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Fc-Receptor-mediated Targeting of Antibody-bearing Liposomes Containing Dideoxycytidine Triphosphate to Human Monocyte/Macrophages

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Abstract—Liposomes bearing surface-attached antibody (L-Ab) molecules can be used for various purposes including the immunospecific delivery of drugs or other materials to antigenic target cells. In this study, L-Ab were prepared to deliver an anti-human immunodeficiency virus (HIV) drug, dideoxycytidine triphosphate (ddCTP) to human monocyte/macrophages. Cells of the monocyte/macrophage lineage are an important reservoir of HIV-1. A mouse monoclonal antibody IgG_{2a} was labelled with ^{125}I and modified using *N* succinimidyl-3-(2-pyridyldithio)propionate (SPDP) as a heterobifunctional reagent in order to conjugate with liposomes to produce a covalent bond (thioether). SPDP-modified antibody was incubated with liposomes containing 5 mol% of maleimido phenyl butyrate phosphatidylethanolamine (MPB-PE) at room temperature (21°C) for 24 h. L-Ab were separated from free and aggregated antibodies by centrifugation. L-Ab were characterized by measuring particle size and binding to anti-mouse IgG-sepharose. Ninety five per cent of the liposomal (L-Ab) lipid label was bound to anti-mouse IgG-sepharose, whereas only 7% of plain liposomes were bound, indicating non-specific binding. Uptake of L-Ab was measured in human monocyte/macrophages as a function of time and compared with that of plain liposomes. The uptake increased with time and it was 4–6 times greater than that of plain liposomes although part of that effect may have been due to unreacted MPB groups.

Acquired immunodeficiency syndrome (AIDS) was initially defined as the development of an opportunistic infection of Kaposi's sarcoma (an unusual neoplasm that had previously been recognized to be associated with certain immunosuppressed states) in a person without a known cause for immunodeficiency (Gottlieb et al 1981; Broder & Gallo 1984). Within three years, it was shown that a retrovirus, which has the capacity to replicate within cells of the immune system and so lead to a profound destruction of $T4^+$ T cells, is the cause of AIDS (Gallo et al 1984; Popovic et al 1984). The current epidemic of AIDS poses a major threat to our population. Urgently needed are both a vaccine to prevent infection with this etiologic retrovirus, human immunodeficiency virus (HIV), as well as safe, effective antiviral agents to treat those individuals already infected. The elucidation of viral replicative mechanisms has allowed the development and testing of several agents active against HIV in-vitro. Inhibitors of reverse transcriptase that have demonstrated activity such as azidothymidine (AZT), dideoxycytidine (ddC), and dideoxyinosine (ddI) have shown activity against HIV. The monocyte has emerged as a potentially important cell line in the pathogenesis of HIV infection.

Interest in liposome-dependent drugs arose from the need to provide liposomes that could deliver drugs to a specific site. Liposomes containing chemotherapeutic agents provide the possibility of selective delivery and selective cytotoxicity (Burchell & Papadimitriou 1985). A relatively new and potentially powerful development has been the use of proteins, such as antibodies that bind to antigens on target cells, to guide drug molecules to their targets (Mayhew & Papahadjopoulos 1983). Such procedures address the major problem of chemotherapy, i.e. of selectivity. Methods of conjugating monoclonal antibodies have resulted in the development of liposomes for specific targeting (Gregoriadis 1982; Weinstein & Leserman 1984). The conjugation of cell-specific antibodies to liposomes containing chemotherapeutic agents has been shown to increase cytotoxicity selectively in several murine tumour cell lines (Machy et al 1982; Martin & Papahadjopoulos 1982). However, there are few reports in the literature examining the application of antibody-bearing liposomes to human cell lines (Matthay et al 1986).

The attachment of proteins to liposomes, like many scientific developments, is a procedure that was envisaged well before its development was fully possible. Several investigators utilized the non-covalent interaction of antibodies with liposomes to create targeted liposomes, and examined their interaction with cells (Weissmann et al 1974; Gregoriadis & Neerunjun 1975). However, it was clear that covalent coupling techniques were necessary for a number of reasons: non-covalent attachment of a protein relies on a fortuitous process; it is difficult to control in terms of the amount of protein that associates; it is useful only for those proteins that will associate non-specifically. For these reasons it is desirable to conjugate proteins to liposomes through covalent linkage.

