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Receptor-mediated endocytosis of antibody-opsonized liposomes by tumor cells

(Fc receptor/fluorescence-activated cell sorter/chemotherapy/lipids/phagocytosis)

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ABSTRACT **Specific receptor-mediated delivery of the contents of small, sonicated liposomes was studied with three murine tumor cell types: an IgG Fc receptor-negative nonphagocytic line (EL4); an Fc receptor-positive phagocytic line ($P388D_1$); and an Fc receptor-positive nonphagocytic line (P388). The liposomes (formed from phosphatidylcholines, cholesterol, and dinitrophenyl-substituted phosphatidylethanolamine) contained carboxyfluorescein as a fluorescent marker and methotrexate as a pharmacologic agent. Binding and internalization of the liposomes were observed by fluorescence microscopy and measured by flow microfluorometry. The hapten-derivatized lipid was used as a binding point on the liposome for the antibody-combining site of the immunoglobulin. In the presence of IgG anti-dinitrophenyl, but not $F(ab')_2$ or IgA anti-dinitrophenyl, liposomes bound to the Fc receptor-bearing cells. The liposomes underwent endocytosis by the $P388D_1$ cells and, to a lesser extent, by the P388 cells. As measured by depression of [^{3}H]deoxyuridine incorporation, methotrexate in IgG-opsonized liposomes had a much greater pharmacologic effect on the $P388D_1$ cells than did the same amount in unopsonized liposomes or in free solution. This observation indicates that an appropriately chosen drug, incorporated in liposomes, can exert its effect on a cytoplasmic target after endocytosis. P388 cells showed a moderate effect of the drug in liposomes. Neither P388 nor $P388D_1$ cells bound or ingested unopsonized liposomes, and the Fc receptor-negative EL4 line neither bound nor ingested opsonized liposomes. The data demonstrate specific interaction of opsonized liposomes with the cells' IgG Fc receptor.**

Many of the therapeutic and biological applications envisioned for liposomes (for recent reviews, see refs. 1–4) require direction of the liposomes to specific target cells or tissues (5, 6). Previously, we explored the possibility of such direction to lymphoid cells: dinitrophenyl (DNP) derivatized liposomes were cross-linked by sheep anti-DNP antibody to human lymphocytes whose membranes had been modified with trinitrobenzenesulfonate (7); MOPC 315 murine myeloma cells, which express surface immunoglobulin with affinity for the dinitrophenyl (DNP) hapten, bound liposomes bearing DNP but not liposomes without the hapten (8); another murine myeloma, TEPC 15, which has affinity for phosphorylcholine (PC), specifically bound liposomes bearing the PC-hapten coupled to phosphatidylethanolamine by a six carbon spacer (unpublished data).

In those studies we used the water-soluble fluorophore carboxyfluorescein (CF), encapsulated within the liposomes during sonication, to assess liposome–cell association (7, 9, 10). Although liposomes became bound to the cells, little or none of their contents entered the cytoplasm. This conclusion was reinforced by the failure of methotrexate (Mtx) encapsulated in the haptenated liposomes to exert a pharmacological effect on the cells.

Because the nonphagocytic cells used did not internalize specifically bound liposomes or their contents, we undertook the present studies to test the possibility of specific delivery by receptor-mediated endocytosis. We used three types of murine cells: an IgG Fc receptor-negative, nonphagocytic line (EL4); an Fc receptor-positive, highly phagocytic line ($P388D_1$); and an Fc receptor-positive line with little or no phagocytic capacity (P388). Hapten-modified lipids incorporated into the liposome bilayer acted as binding points for the antigen-binding sites of immunoglobulin, the Fc portions of which were free to bind to cellular Fc receptors and initiate phagocytosis (11). In a conceptually similar vein, Weissmann and coworkers (12, 13) attached heat-aggregated immunoglobulin nonspecifically to liposome surfaces to enhance phagocytic uptake of enzymes into enzyme-deficient lysosomes. Here we show that receptor-mediated endocytosis can also result in pharmacologically effective delivery of an anti-tumor agent to the cytoplasmic compartment.

