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Report

Neutrophil-Mediated Transport of Liposomes Across the Madin Darby Canine Kidney Epithelial Cell Monolayer

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Targeted drug delivery to peripheral blood neutrophils (PMNs) should be of therapeutic potential in various disease states. In addition, substances taken up by PMNs in the circulation may be delivered to an extravascular site via the naturally occurring cell infiltration. The present study employs an *in vitro* chemotaxis model to test whether particulate drug carriers such as liposomes can be transported across a cellular barrier by migrating PMNs. The system contained 10^7 human PMNs/ml, 0.3-μm liposomes at a total lipid concentration of 2.5 m*M*, and 10% autologous human serum in the apical side of a confluent Madin Darby canine kidney (MDCK) epithelial cell monolayer of 4.71 cm^2. The MDCK cells were grown on a polycarbonate membrane with 3-μm pores without any extracellular matrix, and 10^{-7} *M* f-Met-Leu-Phe was added to the basolateral side as a trigger of chemotaxis. The aqueous phase of the reverse-phase evaporation vesicles (REVs) contained lucifer yellow CH (LY) and [^{14}C]sucrose. The lipid bilayer of the REVs was spiked with [^{3}H]dipalmitoylphosphatidylcholine (DPPC). Transmission electron micrographs showed that, in response to the formyl peptide, PMNs adhered to the apical surface of MDCK cells, emigrated across the MDCK cell layer, passed through the 3-μm pores in the polycarbonate membrane, and finally, appeared in the bottom well. Epifluorescence micrographs showed that most, if not all, of the migrated PMNs contained punctate fluorescence derived from LY. Transport data over a 3.5-hr period indicated that those markers that appeared in the basal side were indeed transported by phagocytosis of REVs by PMNs and that intact serum was an essential component in the process. The PMN-mediated transport of REVs may serve as a possible targeted drug delivery to an extravascular site *in vivo* in various inflammatory diseases.

KEY WORDS: Madin Darby canine kidney (MDCK) cell monolayer; reverse-phase evaporation lipid vesicles; phagocytosis by neutrophils; neutrophil extravasation; targeted drug delivery.

INTRODUCTION

The polymorphonuclear neutrophil (PMN)[2] comprises approximately 60% of the circulating leukocytes in humans. As part of the host defense surveillance function, about one-tenth of the total-blood PMN pool normally emigrates from the bloodstream each hour to extravascular sites. In tissues, they survive for about 4 days. This 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 in a certain disease state in developing more efficient drug delivery to the leukocytes and possibly to an extravascular site by means of submicron particulate drug carriers. If realized, it should be of therapeutic potential not only for pharmacological intervention of the cell functions within the vascular bed but also for targeted drug delivery to an extravascular site mediated by emigrating PMNs. Our earlier study demonstrated that the cellular uptake of the fluid-phase marker lucifer yellow CH (LY) by PMNs was much more efficient when presented to the cells in liposomes than in solutions (2). While the study might be relevant to targeting to circulating phagocytes within the vasculature, it did not provide any clue regarding whether phagocytosed materials can be delivered by PMNs across the endothelial transport barrier. We present some evidence here for the possible PMN-mediated *in vivo* extravasation of particulate drug carriers.

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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-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; LY, lucifer yellow CH; MDCK, Madin Darby canine kidney; PC, phosphatidylcholine, or lecithin; PG, phosphatidylglycerol; PMN, polymorphonuclear leukocyte; REV, reverse-phase evaporation vesicle.

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The idea of using PMNs as a delivery system to an extravascular site has been proposed for some time. For example, PMNs labeled with a γ-emitter were detected in the abscess after autologous iv administration in humans (3). Similarly, finding intravenously administered liposomes in the lung alveolar macrophages in mice was attributed to extravasation mediated by monocytes which were eventually differentiated to macrophages in the alveoli (4). In these types of *in vivo* studies, interpretation of a given observation is not always straightforward. Thus the hypothesis that infiltrating PMNs can deliver drug substances to an extravascular site still remains to be proven.