In a previous study we have reported that liposome-encapsulated dideoxycytidine triphosphate (ddCTP) was not more potent than free ddCTP (Szebeni et al 1990). Therefore, in this study we conjugated antibody to liposomes containing ddCTP through covalent (thioether) linkage, to increase the delivery of ddCTP to human monocyte/macrophages as a first step to demonstrate an improved anti-HIV activity of this drug. Characterization of these conjugates was by measuring the particle size and binding to antimouse

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IgG-sepharose. We also measured the cellular uptake of antibody-bearing liposomes and compared it with liposomes prepared with and without maleimido phenylbutyrate phosphatidylethanolamine (MPB-PE) as control.

Materials and Methods

Chemicals

Egg phosphatidylcholine (EPC), bovine phosphatidyl serine (PS), maleimido phenyl butyrate phosphatidylethanolamine (MPB-PE) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Cholesterol (CH) was obtained from Eastman Kodak Chemicals (Rochester, NY). [^{14}C]Cholesteryl oleate (CH oleate) was obtained from Amersham, Arlington Heights, IL. *N*-Succinimidyl-3-(2-pyridyldithio) propionate (SPDP) was purchased from Pierce, Rockford, IL. Dideoxycytidine triphosphate (ddCTP) was obtained from Pharmacia, Piscataway, NJ. [^{3}H]ddCTP was obtained from Moravek Biochemicals (Brea, CA). Dithiothreitol (DTT) and Protein A-sepharose were purchased from Sigma Chemical Co. (St Louis, MO).

Preparation and characterization of liposomes

Large, unilamellar liposomes containing ddCTP were prepared by modification of the method of Cullis and colleagues (Hope et al 1985; Cullis et al 1987) as follows. Lipids were mixed in chloroform at molar ratios of EPC:PS:CH:MPB-PE:[^{14}C]CH oleate of 32:18:45:5:10^{-5} (0·125 μCi [^{14}C]CH oleate used as a nonexchangeable marker), and were deposited on the walls of glass vials by evaporating the solvent under a nitrogen stream. Solvent traces were subsequently removed by freeze-drying overnight. Liposomes were formed by adding 1 mL of 40 mM ddCTP + 1 μCi [^{3}H]ddCTP to the dry lipid film containing 200 μmol lipid and vortex mixing until all the lipids were dispersed into the phosphate buffer (approx. 10 min). The dispersion was subjected to five freeze-thaw cycles in liquid nitrogen and warm water, and then to ten cycles of pressurized extrusion (at 500–600 psi) through 0·4 μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA). The latter process was carried out in an extruder (Lipex Biomembranes Inc., Vancouver, BC, Canada), maintained at 37°C. Unencapsulated solute was removed by four sequential cycles of centrifugal gel filtration (Fry et al 1978). This method provided >93% recovery of lipids with <3% free drug.

The amount of ddCTP and efficiency of encapsulation in a typical experiment, as estimated from [^{3}H]ddCTP and [^{14}C]CH counts in the final preparation, were 65 μmol ddCTP (mmol lipid)$^{-1}$ (1·6 L (mol EPC)$^{-1}$) and 9%, respectively. Vesicle size distribution, as measured by photon correlation spectroscopy (Coulter Electronics, Inc., Haleigh, FL, Model N4MD), was 215 ± 64 nm (s.d.) in a typical preparation. The inter-experimental mean and coefficient of variation were 207 nm and 5%, respectively (n = 5).

Antibody

Mouse monoclonal IgG_{2a} antibody (H-2-K^k) was prepared and purified as described by Heath et al (1983). Briefly, 2·0 mL of ascites diluted with 2·0 mL of phosphate-buffered saline (PBS; pH 7·4) was applied to a protein A column equilibrated with PBS (pH 7·4). The column was then incubated at room temperature (21°C) for 30 min and washed with PBS (pH 7·4) until the peak at 280 nm returned to baseline. Bound protein was eluted with 0·1 M sodium citrate/citric acid-buffered saline (pH 4·0). Fractions were collected in 0·5 mL 1 M Tris-HCl buffer (pH 9·0). The fractions eluted with sodium citrate/citric acid buffer (pH 4·0) were dialysed against PBS (pH 7·4). The purified antibody was stored in PBS at −20°C.

