Interactions of Immunoliposomes with Target Cells

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We have covalently attached a monoclonal antibody (11-4.1) against the murine major histocompatibility antigen, H-2Kk, on the surface of liposomes. The interaction of these antibody-coated liposomes (immunoliposomes) with target cells, RDM-4 lymphoma (H-2Kk), was investigated. About 90% of the immunoliposomes taken up by target cells at 4 °C could be removed by a mild protease treatment of the cells, whereas only 30% of the uptake at 37 °C was labile to the same treatment. Furthermore, the uptake of immunoliposomes at 37 °C was inhibited by cytochalasin B or by a combination of 2-deoxyglucose and NaF. These results suggest that immunoliposome binding to the target cell surface is the primary uptake event at 4 °C and that the surface-bound liposomes are rapidly internalized by the cells at 37 °C, probably via an endocytic pathway. Studies with fluorescence microscopy of target cells treated with immunoliposomes containing carboxyfluorescein also supported this conclusion. If endocytosis is the mechanism by which immunoliposomes gain entry into target cells, the efficacy of a cytotoxic drug encapsulated would depend on the resistance of the drug to lysosomal inactivation and its ability to escape from the lysosomal system.

Liposomes have been extensively tested as carriers of drugs, enzymes and other biologically important molecules (1-3). One of the major problems of the use of liposomes as carrier systems is their nonspecificity of delivery. In recent years, many successful attempts have been made to covalently couple specific antibody to the surface of liposomes to confer target specificity to the liposomes (4-7). We have developed a method to couple antibody to liposomes by derivatizing antibody molecules with palmitic acid using NHS1 (8). The palmitoyl antibody was subsequently incorporated into liposomal bilayers by a detergent-dialysis method. Using a monoclonal antibody to the murine histocompatibility antigen H-2Kk as a model system, we were able to show that liposomes bearing the antibody (immunoliposomes) interacted specifically with target cells expressing the proper antigen in a mixed culture of target and nontarget cells (9). To use immunoliposomes as a targeted drug carrier system, it is essential to understand the fate of the liposomes and their contents after the initial binding event at the surface of the target cells. In the present report, the mechanism of interaction of immunoliposomes with target cells was investigated.

MATERIALS AND METHODS

Antibody—Anti-H-2Kk antibody was purified with protein A Sepharose affinity chromatography (10) from the culture fluid of a mouse hybridoma cell line 11-4.1 (11) obtained from the Salk Institute. These cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (RC Biological, Lenexa, Kansas) and 1.6 mM sodium pyruvate. IgG secreted by myeloma P3-X63-Ag8 (P3 cells) was purified from ascites fluid by precipitation with 50% saturated ammonium sulfate and chromatography on DEAE-cellose and phosphocellulose (12). IgG was iodinated with 125I or 131I (Amersham, Corp.,) using the chloramine-T method to a specific activity of 1 x 106 to 1 x 107 cpm/μg, respectively.

Lipids—Egg yolk phosphatidylcholine was either purified as described (13) or purchased from Sigma. Cholesterol and brain phosphatidylerine were also obtained from Sigma. Dioleoyl phosphatidylcholine was purchased from Avanti Polar Lipids, Inc., Birmingham, AL. All lipids were stored in chloroform or chloroform-methanol (1:1) at -20 °C. The purity was checked periodically by thin layer chromatography. NHS1 was synthesized by condensing N-hydroxysuccinimide and palmitic acid with dicetylphosphatidylcholine and was recrystallized in anhydrous ethanol (14). NHS1 was stored in anhydrous ethanol (14). NHS1 was stored in anhydrous ethanol (14). NHS1 was stored in anhydrous ethanol (14).

