Specific Interaction of Myeloma Tumor Cells with Hapten-bearing Liposomes Containing Methotrexate and Carboxyfluorescein

Lee D. Leserman,1 John N. Weinstein, John J. Moore,2 and William D. Terry

Immunology Branch [L. D. L., J. J. M., W. D. T.] and Laboratory of Theoretical Biology [J. N. W.], National Cancer Institute, NIH, Bethesda, Maryland 20205

ABSTRACT

Hapten-bearing liposomes containing methotrexate and the fluorescent solute carboxyfluorescein were incubated with murine myeloma tumor cells expressing surface immunoglobulin with affinity for the hapten. Liposomes bearing the diminotriphenyl hapten became bound to MOPC 315 myeloma tumor cells, and liposomes bearing the phosphorylcholine hapten became bound in much larger amounts to TEPC 15 cells. In each case, fluorescence microscopy showed a patchy surface pattern, indicating intact liposomes at the cell surface. Few liposomes were bound to cells in the presence of excess soluble hapten or to cells lacking the relevant surface immunoglobulin. Cell-associated liposomes were quantitated by use of the fluorescence-activated cell sorter, and the pharmacological effect of the methotrexate was assessed from measurement of incorporation of radiolabeled deoxyuridine into the cells. Little inhibition of deoxyuridine incorporation was observed, because contents of the bound liposomes did not enter the cytoplasmic compartments of the cells.

INTRODUCTION

The ideal antitumor chemotherapeutic agent would have activity against malignant but not against normal cells. Conceptually, this might be achieved if the drug were encapsulated in a carrier bearing a ligand for a tumor-specific antigen, enabling it to bind preferentially to tumor cells (7, 8, 22, 33). We have studied this possibility for tumor-specific therapy by taking advantage of the fact that some murine myeloma cells secrete and bear on their surfaces immunoglobulin with known antigen-binding activity (4, 10, 23).

In earlier studies, we showed that liposomes prepared with DNP3-substituted phospholipids bind to MOPC 315 myeloma cells, which secrete and bear on their surface an immunoglobulin with affinity for the DNP radical (14). Binding was specific for the DNP group on the liposome, in that (a) it was inhibited by addition of the cross-reactive soluble hapten TNP-lysine, (b) cells lacking this immunoglobulin bound few liposomes, and (c) nonhaptenated liposomes were not bound. In spite of this specific surface binding, MOPC 315 cells binding liposomes carrying fluorescent molecules within their aqueous spaces did not internalize the fluorophore during the short incubations studied. This suggested that drugs similarly carried might also fail to be transferred to the cytoplasm and might thus exert little or no therapeutic effect.

That work was limited in an important respect. We could not study cell-liposome interactions for long periods of time (i.e., hr to days), since CF does not bind to any intracellular structure and leaves the cell relatively quickly. This efflux would result in an underestimate of the amount transferred to the cytoplasm. In the present study, in addition to fluorescence methods, we use MTX to evaluate the possibility of specific transfer of drug from liposomes to cells. MTX binds essentially irreversibly [binding constant, 1010 to 1011 liters/mol (36)] to the cytoplasmic enzyme DHFR. The ability of MTX to block conversion of deoxyuridine into thymidine thus provides an index of drug delivery to the cytoplasm during longer-term incubations.

For haptenated carriers, we used DNP-bearing liposomes and also liposomes bearing the PC hapten covalently attached to PE by a new synthetic method. We evaluated the specific interaction of MTX-containing liposomes with the myeloma cell lines MOPC 315 and TEPC 15, with respect both to binding of liposomes and to incorporation of radiolabeled deoxyuridine.

MATERIALS AND METHODS

Cells. The mineral oil-induced BALB/c myeloma tumors MOPC 315 and TEPC 15 were gifts of Dr. Michael Potter, NCI. They were maintained by alternate generations of i.p. and s.c. passage. Cells to be used in these experiments were harvested from the peritoneal cavity 1 to 2 weeks following tumor cell inoculation and were depleted of peritoneal macrophages by treatment with carbonyl iron (14).