Radiolabelling of antibody

When required, IgG was radiolabelled with ^{125}I using sodium iodide. Briefly, 10 μL (1·0 mCi) of sodium iodide was added to an Eppendorff tube coated with Iodogen and containing 20 μL 0·05 M sodium phosphate (pH 7·4). To this 100 μL (100 μg) protein was added, and the tube was incubated for 15 min (agitating intermittently). Four hundred microlitres 0·05 M sodium phosphate was added to stop the reaction. The solution was added to a PD-10 (Sephadex G-25) column and the solution allowed to enter the column. PBS (4·3 mL) was added to the column, and 6-drop fractions were collected into polystyrene tubes. The radioactivity was determined in each fraction and the three samples with greatest radioactivity were pooled for use.

Modification of antibody by SPDP

To 1 mL of HEPES-buffered saline containing 1 mg antibody in a glass tube was added 1·6 μL 20 mM SPDP. This gives a molar ratio of 5 mol SPDP (mol mouse IgG)$^{-1}$. The reaction mixture was incubated for 30 min at room temperature (21°C). Unreacted SPDP was separated from labelled antibody by column chromatography using a Sephadex G 25 column equilibrated with acetate buffer (pH 4·5). Fractions

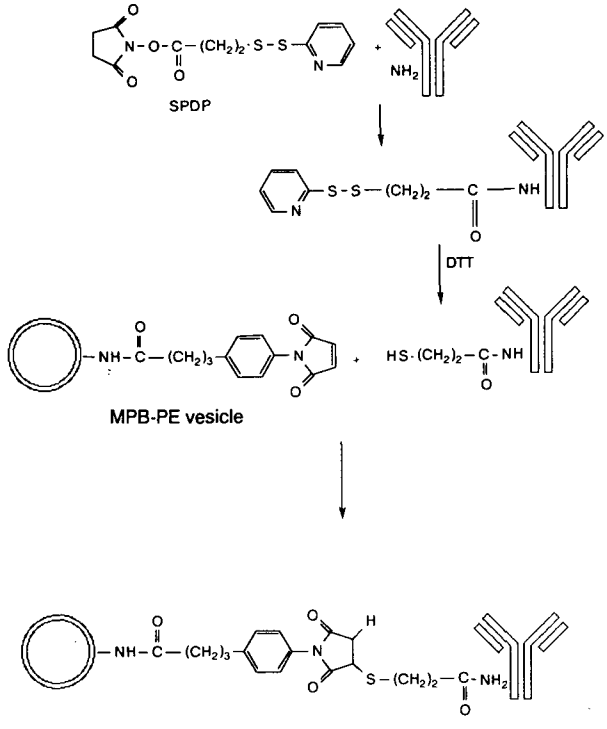

FIG. 1. Schematic representation of covalent coupling of IgG to MPB-PE liposomes.

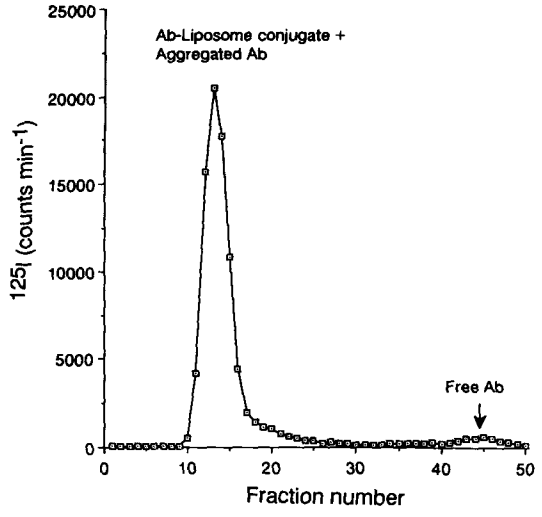

FIG. 2. Five hundred microliters of a reaction mixture of liposomes plus the murine hybridoma antibody 36.7.5 modified by SPDP and reduced by DTT was passed over the column of sepharose 4B (5 cm, 1·5 cm, 1 mL min^{-1}) after 24 h incubation. The 0·5 mL fractions were followed by ^{125}I.