The abbreviations used are: NHS1, N-hydroxysuccinimide ester of palmitic acid; MTX, methotrexate; araC, cytosine-β-d-arabinofuranoside; CF, carboxyfluorescein; PBS, phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 1 mM Na2HPO4, pH 8.0); P3 cells, BALB/c mouse myeloma cells P3-X63-Ag8; IgG, immunoglobulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; SDS, sodium dodecyl sulfate; P3 IgG, immunoglobulin secreted by P3-X63-Ag8 cells; [3H]dUTp, deoxy-[3H]uridine; [3H]araC, [5-3H]cytosine-β-d-arabinofuranoside; [3H]dT, [methyl-3H]thymidine; anti-H-2Kk-lipo-MTX, liposomes carrying anti-H-2Kk antibody in the bilayer with entrapped MTX; P3-IgG-lipo-MTX, liposomes carrying P3 IgG in the bilayer with entrapped MTX; lipo-MTX, liposomes containing no IgG but with entrapped MTX; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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drous dioxane (doubly distilled over metallic sodium) in a desiccator at room temperature. Hexadecyl [3H]cholestanyl ether was obtained by a catalytic reduction of hexadecyl cholesteryl ether with tritium gas (ICN Radiochemicals, Irvine, CA). This radiolabeled lipid behaves similarly to hexadecyl [3H]cholesteryl ether which has been shown to be a faithful marker for liposomes and it is neither exchangeable with cellular lipids nor metabolizable by cells (16). In most experiments, MTX, chlorambucil, azide, colchicine, cytochalasin B, and 2-deoxyxglycose were purchased from Sigma. Cyto-
osome-β-d-arabinofuranoside hydrochloride (araC HCl) was purchased from P-L Biochemicals. [5-3H]araC, [3', 5', 7-3H]MTX, and deoxy[6-
H]uridine were obtained from Amersham Corp. [methyl-3H]Thymi-
dine was obtained from ICN Radiochemicals. CF was purchased from Eastman, recrystallized according to Ralston et al. (16) and purified on a LH-20 column (Pharmacia) equilibrated and eluted in 50% ethanol. Other miscellaneous chemicals were obtained from commercial sources as A.C.S. certified or higher grades.

**Coupling of Antibody with Palmitic Acid**—The palmitic acid derivatization of antibody using NHS-P was performed as previously described (8). Briefly, the antibody was derivatized with a NHS-P to antibody molar ratio of 10:1. Palmitoyl antibody was purified with Sephadex G-75 chromatography. The antibody was eluted in PBS containing 0.15% deoxycholate for 2 liters of PBS to remove sucrose. Immunoliposomes were collected from PBS at 4 °C before observation under a Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera. All fluorescence pictures were taken with an Orthomat-W camera. All fluorescence pictures were taken with an Orthomat-W camera.

**Liposome Preparation**—A solvent-free mixture of egg yolk phosphatidylcholine (or dioleoyl phosphatidylcholine), cholesterol, and phosphatidylserine (molar ratio 57:33:10) in PBS (20 mg/ml) was solubilized for 30 min in a probe-type sonifier (Heat Systems, model W-375) at 1 °C under argon. In some experiments, a trace of [3H]cholesteryl ether was included in the lipid mixture to a final specific activity of 3.0 × 10^6 cpm/mg of lipid. Palmitoyl IgG was then added to the sonicated lipid at a lipid to protein ratio of 10:1 (w/w). Concentrated deoxycholate was added to bring the final deoxycholate to pH 0.751 (w/w). After vortexing, the mixture was dialyzed for 45 h at room temperature using Spectrator-2 dialysis tubing (Spectrum, Los Angeles, CA) against a change of 2 liters of PBS. The turbid liposomal suspension was then chromatographed on a column of Sepharose 4B to remove unincorporated IgG (8). The liposomes collected in the void volume fractions were further purified by preparative sucrose gradient centrifugation to remove aggregated free antibody molecules (8). The gradients consisted of 23.5 ml of 0–20% linear sucrose gradient with a 1.0-mL 65% sucrose as cushion. The gradients were spun at 85,000 × g for 16 h at 4 °C. The fractions containing liposomes were collected and dialyzed at 4 °C against 3 changes of 2 liters of PBS to remove sucrose. Immunoliposomes prepared with this procedure contained an average of 48 IgG molecule per liposome (8).