MATERIALS AND METHODS

Tumors. The methylcholanthrene-induced DBA/2 tumor P388, obtained from the Division of Cancer Treatment (National Cancer Institute) was maintained by serial intraperitoneal passage in female DBA/2 mice from The Jackson Laboratory. $P388D_1$ (14, 15) originated from cells cultured from a DBA/2 mouse in which the P388 tumor was being passed (16). The $P388D_1$ cells, maintained in medium containing 10% heat-inactivated (56°C, 30 min) fetal calf serum, were a kind gift of J. F. Jones (National Cancer Institute). The C57BL/6 lymphosarcoma EL4 was a gift from John Wunderlich (National Cancer Institute). More than 80% of $P388D_1$ cells phagocytosed latex particles in a 4-hr assay. P388 and EL4 cells were obtained from ascites 6–7 days after intraperitoneal injection of 10^6 tumor cells. Fewer than 10% of cells so obtained ingested latex beads, and these appeared morphologically to be peritoneal macrophages. Most of these macrophages were removed with iron. P388 and EL4 gave essentially the same results when the carbonyl iron step was omitted.

Abbreviations: Mtx, methotrexate; CF, carboxyfluorescein; d[^{3}H]Urd, deoxy[6′-^{3}H]uridine; DNP, dinitrophenyl; TNP, trinitrophenyl; Ste_2-PtdCho, distearoyl phosphatidylcholine; Pam_2-PtdCho, dipalmitoyl phosphatidylcholine; Ole_2-PtdCho; dioleoyl phosphatidylcholine; Chol, cholesterol; DNP-cap-PtdEtn, DNP coupled through a six-carbon spacer to phosphatidylethanolamine; lipos, liposomes.

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Antibody. Rabbit IgG anti-trinitrophenyl (TNP) was prepared as described (17) and was purified on a column of DNP-lysine-Sepharose 4B, coupled by CNBr. A portion of the antibody was digested with pepsin to make $F(ab')_2$ fragments. Undigested whole antibody was removed on a *Staphylococcus aureus* protein A-Sepharose column (Pharmacia) (18). The intact Ig and $F(ab')_2$ anti-DNP antibodies had hemagglutinin titers of 2^{12} and 2^{11}, respectively, against TNP-modified sheep erythrocytes for equimolar concentrations of antibody, as determined by A_{280} measurements of the affinity-purified protein. IgA anti-TNP was obtained from ascites fluid of BALB/c mice carrying the MOPC 315 tumor. It was purified by the technique of Goetzl and Metzger (19). Heat-aggregated rabbit immunoglobulin was prepared by a modification of the method of Weissmann *et al.* (12), using serum from unimmunized rabbits. It was used at a final concentration of 1 mg/ml.

Liposomes. Liposomes containing 10 mM CF (Eastman) were prepared by sonication using techniques and materials as described (7–10). Mtx (NSC 740) was provided by the Division of Cancer Treatment. To prepare 25 mM Mtx solution, dry Mtx was added to a solution containing 10 mM CF and 1.25% $NaHCO_3$. Dioleoyl, dipalmitoyl, and distearoyl phosphatidylcholines (Ole_2-PtdCho, Pam_2-PtdCho, and Ste_2-PtdCho, respectively) and DNP coupled through a six-carbon spacer to phosphatidylethanolamine (DNP-cap-PtdEtn) (20) were obtained from Avanti Biochemicals (Birmingham, AL). Cholesterol (Chol) was obtained from Sigma and recrystallized twice from ethanol. The lipid mixtures used were Ole_2-PtdCho/Chol/DNP-cap-PtdEtn, 50:47.5:2.5 (molar), and Ste_2-PtdCho/Pam_2-PtdCho/Chol/DNP-cap-PtdEtn, 30:30:37.5:2.5 (molar). Thin-layer chromatography of 100 μg of each mixture gave only the expected spots. Liposome preparations usually contained 8–12 mg of lipid and were sonicated with the microtip of a W-350 probe sonicator (Heat Systems/Ultrasonics, Plainview, NY). Sonications were at 5–10°C (Ole_2-PtdCho liposomes) or 45–50°C (Pam_2-PtdCho and Ste_2-PtdCho liposomes) for 40 min under argon in 3–4 ml of aqueous solution of 10 mM CF, with or without 25 mM Mtx. Liposomes were used within 24 hr of separation from solute on a PD-10 Sephadex G-25 column (Pharmacia). All experiments reported here gave similar results with both lipid preparations.

