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Research Article

Cellular Uptake of a Fluid-Phase Marker by Human Neutrophils from Solutions and Liposomes

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In assessing the feasibility of utilizing the phagocytic activity of polymorphonuclear leukocytes (PMNs) for a more efficient drug delivery to the cell, the uptake of the fluid-phase marker lucifer yellow CH (LY) at 37°C by human PMNs from LY-containing liposomes was compared with that from solutions. In the presence of 10% autologous serum, the LY uptake at 37°C via phagocytosis of LY-containing liposomes was generally two orders of magnitude greater than that via pinocytosis for a given PMN source when the concentrations of PMN, LY, and total lipid were in the range of 10^7 cells/ml, 0.5 mg/ml, and 50 μmol/ml, respectively. As expected, the LY uptake via phagocytosis was critically dependent upon the LY entrapment efficiency in the liposome preparation. Interestingly, little LY uptake was found when the serum was heat inactivated (56°C × 30 min). The serum effect was upon liposome vesicles rather than upon the cells. The present study demonstrates that the use of particular drug carriers for targeted drug delivery to PMNs and possibly to an extravascular site mediated by the cell infiltration is a viable approach.

KEY WORDS: human neutrophils; phagocytosis; fluid-phase pinocytosis; particulate drug carriers; liposomes; lucifer yellow CH.

INTRODUCTION

As part of the host defense mechanism, leukocytes such as polymorphonuclear neutrophils (PMNs)[2] carry on surveillance function through continuous emigration from the circulation to extravascular sites. The normal interaction between circulating PMNs and vascular endothelium is, however, dramatically altered by a chemotactic signal generated by extravascular stimuli. In acute inflammation, for example, PMNs attach tightly to the endothelium closest to the site of inflammation, successfully maintain the adhesion, undergo diapedesis at interendothelial junctions, and accumulate at the site of inflammation through extravascular migration. Concurrent with these events are enhanced phagocytosis by activated PMNs toward appropriately opsonized particulate materials such as invading pathogens and microvascular permeability toward macromolecules (1).

We have been interested in the potential utility of enhanced phagocytosis and infiltration of PMNs under certain disease states in targeted drug delivery to the leukocytes and possibly to an extravascular site by means of submicron particulate drug carriers. While future studies will address more relevant questions regarding the neutrophil-mediated extravasations, we wish first to demonstrate that the phagocytic function of our target cell is indeed a sufficient condition for an improved drug delivery by comparing the cellular uptake of a fluid-phase marker from solutions and liposomes. The difference in the uptake between baseline pinocytosis and receptor-mediated phagocytosis should provide some measure of target specificity.

Lucifer yellow CH (LY), a highly fluorescent aromatic sulfonate, was used exclusively in the present study. The compound was recently proven to be a fluid-phase marker toward mouse peritoneal macrophages (2). For a model particulate drug carrier, reverse-phase evaporation vesicles (REVs) (3) were used. In some instances, the liposome was also labeled with [^{3}H]dipalmitoylphosphatidylcholine (DPPC). Human PMNs were harvested from various donors and a large intersubject variation in the uptake rate was noticed. The uptake was monitored at 4 and 37°C in the presence and absence of autologous serum. Implications of LY-entrapment efficiency and other pharmaceutics-related factors associated with a liposome preparation are discussed in comparing the uptake rate from solutions and liposomes.

MATERIALS AND METHODS

Materials

Lipids used to prepare REVs were egg L-α-lecithin and phosphatidylglycerol (Avanti Polar Lipids, Birmingham, Ala.), [^{3}H]DPPC (New England Nuclear, Boston, Mass.), and cholesterol (ICN Biochemicals, Costa Mesa, Calif.).

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[2] Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; HBSS, Hanks' balanced salt solution; HBSS/HEPES, HBSS containing 0.2 *M* HEPES; HEPES, *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid; LY, lucifer yellow CH; PC, phosphatidylcholine or lecithin; PG, phosphatidylglycerol; PMN, polymorphonuclear leukocyte; REV, reverse-phase evaporation vesicle; RES, reticuloendothelial system.