**Encapsulation of Solutes in Liposomes**—Immunoliposomes or control liposomes were incubated in a solution containing solutes in the presence of 0.15–0.18% deoxycholate for 2 h at 37 °C (final concentration of the trapping mixture 30–35 mg/ml). When trapping MTX and araC, the drugs were [3H]labeled to facilitate the assay of drug encapsulated in the liposomes. The concentration of cytotoxic drugs in the trapping mixture was from 4.5–7.0 mg/ml at a specific activity of 5 × 10^6–1.2 × 10^7 cpm/μg in the case of MTX and 10.6 mg/ml at a specific activity of 8 × 10^6 cpm/μg for araC. CF trapping was done either in a 10 or 200 mM solution of CF. At the end of incubation, untrapped solutes and deoxycholate were removed from the liposomes using a Sepharose 4B column equilibrated and eluted in PBS. Sucrose gradient centrifugation analysis indicated that the IgG remained liposome-associated after this trapping procedure. More than 90% of the trapped solute remained liposome-associated when stored in PBS at 4 °C for at least 72 h as judged by gel filtration analysis.

**Immunoliposome Uptake by RDM-4 Cells**—RDM-4 cells (2 × 10^6 cells/ml) were incubated with anti-H-2K^k^ immunoliposomes at either 4 or 37 °C for 4 h. After washing, they were exposed to 1 ml of 0.04% protease ( Pronase E, Sigma) in (Ca²⁺, Mg²⁺)-free Heps buffer containing 1 mM EGTA for 15 min at 37 °C. The cells were then washed three times with 3 ml of ice-cold PBS containing 0.1% bovine serum albumin. The final pellets were solubilized in 200 μl of 2% SDS and processed for radioactivity counting.

**Effects of Inhibitors on Immunoliposome Uptake by RDM-4 Cells**—RDM-4 cells (2 × 10^6 cells/ml) were preincubated for 30 min at 37 °C with various inhibitors. Anti-H-2K^k^ immunoliposomes were added (5 μg/ml of IgG, 100 μg/ml of lipid). The samples were incubated at 37 °C for an additional 4 h. The cells were washed three times in 3 ml of ice-cold PBS containing 0.1% bovine serum albumin, solubilized in 200 μl of 2% SDS, and processed for radioactivity counting.

**Incubation of CF-containing Immunoliposomes with Cells**—RDM-4 and P3 myeloma cells (H-2K^k^) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1.5% sodium pyruvate. Liposomes carrying either P3 IgG (IgG secreted by P3 cells) or anti-H-2K^k^ (final protein concentration, 20 μg/ml) with entrapped CF were incubated with 2 × 10^6 RDM-4 or 1 × 10^6 P3 cells in complete medium at 4 or 37 °C for 6 h (final volume 1 ml). The cells were washed three times with 3 ml of ice-cold PBS before observation under a Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera. All fluorescence pictures were taken with an Orthomat-W camera.

**Encapsulation of Cytotoxic Drugs Encapsulated in Immunoliposomes**—Various doses of cytotoxic drugs (araC or MTX) entrapped in anti-
H-2K^k^ or P3 IgG liposomes were incubated with RDM-4 (2 × 10^6 cells/ml) or P3 (1.5 × 10^6 cells/ml) cells in 96-well microtiter plates for 3 h at 37 °C (total volume, 200 μl/well). The cells were then washed three times with 250 μl of medium after which 200 μl of fresh medium was added and the plates incubated at 37 °C for 30 min. [methyl-3H]Thymidine (araC cytotoxicity assay) or deoxy[6-3H]Thymi-
dine (MTX cytotoxicity assay) was added to each well (0.75 μCi/well) and the plates were incubated at 37 °C for an additional 16 h. The cells were then harvested onto filter paper strips using a cell harvester (Tietzert Cell Harvester, Flow Laboratories, Rockville, MD). The filters were submerged in 5% trichloroacetic acid for 30 min at 4 °C followed by washing in ice-cold 95% ethanol and acetone. The filters were then dried and counted. Measurements were done in quadruplicates. Standard deviations from the mean were generally between 5 and 10%.

In some experiments where liposome-entrapped MTX was used, various concentrations of chloroquine were included in the medium throughout the experiments to assess the effect of this lysosomotropic amine on the cytotoxicity elicited by liposome-entrapped MTX.

**Other Analytical Methods**—Quantitation of CF fluorescence was done on an Aminco-Bowman spectrophotofluorometer using excita-
tion and emission wavelengths of 490 and 526 nm, respectively. γ-Counting was done on a Beckman Gamma II γ-counter. Liquid scintillation counting was done on a Beckman LS-230 counter using Triton-toluene fluor.