In the present study, a literature chemotaxis model (5) was adopted after some modifications. The system uses a Madin Darby canine kidney (MDCK) cell monolayer grown on a polycarbonate membrane with 3-μm pores. It provided the two compartments needed for the chemotaxis. The membrane, 2.45 cm in diameter, is part of a commercially obtained presterilized culture insert for a regular six-well plate. The apical-to-basolateral transport across the MDCK cell layer of two fluid-phase markers entrapped in liposomes as well as a lipid component marker in the liposome was monitored in the presence of PMNs. Chemotaxis was induced by introducing f-Met-Leu-Phe to the basolateral side of the cell layer.

MATERIALS AND METHODS

Materials. Normal and Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (10×) and a powder form of Eagle's minimum essential medium with Earle's salts and L-glutamine (MEM) were obtained from Gibco Laboratories (Grand Island, N.Y.). Medium at normal strength was prepared following the manufacturer's recommended procedure. Fetal bovine serum from HyClone Laboratories (Logan, Utah) was heat-inactivated in a water bath at 56°C for 30 min after being thawed at room temperature. A penicillin/streptomycin mixture, at 5000 U/ml and 5 mg/ml, respectively, and a trypsin/EDTA mixture, 1:250 (1×), were obtained from Flow Laboratories (McLean, Va.) The Transwell, PVP-free, of 24.5-mm diameter and 3.0-μm pore size was obtained from Costar (Cambridge, Mass.) Other plasticware for standard cell culture was from commerical sources such as Fisher Scientific (Pittsburgh, Pa.) and Nalgen Co. (Rochester, N.Y.). The medium for MDCK cell culture was prepared by mixing 900 ml of MEM, 100 ml of heat-inactivated fetal bovine serum, 10 ml of the antibiotic solution, and 10 mmol of each of *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic (HEPES) acid and sodium salt (U.S. Biochemical Corp., Cleveland, Ohio) and sterilizing using 0.2-μm filtration. This mixture is referred to simply as medium hereafter. Similarly normal and Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solutions used in the present study contained 20 m*M* HEPES. These solutions are referred to as HBSS and CMF/HBSS, respectively. Lucifer yellow CH (LY) was obtained from Molecular Probes (Eugene, Ore.) and used without further purification. [^{14}C]Sucrose and [^{3}H]inulin were obtained from New England Nuclear (Boston, Mass.), whereas [^{3}H]dextran was from Amersham (Arlington Heights, Ill.). The latter two nuclides were purified using a Sephadex G-100 (Pharmacia, Piscataway, N.J.) column and HBSS as an eluent just prior to use. Cold inulin and sucrose were obtained from Sigma (St. Louis, Mo.) and Mallinckrodt (Paris, Ky.), respectively. The chemotactic peptide f-Met-Leu-Phe was obtained from Sigma and used without further purification. When the eluent was monitored at 220 nm, the major peak corresponded to 95% purity in a high-performance liquid chromatographic assay with an acetonitrile gradient system containing 0.1% trifluoroacetic acid. [^{3}H]f-Met-Leu-Phe was obtained from New England Nuclear and used as received. Lipids used to prepare reverse-phase evaporation vesicles (REVs) were egg L-α-lecithin and phosphatidylglycerol (Avanti Polar Lipids, Birmingham, Ala.), [^{3}H]dipalmitoylphosphatidylcholine (DPPC) (New England Nuclear, Boston, Mass.), and cholesterol (ICN Biochemicals, Costa Mesa, Calif.).

Cells. The MDCK cell line was obtained from the American Type Culture Collection, Rockville, Md., at serial passage 52. They were subcultured and kept frozen. Cells were thawed and seeded on the Transwell on an "as-needed" basis. Preparation of the cell monolayer was described in detail in a previous study (6). Essentially the same procedure was adopted in the present study, however, the following modifications were made. The seeding concentration was 7.5×10^4 cells/cm^2 and cells were introduced to the Transwell in 1.5 ml of the medium defined earlier. Transport studies were carried out on day 5. General cell morphology, electrical resistance across the cell layer, and cell counts were determined as described before (6).

