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Membrane phospholipid asymmetry as a determinant of erythrocyte recognition by macrophages

(drug delivery/phagocytosis/two-phase polymer partitioning/merocyanine 540/lipid packing)

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ABSTRACT **To assess the role of transbilayer phospholipid distribution in the recognition and phagocytosis of erythrocytes by macrophages, human erythrocytes with either a symmetric or asymmetric distribution of membrane phospholipids were prepared by hypotonic hemolysis and then incubated with cultures of human monocyte-derived macrophages. Erythrocytes with an abnormal, symmetric distribution were phagocytosed 4 times more readily than their counterparts with an asymmetric distribution or than normal, asymmetric intact erythrocytes. This enhanced phagocytosis correlated with two biophysical properties of the membrane: the spacing of phospholipids, as assessed by binding of the dye merocyanine 540, and the relative hydrophobicity, as measured by aqueous two-phase polymer partitioning. These results suggest a mechanism by which loss of membrane asymmetry is translated into recognition by macrophages and provide guidelines in loading erythrocytes that may be useful in manipulating the mode of delivery when erythrocytes are used as drug carriers *in vivo*.**

Erythrocytes loaded by hypotonic hemolysis (erythrocyte-carriers‡) can be reinfused into the circulation for delivery of pharmacologically active substances (2–4). Among several advantages of this approach, one may be versatility with respect to the mode of drug delivery. In some regimes, long-lived erythrocyte-carriers are required to provide for the slow, sustained release of drugs into the circulation. In other instances, it may be more efficacious to target carriers to tissues or organs. In these latter cases, erythrocytes must be altered in such a way as to trigger their removal from the circulation by macrophages resident in these tissues. The extent to which erythrocytes are recognized as abnormal can affect both the primary site and rate of sequestration (2). Within this context, it would be highly desirable to be able to control the recognition of erythrocyte-carriers by the reticuloendothelial system.

The phospholipids of the human erythrocyte membrane are not symmetrically distributed between the two halves of the bilayer. The outer monolayer predominantly contains the neutral phospholipids phosphatidylcholine and sphingomyelin, whereas the anionic phosphatidylethanolamine and phosphatidylserine are mainly restricted to the inner leaflet of the bilayer (5). Asymmetry with respect to lipid headgroup creates a second type of asymmetry, since the fatty acyl chains of the predominantly exterior phospholipids are more highly saturated than the acyl chains of the predominantly interior phospholipids (6). Because saturated fatty acyl chains are able to pack more efficiently than unsaturated ones (7), the lipids of the outer monolayer are more closely spaced than the lipids of the inner leaflet of the membrane (8). This difference in spacing can be recognized by the naturally fluorescent probe merocyanine 540 (MC540), which is sensitive to lipid packing: the dye binds to membranes with loosely packed lipids but not to more ordered lipid bilayers (9). When applied externally to normal erythrocytes, the impermeant dye fails to bind (10). If, however, it is applied to erythrocyte membranes whose asymmetry has been lost, the probe is able to detect the loosened lipid packing of the outer leaflet, produced by the introduction of unsaturated acyl chains from the inner leaflet, and is able to bind (11, 12).

When erythrocytes are hypotonically lysed and resealed, normal asymmetry is not necessarily maintained (13). When Mg^{2+} is the only divalent cation present during lysis and resealing, and the volume of lysis buffer is about 4-fold greater than the volume of the erythrocytes lysed, lipid asymmetry is maintained, as judged by phospholipase digestion assays and the inability of the cells to be stained by MC540. If 1 mM Ca^{2+} is present during lysis and resealing, asymmetry is abolished, as judged by the observations that much more phosphatidylethanolamine becomes accessible to digestion by externally added lipase and that MC540 is able to bind to the external leaflet. At lower ratios of lysing buffer to packed erythrocytes, the effect of calcium is not as pronounced. If the volume of lysing buffer is equal to that of the erythrocyte pellet, asymmetry is largely maintained, with only a small increase in the fraction of externally exposed phosphatidylethanolamine even when Ca^{2+} is present. Under these same conditions, when the lysing volume is increased to 4 times that of the erythrocyte pellet, asymmetry is completely abolished. In summary, two pairs of symmetric and asymmetric erythrocyte-carriers can be prepared: either the volume of the lysing buffer is held constant while varying the concentration of divalent cations or the volume is varied without altering divalent-cation concentration.