**RESULTS**

**Temperature Sensitivity of Immunoliposome Uptake**—The uptake of anti-H-2K^k^ immunoliposomes by RDM-4 cells at 4 and 37 °C was quantitated using liposomes doubly labeled with [3H]cholesteryl ether and [3H]anti-H-2K^k^). Consistent with previously published results from this laboratory (18), these immunoliposomes only associated with RDM-4 cells (k haplotype), but not with P3 cells (d haplotype) (data not shown). Fig. 1 shows the time course of the [3H]uptake by these cells at the two temperatures. The uptake of CF followed a similar pattern (data not shown). At 4 °C, the uptake reached saturation within an hour and remained at a constant level in the 6-h incubation period. The uptake at 37 °C showed an initial phase at a rate significantly higher than that ob-
erved at 4 °C. This was followed by a second slower phase of uptake which continued throughout the 6-h incubation period. To gain better insight into the uptake processes, an experi-
ment was performed to estimate what proportion of the im-

**Protease Treatment of Cells**—RDM-4 cells (2 × 10^6 cells/ml) were incubated with immunoliposomes at either 4 or 37 °C for 4 h. After washing, they were exposed to 1 ml of 0.04% protease ( Pronase E, Sigma) in (Ca²⁺, Mg²⁺)-free Heps buffer containing 1 mM EGTA for...
munoliposomes taken up by RDM-4 cells was located externally. Since the liposome-cell binding is mediated by the antigen-antibody complexes (8), protease treatment would be an effective method to release the externally bound liposomes. RDM-4 cells were allowed to take up immunoliposomes for 4 h at either 4 or 37 °C, the unbound liposomes were washed away and the cells were subjected to a mild protease treatment to see what fraction of the cell-associated liposomes could be released. When the liposome-cell incubation was done at 4 °C, 90% of the bound liposomes could be released by the protease treatment (Table I). In contrast, 70% of the liposomes taken up by cells at 37 °C were not released by the same protease digestion. The results suggest that the liposomes taken up by RDM-4 cells at 4 °C are primarily associated with the cell surface while a large per cent of those taken up at 37 °C are internalized and, therefore, are inaccessible to the protease digestion.

Effects of Endocytosis Inhibitors on Immunoliposome Uptake—The effects of endocytosis inhibitors on the uptake of anti-H-2Kk immunoliposomes at 37 °C were investigated in an attempt to elucidate the mechanism of internalization of immunoliposomes by RDM-4 cells. Endocytosis has been shown to be dependent on microfilament functions (19) and immunoliposomes by RDM-4 cells. Endocytosis has been shown to be dependent on microfilament functions (19) and

| Treatment | 4 °C | 37 °C |
|-----------|-----|------|
|           | $^{3}H$-cpm | $^{3}H$-cpm |
| Control   | 14,464 ± 867 | 29,609 ± 2,264 |
| Protease  | 1,724 ± 744 | 20,670 ± 1,899 |
| % released by protease | 88.1 | 30.2 |

TABLE I
Sensitivity of immunoliposome uptake by RDM-4 cells to protease

IgG concentration was 5 μg/ml (1.4 × 10$^6$ cpm/μg), and lipid concentration was 100 μg/ml (3.0 × 10$^6$ cpm/μg) in cell-liposome incubation mixture.

(6). These data suggest that endocytosis is an important mechanism by which anti-H-2Kk immunoliposomes are taken up into RDM-4 cells at 37 °C.

Incubation of CF-containing Immunoliposomes with Cells—CF was trapped inside anti-H-2Kk or P3 IgG liposomes in a 10 or 200 mM CF solution. By measuring the fluorescence intensities of the liposome preparations in the presence and absence of Triton X-100 (final concentration 0.2%), one can determine the degree of self-quenching of CF in the vesicles (23). The CF trapped in liposomes prepared in a 10 mM CF solution was 60% quenched while that prepared in a 200 mM solution was 90–95% quenched. The degree of CF quenching in these vesicle preparations remained constant during a period of 3 to 4 days when the vesicles were stored at 4 °C in PBS, indicating that the CF was stably trapped inside the liposomes and very little leakage had occurred. Relatively high concentrations of CF were used in these experiments, because it has been shown that once CF is delivered to the cytoplasm of the cells it is greatly diluted and becomes highly fluorescent (24), thus maximizing the chance of detection by fluorescence microscopy.