Human neutrophils were harvested from citrated blood from healthy volunteers. A literature procedure (7) was slightly modified for our studies, which was described in detail elsewhere (2). Briefly, after platelets were eliminated by centrifugation, the plasma was subject to dextran sedimentation, Histopaque-1077 (Sigma) density centrifugation, and hypotonic red cell lysis. Autologous serum was obtained from whole blood following a normal procedure.

Preparation and Characterization of REVs. Reverse-phase evaporation vesicles were prepared following the literature procedure (8) after slight modifications. The procedure was described in detail elsewhere (2). Throughout the study, the lipid composition was maintained at a 2:2:1 molar ratio of egg L-α-lecithin, cholesterol, and phosphatidylglycerol and spiked with [^{3}H]DPPC. The lipid mixture in ether was emulsified in HBSS containing LY and [^{14}C]sucrose in a cup horn sonicator under an argon stream at 4°C. After ether was evaporated under a controlled vacuum, the resulting REVs were separated from unentrapped markers in solution by means of size-exclusion chromatography. A combined REV fraction was then further diluted to a mixture with PMNs and serum immediately prior to the transport study. In this final mixture, the radioactivity was typically 10^7 and 10^6 dpm/ml for ^{3}H and ^{14}C, respectively. Likewise the LY and total lipid concentration was in the range of 0.5 mg/ml and 2.5 μmol/ml, respectively. A liposome preparation, once separated by size-exclusion chromatography, was used within 5 hr. The average diameter of vesicles was determined to be in the range of 0.3 to 0.5 μm by a photon correlation spectroscopy technique (2).

Transport of REVs Across MDCK Cell Layers. On day 5, the medium in which the MDCK cell monolayers had been grown was replaced with 37°C HBSS 60 min before

f-Met-Leu-Phe was introduced. A mixture of REVs, PMNs, and serum was prepared at 37°C 15 min prior to the addition of f-Met-Leu-Phe. The PMN concentration was always 10^7 cells/ml. In some control experiments, REVs were replaced with an HBSS solution containing LY and [^{14}C]sucrose, or serum was omitted. A few minutes prior to the addition of f-Met-Leu-Phe, the HBSS in the Transwell (i.e., apical side of an MDCK cell layer) was replaced with 1 ml of the above mixture. At t = 0, 0.1 of 2.6 ml of HBSS in the bottom well (i.e., basolateral side of an MDCK cell layer) was replaced with 0.10 ml of an f-Met-Leu-Phe stock solution in HBSS at a concentration of 2.6×10^{-6} M. The peptide stock solution was always prepared immediately before use from a solution in dimethyl sulfoxide (Aldrich) at a concentration of about 0.1 mg/ml. In some experiments in which the transport property of the peptide was estimated, [^{3}H]f-Met-Leu-Phe and [^{14}C]sucrose were used. In all cases, the peptide stock solution was kept in an ice bath until a 0.10-ml aliquot was added to 2.50 ml of HBSS at 37°C in a bottom well.

At a given time interval, a Transwell containing the MDCK cells was transferred to another bottom well which had just received 0.10 ml of the peptide stock solution. This manipulation, generally with 30-min intervals, was continued over a 210-min period. After examination under an inverted microscope (see below), PMNs present in the bottom well were detached from the bottom surface by means of a cell scraper (Gibco Catalog No. 925-1150 XT). An aliquot, either 0.5 or 1.0 ml, was subjected to dpm measurement, while another was diluted in 75% isopropanol for LY determination. The latter was shaken overnight to assure complete cell destruction and cell debris were spun down prior to measurement of fluorescence intensity. Conditions for the LY measurement as well as DPM determination were described elsewhere (2,6). Concentrations of markers, LY, sucrose, and DPPC found in the bottom well were converted to a mass unit accumulated in 2.60 ml up to a given point in time.