Radionuclides. Deoxy[6′-^{3}H]uridine (d[^{3}H]Urd; specific activity, 15 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), and [3′,5′,7(*n*)-^{3}H]Mtx were obtained from Amersham. Labeled Mtx for these studies was further purified by DEAE-cellulose chromatography by D. S. Zaharko (National Cancer Institute).

Measurement of Mtx and CF. The Mtx content of each liposome preparation was assessed from the known molar ratio of Mtx to CF. Actual liposome Mtx content, as determined by high-pressure liquid chromatography and by incorporation of radiolabeled Mtx, confirmed that Mtx and CF were encapsulated commensurately. There is some uncertainty about the size of liposomes made with more than 30 mol % Chol (21); if the ones described here were of average diameter (250 Å), each would contain 13 molecules of CF and 33 molecules of Mtx. The fluorescence of CF in liposomes also containing Mtx was decreased by a factor of about 2 with respect to the same concentration of CF encapsulated alone. The true CF content was determined after detergent lysis of liposomes, which eliminates fluorescence quenching (9, 22).

Mtx-Mediated Inhibition of d[^{3}H]Urd Incorporation. Cells (5×10^5/ml) were incubated in triplicate in flat-bottomed wells of 96-well tissue culture plates (Costar) in 0.1 ml of Eagle's minimal essential medium supplemented with 2 mM glutamine, 10 mM Hepes buffer, antibiotics, and 10% heat-inactivated fetal calf serum. Antibody was added at the beginning of incubation; 15 min later, free Mtx, liposomes containing CF, or liposomes containing both CF and Mtx were added. Mtx concentration was varied by changing the concentration of liposomes loaded with 25 mM Mtx. After 1–3 hr of incubation at 37°C, 1 μCi of d[^{3}H]Urd was added to each well. After an additional 10–18 hr, cells were collected with an automatic harvester, and incorporation of ^{3}H was determined by liquid scintillation. The Mtx-induced depression of d[^{3}H]Urd uptake was stable during this interval.