CF-containing anti-H-2Kk immunoliposomes were incubated with RDM-4 or P3 cells at 4 °C. No detectable fluorescence was observed on the P3 cells (micrographs not shown). In contrast, RDM-4 cells incubated with anti-H-2Kk immunoliposomes containing CF showed bright fluorescence on the cell peripheries (Fig. 2A). The uniform ring appearance of fluorescence indicated that the liposomes were binding on the surface of the cells. When the liposome-cell incubation was done at 37 °C, a distinctly different pattern of fluorescence was observed (Fig. 2B). First of all, a nonuniform distribution of fluorescence appeared, the fluorescence was much more punctate and there were patches of strong fluorescence on the cells. Secondly, one began to see diffused fluorescence occurring intracellularly, indicating the release of CF inside the cells. P3 cells incubated with the same immunoliposomes at 37 °C did not show significant fluorescence, once again demonstrating the specificity of cell-immunoliposome interaction (micrographs not shown). In some experiments, CF-containing anti-H-2Kk immunoliposomes were incubated with RDM-4 cells at 4 °C for 3 h, at which time the unbound liposomes

![Graph](image-url)

**FIG. 1.** The uptake kinetics for anti-H-2Kk immunoliposomes by RDM-4 cells. •—•, uptake at 4 °C; ○—○, uptake at 37 °C.
FIG. 2. Fluorescence labeling of RDM-4 cells by CF-containing anti-H-2K<sup>a</sup> immunoliposomes. RDM-4 cells were incubated with (A) 10 mM CF-containing anti-H-2K<sup>a</sup> liposomes for 6 h at 4 °C (a–h), (B) 200 mM CF-containing anti-H-2K<sup>a</sup> liposomes for 6 h at 37 °C (i–n); unbound liposomes were washed away and cells were observed under a fluorescence microscope. C, cells were incubated with 200 mM CF-containing anti-H-2K<sup>a</sup> liposomes at 4 °C for 3 h, incubation was continued for an additional 3 h at 37 °C after unbound liposomes were washed away (o–t). a, b, c, d, i, j, m, o, p, s are phase contrast micrographs (exposure time 1 s) and e, f, q, h, k, l, n, q, r, t are fluorescence micrographs (exposure time 30 s). Bar is 5 μm.

were washed away and the incubation continued for an additional 3 h at 37 °C. RDM-4 cells treated in this fashion showed primarily diffused intracellular fluorescence (Fig. 2C), and the patchy fluorescence appearance was absent. In other control experiments, P3 IgG liposomes with entrapped CF did not label RDM-4 nor P3 cells under any of the incubation conditions used (micrographs not shown), demonstrating the immunospecificity for the internalization of the liposomal contents into cells.

Cytoxicity of MTX Entrapped in Anti-H-2K<sup>a</sup> Immunoliposomes—Experiments were performed to determine whether anti-H-2K<sup>a</sup> immunoliposomes could serve as effective drug carriers to accomplish specific target cell cytotoxicity. MTX, a potent inhibitor of the enzyme dihydrofolate reductase, was trapped inside immunoliposomes for this purpose. MTX could be quite stably trapped in immunoliposomes prepared by our method. Very little leakage occurred when the preparation was stored in PBS at 4 °C for 3–4 days, as judged by Sephadex gel filtration. When incubated in RPMI 1640 growth medium with 10% fetal calf serum at 37 °C, 80–85% of the MTX remained entrapped after a 24-h period. The presence of either RDM-4 or P3 cells in the incubation medium did not appear to affect the rate of leakage.