Microscopic Examination. For rountine observation of PMNs that had appeared in the bottom well, a Nikon Model Diaphot inverted microscope was used, normally with a 10DL or 20DL objective. Epifluorescence microscopy was carried out with a B1 filter combination. For photomicroscopy, a Nikon Model Optiphot upright microscope was used with a Fluor 40 objective. After HBSS was suctioned off from a well, attached PMNs were fixed with 4% paraformaldehyde (Aldrich) in phosphate buffer for 30 min at room temperature. After gently rinsing in HBSS, one drop of 75% glycerol was added to the specimen before a cover slide was placed. Exposure time for a fluorescent specimen was in the range of 50 sec when Tri-X Pan with ASA400 was used.

For transmission electron microscopy (TEM), an MDCK cell monolayer from the transport experiment was rinsed briefly in a warm 0.1 M cacodylate buffer at pH 7.2 containing 5% sucrose and 0.01% $CaCl_2$ and immediately fixed at room temperature with 3.0% glutaraldehyde (Polyscience, Warrington, Pa.) in the same cacodylate buffer for 45 min. After rinsing three times for 5 min each in the buffer, the specimen was fixed with 1.0% osmium tetroxide (Polyscience) in the cacodylate buffer for 45 min. The specimen was again rinsed with the cacodylate buffer three times for 5 min each and dehydrated through serial ethanol solutions beginning at 50%. Dehydration in 100% ethanol at the end was carried out three times for 2 min each. The specimen was then infiltrated in a 1:1 mixture of Poly/Bed 812 kit (Polyscience):ethanol for 30 min, followed by three changes with fresh Poly/Bed 812 resin for 30 min each. The sample was allowed to stand at room temperature overnight, followed by polymerization at 60°C for 48 hr. Ultrathin sections were obtained using a DuPont/Sorvall MT6000 ultramicrotome equipped with a diamond knife. The sections were then stained with 3% uranyl acetate for 10 min and Reynold's lead citrate (9) for 7 min. Sections were photographed using a JEOL 1200EX electron microscope at 60 kV with Type 4489 Kodak film.

RESULTS AND DISCUSSION

The chemotaxis model adopted in the present study consisted of 10^7 PMNs/ml, REVs at a total lipid concentration of approximately 2.5 mM, and 10% autologous human serum in the apical side of a confluent MDCK cell layer and 10^{-7} M f-Met-Leu-Phe in the basal side. The latter was added at the beginning of a transport experiment. The appearance of liposome-associated markers in the basal side was then continuously monitored for a 3.5-hr period. The basic experimental protocol was such that each Transwell was exposed to a fresh f-Met-Leu-Phe solution at 10^{-7} M every 30 min.

In a series of preliminary experiments, we determined the concentration of the formyl peptide along with a fluid-phase marker, sucrose, in the apical side over a 3.5-hr period. This was carried out using HBSS containing [^{3}H]f-Met-Leu-Phe and [^{14}C]sucrose in the basolateral side.