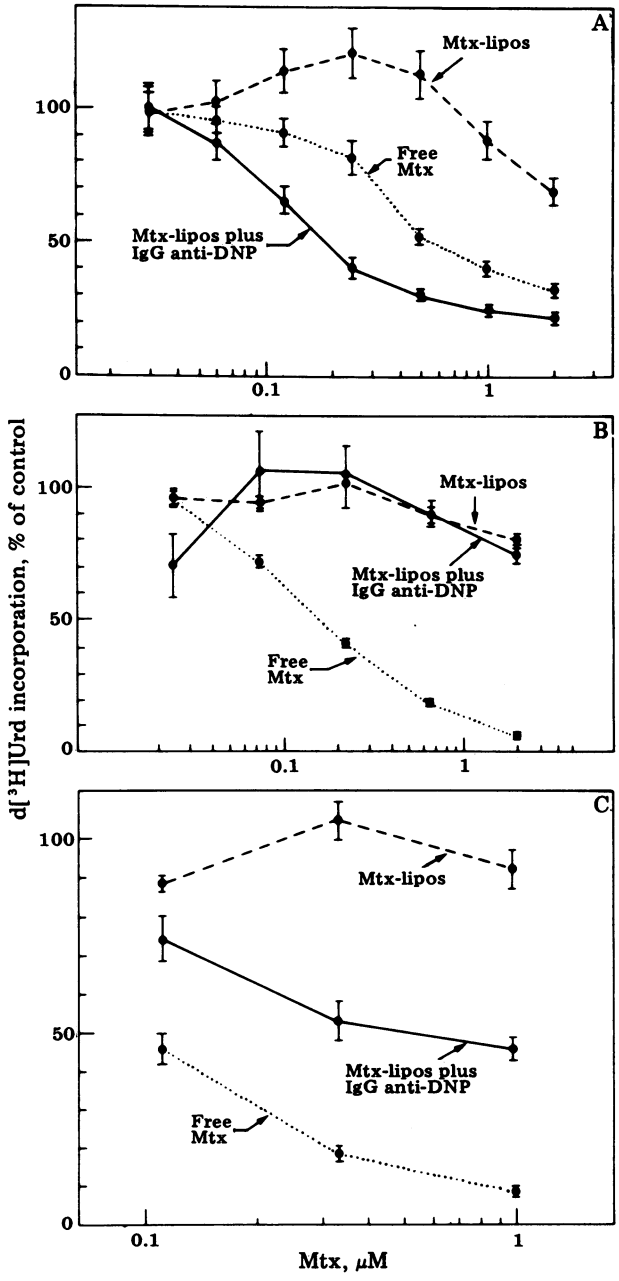

FIG. 1. Inhibition of d[^{3}H]Urd incorporation into murine tumor cells (50,000 cells per incubation) by Mtx free in solution or in DNP-bearing liposomes (lipos) in the presence or absence of IgG anti-DNP. The cells—(*A*) $P388D_1$; (*B*) EL4; (*C*) P388. d[^{3}H]Urd was added after 3 hr, and cultures were harvested after overnight incubation. Results are expressed as percentage of d[^{3}H]Urd incorporation relative to controls incubated without Mtx. Liposomes used were Ole_2-PtdCho/Chol/DNP-cap-PtdEtn, 50:47.5:2.5 (molar). Results are shown as mean ± SD.

Analysis of Cell Fluorescence. Cells (3×10^6) were preincubated with or without antibody for 15 min in polystyrene tubes containing 0.5 ml of Eagle's medium with 10% heat-inactivated fetal calf serum. Liposomes were then added in small volumes, the tubes were gently shaken, and incubation was continued for an additional 20 min at the same temperature (4°C or 37°C). Use of fetal calf serum largely eliminates nonspecific binding and transfer of solute to the cells (10). In some experiments, heat-aggregated IgG from unimmunized rabbits or a combination of metabolic inhibitors (0.1% NaN_3 and 50 mM 2-deoxy-D-glucose) was added 30 min prior to the antibody. After incubation the cells were washed several times in Eagle's medium/10% fetal calf serum, and cell-associated fluorescence was evaluated by fluorescence microscopy and flow microfluorometry. Our use of the FACS-II (Becton-Dickinson Electronics, Mountain View, CA) for analysis of cell-associated fluorescence has been described in detail (7, 9, 10). After the cells were washed, 5-μl samples were taken for fluorescence microscopy at ice or room temperature (8).

RESULTS

Inhibition of d[^{3}H]Urd Incorporation. All tumors were sensitive to Mtx free in solution, but they were much less sensitive to liposome-encapsulated Mtx (Fig. 1). Liposomes made without Mtx had no effect on d[^{3}H]Urd incorporation by any of the tumor cells in the presence or absence of anti-DNP antibody and did not alter their sensitivity to free Mtx (data not shown). With IgG anti-DNP antibody, however, P388D_1 tumor cells showed considerably increased inhibition of d[^{3}H]Urd uptake when incubated with Mtx-containing liposomes. In a series of experiments of the type shown in Fig. 1, Mtx in IgG-opsonized liposomes caused 50% inhibition at one-fourth to one-third the concentrations than did free Mtx and at one-tenth the concentrations than did Mtx in unopsonized liposomes. In marked contrast, addition of IgG anti-DNP antibody did not alter the effect of liposomal Mtx on EL4 tumor cells (Fig. 1*B*). P388 showed an intermediate pattern (Fig. 1*C*)—that is, antibody increased the activity of liposome-bound Mtx, but inhibition of d[^{3}H]Urd uptake was never as pronounced as that caused by the equivalent amount of Mtx free in solution.