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Hanks' balanced salt solution (HBSS) with or without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, N.Y.) was slightly modified to accommodate 20 mmol of sodium salt of *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES; US Biochemicals, Cleveland, Ohio). It was prepared by dissolving, in a final volume of 1.0 L of glass-distilled water, 5.206 g of Na-HEPES and 87.5 ml of 10× HBSS and adjusting the final pH to 7.30 with 1.0 *N* HCl. The osmotic pressure of the modified buffer (HBSS/HEPES) was generally in the range of 290 to 310 mOsmol/kg H_2O. Lucifer yellow CH (LY) was obtained from Molecular Probes (Eugene, Ore.) and used without further purification. Plastic rather than glassware was exclusively used in handling LY solutions to avoid adsorption on glass surfaces (2).

Harvest of Human PMNs

Human PMNs were isolated from citrated blood of healthy volunteers, ages 18 to 55 years, following a slightly modified literature procedure (4). Approximately 500 ml of blood containing 40 ml of 3.8% sodium citrate was centrifuged at 300*g* for 20 min in 50-ml aliquots. The upper plasma layer was removed, and to the resulting cell pellets, 5.0 ml of 6.0% dextran T-500 (Pharmacia, Piscataway, N.J.) in 0.9% NaCl USP (Kendall McGaw, Irvine, Calif.) was added. The final volume of each tube was adjusted to 50 ml with saline and allowed to sediment for at least 60 min in a 50-ml plastic graduated cylinder. In the meantime, approximately 15 ml of plasma saved from the first centrifugation was further spun at 2800*g* for 20 min at 4°C to obtain platelet-poor plasma, of which 10 ml was added to 30 ml of 0.9% NaCl to make 25% platelet-poor plasma in saline. This was for later use.

When sedimentation of the red blood cells was complete, the top layer was transferred into 4×50-ml plastic tubes and centrifuged at 275*g* for 15 min. To each resulting pellet, 8.0 ml of 25% platelet-poor plasma was added. After gentle trituration with a pipette, each cell suspension was carefully layered over separate 3.0-ml aliquots of Histopaque-1077 (Sigma, St. Louis, Mo.) contained in 15-ml conical plastic centrifuge tubes with screw caps. Upon centrifugation at 750*g* for 25 min, three distinct bands became apparent: a small dark pellet of erythrocytes at the very bottom, a gray layer of PMNs in the middle, and a narrow cloudy white band of monocytes and lymphocytes located about one-third from the bottom. The supernatant all the way to the PMN layer was suctioned off and the side of the tube near the lymphocyte band was wiped with Q-tips. Cell pellets were all combined into 4.0 ml of 0.9% NaCl and transferred into a 50-ml conical plastic tube. Hemolysis of remaining red blood cells was achieved by rapid mixing of the cell suspension with 20 ml of distilled water for 20 sec. Tonicity was then restored by rapidly adding 8.0 ml of 3.6% NaCl. The cell suspension was centrifuged at 300*g* for 5 min, and the hemoglobin-rich supernatant was discarded. The PMNs thus obtained were once again washed with Ca^{2+}- and Mg^{2+}-free HBSS/HEPES before being suspended in HBSS/HEPES for the LY uptake experiments, which usually began within 2 hr of the cell isolation.

The procedure described above generally yielded approximately 5×10^8 cells from 400 ml of citrated blood with greater than 95% purity. The final volume of PMN suspensions was adjusted in such a way that the cell density in the uptake study, 10^7 cells/ml, can be achieved by a convenient dilution. Cell concentration was routinely determined using a Coulter Counter Model ZM after proper dilution in Isoton II (Curtin Matheson Scientific, Houston, Tex.). Cells were identified under a microscope after being stained with Leukostat (Fisher Scientific, Orangeburg, N.Y.).

Preparation and Characterization of REVs

A literature procedure (3) was adopted with some modifications in preparing REVs using a 2:2:1 molar ratio of phosphatidylcholine (PC), cholesterol, and phosphatidylglycerol (PG). Typically, for a 2-ml final preparation, 1.57 ml of 20 mg/ml of PC in $CHCl_3$ (40 μmol), 15.55 mg of cholesterol (40 μmol), and 0.77 ml of 20 mg/ml of PG in $CHCl_3$ (20 μmol) were added to a 50-ml round-bottom flask and the solvent was evaporated using a rotary evaporator. If necessary, [^{3}H]DPPC in a 1:1 mixture of toluene and ethanol was also added at this point. The dried lipids were then dissolved in 6.0 ml ethyl ether which had been kept over a 1.0% sodium bisulfite solution. To this, 2.0 ml of HBSS/HEPES with or without LY was added. The concentration of LY was usually about 10 mg/ml. After the head space was replaced with argon gas (Union Carbide, Linde Division, Danbury, Conn.), the contents were sonicated in a cup horn attached to a Branson Model 350 sonifier (Branson Sonic Power, Danbury, Conn.) for 60 sec with a 50% duty cycle using the power setting of 8. This emulsification step was carried out with 4°C water circulating through the cup horn. The resulting emulsion was further mixed by means of a 10-ml Mixxor (Rainin, Woburn, Mass.). It was then transferred back to the round-bottom flask and sonicated again for 2.0 min. A glass marble of 1.5-cm diameter was placed into the flask and a 75-mm Hg vacuum was introduced for 30 min while the flask was rotated conveniently on an evaporator. This was followed by evaporation at 30 mm Hg for 10 min. In both cases, the controlled evaporation was done at room temperature.