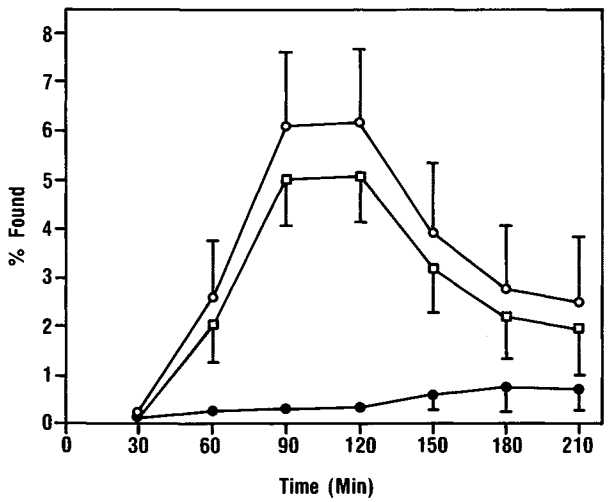

Fig. 1. Concentrations of f-Met-Leu-Phe (circles) and sucrose (squares) found in the apical side of 4.71-cm^2 MDCK cell monolayers with (open symbols) or without (filled symbols) 10% human serum when the concentrations were held constant in the basal side at 10^{-7} and 10^{-6} M for the peptide and sucrose, respectively. Data obtained in the presence of 10% heat-inactivated serum were identical to those obtained without serum within experimental errors. Concentrations are expressed as percentages of the concentrations in the basal side and were calculated from radioactivity measurements. Bars represent one standard deviation for eight and six determinations for the transport experiment with and without serum, respectively.

The appearance of radioactivity was then followed in the apical side, which contained HBSS with or without 10% serum. Here again, every 30 min, the whole content was withdrawn for radioactivity determination and replaced with a fresh solution. As shown in Fig. 1, the concentration of neither marker was at steady state when serum was present. On the other hand, in the absence of serum or in the presence of 10% heat-inactivated (56°C × 30 min) serum, the transport of markers was minimal. Serum was previously reported to increase the permeability of an MDCK cell monolayer (5). At present, we are not able to explain why the permeability decreased again after 2 hr. It is also interesting to note that the peptide transported slightly faster than sucrose. Although the chemical identity could not be determined due to the low concentration recovered in the apical side, decomposition of the peptide would have been minimal, since the molecules are believed to have migrated via a paracellular shunt pathway (6).

In response to a chemotactic profile such as the one depicted in Fig. 1, PMNs adhered to the apical surface of MDCK cells, emigrated across the cell layer, presumably through intercellular junctions, passed through 3-μm pores in the polycarbonate membrane, and finally, appeared in the basal side (Figs. 2 and 3). This series of observations is analogous to the PMN infiltration occurring *in vivo* at an inflammatory lesion (10). The hypothesis we wished to test with the model was that PMNs migrating across the MDCK cell layer can carry liposomes with entrapped substances.

As shown in Fig. 4, most, if not all, of the PMNs recovered in the basal side of the MDCK cell layer were fluorescent with LY originally entrapped in liposomes. Establishing that the markers associated with liposomes which ended up in the basal side were indeed delivered by migrated PMNs required a series of control experiments. Table I lists all possible combinations in the experimental design with PMN, 10% intact human serum, and f-Met-Leu-Phe as independent variables. In the present study, these eight protocols were considered with both liposomes and solution in the donor compartment of the system. They are conveniently designated R and S, respectively.

Some of the quantitative transport data are summarized in Fig. 5. In the 8S series of experiments, in which a sucrose/LY solution was placed in the donor compartment under a fully chemotactic condition, both markers appeared in the basal side extremely rapidly. It has been reported that in the absence of serum, an MDCK cell monolayer still maintains close cell–cell contacts, preventing the leakage of the tracer horseradish peroxidase even with migrating PMNs (11). Thus, the serum must not only enhance the leakage of dissolved solute molecules across the cell monolayer as found in Fig. 1, but also permit the medium drag along the migrating PMNs. In short, the high permeability observed for sucrose (~5% hr^{-1} 4.71 cm^{-2}) and LY (not shown here) most likely represents escape through the epithelial junctions along with migrating PMNs. Such a leakage concurrent with the leukocyte extravasation *in vivo* has been reported to occur without much structural damage to the vasculature (1,12–14).