MTX. MTX (NSC 740), Monsanto Lot 77-1122-01, was provided by the Division of Cancer Treatment, NCI.

Radioisotopes. [3H]dUrd, specific activity 15 Ci/mmol, and [3',5',7-3H]MTX were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Labeled MTX was further purified by DEAE-cellulose chromatography by Dr. D. S. Zaharko, NCI, who kindly provided it to us. [3H]MTX was diluted with unlabeled MTX to a specific activity of 146 mCi/mmol for use.

Liposomes. Liposomes containing 10 mm CF (Eastman Kodak Co., Rochester, N. Y.) were prepared by sonication, using techniques and materials described in detail previously (14, 33). MTX solution (25 mM) was made by adding dry MTX to a solution containing 10 mm CF and 1.25% NaHCO3. Non-hapten-bearing liposomes were made at DOPC:cholesterol molar ratios of 50:50. Hapten-bearing liposomes were made at DOPC:cholesterol:hapten-bearing PE molar ratios of 50:47.5:2.5. All lipids used gave only the expected spots on thin-layer

1 To whom requests for reprints should be addressed, at Centre d’Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, F-13288 Marseille Cedex 2, France.
2 Present address: Department of Medicine, Walter Reed Army Medical Center, Washington, D. C. 20012.
3 The abbreviations used are: DNP, dinitrophenyl; TNP, trinitrophenyl; CF, carboxyfluorescein; MTX, methotrexate; DHFR, dihydrofolate reductase; PC, phosphorylcholine; PE, phosphatidylethanolamine; [3H]dUrd, deoxy(y5-3H)uridine; DOPC, dioleoyl phosphatidylcholine; DNP-caproyl-PE, N-dinitrophenylcaproylphosphatidylethanolamine; PC-caproyl-PE, N-phosphorylcholine:caproylphosphatidylethanolamine; MEM, Eagle’s minimal essential medium; FCS, fetal calf serum.
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4 R. Blumenthal et al., unpublished observations.

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Liposome Binding to Myeloma Cells

Analysis of Tumor Cell Fluorescence. Tumor cells were preincubated in polystyrene tubes at 0 or 37 °C for 30 min in MEM with 10% heat-inactivated (56 °C, 30 min) FCS. The FCS eliminates nonspecific binding. Each tube contained approximately 3 million cells in 0.5 ml incubation medium. In inhibition experiments, PC-chloride (Sigma Chemical Co.) was added to a final concentration of 100 μg 15 min before addition of liposomes. The liposomes were added in 50-μl volumes. Tubes were shaken gently to resuspend cells and were incubated for 20 min at the same temperature as during preincubation. Following incubation, cells were washed several times in MEM containing 10% FCS, and cell-associated fluorescence was evaluated by fluorescence microscopy and flow microfluorometry. Our use of the FACS-II (Becton-Dickinson Electronics Laboratories, Mountain View, Calif.) for analysis of cell-associated fluorescent liposomes has been described in detail (1, 14, 33). Appropriate corrections were made for the quantum efficiency of CF in liposomes and in the cell cytoplasm. Cells for fluorescence microscopy were placed on glass slides under coverslips in 5-μl volumes in MEM with 10% heat-inactivated FCS. They were examined by fluorescence and phase optics with a Zeiss universal microscope. Samples from incubations at 4 °C were initially examined on chilled slides within a few sec after removal from ice. Otherwise, observations were at room temperature.