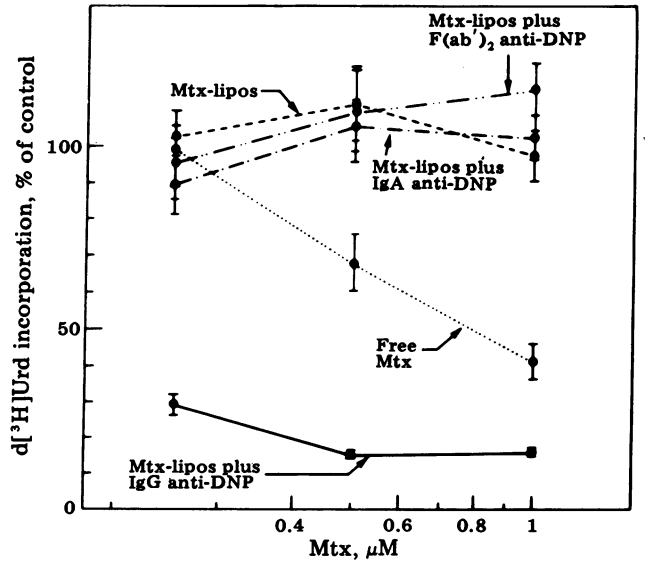

FIG. 2. Effect of various forms of anti-DNP antibody on uptake of Mtx-containing liposomes by 50,000 P388D_1. The cells were incubated in the presence of free Mtx or Mtx in DNP-bearing liposomes. IgG, F(ab')$_2$, or IgA anti-DNP was added (to 40 μg/ml) to wells containing liposomes. After 3 hr, d[^{3}H]Urd was added. Liposome composition, protocol, and data presentation are as in Fig. 1.

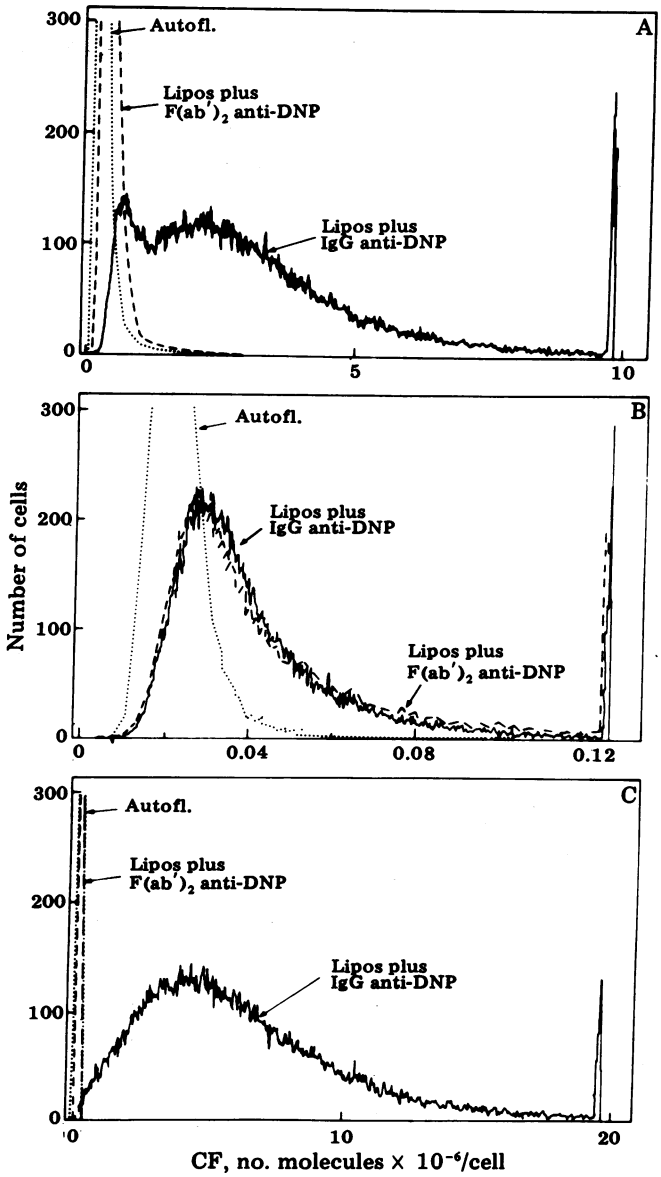

FIG. 3. Flow microfluorometric profiles of P388D_1 (*A*), EL4 (*B*), and P388 (*C*) cells incubated with DNP-liposomes in the presence of IgG or F(ab')$_2$ anti-DNP. Liposomes contained 10 mM CF; the concentration of CF in the incubations was 1.3 μM. Liposome composition was Ste_2-PtdCho/Pam_2PtdCho/Chol/DNP-cap-PtdEtn, 30:30:37.5:2.5 (molar). Note the differences in abscissa scales for *A*, *B*, and *C*.