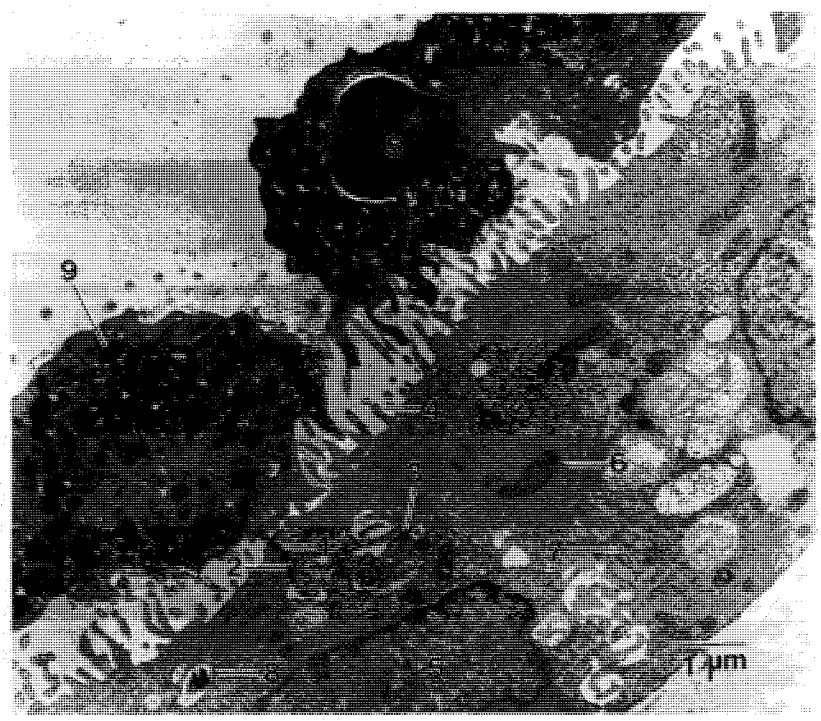

Fig. 2. TEM of human PMNs adhering to the apical surface of confluent MDCK cells: (1) zonula occludens (tight junction); (2) zonula adherens; (3) macula adherens (desmosome); (4) microvilli; (5) nucleus; (6) mitochondria; (7) Golgi apparatus; (8) secondary lysosome; (9) azurophilic granule. This particular sample was obtained 10 min after 10^{-7} *M* f-Met-Leu-Phe was introduced to the basolateral side of the MDCK cell monolayer (i.e., bottom corner of the right). ×15, 500; reduced 40% for reproduction.

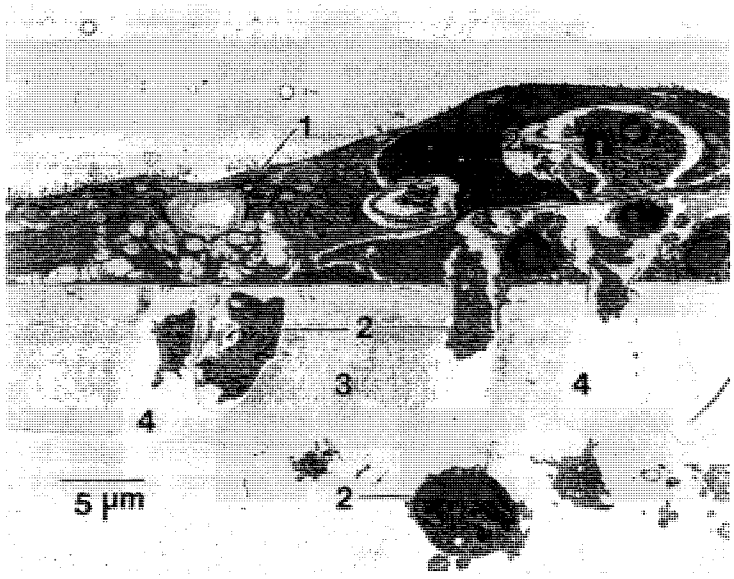

Fig. 3. PMN migration across confluent MDCK cells, 60 min after 10^{-7} *M* f-Met-Leu-Phe was introduced: (1) MDCK cells; (2) PMNs; (3) polycarbonate membrane in a Transwell; (4) 3-μm pores in the membrane. Some PMNs are in the pores and some have already passed through the pores. ×2000; reduced 35% for reproduction.

