0·1 m sodium phosphate buffer containing 0·1 M NaCl, pH 7·5, for 30 min. The reaction mixture was dialysed extensively against the above buffer at 4 °C in order to remove low-molecular-weight substances. The content of 2-pyridyl disulphide groups, as determined by the method of Carlsson et al (1978), was 6–8·2 per mole of

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IgG. The modified IgG was stored at −20 °C until needed.

Covalent coupling of antibodies to liposomes. PDP-IgG was bound to liposomes by the method of Barbet et al (1981). PDP-IgG was reduced with dithiothreitol (DTT) in 0·1 M sodium acetate buffer containing 0·1 M NaCl, pH 4·5 for 40 min. Excess DTT and pyridine-2-thione were removed by gel filtration on a Sephadex G-25 M column equilibrated with 0·5% $NaHCO_3$. Thiolated IgG (8–12·6 mg) and liposomes (26 μmol of phospholipid) were stirred together overnight at room temperature and pH 8. Liposomes were separated from unbound antibody twice by centrifugation at 22 000*g* for 25 min followed by washing of the pellet with 0·5% $NaHCO_3$. In order to determine non-specific adherence of antibodies to liposomes, 9 mg of PDP-IgG that had not been reduced by DTT was mixed with liposomes as above. The protein content of the liposome fraction was analysed by the method of Lowry et al (1951).

Determination of antibody activity. The antigen-binding capacity of the anti-BSA IgG was determined using [^{125}I]BSA as antigen by a method based on Farr assay (Hudson & Hay 1980). For determining the antigen-binding capacity of the IgG linked to liposomes, an aliquot of liposome-anti-BSA IgG containing 314 μg of IgG was incubated with an excess of [^{125}I]BSA (400 μg, 55 000 counts min^{-1}) for 2 h at 37 °C with continuous shaking. This was followed by incubation at 4 °C for an additional 2 h. Liposome-antibody-antigen complex was then sedimented by centrifugation at 22 000*g* for 30 min. The pellet was washed twice with 0·1 M PBS. The amount of BSA bound to liposomes was calculated by determining the ^{125}I associated with the pellet. In control experiments the non-specific binding of [^{125}I]BSA to normal rabbit IgG or normal rabbit IgG-liposomes was determined. Values for specific binding to anti-BSA IgG were corrected by subtracting the amounts of radioactivity that bound non-specifically.

Results and discussion

About 24–32% of the thiolated IgG became bound to liposomes (96–123 μg IgG μmol^{-1} phospholipid), a binding efficiency comparable to that reported by other investigators (Barbet et al 1981; Martin et al 1981). The non-specific adherence of antibodies to liposomes was only 0·9% (3·1 μg IgG μmol^{-1} phospholipid). This confirms that the chemical reaction between thiolated IgG and liposomes (i.e. the thiol-disulfide exchange reaction) was responsible for increased binding of IgG to liposomes.

For immunospecific liposomal targeting it is essential that the liposome-bound antibody retain its immunological reactivity. The antigen-binding capacity of anti-BSA IgG before incorporation in liposomes was found to be 0·1641 μg BSA/μg anti-BSA IgG. The antigen-binding capacity of the rabbit anti-BSA IgG was reduced by only about 20% after its linkage to liposomes.

By virtue of the primary amino group, stearylamine can react readily with the heterobifunctional reagent SPDP to give rise to another reactive compound: PDP-SA.

Stearylamine, in contrast to phosphatidylethanolamine, has a well-defined and simple molecular structure, i.e. it has a single hydrocarbon chain whereas phosphatidylethanolamine (PE) has two fatty acyl chains of varying length and saturation. Because of its single chain, PDP-SA may have a better fit into the lipid bilayers of the liposomes than the two-'tailed' PDP-PE in which the length of the two chains are often unequal. Further, because its chain is saturated, PDP-SA is likely to be more stable than PDP-PE with its unsaturated fatty acyl chains. An oxidative reaction involving the PDP-PE could affect the integrity of liposomes and the stability of their linkage to antibodies. Other advantages of using PDP-SA for coupling antibodies to liposomes are the low cost of SA, the simple synthesis procedure and the almost quantitative conversion of SA to PDP-SA. Only about 20% of entrapped MTX leaked during the overnight binding reaction. Thus, by the linkage of appropriate antitumour antibodies to MTX-containing liposomes, it may be possible to add to the tumour-inhibitory effect of MTX linked directly to anti-tumour IgG (Kulkarni et al 1981).

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REFERENCES

Barbet, J., Machy, P., Leserman, L. D. (1981) J. Supramolec. Struct. Cell. Biochem. 16: 243–258

Carlsson, J., Drevin, H., Axen, R. (1978) Biochem. J. 173: 723–737

Endoh, H., Suzuki, Y., Hashimoto, Y. (1981) J. Immunol. Methods 44: 79–85

Goldmacher, V. S. (1983) Biochem. Pharmacol. 32: 1207–1210

Hudson, L., Hay, F. C. (1980) Practical Immunology. Blackwell Scientific Publications, Oxford, 2nd ed., pp 94–98

Jansons, V. K., Mallett, P. L. (1981) Anal. Biochem. 111: 54–59

Kulkarni, P. N., Blair, A. H., Ghose, T. I. (1981) Cancer Res. 41: 2700–2706

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193: 265–275

Martin, F. J., Hubbell, W. L., Papahadjopoulos, D. (1981) Biochemistry 20: 4229–4238

Shen, D., Huang, A., Huang, L. (1982) Biochim. Biophys. Acta 689: 31–37

Szoka, F. Jr., Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75: 4194–4198