The REV preparation obtained as described above, which is referred to as a primary preparation hereafter, was loaded onto a size exclusion chromatography column containing 60 ml (1.6-cm diameter × 30-cm height) of Sephacryl S-1000 (Pharmacia, Piscataway, N.J.) (5) and eluted with HBSS/HEPES at a flow rate of approximately 0.5 ml/min. The eluent was continuously monitored at 274 nm, where the UV absorption maximum occurs, using a Gilson Model 250 spectrophotometer (Oberlin, Ohio). The fractions containing liposomal suspension were subjected to size analysis. A Nicomp Model 200 laser particle sizer (Nicomp Instruments, Santa Barbara, Calif.) was used with the relative area mode of analysis. Zeta potentials were measured for certain REV preparations using a Model 501 Lazer Zee Meter (Pen Kem, Bedford Hills, N.Y.). The concentration of LY entrapped in and free from liposomes was determined spectrofluorometrically as described below. Once separated, the LY-containing REV preparation was used in the uptake experiment within 5 hr.

Spectrofluorometric Determination of LY Concentration

The fluorescence intensity derived from LY was measured using an SLM/Aminco Model SPF-500 spectrofluo-

rometer (Urbana, Ill.) with the excitation and emission wavelengths set at 430 and 540 nm, respectively. Corresponding bandpasses were 10 and 20 nm. For LY-containing REV preparations, samples were diluted in 75% isopropanol (Burdick and Jackson, Muskegon, Mich.). The magnitude of dilution was such that all lipids were in solution. Standard curves for both aqueous and isopropanol solutions containing known amounts of LY were found to be linear with respect to LY concentration over a range of 0.1 to 50 ng/ml. The assay sensitivity (i.e., the slope of the standard curve) for isopropanol solutions was approximately 1.6-fold greater than for aqueous solutions. Stock solutions of LY were prepared by weighing LY in a room of 20% relative humidity using plasticware.

Uptake of LY by Human PMNs from Solution and REVs

An uptake kinetic experiment was initiated by mixing a PMN suspension in serum-containing HBSS/HEPES and a LY solution or REV preparation in the same buffer, both preincubated at 37°C. Serum was obtained from the blood of the same PMN donor and its final concentration in the mixture was always 10%. When necessary, heat inactivation of serum was carried out by 30-min incubation at 56°C prior to use. The final PMN concentration in the mixture was maintained at 10^7 cells/ml. The experiment was conveniently carried out in a 15-ml plastic tube with a round bottom, which accommodates a small circular magnetic stirrer. The tube was kept in a jacketed beaker through which 37°C water was circulated. In some experiments, samples were analyzed only at two time points, usually at 20 and 40 min, but always in quadruplicate. The procedure adopted for separating PMNs from entrapped LY either from a solution or from a liposomal suspension was essentially the same as the one described in the literature (6). At a given time, an aliquot of 0.5 ml from the mixture was gently layered over, in duplicate or quadruplicate, 0.5 ml of a silicone oil at density 1.05 (General Electric VersilubeR F-50) which had been, in turn, placed over 0.25 ml of 25% sucrose at density 1.10 in a 1.5-ml microcentrifuge tube. It was then centrifuged at 8200*g* for 60 sec using an Eppendorf Model 5415 microcentrifuge (Brinkmann, Westbury, N.Y.). After the liquid layers were suctioned off, the bottom of the plastic tube which contained PMNs was cut away with a razor blade. It was added to 1.0 ml of 0.02% sodium deoxycholate (Sigma, St. Louis, Mo.) containing 0.02% sodium azide (Sigma) in a microfuge tube and was continuously agitated in a wrist shaker for 16 to 20 hr in a 4°C room.