In the 8R series, the transport of liposome-associated markers across the MDCK cell layer was monitored in the presence of all chemotactic components. As shown by the open symbols in Fig. 5, the transport was much faster than that observed in the 6R series without serum (filled squares, for example). Once again, as in the experiments with solutions, serum enhanced the transport of liposome-associated markers. Its role can be multifaceted. In our previous study (2) it was demonstrated that the phagocytosis of lipsomes by human PMNs requires intact serum. Thus truly PMN-mediated transport of liposomes would have occurred only in the presence of serum. This was consistent with the finding that the 8R series of experiments with 10% heat-inactivated serum resulted in minimum transport of liposomes (data not shown). Also, as shown in Fig. 1, serum provided a higher concentration profile of f-Met-Leu-Phe in the apical side thoughout the study period than without serum. This could have activated PMNs to a greater extent than in the 6R series and resulted in a higher degree of PMN emigration.

As pointed out repeatedly thus far, serum enhances the permeability of a solute in solution across an MDCK cell monolayer. However, the increased permeability was not leaky enough for liposomal particles to pass through the

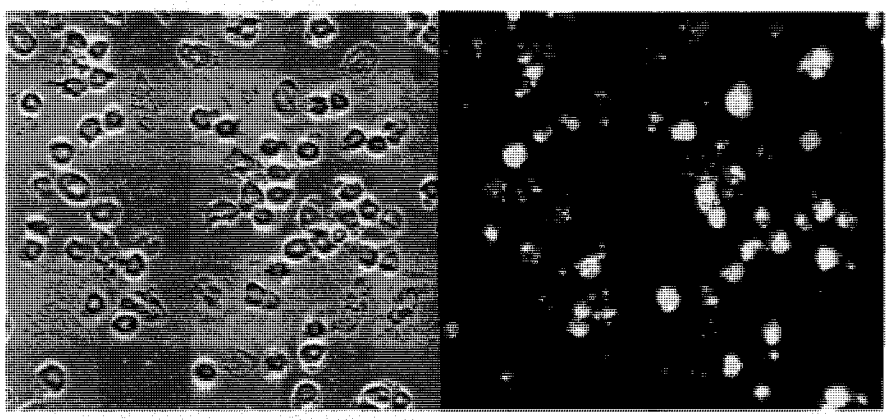

Fig. 4. Phase-contrast light micrograph and the corresponding fluorescence image of human PMNs which have migrated across confluent MDCK cells in response to f-Met-Leu-Phe. PMNs in this particular sample were those recovered in the basolateral side of the cell layer during a 30-min period, from 90 to 120 min after the initiation of chemotaxis, with LY-containing liposomes and 10% human serum in the apical donor side. The PMNs were briefly fixed in paraformaldehyde, rinsed, and immobilized in glycerin. ×475; reduced 35% for reproduction.

Table I. All Possible Experimental Designs for Investigating the Effects of PMN, Serum, and f-Met-Leu-Phe on the Transport Across Confluent MDCK Cell Layers[a]

	Apical side		Basolateral side
No.	PMN	Serum	f-Met-Leu-Phe
1	−	−	−
2	+	−	−
3	−	+	−
4	−	−	+
5	+	+	−
6	+	−	+
7	−	+	+
8	+	+	+

[a] Transport of markers was studied by placing either liposomes (R) or solutions (S) in the apical side of the MDCK cell monolayer. For example, 6R and 7S represent studies on liposome transport in the absence of serum and on spontaneous transport of LY and [^{14}C]sucrose in a solution under a chemotaxic condition without PMNs, respectively.