The inhibition of [3H]dUrd incorporation into cellular DNA was used as an assay for MTX action. This assay faithfully reported the cytotoxicity of MTX, since its result correlated very well with the decrease in cell growth as measured by the enumeration of viable cells and assay of total cellular protein in the culture (data not shown). As controls, the effects of free MTX, empty anti-H-2K<sup>a</sup> liposomes not containing MTX (anti-H-2K<sup>a</sup>-lipo) and P3-IgG-lipo-MTX or in bare liposomes not carrying any IgG in the bilayers (lipo-MTX) but with free native anti-H-2K<sup>a</sup> added externally, were also determined. RDM-4 cells were incubated with various doses of different MTX preparations at 37 °C for 3 h, the unbound liposomes and free drug were washed away, [3H]dUrd was added with fresh medium and the extent of [3H]dUrd incorporation into cellular DNA was assayed. Under these conditions, empty anti-H-2K<sup>a</sup> liposomes, P3-IgG-lipo-MTX and lipo-MTX with free anti-H-2K<sup>a</sup> did not show any significant effect on the [3H]dUrd incorporation into RDM-4 cells (Fig. 3a). Free MTX inhibited [3H]dUrd incorporation in a dose-dependent fashion with an ID₅₀ of 2.3 μM (Fig. 3a). Anti-H-2K<sup>a</sup>-lipo-MTX was more potent than free MTX, with an ID₅₀ of 1.3 μM. At a dose of 4.5 μM of anti-H-2K<sup>a</sup>-lipo-MTX, the incorporation of [3H]dUrd was inhibited 90%. When the same experiment was done on P3 cells, the only effective inhibitor of [3H]dUrd incorporation was free MTX, with an ID₅₀ of 1.2 μM (Fig. 3b). Anti-H-2K<sup>a</sup>-lipo-MTX, while very effective in RDM-4 cells, was completely ineffective in P3 cells. This clearly demonstrated the target-specific cytotoxicity of MTX encapsulated in the anti-H-2K<sup>a</sup> liposomes.

The inhibitor studies mentioned before suggested that a significant proportion of the immunoliposomes interacting with target cells was probably endocytosed into the target cells (Table II). Consequently, experiments were performed
a significant portion of the target-specific cytotoxicity induced by anti-H-2K<sup>k</sup>-lipo-MTX is due to a chloroquine-sensitive process, most likely related to the endocytic entry of the anti-H-2K<sup>k</sup> liposomes into target RDM-4 cells.

Cytotoxicity of Anti-H-2K<sup>k</sup> Immunoliposomes Containing araC—If the endocytosis of immunoliposomes plays a major role in the target-specific cytotoxicity of anti-H-2K<sup>k</sup>-lipo-MTX, a cytotoxic drug that is labile to lysosomal hydrolases should be ineffective in target-specific cytotoxicity when en-

![Diagram](http://www.jbc.org/)

**FIG. 4.** Reversal of cytotoxicity induced by MTX-containing anti-H-2K<sup>k</sup> liposomes by chloroquine. Effects of free MTX (●—●), anti-H-2K<sup>k</sup> liposomes containing MTX (○—○) and P3 IgG liposomes containing MTX (△—△) on [H]dUrd incorporation into RDM-4 cellular DNA in the presence of chloroquine were assayed according to "Materials and Methods." Effect of chloroquine alone (□—□) was also assayed.

![Diagram](http://www.jbc.org/)

**FIG. 5.** Cytotoxicity of anti-H-2K<sup>k</sup> immunoliposomes containing araC. RDM-4 cells (a) or P3 cells (b) were incubated with free araC (●—●), anti-H-2K<sup>k</sup> liposomes containing araC (○—○), P3 IgG liposomes containing araC (△—△), bare liposomes containing araC incubated with free native anti-H-2K<sup>k</sup> antibody (▲—▲), or empty anti-H-2K<sup>k</sup> liposomes (□—□) for 3 h at 37 °C. Unbound liposomes were washed away; [H]dT incorporation into cellular DNA was assayed as described under "Materials and Methods."
trapped in immunoliposomes. To test this hypothesis, we entrapped araC, which has been shown to be degraded by lysosomal enzymes, into anti-H-2K^k liposomes and tested its effect on the incorporation of [3H]dT into RDM-4 and P3 cells. Under the experimental conditions, free araC inhibited [3H]dT incorporation into RDM-4 cells with an ID50 of 5.6 μM (Fig. 5a). Unlike liposome-entrapped MTX, araC showed significant effect on [3H]dT incorporation when entrapped into different kinds of liposomes, including anti-H-2K^k liposomes, P3-IgG liposomes and liposomes which were devoid of IgG in the bilayers (Fig. 5a). Importantly, araC showed the same dose-response curves with ID50 = 20 μM regardless of what type of liposomes it was entrapped in. Anti-H-2K^k-lipo-
arac demonstrated the same ability to inhibit [3H]dT incorporation as the other two types of liposomes in spite of its unique ability to interact with RDM-4 cells. Similar results were obtained with P3 cells (Fig. 5b); araC entrapped in liposomes again was less potent when compared with free drug and it had about the same potency in all three types of liposomes (ID50 = 13 μM).