To assess the role of the IgG Fc region in the enhanced Mtx effect we incubated P388D_1 cells with MTX-containing DNP-bearing liposomes and added 40 μg of intact rabbit IgG anti-DNP, of IgA anti-DNP (MOPC 315 protein), or of the F(ab')$_2$ fragment of IgG anti-DNP per ml (Fig. 2). Only the intact IgG anti-DNP antibody augmented the inhibition of d[^{3}H]Urd incorporation. A pattern similar to that in Fig. 2 was obtained for different concentrations of antibody from 20–80 μg/ml. Anti-DNP antibody did not augment the effect of free Mtx or the uptake of Mtx from liposomes made without DNP (data not shown).

Fluorescence Analysis of Cells Incubated with Liposomes. Fig. 3*A* shows fluorescence profiles obtained by flow microfluorometry for P388D_1 cells incubated with DNP-vesicles and either intact IgG anti-DNP or F(ab')$_2$ anti-DNP. With the F(ab')$_2$ there was little signal beyond the intrinsic fluorescence of the cells; in the former, most cells were brightly fluorescent.

Table 1. Flow microfluorometric measurements on cells incubated with DNP-liposomes and anti-DNP immunoglobulin

Cell line	Temp., °C	Mean cell fluorescence: No Ig*	IgA anti-DNP (MOPC 315)	$F(ab')_2$ anti-DNP	IgG anti-DNP
$P388D_1$	4	55	37	27	3,130
	37	183	171	86	4,530
P388	4	19	12	8	4,520
	37	17	17	17	10,200
EL4	4	3	3	3	6
	37	25	22	29	27

Pam_2-PtdCho/Ste_2-PtdCho/Chol/DNP-cap-PtdEtn liposomes were incubated with cells for 20 min. Mean cell fluorescence is expressed as the number of CF molecules $\times 10^{-3}$/cell.

* Background "autofluorescence" was as follows: $P388D_1$, 334; P388, 60; EL4, 38. These values have been subtracted from the data to obtain the numbers above.

Fig. 3B shows little fluorescence in either case for EL4 cells (note the difference from Fig. 3A in fluorescence scale). Fig. 3C shows a pattern for P388 similar to that for $P388D_1$. Means of cell fluorescence obtained from histograms similar to those in Fig. 3 are given in Table 1. Both at 4°C and at 37°C, very little fluorescence was associated with the cells, except in the cases of P388 and $P388D_1$ in the presence of whole IgG anti-DNP. A subpopulation of $P388D_1$ cells, perhaps the same as that not taking up latex, showed little fluorescence.