Lowry's protein assay was run for a 0.1-ml aliquot from the cell debris suspension. When the uptake of lipids was also monitored with [^{3}H]DPPC, a 0.3-ml aliquot from the cell debris was subjected to DPM determination on a Beckman Model LS5801 liquid scintillation counter (Irvine, Calif.), after dilution in 10 ml of ACS (Amersham, Arlington Heights, Ill.). The remaining sample in the microfuge tube was centrifuged at 16,000*g* for 2 min. The concentration of LY in the supernatant was determined from the fluorescence intensity measured after proper dilution in water as described earlier. The total amount of LY taken up by PMN at a given time interval was normalized per milligram cell protein. All calculations were based on recovery from the 0.5-ml aliquot withdrawn from the reaction mixture. Uptake rate is expressed in terms of $\mu g\ LY \cdot mg\ protein^{-1} \cdot hr^{-1}$.

RESULTS

Characterization of REV

In the present study, the primary REV preparation was separated by size exclusion chromatography using Sephacryl S-1000. As shown in Fig. 1, separation of the liposomal fraction from unentrapped LY was almost complete. When a total of 50 μmol of a lipid mixture (20 μmol of PC, 20 μmol of cholesterol, and 10 μmol of PG) was processed with 1.0 ml of 10 mg/ml LY solution in HBSS/HEPES, approximately 40% of the total LY was eluted with the REV fraction [i.e., (A + B + C)/(A + B + C + D) = 0.4 in Fig. 1]. The concentration of LY in the combined REV fraction used in the LY uptake experiments, for example, fraction B in Fig. 1, was approximately 0.5 mg/ml. The size of REVs varied depending on the elution time but generally ranged from 0.05 to 1.0 μm. For the LY uptake experiments, fractions were combined in such a way that the average diameter of REVs was in the range of 0.3 to 0.5 μm. Zeta potential was generally −25 mV. When a primary REV preparation was incubated in serum for 30 min at room temperature prior to the size exclusion chromatography, only about 30% of the total LY was associated with the lipids, the zeta potential became more negative (−30 mV), and the average size appeared to be slightly smaller (e.g., 0.49 vs 0.45 μm).

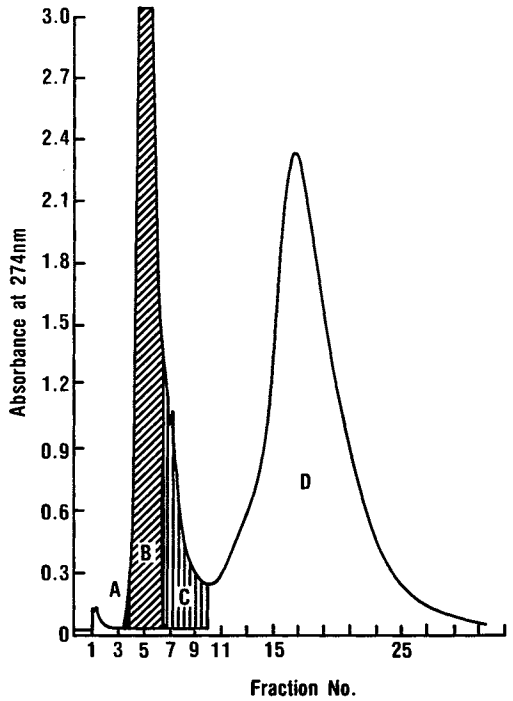

Fig. 1. Typical size exclusion chromatography of 1.0 ml of a REV preparation containing 10 mg of LY and 50 μmol of lipids on a 60-ml Sephacryl S-1000. Eluent was continuously monitored at 274 nm in a flow-through cell of 0.4-cm optical path. Fraction size was approximately 4.5 ml. See text for more details.