Incubation of Tumor Cells with MTX or MTX-containing Liposomes. Tumor cells at a concentration of 5 × 10^5/ml were incubated in 0.1-ml volumes in 96-well flat-bottom tissue culture plates in MEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and antibiotics. Free MTX, liposomes containing CF, or liposomes containing both CF and MTX were added at the indicated concentration to triplicate wells. Liposomal MTX concentration was varied by changing the concentration of liposomes, which always contained 25 mM MTX. Following 1 to 2 hr of incubation at 37 °C, 1 μCi of [3H]dUrd was added to each well. After an additional 10- to 18-hr incubation, wells were harvested with an automated harvester, and incorporated [3H]dUrd was determined in a liquid scintillation counter. Results are presented as the percentage of [3H]dUrd incorporation with respect to control cells incubated with medium alone.

Measurement of Cellular DHFR Levels. We used a procedure similar to that of Goldman et al. (6). TEPC 15 cells (10^6) in 1 ml MEM with 10% heat-inactivated FCS were incubated with 10 μl of [3H]MTX for 2 to 4 hr at 37 °C. The cells were then washed 3 times by centrifugation and resuspended in fresh medium without MTX for an additional 2 to 4 hr. By this time, the level of intracellular MTX had stabilized. After an additional wash, the cells were resuspended in scintillation fluid and counted for incorporated [3H].

RESULTS

Binding of Hapten-bearing Liposomes to TEPC 15 and MOPC 315 Myeloma Cells. Cells of the murine myeloma tumor MOPC 315 secrete and bear surface immunoglobulin with affinity for DNP (4, 10). We have shown that these cells specifically bind liposomes with membrane phospholipids that contain PE to which the DNP hapten has been covalently attached. This binding was visualized by inclusion of the fluorophore CF inside the liposomes. TEPC 15 cells, which bear surface immunoglobulin with affinity for PC, bound few DNP liposomes (14).

The new synthesis of a PC-substituted derivative of PE enabled us to evaluate the binding to TEPC 15 cells of liposomes bearing this hapten. Liposomes were prepared with 2.5 mol % of the phospholipid substituted with the PC hapten (PC liposomes), and were incubated with TEPC 15 or MOPC 315 tumor cells. The results of a representative experiment using PC liposomes are presented as a histogram of fluorescence intensity in Chart 1. PC liposomes, but not liposomes without

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6 J. J. Moore. Biological properties of the phosphorylcholine ester PCCA, manuscript in preparation.
haptens, were bound by TEPC 15 cells. This binding was specific for the PC determinant in that it was inhibitable by the free hapten PC-chloride. The average TEPC 15 cell bound approximately 10 million molecules of CF (corresponding to 770,000 liposomes if each is assumed to be 250 Å in diameter, containing 13 molecules of dye). MOPC 315 cells failed to bind significant numbers of PC-substituted liposomes (Chart 2).

Examination of the TEPC 15 cells by fluorescence microscopy (Fig. 2) showed the same patchy, noncapped pattern of surface binding of PC liposomes as that seen for MOPC 315 cells and DNP liposomes in our previous study (14), except that the TEPC 15 fluorescence was much brighter. The fluorescent patches appeared less aggregated and were dimmer after incubation on ice rather than at 37°. Binding was effectively abolished by inclusion of PC-chloride as an inhibitor, by omission of the PC hapten, or by substitution of DNP for the PC. Not all of the cells bound vesicles. See Ref. 14 for further discussion of the morphology and its interpretation.

Inhibition by MTX of \[^{3}H\]dUrd Incorporation into MOPC 315 and TEPC 15 Cells. Charts 3 and 4 summarize the results of experiments in which we addressed 2 hypotheses: (a) that liposome encapsulation would enhance the antimetabolite effect of MTX on myeloma cells in the absence of specific binding; and (b) that hapten-specific binding would enhance the MTX effect.