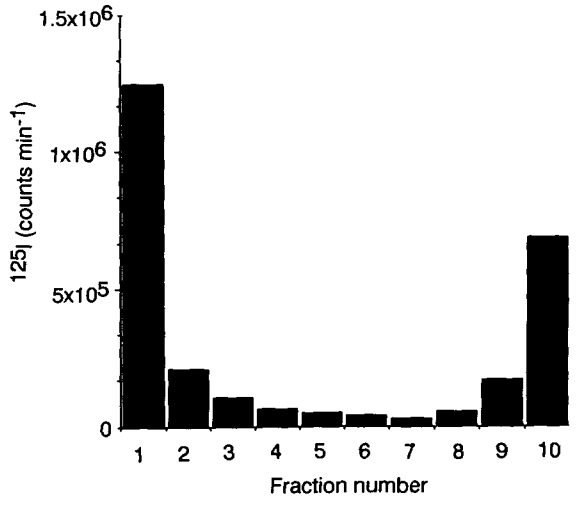

FIG. 3. Metrizamide solution (40%; 2·5 mL) was placed over 0·5 mL of a reaction mixture of liposomes plus the murine hybridoma antibody 36.7.5 modified by SPDP and reduced by DTT. This was followed by 1.5 mL of 20% metrizamide solution and 0·5 mL of phosphate-buffered saline, then centrifuged at 143000 *g* for 16 h. Fractions (0·5 mL) starting from the top layer were placed in Falcon tubes and were followed by ^{125}I.

were followed by ^{125}I-labelled antibody as a marker. The SPDP was reduced to the thiol form by addition of DTT to a final concentration of 50 mM. After 20 min at room temperature the antibody was separated from DTT by a passage on a column pre-equilibrated with 10 mM HEPES-buffered saline, pH 8·0.

Conjugation of modified antibody to liposomes

The antibody (100 $\mu g\ mL^{-1}$) modified with SPDP was mixed with 50 μL liposomes and incubated at room temperature for 24 h with constant stirring under a nitrogen stream. Schematic representation of the conjugation process is shown in Fig. 1.

Separation of liposomes from noncoupled antibodies

Separation of the conjugated liposomes from noncoupled protein is one of the most important steps in characterizing the product. The most commonly used method for the separation has been gel chromatography (Sepharose-4B) as shown in Fig. 2. An alternative method has been developed by Heath et al (1981) using an isotonic density medium. On centrifugation, the liposomes migrate out of the lower layer up to the interface between the second medium layer and a small buffer layer on the top of the gradient. The unbound protein does not float, because it is too dense. In this study 2·5 mL 40% w/v solution of metrizamide was placed over the reaction mixture in a centrifuge tube followed by 1·5 mL 20% (w/v) solution of metrizamide and 0·5 mL PBS on top. Centrifugation was for 16 h at 143000 *g* (Fig. 3).

Stability of antibody-liposome conjugates

Antibody-liposome conjugates were incubated in Dulbecco's modified eagle's medium (DMEM) in the presence of 10% foetal calf serum (FCS), 100% FCS and 500 mM DTT at 37°C. Samples were subjected to metrizamide density gradient separation as described above. Fractions (0·5 mL) starting from the top layer were placed in Falcon tubes and were followed for the presence of ^{125}I.

Binding of antibody-liposome conjugates to antimouse IgG-sepharose

Antimouse IgG-sepharose was packed in a small (1·0 mL) column. Antibody-liposome conjugates were applied to the column and allowed to stand for 30 min. The column was eluted with PBS (pH 7·4) and the radioactivity was measured by counting the fractions in a liquid scintillation counter.