Fluid-Phase Pinocytic Uptake of LY by Human PMNs

In fluid-phase pinocytosis, solutes present in the medium enter the cell along with the surrounding medium without any adsorption onto the cell membrane. The process is therefore characterized by a rate which is directly proportional to the solute concentration in the medium (7). As shown in Fig. 2, the accumulation of LY in PMNs followed a curvilinear profile, with the rapid initial uptake representing largely influx and the linear portion after about 30 min representing a dynamic equilibrium between influx and efflux. For a given source of PMNs, both initial and steady-state rates of LY accumulation were found to be proportional to the initial concentration of LY. Depending on the PMN donor, however, both uptake rates were found to vary significantly. A total of 15 PMN donors was employed in the present study, which resulted in 25 sets of initial and steady-state rates. When the rates of all 25 determinations were normalized to an initial LY concentration of 1.0 mg/ml, the average initial and steady-state rates were 423 and 126 ng LY · mg protein^{-1} · hr^{-1}, respectively. The corresponding, standard deviations were as large as 235 and 65 ng LY · mg protein^{-1} · hr^{-1}. The variability in pinocytic activity of PMNs from healthy adults observed in the present study may be added to a long list of functional heterogeneity which has been appreciated for many years (8). Due to the wide range of intersubject variation, all subsequent comparative kinetic experiments were run using a single source of PMNs.

The ratio of the initial to the steady-state rates was significantly less dependent upon the donor [ave. = 3.4 ± 0.86 (SD)]. If one assumes that the initial rate observed represents the true influx rate throughout the time course, it can be estimated that approximately 70% of the internalized LY is returning to the medium when the steady state of accumulation is observed. This is in a fair agreement with the estimate of 80% reported for LY uptake by thioglycollate-elicited mouse peritoneal macrophages (2), although the initial and steady-state LY accumulations in the latter case were about 3.3- and 2.0-fold greater than those determined in the present study.

Phagocytosis of LY-Containing REVs by Human PMNs

In contrast to the fluid-phase pinocytosis of LY discussed above, LY accumulation in PMNs from REVs revealed a sigmoidal profile, when the cells, REVs, and normal autologous serum were mixed together at time zero (Fig. 3). At $t > 30$ min, however, an apparent steady state was observed. The steady-state LY accumulation rate shown in Fig. 3 was approximately 90-fold greater than that observed with the fluid-phase uptake when both rates were normalized to a unit LY concentration.

That the initial lag phase of uptake was due to the time required for the interaction between REVs and the serum component(s) was established by simple preincubation experiments. As shown in Fig. 4A, LY accumulation did not show any initial latency when REVs were preincubated in dilute serum for 15 min at 37°C prior to being added to a PMN suspension. On the other hand, at $t > 5$ min, the LY uptake rate became slower than that observed when the REVs were directly added to a PMN suspension at $t = 0$. This difference is attributed to the instability of REVs in dilute serum, a subject briefly discussed earlier as well as reviewed in the literature (9). Once serum facilitates the leakage of LY from REVs, LY in the medium would enter the cells only via fluid-phase pinocytosis, a cellular uptake process much inferior to phagocytosis. In support of this conclusion, the rate of REV lipid uptake at $t > 5$ min was virtually identical (Fig. 4B). The difference in the lipid uptake at $t < 5$ min shown in Fig. 4B once again indicates the importance of the interaction between REVs and serum. Since all other experiments in the present study were done without preincubation of REVs with serum, subsequent rate comparisons were all made at the apparent steady state.

As shown in Fig. 5, the fluid-phase uptake of LY by human PMNs was affected very little by 10% serum (D vs

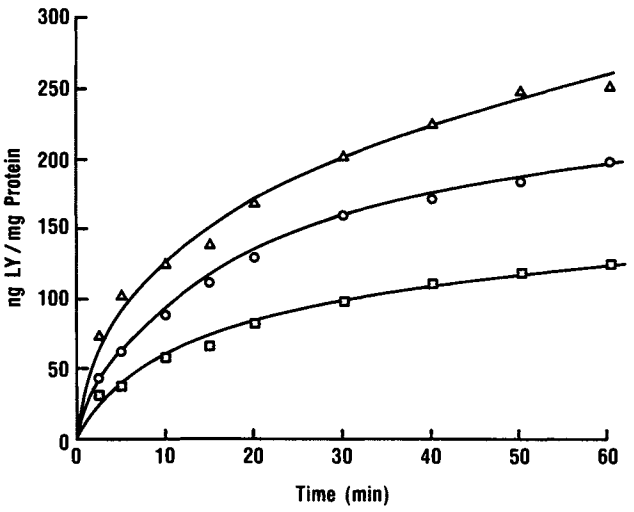

Fig. 2. Fluid-phase pinocytosis of LY by human PMNs at 37°C at initial LY concentrations of 0.817 mg/ml (△), 0.642 mg/ml (○), and 0.408 mg/ml (□). Each point represents the average of duplicate determinations.