\[^{3}H\]dUrd incorporation by both TEPC 15 and MOPC 315 tumor cells was significantly (P < 0.01) inhibited by free MTX at concentrations as low as 30 nM, indicating sensitivity of both tumors to the drug. Vesicles made with 2.5 mol % PC- or DNP-substituted PE were sonicated in the presence of 25 mM MTX mixed with 10 mM CF or with 10 mM CF alone. Fluorescence microscopy confirmed that the presence of MTX in liposomes did not change the selective binding of PC liposomes to TEPC 15 cells, and of DNP liposomes to MOPC 315 cells. Charts 3 and 4 indicate that MTX in liposomes inhibited \[^{3}H\]dUrd uptake by the tumor cells, but to a lesser extent than the same quantity of MTX in free solution. Incubation of cells with MTX-containing liposomes for as long as 20 hr before addition of \[^{3}H\]dUrd did not enhance the drug effect (data not shown). PC liposomes made without included MTX were slightly inhibitory only at the highest concentration (Chart 3).

Presumably, liposomal MTX was less effective than an equivalent concentration in free solution because it was less available to the usual MTX transport mechanisms of the cells. As part of the same experiment, we determined in the following way the effect of liposomes containing MTX was not a consequence of specific binding. PC liposomes containing MTX were added to TEPC 15 cells in the presence of 1000 µM PC-chloride, which did not change the selective binding of PC liposomes to TEPC 15 cells. This binding was specific for the PC determinant in that it was inhibitable by the free hapten PC-chloride. The average TEPC 15 cell bound approximately 10 million molecules of CF (corresponding to 770,000 liposomes if each is assumed to be 250 Å in diameter, containing 13 molecules of dye). MOPC 315 cells failed to bind significant numbers of PC-substituted liposomes (Chart 2).
with cells incubated without the soluble hapten. This finding is with MOPC 315 cells in the presence of 50 JUM TNP-lysine.

chloride, and DNP liposomes containing MTX were incubated with [3H]MTX, TEPC 15 cells were found to contain (2.7 ±0.5)
cytic process. consistent with a nonspecific mechanism for the action of the tumor cells (as confirmed by fluorescence microscopy), [3H]
of 50 /UM. After a 3-hr incubation at 37°, 1 ¿iCi of [3H]dUrd was added to each

or in the presence of MTX in free solution, or in DNP liposomes at concentrations of 30 to 2000 nm. TNP-lysine was added to some wells to a final concentration of 50 μM. After a 3-hr incubation at 37°, 1 μCi of [3H]dUrd was added to each

well, and incorporated radionucleide was measured following overnight incubation. Data are expressed as in Chart 3. Control wells incorporated 18,055 ± 551 cpm.

chloride, and DNP liposomes containing MTX were incubated with MOPC 315 cells in the presence of 50 μM TNP-lysine. Despite the fact that these soluble haptenes blocked binding to tumor cells (as confirmed by fluorescence microscopy), [3H] dUrd incorporation was inhibited to exactly the same extent as with cells incubated without the soluble hapten. This finding is consistent with a nonspecific mechanism for the action of the liposomal MTX, as by leakage of contents or by a slow endo-
cytic process.

Estimation of Intracellular DHFR Levels. From incubations with [3H]MTX, TEPC 15 cells were found to contain (2.7 ± 0.5) 
× 10^5 molecules of DHFR per cell. This figure (corresponding to an intracellular concentration of 3 × 10^{-7} M, assuming a mean cell diameter of 15 μm) is comparable with the DHFR levels of other murine tumors (27). This amount of MTX would have been delivered to the cytoplasma by incorporation of the contents of about 10^4 250-Å liposomes, fewer than 2% of the liposomes specifically associated with the surface of the TEPC 15 tumor cells. Thus, the amount of MTX bound on the surface would easily have been adequate to saturate the cellular DHFR if a major fraction of the drug had been delivered to the cytoplasma.