Elutriated monocyte/macrophages

Peripheral blood monocytes were obtained from leukapheresed healthy, HIV-1 antibody-negative donors by Ficoll-Hypaque (Pharmacia) separation and were purified by counterflow centrifugal elutriation using a Beckman elutriation system (Wahl et al 1984a, b). This method provides monocytes with >95% positivity for Leu M3 and nonspecific esterase. Throughout the process, pyrogen-free PBS was used. The cells were then washed and plated in DMEM with 10% FCS, 2 mM glutamine and antibiotics.

Cellular uptake

Monocyte/macrophages were incubated with antibody-bearing liposomes or plain liposomes in 6-well Costar plates (5×10^6 cells/well). Uptake of plain liposomes and antibody-bearing liposomes was estimated by counting cell-associated ddCTP radioactivity after washing the cells 4 times with ice-cooled saline and detaching them from the plates with 0·5% Triton X-100.

Results and Discussion

Conjugation of antibody to liposomes increased linearly with antibody concentration up to 2 mg mL^{-1}, and the

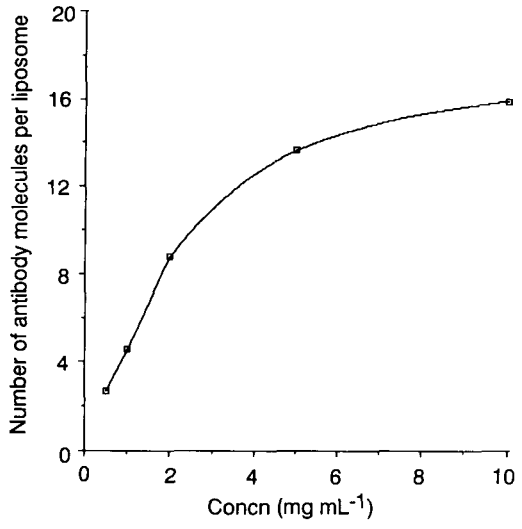

FIG. 4. Effect of antibody concentration on coupling of IgG to MPB-PE liposomes.

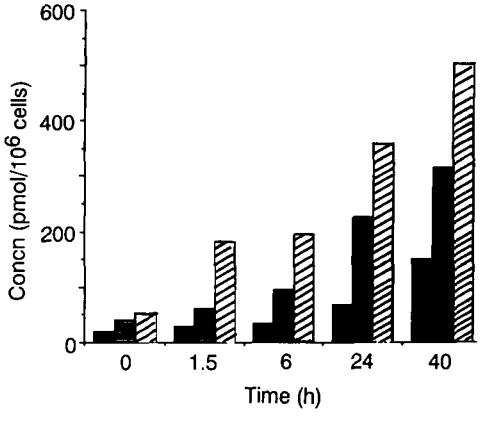

FIG. 5. Binding of plain liposomes (■), MPB-PE liposomes (■) and antibody-bearing liposomes (■) to human monocyte/macrophages.

conjugation was saturated at about 10 mg mL^{-1}, as shown in Fig. 4. Binding of L-Ab and plain liposomes to anti-mouse IgG-sepharose showed that seven percent of label in plain liposomes compared with >95% in immunoliposomes was bound to antimouse IgG-sepharose, indicating non-specific binding. Stability studies indicated that the thioether linkage is stable after incubation with DMEM (10% FCS), 100% FCS and DTT. However, disulphide linkage between antibody and liposome is unstable in the presence of DTT (Machy et al 1982).

The uptake of plain, MPB-PE and antibody-bearing liposomes by cultured human monocyte/macrophages increased with time. The uptake of L-Ab was 4–6 times higher than that of plain liposomes (Fig. 5). Uptake of liposomes containing MPB groups was also higher than that of plain liposomes (Fig. 5). This observation suggested that unreacted MPB groups have a tendency to bind to the cells. The uptake of ddCTP was 2–7 times higher than that of plain liposomes and 1·5–2 times higher than that of MPB-PE liposomes as shown in Fig. 6. In our previous study, the