Recent experiments suggest that disruption of the normal asymmetric distribution of phospholipids between the hemileaflets of the erythrocyte membrane has a pronounced effect on the interaction of these normally inert cells with other cell types (14). When otherwise comparable asymmetric and symmetric pairs were presented to a murine macrophage cell line, symmetric erythrocytes were phagocytosed in greater numbers than their asymmetric counterparts (14). However, the functional relationship between the immortalized, cultured cell line and primary macrophages is not known; in addition, the interactions observed crossed species boundaries. In this report we present evidence that human macrophages derived directly from primary monocytes preferentially phagocytose human erythrocyte-carriers whose lipid asymmetry has been disrupted. In addition, we quantify two biophysical properties of the erythrocyte membrane that are correlated with this preference.

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Abbreviation: MC540, merocyanine 540.
‡Erythrocytes prepared so that they entrap substances while retaining a large fraction of their cellular contents (1).

MATERIALS AND METHODS

Cells and Phagocytosis Assays. Erythrocyte-carriers with asymmetric or symmetric membranes were prepared from human erythrocytes as described (13), with the following modifications: 1:1 and 4:1 erythrocyte-carriers were resealed with 10× phosphate-buffered saline (1.37 M NaCl/57 mM KCl/54 mM Na_2HPO_4/45 mM KH_2PO_4/15 mM $CaCl_2$/15 mM $MgCl_2$); Ca^{2+} and Mg^{2+} erythrocyte-carriers were prepared by the same method as 1:1 and 4:1 erythrocyte-carriers but were lysed in 10 mM Tris/0.1 mM EGTA, pH 7.2, containing 1 mM $CaCl_2$ or 1 mM $MgCl_2$ and were resealed with 10× phosphate-buffered saline containing either 15 mM $CaCl_2$ or 15 mM $MgCl_2$. All erythrocyte-carriers were loaded with bovine serum albumin (fraction V, Sigma) by inclusion of the protein in the lysis buffer at 0.1% (wt/vol). Such cells have been previously shown to retain their impermeability to both small ionic compounds and to macromolecules (13). Human peripheral blood monocytes were isolated, cultured, and used in phagocytosis assays performed as described in detail elsewhere (15). In visual assays, coverslip cultures were fixed by methanol, stained for hemoglobin with 3,3'-dimethoxybenzidine in methanol, counterstained with Giemsa stain, and then mounted for viewing.

MC540 Staining Assays. Cells were stained with MC540 and examined by fluorescence microscopy as described (15). To quantify dye binding, MC540 was added at 10 μg/ml to 4 × 10^8 cells in 1 ml of buffer containing 0.05% (wt/vol) bovine serum albumin (15). After 10 min at room temperature, cells were pelleted by centrifugation and washed several times, and dye was recovered from the combined supernatants by extraction with 1 ml of pentanol. Triplicate 100-μl samples were diluted to 1 ml with 50% (vol/vol) ethanol and immediately sealed to prevent evaporation. The amount of dye in these samples was measured by absorption at 555 nm, and the amount of dye bound to cells was obtained by subtracting the experimentally determined values from the original quantity of dye added to the cells. Since hemoglobin also absorbs at 555 nm, its presence in the extracted sample was checked by washing and extracting cells identical to the sample but without MC540; optical density at 555 nm was always found to be negligible.

Partitioning. Phase partitioning was performed essentially according to the methods of Walter (16). The phase system consisted of 3.5% (wt/wt) poly(ethylene glycol) (PEG; molecular weight 6000; Pharmacia), 5% (wt/wt) dextran (molecular weight 500,000; Pharmacia), 0.15 M NaCl, and 0.01 M sodium phosphate (pH 7.0). After the two phases were allowed to equilibrate and separate overnight at room temperature, each was collected. To tubes containing 10^6 erythrocytes was added 0.2 ml of each phase, and tubes were inverted several times for mixing. After 10 min at room temperature to allow separation of the phases and partitioning of cells, 0.1 ml was removed from the upper phase and diluted with 9.9 ml of Isoton (Coulter Electronics), and the cells were counted using a Coulter counter. The partition coefficient is expressed as the percent of the total cells found in the upper phase.

RESULTS

The ability to manipulate the transbilayer distribution of the phospholipids of erythrocytes provides a useful system with which to test the effects of phospholipid packing on interactions with macrophages. Two sets of asymmetric and symmetric erythrocyte-carriers were therefore prepared. Erythrocytes were lysed in equivalent volumes of buffer differing in divalent cations, producing asymmetric (Mg^{2+}) or symmetric (Ca^{2+}) erythrocyte-carriers. Alternatively, erythrocytes were lysed in a volume equal to or 4-fold greater than the volume of packed erythrocytes, without controlling divalent cation concentration, producing asymmetric (1:1) or symmetric (4:1) erythrocyte-carriers.