DISCUSSION

In previous communications (8, 9), we reported a method for the covalent coupling of monoclonal antibody to liposomal bilayers and that the coupling resulted in specific interaction between the antibody-containing liposomes and target cells. It is of great interest to investigate about the fate of these immunoliposomes after the initial binding on the target cell surface. For the successful use of immunoliposomes as carriers for therapeutic agents, the entrapped contents must gain entry to intracellular sites of action. This can be accomplished in several ways: the liposomal contents could leak out of the liposomes after the binding event and enter the cells as free drugs; or the liposomal bilayer could fuse with the cell membrane with consequent injection of contents into the cytoplasm; or the contents could enter via cellular endocytosis. In this study, we attempted to examine these possibilities using the H-2K^k antigen as a model system.

The uptake of immunoliposomes by target cells is temperature dependent. The uptake at 37 °C indicated a complex time course and the amount taken up at 37 °C was much higher than that at 4 °C (Fig. 1). Protease treatment experiments showed that approximately 90% of the immunoliposomes taken up at 4 °C was susceptible to protease treatment while about 70% of that taken up at 37 °C was protected (Table I). Endocytic inhibitors such as cytochalasin B and a combination of 2-deoxyglucose and sodium azide inhibited uptake of anti-H-2K^k immunoliposomes by RDM-4 cells at 37 °C by about 50% (Table II). These data strongly supported that endocytosis is an important mechanism of immunoliposome uptake at 37 °C; about half of the cell-associated liposomes are internalized after 4-h incubation. Leserman and his group, who used a different approach to attach antibody molecules to sonicated vesicles, also suggested the involvement of endocytosis as an uptake mechanism of liposomes by the target cell system he used (27, 28). The sonicated vesicles Leserman used (500–600 Å average diameter) (4) were smaller than the ones prepared by our method (approximately 1,000 Å average diameter) (17), and in both instances, endocytosis seems to play an important role in vesicle uptake by target cells.

If immunoliposomes are taken up by target cells via an endocytic pathway, they will most likely be delivered into the lysosomal compartment. A weakly acidic compound such as CF, when delivered to the lysosomes, would partition into the cytoplasm from the acidic environment of the lysosomes. Indeed, when RMD-4 cells were incubated with immunoliposomes containing CF at 37 °C, strong intracellular fluorescence was observed (Fig. 2, B and C). Such intracellular release of CF only occurred at 37 °C. At 4 °C only peripheral ring fluorescence could be seen, representing the cell surface binding of immunoliposomes (Fig. 2A). Internalization of the liposomal content was probably not due to the uptake of free drug after it had been released from the liposomes, as CF-containing liposomes carrying P3 IgG in the bilayers did not label RDM-4 cells under identical incubation conditions, although the possibility that the leakage of contents was induced by specific antibody-antigen interaction could not be ruled out. The transfer of the liposomal content was target cell specific; the same anti-H-2K^k immunoliposome preparation did not label P3 cells (H-2K^d). At 37 °C, capping and patching of immunoliposomes were observed (Fig. 2B). This is not surprising in view of the multivalent nature of these immunoliposomes (9). In experiments where RDM-4 cells were allowed to take up CF-containing immunoliposomes at 4 °C and were then incubated at 37 °C after the unbound liposomes were washed away, significantly less patching and capping were seen. Mostly diffused intracellular fluorescence was observed in this case. This suggests that the caps and patches are not stable structures.