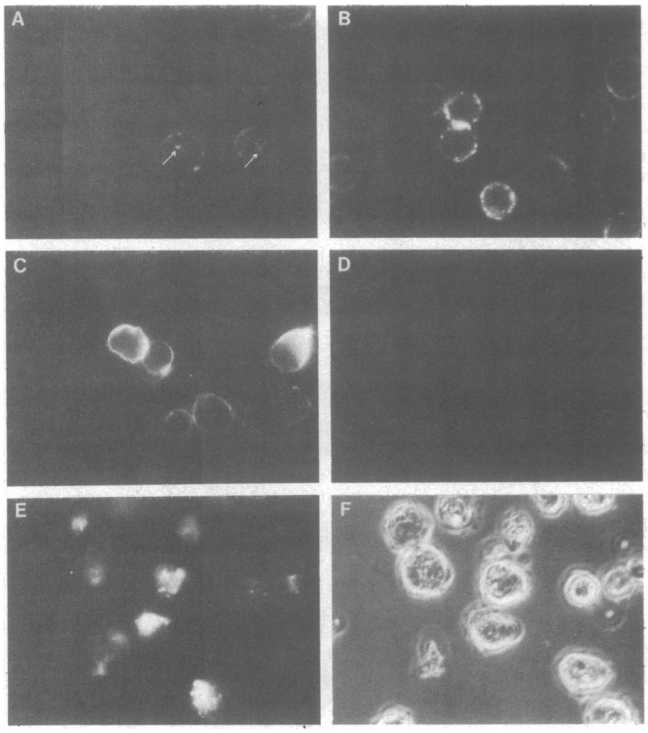

FIG. 4. Fluorescence micrographs of tumor cells incubated at 4°C or 37°C for 20 min with DNP-liposomes containing 10 mM CF; the concentration of CF in the incubation was 1.3 μM. Liposomes of composition Ste_2-PtdCho/Pam_2-PtdCho/Chol/DNP-cap-PtdEtn 30:30:37.5:2.5 (molar) were used. (A) P388 with IgG anti-DNP at 37°C. Arrows indicate punctate accumulations of CF determined, by through-focusing, to be within the cell near the nucleus. (B) Same as A but with 0.2% NaN_3 and 50 mM 2-deoxyglucose. (C) Same as A but incubated at 4°C. (D) Same as A but with $F(ab')_2$ instead of whole IgG anti-DNP. (E) $P388D_1$ cells with IgG anti-DNP at 37°C. (F) Same field as in E but with phase-contrast optics. Not all $P338D_1$ cells showed fluorescence (as expected, given the biphasic profile in Fig. 3A). One cell in E had moved out of the field before the picture in F was taken. (×250.)

Fluorescence Microscopy. Cell samples examined by flow microfluorometry were also examined by fluorescence microscopy (Fig. 4). Little or no fluorescence (above autofluorescence) was seen when any of the three cell types was incubated with DNP-liposomes and IgA anti-DNP, $F(ab')_2$ anti-DNP, or no antibody. EL4 cells failed to bind liposomes even when intact IgG anti-DNP was added (except for a minor population of cells which morphologically resembled peritoneal macrophages). In incubations at 37°C with IgG anti-DNP, $P388D_1$ and P388 cells were of roughly equivalent brightness. Most of the liposomes remained in a patchy pattern at the surface of the P388 cells, but a well-localized aggregation of small fluorescent specks was seen internally, near the nucleus of most cells. Most of the bound liposomes were rapidly internalized by $P388D_1$. NaN_3 plus 2-deoxyglucose eliminated the fluorescence internalization in incubations of P388 and $P388D_1$ with IgG anti-DNP but did not prevent binding. Heat-aggregated IgG blocked binding to $P388D_1$ cells. Incubation at 4°C with IgG anti-DNP resulted in a rather even ring-pattern staining of both P388 and $P388D_1$. The pattern of uptake seen by fluorescence microscopy correlated well with the pharmacologic effects of Mtx in liposomes (Figs. 1 and 2).

DISCUSSION

In previous studies we showed that small liposomes bearing hapten-modified phospholipid could be bound specifically via IgG to the surface of haptenated lymphocytes (7) and via surface immunoglobulin to murine myeloma cells (ref. 8; unpublished data). However, in neither case were contents of the bound liposomes incorporated into the cytoplasm of the cells in significant amounts. The present paper extends these observations to other cell types and demonstrates that specific targeting of a drug to the cells' cytoplasm can be achieved by means of receptor-mediated endocytosis. The results will be discussed in terms of two separate stages: binding and internalization of liposome contents.

In the absence of anti-DNP antibody, there was little or no binding to any of the cell types studied. IgG-opsonized liposomes, however, were bound in large numbers to P388 and $P388D_1$ cells, as determined by fluorescence microscopy and flow microfluorometry. Several lines of evidence indicated involvement of the IgG Fc receptor in this binding: (a) there was no binding to the Fc receptor-negative EL4 cells, whereas P388 and $P388D_1$ (>90% Fc receptor-positive, by rosetting of IgG-opsonized sheep erythrocytes) bound large numbers of liposomes; (b) binding was not seen when liposomes were opsonized with $F(ab')_2$ anti-DNP or with the IgA anti-DNP myeloma protein MOPC 315; (c) binding to $P388D_1$ cells at 4°C was blocked by heat-aggregated rabbit IgG; and (d) nephelometric measurements (unpublished observations) showed that haptenated liposomes were aggregated to approximately the same extent by IgA, IgG, and $F(ab')_2$ anti-DNP, indicating that simple aggregation of liposomes by the IgG antibody cannot account for the binding. An additional indication that the size of the particle does not account for apparent specificity is the finding that hand-shaken multilamellar liposomes also require IgG opsonization for binding to these cells (data not shown). Because the only serum components in the incubations were heat-inactivated or affinity-purified, participation of complement receptors is unlikely.