Since homologous human macrophages are not readily available from tissue, human monocytes were isolated from peripheral blood and cultured in autologous serum for 6–7 days, during which time they acquired the morphological and functional characteristics of activated tissue macrophages (15, 17). Erythrocyte-carriers loaded with bovine serum albumin (or intact, unmanipulated erythrocytes, as controls) were layered over monolayers of these human monocyte-derived macrophages at a ratio of 100:1. Cultures were incubated at 37°C for 1 hr and washed to remove the majority of erythrocytes. Residual nonadherent erythrocytes and adherent but unphagocytosed erythrocytes were lysed with 0.17 N NH_4Cl. When cultures were fixed and stained for hemoglobin and then examined visually, macrophages in cultures that had been presented with either asymmetric erythrocyte-carriers or intact, unmanipulated erythrocytes contained zero or one internalized erythrocyte per cell (Fig. 1*A*). In contrast, macrophages in cultures that had been presented with symmetric erythrocyte-carriers (Fig. 1*B*) contained on average 3–5, but occasionally up to 10, phagocytosed erythrocytes. To provide a quantitative esti-

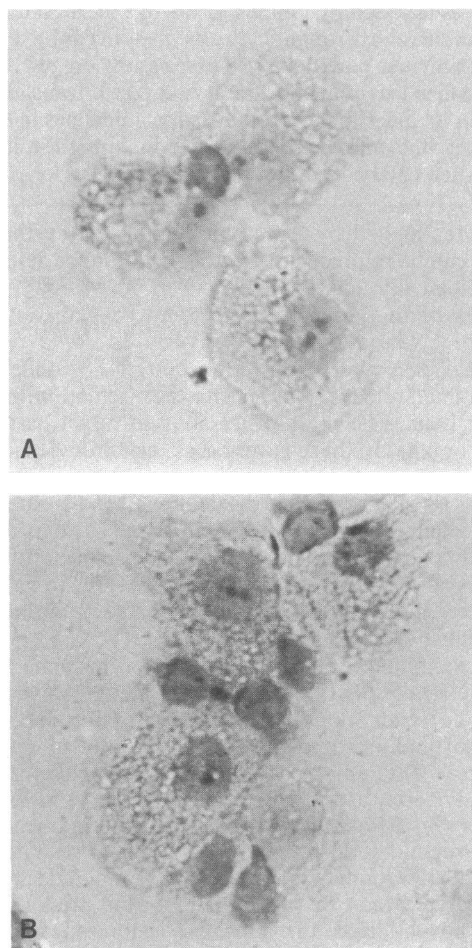

FIG. 1. Phagocytosis of erythrocyte-carriers by monocyte-derived macrophages. Asymmetric (*A*) or symmetric (*B*) erythrocyte-carriers were incubated with human monocyte-derived macrophages for 1 hr at 37°C; noningested erythrocytes then were lysed, and macrophages were fixed, stained for hemoglobin, and photographed. Vacuolization of macrophages is produced by the NH_4Cl lysis step. In *A*, three macrophages have ingested one erythrocyte; nuclei can be identified by their two nucleoli. In *B*, four macrophages have ingested seven erythrocytes. (×1800.)

mate of the preference of macrophages for symmetric versus asymmetric erythrocytes, the numbers of erythrocytes ingested by macrophages in random microscopic fields in parallel cultures were compared. As seen in Table 1, macrophages phagocytosed symmetric erythrocyte-carriers at 3–4 times the number of asymmetric erythrocyte-carriers, and 5 times the number of intact erythrocytes, regardless of the route by which the symmetric carriers were prepared.

To confirm these values derived from visual examination of macrophages, phagocytosis by the entire population was measured quantitatively by using erythrocytes labeled with ^{51}Cr. Labeled erythrocytes were incubated with macrophages, and noningested erythrocytes were lysed as before. The percent of added radioactivity that remained associated with macrophages after the lysis step was used as a measure of phagocytosis. As seen in Table 1, values obtained by this method were in general agreement with those obtained visually and indicate that asymmetric erythrocyte-carriers (1:1 and Mg^{2+}) and intact erythrocytes were recognized and phagocytosed in approximately equal numbers, whereas symmetric erythrocyte-carriers (4:1 and Ca^{2+}) were phagocytosed in significantly greater numbers.