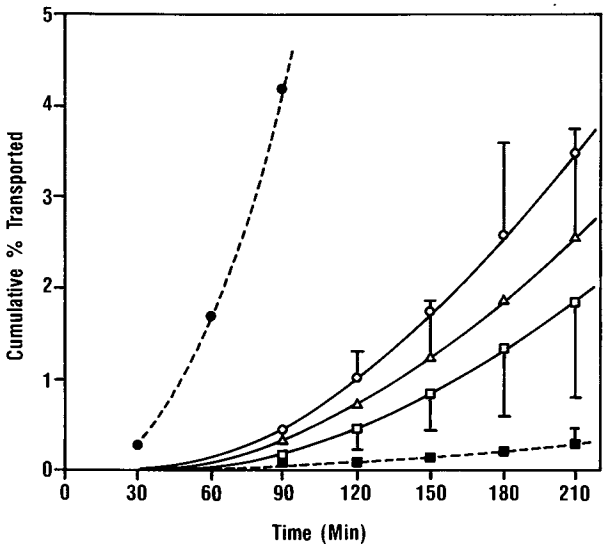

Fig. 5. Apical-to-basolateral transport of sucrose (circles), LY (triangles) and DPPC (squares) across 4.71-cm^2 MDCK cell monolayers under various chemotaxis conditions for human PMNs. Data obtained from the 8R series of experiments are represented by open symbols (N = 15), where the transport of the markers in liposomes was monitored in the presence of 10% serum and PMNs activated by f-Met-Leu-Phe. Filled circles represent the transport of sucrose under the same conditions but using solutions in the apical donor side (i.e., 8S series; N = 6). Filled squares show the appearance of DPPC in the 6R series of experiments (N = 18), in which the donor compartment did not have any serum. For simplicity, data obtained at $t < 90$ min are not shown. Bars indicate one standard deviation for N determinations.

MDCK cell layer in the absence of PMNs. This observation was independent of f-Met-Leu-Phe, namely, the 3R and 7R series (data not shown). Serum is, however, known to cause leakage of substances entrapped in REVs such as LY and sucrose under the present experimental conditions (2). Once leaked out, the marker molecules present in the medium would pass through the cell layer at the same rate as in the 8S series.

In our previous study (6), sucrose was found to traverse the MDCK cell layer somewhat faster than LY. The same trend was observed in the 8R series of experiments in the present study. It thus supports the likelihood of serum-caused leakage of the markers from liposomes and subsequent transport in solution along with migrating PMNs. The true liposome transport which was mediated by PMNs would therefore be represented by that of DPPC (open squares in Fig. 5). The possibility of spontaneous DPPC transfer from liposomes to PMNs rather than phagocytosis was ruled out previously (2). The foregoing analysis establishes that LY, sucrose, and DPPC found in the basal side of an MDCK cell monolayer under a chemotaxis condition were indeed transported by PMNs. In the process, serum played a multifaceted role.

The PMN-mediated transport of particulate drug carriers across a cellular barrier we observed in the present study supports possible targeted drug delivery to an extravascular site *in vivo*. In concept, it can be achieved by autologous iv administration of the leukocytes subsequent to *ex vivo* loading of a biologically active substance. In the drug loading, utilizing the phagocytic activity of the target cell should be much more efficient than through a simple fluid-phase pinocytosis for water-soluble polar substances (2). In practice, however, direct iv administration of submicron particulate drug carriers would be more desirable. In such a situation avoiding the uptake by the reticuloendothelial system would become a challenging task, a subject recently reviewed in the literature (15). In addition, the cell infiltration kinetics in the progression of an inflammation should be taken into consideration (16,17). Lack of extravasation of liposomes reported in a granuloma pouch assay for an inflammation model (18) could well be due to inappropriate timing of the liposome administration and/or tracer assay. Finally, but not in the least, it should be taken into consideration that phagocytosed materials would be exposed to a hostile environment of endosomes and lysosomes in terms of acidic pH's and degradative enzymes (19,20). Thus either a mechanism by which the drug substance can escape from these organelles should be developed or only substances which can survive the hostile environment should be selected in further testing in an animal disease model.

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