Uptake after binding was assessed by fluorescence microscopy and by the effect of encapsulated Mtx on cellular incorporation of d[^{3}H]Urd. Any effect of Mtx almost certainly indicates entry of the drug into the cell cytoplasm and binding

to dihydrofolate reductase there. When incubated with Mtx-containing liposomes, EL4 cells showed no antibody-mediated increase of fluorescence and, correspondingly, no Mtx effect beyond that explainable by the small amount of leakage of drug from liposomes into the medium during incubation. Thus, the nontargeted cells were protected from a cytotoxic drug when it was encapsulated in liposomes. A marked increase in the Mtx effect on $P388D_1$ cells was seen when they were exposed to Mtx in IgG-opsonized liposomes, compared with the same concentration of drug free in solution or in unopsonized liposomes. This functional effect corresponds to our observations of rapid internalization by $P388D_1$ cells of IgG-opsonized fluorescent liposomes. The fluorescence observed represents a lower limit on uptake, because flow microfluorometric studies showed considerable efflux of CF from the cytoplasm during the time of incubation (data not shown). Internalization, but not binding, was inhibited when incubations were performed in the cold or at 37°C in the presence of 2-deoxyglucose and NaN_3. The drug effect on the nonphagocytic P388 line was increased when liposomes were mixed with IgG antibody, but it was not as great as for an equivalent amount of free Mtx. We did observe patching of bound opsonized liposomes on the surface of the P388 cells and also a small amount of internalized fluorescence in specks near the nucleus. The specks probably represented CF that had undergone endocytosis because they were abolished by metabolic inhibitors.

The absence of an enhanced drug effect when the highly Mtx-sensitive EL4 cells were exposed to Mtx-containing liposomes and antibody rules out the possibility that liposomes simply leaked their contents into the medium in the presence of antibody. Similarly, augmentation of the effect of liposome-encapsulated Mtx by antibody could not be explained merely by liposome leakage induced by $P388D_1$ cells—for instance, by antibody-dependent cell-mediated cytolysis (23, 24); this could only result in inhibition of d[^{3}H]Urd incorporation equivalent to that mediated by the same amount of free Mtx. Experiments not presented here showed that the presence of anti-DNP antibody and DNP-bearing liposomes made without Mtx did not increase the sensitivity of $P388D_1$ cells to free Mtx. Therefore, the cells are not more sensitive to Mtx when engaged in phagocytosis. The enhanced drug effect suggests that Mtx survived uptake into phagolysosomes and was released from there into the cytoplasm (as would be expected for a relatively small weak anion such as Mtx).

In a seminal series of studies, Weissmann *et al.* (12, 13) used large multilamellar liposomes coated with heat-aggregated immunoglobulin as a means of introducing enzymes into cells. Our studies differed from those in a number of respects: (*a*) use of the hapten-derivatized phospholipid as a binding point for the antibody combining site of the immunoglobulin; (*b*) use of small, highly sonicated liposomes; (*c*) direct demonstration that binding and internalization depend on the Fc receptor (rather than on size of the aggregates, for example); (*d*) inclusion of anti-tumor drug and assay for its biochemical effect; and (*e*) the finding that a small molecule taken up by phagocytosis can escape the phagolysosomal system to reach a cytoplasmic target.

It is not clear at this point to what extent other phagocytic cell types will vary in their uptake of liposomes by means other than the Fc receptor. Preliminary experiments (unpublished data) under conditions of incubation similar to those used here suggest a larger component of Fc receptor-independent uptake by mouse peritoneal macrophages. Therapeutic use of the Fc receptor for liposome uptake by tumor cells might be complicated by the presence of Fc receptors on large numbers of normal cells. However, it might prove possible to exploit differences in ligand specificities of Fc receptors on different cell types.

Note Added in Proof. We recently found that some commercial batches of CF contain hydrophobic impurities not removed by the repurification method in ref. 10 and capable of giving spuriously high numbers for background transfer to cells in the absence of serum. An additional step of purification (on a Sephadex LH-20 column; Pharmacia) is required to remove these impurities. However, they would not significantly affect results in studies such as the ones described here even if present.

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