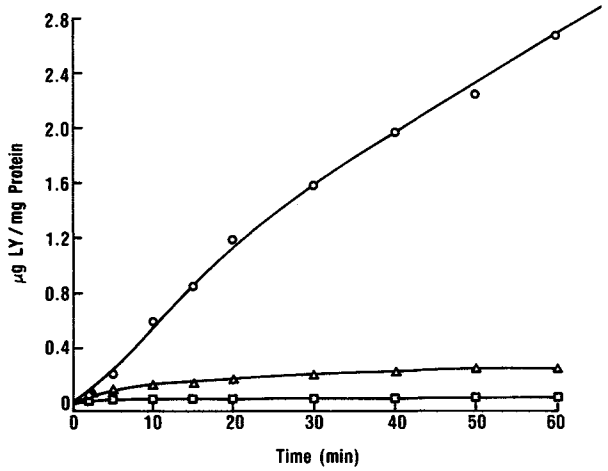

Fig. 3. Accumulation of LY in human PMNs from a LY-containing REV preparation at an initial LY concentration of 0.203 mg/ml at 37°C (○) and 4°C (□) and from a 0.817 mg LY/ml solution at 37°C (△). In all cases, the incubation medium contained 10% autologous serum, and at time zero each component was mixed together at once.

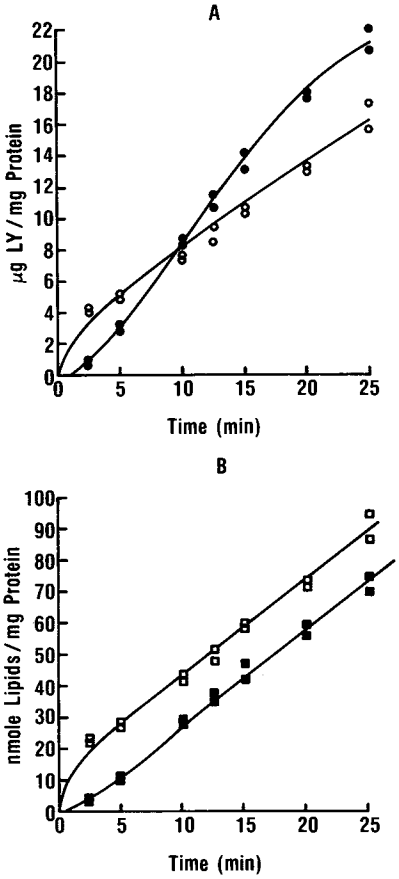

Fig. 4. Uptake of LY from REVs (A) and the lipids in the REV preparation (B) by human PMNs at 37°C. Open symbols represent the uptake from a system in which 2.0 ml of a stock PMN suspension was added at $t = 0$ to a mixture of 1.0 ml of serum and 7.0 ml of a LY-containing REV preparation which had been preincubated at 37°C for 15 min. Filled symbols represent the uptake from a system in which PMNs were preincubated with serum. The concentrations of LY and total lipids in the REV preparation, which was also spiked with [^{3}H]DPPC, were 1.23 mg/ml and 50 μmol/ml, respectively.

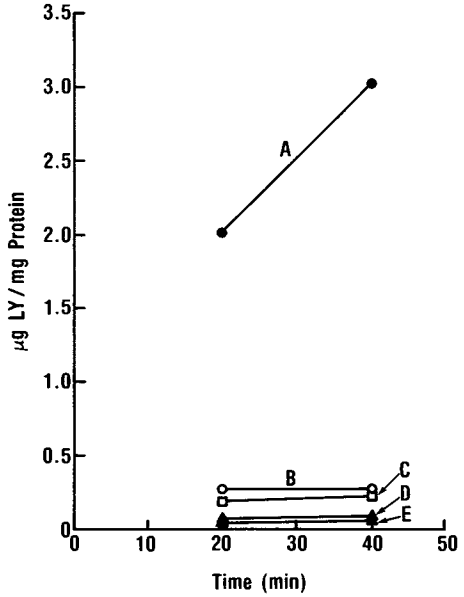

Fig. 5. Effect of 10% serum on the LY uptake by human PMNs at 37°C from a solution (0.81 mg/ml) and from a REV preparation (0.81 mg/ml): A, REVs with intact serum; B, REVs without serum; C, REVs with heat-inactivated serum; D, solution with serum; E, solution without serum. Each data point is an average of quadruplicate determinations.