Since endocytosis is an important pathway of uptake of immunoliposomes by target cells, it becomes important to consider carefully the agents that are entrapped for therapeutic purposes. First of all, the drug would have to be able to survive the hostile hydrolytic environment of the lysosomes. Secondly, for a drug that has its site of action other than the lysosomal system, it must have the means to transverse the lysosomal barrier membrane and escape into the cytoplasm before it can get to its site of action. MTX, a drug clinically used in cancer chemotherapy, exhibited target cell-specific cytotoxicity when entrapped in anti-H-2K^k immunoliposomes (Fig. 3). Chloroquine, a lysosomotropic amine known to raise the intralysosomal pH of mammalian cells, partially reversed this cell-specific cytotoxicity (Fig. 4). These data are consistent with the idea that a significant portion of the anti-H-2K^k-lipo-MTX-induced cytotoxicity is due to the entry of vesicles via the endocytic pathway and the subsequent escape of MTX from the lysosomes into the cytoplasm. MTX could be transported to the cytoplasm via a folate transport system in the lysosomal membrane. A more likely mechanism is that MTX, being a weakly acidic molecule, would simply partition into the cytoplasm from the lysosomal compartment. In the presence of chloroquine, when the intralysosomal pH is raised to above the pK of the acidic groups on MTX, the partition process should be effectively blocked. There is also the possibility that the lysosomal hydrolases may be inhibited by the chloroquine treatment, therefore preventing the degradation of liposomes and the release of entrapped MTX.

Following a similar line of reasoning, one would expect a drug that is degraded in the lysosomal system to be ineffective when entrapped inside immunoliposomes. AraC was trapped in anti-H-2K^k liposomes to test this hypothesis. The N-glycosidic linkage in araC has been shown to be labile to lysosomal hydrolases and, therefore, araC is not expected to survive as an active drug once it reaches the lysosomes. Results showed that araC entrapped in various types of liposomes was active when incubated with RDM-4 or P3 cells although the potency was lower when compared to free araC (Fig. 5). This cytotoxicity was most likely due to the leakage of araC from the liposomes during the incubation period with cells. This phenomenon was not seen with liposome-entrapped MTX. Juliano observed that the leakage rate of MTX
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from liposomes was 4- to 5-fold slower than that of araC and he suggested that this difference may be due to the fact that a dianion (MTX) was likely to permeate lipid bilayers more slowly than an uncharged molecule such as araC (29). It is important to note that there was no improvement in drug potency when araC was entrapped in anti-H-2K\(^{k}\) liposomes in either the target (RDM-4) or nontarget (P3) cell system. Thus, the anti-H-2K\(^{k}\) immunoliposomes have lost their ability to induce target cell-specific cytotoxicity when the entrapped agent is araC, a drug that is degraded in the lysosomal system.

From the data in Fig. 3, it is possible to estimate the level of intracellular MTX which is required for cytotoxicity. Assumption is made that under the incubation conditions the intracellular MTX concentration has equilibrated with that of the extracellular medium for RDM-4 cells treated with free MTX. At 2.3 \(\mu\)M of the free MTX, the total intracellular MTX is 2.5 \(\times\) 10\(^{-10}\) mol per 4 \(\times\) 10\(^5\) cells assuming a cell diameter of 8 \(\mu\)m. Although this amount may be an underestimate since MTX might be accumulated intracellularly, immunoliposomes can easily deliver this level of drugs to the cells. For example, at the ID\(_{50}\) of anti-H-2K\(^{k}\)-lipo-MTX (1.3 \(\mu\)M), it represents about 0.1% of the total immunoliposomes in the incubation medium. The cellular uptake of immunoliposomes is generally in the range of a few per cent. Thus the immunoliposomes are effective in delivering drugs to the target cells, provided that the drug can escape from the lysosomes.

In conclusion, this study has demonstrated the feasibility of using antibody-coated liposomes as targeted carriers of pharmaceutical agents. Endocytosis appears to be a very important pathway of immunoliposome uptake by target cells. In view of this, it is essential to carefully consider the type of compounds that are entrapped for therapeutic purposes. For agents with sites of action other than the lysosomal system, they must be evaluated on the basis of their stability in the extracellular medium. For example, at the ID\(_{50}\) of anti-H-2K\(^{k}\)-lipo-MTX, they must be evaluated on the basis of their stability in the extracellular medium. For example, at the ID\(_{50}\) of anti-H-2K\(^{k}\)-lipo-MTX, the total intracellular MTX is 4- to 5-fold slower than that of araC (29).

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