DISCUSSION

Liposomes have become increasingly prominent in recent years as drug carriers and as tools in cell biology and immu-
nology. In each of these contexts, a major challenge has been that of specifically “targeting” the liposomes for interaction with particular cells. In previous studies, in our own laboratory and in others, antibody has been used to achieve this specificity (2, 7, 8, 14–16, 33, 35). Conceptually, a tumor-specific ther-
peutic effect might be realized if liposomes containing drugs were directed to cell surface determinants unique to the tumor. Most B-cell tumors, including the myelomas and chronic lymphocytic leukemias, bear clonally expressed surface immuno-
globulin, which might act as target for such liposomal therapy (25, 26). In the present study, we have used murine myeloma tumors with known hapten-binding specificity to investigate requirements for, and consequences of, the targeting of hap-
tenated liposomes.

Liposomes bearing a newly synthesized haptenated lipid, PC-caproyl-PE, bound in large numbers to the surfaces of TEPC 15 cells, which bear surface immunoglobulin with affinity for PC. Cell-associated fluorescence was observed by fluores-
cence microscopy and was quantitated by the fluorescence-activated cell sorter. The binding of PC liposomes to TEPC 15 cells was approximately 30 times as great as that of DNP liposomes to MOPC 315 cells in our previous studies. This observation presumably reflects a larger amount of surface immunoglobulin on the TEPC 15 tumor line that we used, but the density of cell surface immunoglobulin has not been evaluated by other techniques. Specificity of liposome binding was indicated by (a) inhibition by an excess of the soluble hapten, PC-chloride; (b) lack of binding to MOPC 315 cells; and (c) lack of binding of DNP-bearing liposomes to TEPC 15 cells (9).

A recent report (20) suggests that TEPC 15 immunoglobulin will bind to the PC head groups of phospholipid micelles. Since we saw no liposome binding without addition of PC-caproyl-PE, apparently the PC head groups of the lipid bilayer itself were not sterically available for binding of liposomes, to TEPC 15 surface immunoglobulin. In liposomes made with the PC-
caproyl-PE hapten, the PC group is separated from the bilayer by a 6-carbon spacer (see Fig. 1).

Liposomes used in the present study contained MTX in their aqueous spaces (3, 5, 8, 11–14, 30). For a drug delivered in a liposome to be therapeutically effective, it would generally be required to enter the cell. This would occur, for example, if binding were followed by fusion with, or endocytosis of, the liposomes and release of their contents into the cell cytoplasma. Of the MTX bound in PC liposomes to the surface of TEPC 15 cells, 2% would have sufficed to saturate the DHFR of the myeloma cells.

In the present experiments, we were unable to detect a significantly enhanced effect of encapsulated MTX as a con-
sequence of specific binding to either MOPC 315 or TEPC 15 cells, and MTX in liposomes was much less effective than an equivalent amount of free drug. Binding of liposomes to the cells was inhibited by soluble hapten, but the hapten did not reduce the drug effect, and non-hapten-bearing liposomes were just as effective as were hapten-bearing liposomes in inhibiting [3H]dUrd uptake (data not shown). This was true whether we used liposomes made with the diunsaturated phospholipid DOPC or liposomes made with the fully saturated distearoyl and dipalmitoyl PC's. This suggests that the effective MTX was the small amount of drug leaked from the lipo-
somes into the solution, or that transferred to the cells in a nonspecific manner, e.g., by endocytosis or fusion. The exper-
iments described here were done in the presence of 10% heat-
inactivated FCS, which inhibits both nonspecific adsorption of liposomes and transfer of their contents to lymphoid cells (1). Qualitatively similar results were, however, obtained in the absence of FCS. Thus, for these 2 BALB/c myeloma tumors, liposomes specifically bound to the cell surface did not deliver their contents into the cell. In 2 recent reports (12, 30), lipo-
some-encapsulated MTX was also less effective than free drug, under circumstances where liposomes were not specifically targeted to cell surface determinants.