E). In contrast, phagocytic uptake was significantly influenced by serum (A vs B). Most interestingly, in the presence of heat-inactivated serum, the phagocytic uptake became indistinguishable from that observed without serum (C vs B). Thus, the present study demonstrates intimate involvement of the heat-labile (at 56°C × 30 min) serum component(s) in the REV opsonization. All other kinetic experiments were carried out in the presence of 10% intact autologous serum.

As in the case of fluid-phase pinocytosis, the uptake of LY from REVs was proportional to the initial concentration of LY in the incubation mixture when the concentration of total lipid from REVs and thus the total number of vesicles were maintained constant (Fig. 6). This experiment was conveniently carried out using a series of mixtures of LY-containing REVs and blank REVs, both containing identical radioactivity derived from [^{3}H]DPPC. As expected, the concurrent lipid uptake was identical to one another within experimental error (inset in Fig. 6). Effects of the initial lipid concentration on the uptake of LY and the lipids for the first 20 min are shown in Fig. 7 when the LY concentration was maintained constant. This experiment was carried out by simply diluting an LY-containing REV preparation with a blank liposome preparation prior to incubation with PMNs. For the lipid concentration range studied, the lipid uptake was proportional to its initial concentration without any indication of saturation. On the other hand, LY uptake decreased exponentially as the total lipid concentration increased, reflecting competitive binding and subsequent internalization.

DISCUSSION

Targeted delivery of biologically active substances to peripheral blood PMNs is of therapeutic potential in terms of pharmacological intervention of the roles the leukocytes play within the vascular bed in the pathogenesis of various tissue injuries (10). In addition, it is an intriguing question from a drug-targeting viewpoint whether substances taken up by PMNs can be delivered to an extravascular site to which PMNs are migrating in response to certain inflammatory mediators. It has been known for some time that radiolabeled autologous PMNs when reinjected accumulate in an inflamed site (11). In theory, any property uniquely associated with the target cell should provide a means of targeted drug delivery. The present study is concerned with a hypothesis that the phagocytic function of PMNs, especially when activated by a variety of stimuli, can be utilized for an effective drug delivery to PMNs and possibly to an extravascular site mediated by the migrating cells.

The concentration-dependent uptake observed (Fig. 2), together with a similar study in mouse peritoneal macro-

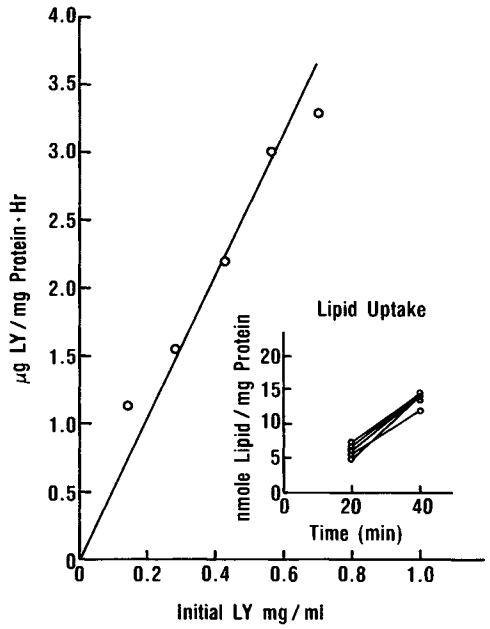

Fig. 6. Rate of LY uptake at 37°C from REVs by human PMNs as a function of the initial LY concentration in the liposome at a constant total lipid concentration, 2.24 μmol/ml. The uptake rates were determined from LY accumulated in PMNs at t = 20 and 40 min. Concurrent lipid uptake as measured using [^{3}H]DPPC is shown in the inset for the same series of REV preparations.