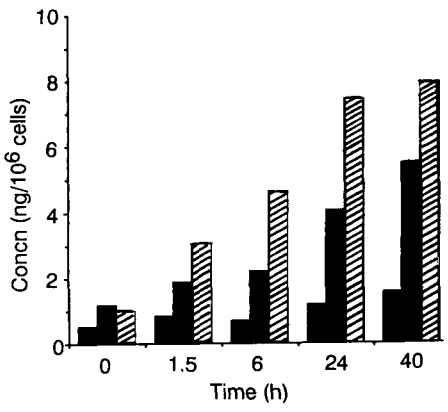

FIG. 6. Uptake of liposomal encapsulated ddCTP from plain liposomes (■), MPB-PE liposomes (■) and antibody-bearing liposomes (■) by human monocyte/macrophages.

uptake of liposome-encapsulated ddCTP was similar to free ddCTP; therefore, the liposomal ddCTP was not more potent than free ddCTP (Szebeni et al 1990).

Early in-vitro investigations into the interaction of liposomes with a variety of phagocytic cells indicated that the majority of liposomes that become cell-associated are internalized by the cells (Gregoriadis & Buckland 1973; Weissmann et al 1975; Poste et al 1979). However, the extent of liposome binding and subsequent ingestion by macrophages is greatly influenced by the type of liposome employed. Multilamellar vesicles for example, are better recognized than small unilamellar vesicles of identical lipid composition (Schroit & Fiddler 1982). In addition, the lipid composition of the liposomes also influences the rate and extent of phagocytosis. For example, the negatively charged liposomes are phagocytosed better than liposomes containing exclusively neutral phosphatidylcholine (Schroit et al 1986). Similar degrees of lipid-dependent phagocytic enhancement have been shown to occur with many cells of mononuclear phagocyte series, including Kupffer cells (Dijkstra et al 1985), alveolar macrophages (Schroit & Fiddler 1982; Schroit et al 1983), and human peripheral blood monocytes (Kleinerman et al 1983; Schroit et al 1983). Negative charge does not, however, seem to be entirely responsible for the apparent recognition of the vesicles by macrophages, since phosphatidic acid is not effective in enhancing phagocytosis (Schroit et al 1986).

In 1968, Huber and Fudenberg showed that macrophage membranes possess receptors for the Fc portion of immunoglobin molecules. This observation explained a variety of macrophage recognition mechanisms, and the Fc receptor provides a recognition site to-Ab which are rendered more susceptible to phagocytosis (opsonized liposomes) can be directed.

The success of antibody-dependent liposome-mediated targeting depends on efficient, stable and irreversible association of the protein with the liposome. Ideally, liposome delivery systems of this type should not suffer from leakage, aggregation, or loss of bound ligand. A variety of procedures has been employed to conjugate antibodies to liposomes (Heath & Martin 1986), ranging from simple co-sonication of the antibody in the presence of the liposome lipid (Huang

& Kennel 1979) to highly specific covalent attachment of the antibody to pre-derivatized lipids (Huang et al 1980; Martin et al 1981; Martin & Papahadjopoulos 1982) or directly to liposomes (Leserman et al 1980). Antibodies can be covalently attached to pre-formed liposomes by generation of active aldehyde groups on liposomal glycolipids (Heath et al 1980), by gluteraldehyde cross-linking (Torchilin et al 1978) or by heterobifunctional cross-linking reagents such as SPDP (Leserman et al 1980; Martin et al 1981) and succinimidyl-4-(*p*-melimidophenyl)-butyrate (SMPB) (Martin & Papahadjopoulos 1982). The use of both SPDP and SMPB are elegant techniques which facilitate high-efficiency coupling without significant vesicle aggregation, protein denaturation, or loss of ligand activity. It would appear, therefore, that the most direct approach towards targeting liposomes to macrophages would be to use specific anti-macrophage antibodies coupled to liposomes by the above mentioned procedures.

In conclusion, the antibody can be conjugated to liposomes through a covalent (thioether) linkage, which is more stable even if traces of DTT are present in the sample, without affecting the specificity. The uptake of L-Ab and delivery of ddCTP to human monocyte/macrophages can be increased over that of plain and MPB liposomes by Fc receptor-mediated binding. Further studies will be required to explore the use of antibody-bearing liposomes to deliver ddCTP and treat HIV-infected monocytes.

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