7 L. D. Leserman and J. N. Weinstein, unpublished observations.
Microscopic observation of TEPC 15 tumor cells incubated with liposomes for as long as 24 hr showed only patchy surface, and not cytoplasmic, fluorescence. An analogous observation was previously reported by Hannestad and Gaudernack (9) for with liposomes for as long as 24 hr showed only patchy surface, specifically bound liposomes may relate to the lack of capping and endocytosis of surface immunoglobulin. It has been suggested that the absence of capping may, in turn, relate to the active secretion of immunoglobulin by the myeloma cells (24).

Fig. 2 shows that some cells do not bind sufficient fluorescent liposomes to be photographed; nevertheless, the number of liposomes bound to most cells would have been adequate to inhibit [3H]Urd uptake if the encapsulated MTX had entered the cells.

In contrast to myeloma tumor cells, antigen-reactive B-cells cap and endocytose antigen bound to surface immunoglobulin (21). Since liposome-associated antigens will induce immune responses (31, 32), they presumably bind to antigen-reactive cells. This would permit specific delivery of drugs in liposomes to cells responding to liposome-associated antigens. Experiments to evaluate this possibility are in progress.

This in vitro study does not address a number of difficulties that might arise in applications in vivo of liposome-mediated immunotherapy: (a) patients carrying surface immunoglobulin-positive myeloma tumors usually have high blood levels of secreted immunoglobulin which would be expected to bind to the liposomes in the circulation and prevent interaction of these liposomes with the tumor cell surface. Indeed, the circulation time of DNP-bearing or PC-bearing liposomes is much reduced in the bloodstream of mice bearing the MOPC 315 tumors, respectively, and few liposomes would be expected to reach the tumor; (b) immunogenicity of the targeting moiety would require consideration, as an immune response to the liposomes would similarly be expected to reduce their circulation time and perhaps result in allergic reactions; (c) many tumors express unique surface determinants only at some stages in the cell cycle or they include subpopulations not expressing the determinant (17). The fluorescence profile in Chart 1 suggests such a subpopulation among the TEPC 15 cells; (d) investigation is just beginning on the ability of liposomes to pass capillary endothelial barriers to reach the cells of solid tumors; we therefore feel that hematogenous tumors would be more appropriate than solid tumors for initial therapeutic efforts with specifically targeted liposomes.

For those tumors which do not secrete competing ligand, direction of liposomes to the surface of tumor cells might, nevertheless, be achieved. For diagnostic localization (e.g., with γ-emitting radionuclides), binding would suffice. Binding might also be therapeutically useful if the liposome carried a compound capable of acting on the cell without entry, such as an α emitter (29). In a separate report (16), we describe the use of the hapten bound to the liposome membrane as a means of opsonizing liposomes with IgG anti-hapten antibody. In this context, we have found liposome-bound MTX to be more effective than free drug against an IgG Fc receptor-bearing phagocytic tumor. This study confirms that MTX taken up in liposomes can be released in active form. However, the Fc receptor is ubiquitous, and significant delivery to normal Fc receptor-bearing cells would also be expected. The challenge remains to be able to specifically direct liposomes (as by covalently coupled antibody) to those specific tumor cell surface determinants which permit entry.

ACKNOWLEDGMENTS

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Liposome Binding to Myeloma Cells


Fig. 1. Hapten-modified lipids. Structures of the hapten-bearing lipids DNP-caproyl-PE and PC-caproyl-PE. When one of these lipids is included in a liposome, the hapten is separated from the bilayer by a 6-carbon spacer. The haptenated lipids used in our experiments contained natural mixtures of fatty acid chains, of which the palmitic and oleic chains shown are representative.
Fig. 2. Binding of fluorescent liposomes to TEPC 15 cells. 

A. fluorescence micrograph after incubation with PC liposomes. The large patches or aggregates seen are at the cell periphery, as verified by through-focusing. Some patches are out of focus. Not all cells show binding. 

B. fluorescence micrographs after incubation with PC liposomes and 100 μM PC-chloride. No binding can be seen. 

C and D are phase images of the fields in A and B, respectively. See text for protocol.
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