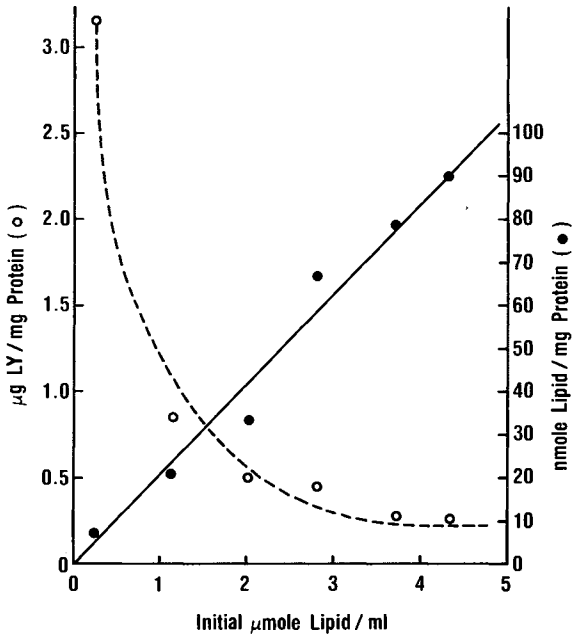

Fig. 7. Amounts of LY (○) and lipids (●) accumulated in human PMNs from REVs for the first 20 min of incubation at 37°C as a function of total lipid concentration at a constant LY concentration, 0.083 mg/ml. The lipid concentration was estimated from [^{3}H]DPPC in the REVs.

phages (2), supports the idea that the LY uptake by human PMNs from solutions is via fluid-phase pinocytosis. That the LY accumulation in PMNs when LY-containing REVs were fed is due to phagocytic internalization, not due merely to binding of the REVs on the cell surface, is supported by the absence of significant LY accumulation in the cells at 4°C (Fig. 3) and the seemingly specific serum effect (Fig. 5). A heat-labile substance(s), possibly C3b complement (12), apparently opsonized the REVs prior to endocytosis. That the serum effect was upon REVs rather than upon PMNs was established in Fig. 4, where the order of preincubation of components was changed. Enhanced uptake by human PMNs of an aqueous marker entrapped in multilamellar vesicles in the presence of serum or heat-aggregated IgG has been known (6), but its effect was far much less significant than in Fig. 5.

Results obtained from experiments, in which the uptake of [^{3}H]DPPC-labeled lipids as well as LY was monitored (for example Figs. 4, 6, and 7), provided an opportunity to test if there existed other lipid transfer mechanisms in addition to phagocytosis. The lipid-to-LY ratio in a liposome preparation used in an uptake experiment was compared with that found in PMNs after incubation for a certain period of time. In general, higher ratios were found in the PMN pellets than in the liposome preparations, especially at a longer time of incubation. For example, the starting lipid-to-LY ratio for experiments shown in Fig. 4 was 3.90 μmol lipids/mg LY. For the experiment in which no preincubation of REVs with serum was made (filled symbols in Fig. 4), the value changed very little throughout the experiment, whereas it increased to as high as 5 at t = 25 min for the experiment in which the liposome was preincubated with serum (open symbols). This observation is consistent with a view that the change in the lipid-to-LY ratio is due to LY leakage from REVs caused by serum and that [^{3}H]DPPC enters the cell at the same rate as LY.

By virtue of the high solute concentration at the cell surface brought about by the binding of molecular aggregates, REVs in the present case, or by a favorable partition onto the cell membrane as expected for many lipophilic substances, phagocytosis and adsorptive pinocytosis provide a much more efficient cellular uptake than fluid-phase pinocytosis (13). Therefore, a priori, a more efficient delivery to phagocytes such as PMNs or macrophages is expected when drug molecules are presented in a form of concentrated molecular aggregates. This will be particularly significant for water-soluble polar substances such as markers of fluid-phase pinocytosis. The difference in the uptake rate between those two different routes of delivery could serve as a measure of target specificity in drug delivery to phagocytes.

Submicron particulate drug carriers appear to be ideal in many aspects for drug delivery to phagocytes (14,15), provided that the carrier is of a high drug loading. In the present study, REV (3) was chosen as a model system, which demonstrated as high as 40% LY entrapment. The importance of the loading efficiency in the cellular uptake of LY is clearly demonstrated in Figs. 6 and 7; the lower the lipid-to-LY ratio, the higher the LY uptake. The most ideal delivery system would be a hypothetical situation in which the system consists of pure solute molecules alone but is still recognized by a receptor-mediated endocytic uptake mechanism. In the present study, the difference in the LY uptake, as measured in terms of the apparent steady-state accumulation rate at $t > 20$ min, between the phagocytosis of LY-con-

taining REVs and the fluid-phase uptake of an LY solution was generally in the range of 100 for a given source of PMNs in the presence of 10% intact serum when the concentrations of PMN, LY, and total lipids per milliliter were approximately 10^7, 0.5 mg, and 5 μmol, respectively. Whether this magnitude of difference will result in a significant difference in drug disposition favorable to PMNs *in vivo* remains to be seen.

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