Although these results point strongly to an effect of lipid distribution on susceptibility to phagocytosis, two other potential contributions were also considered but ruled out. First, the results might be a consequence of differences in shape or morphology between symmetric and asymmetric erythrocyte-carriers. However, examination of aliquots revealed that all the erythrocyte-carriers, regardless of the route of preparation, retained normal discoid morphology throughout the experiment when maintained in physiological saline containing 0.1% (wt/vol) glucose. Second, phagocytosis might be induced by binding of immunoglobulins to antigens exposed preferentially on symmetric, but not asymmetric, membranes. To preclude this possibility, erythrocytes were never exposed to serum which might contain immunoglobulins, and macrophages were thoroughly washed to remove traces of serum before erythrocytes were added. Therefore, the results observed represent generalized phagocytosis rather than Fc receptor-mediated phagocytosis.

The transbilayer disposition of phospholipids in each type of erythrocyte-carrier in all experiments was verified by visual examination of cells stained with MC540, using a fluorescence microscope. To quantify the actual amount of dye bound by each population of cells, stained cells were separated from unbound dye by centrifugation and the amount of unbound dye remaining in the supernatant was determined (10, 15). The results of such analyses (Table 2) indicate that both types of symmetric erythrocyte-carriers bound ≈4 times as much dye as intact erythrocytes. As perhaps expected from the somewhat increased exposure of phosphatidylethanolamine in asymmetric 1:1 erythrocyte-carriers versus asymmetric Mg^{2+} erythrocyte-carriers and intact erythrocytes (13), this former species of erythrocyte-carrier bound more dye. Using a linear least-squares fit, a good correlation ($P < 0.001$) was found between the phagocytosis ratio (determined from whole-population analysis with ^{51}Cr) and the amount of MC540 bound for each population of erythrocytes.

Table 2. Binding of MC540 by intact erythrocytes and erythrocyte-carriers

Cell type*	MC540 bound, μg per 4×10^8 cells†	Binding index‡
Intact erythrocytes (A)	0.89 ± 0.06	1.0
1:1 erythrocyte-carriers (A)	1.16 ± 0.08	1.3
4:1 erythrocyte-carriers (S)	3.38 ± 0.24	3.8
Mg^{2+} erythrocyte-carriers (A)	0.85 ± 0.05	1.0
Ca^{2+} erythrocyte-carriers (S)	3.92 ± 0.26	4.4

*(A) denotes asymmetric and (S) denotes symmetric distribution of phospholipids.
†Mean ± SD of four separate experiments.
‡Amount bound relative to intact erythrocytes.

In nonimmune phagocytosis of bacteria, surface hydrophobicity has been suggested to play a dominant role. Since alterations in the lipid composition of the external leaflet of erythrocytes might be expected to affect this property, relative hydrophobicity was compared for symmetric versus asymmetric erythrocyte-carriers and intact erythrocytes by measuring partitioning in an aqueous, two-phase system of PEG and dextran. When aqueous solutions of dextran and PEG are mixed above certain concentrations, two immiscible phases result, a PEG-rich upper phase and a dextran-rich lower phase (18). This system can be buffered and made isotonic so that the partitioning properties of cells can be examined. When phosphate is maintained at a low concentration (<10 mM), no electrostatic potential exists between the phases (19) and partitioning provides a measure of relative surface hydrophobicity (20). The partition coefficient, defined as the percent of added cells found in the upper, more hydrophobic, PEG-rich phase of the system after partitioning, was determined for erythrocytes (Table 3). The higher partition coefficients of symmetric erythrocyte-carriers, as compared to asymmetric erythrocyte-carriers and intact erythrocytes, indicate that these cells have a more hydrophobic surface. Again, the somewhat increased value for 1:1 erythrocyte-carriers is consistent with the minor perturbation of normal asymmetry found in this type of carrier. Since properties that determine partitioning have

Table 1. Phagocytosis of intact erythrocytes and erythrocyte-carriers by monocyte-derived macrophages

	Visual assay		^{51}Cr assay	
Cell type*	No. of cells ingested per macrophage†	Phagocytic index‡	% input ^{51}Cr ingested§	Phagocytic index¶
Intact erythrocytes (A)	0.6	1.0 ± 0.1	0.41	1.00 ± 0.03
1:1 erythrocyte-carriers (A)	0.9	1.5 ± 0.2	0.51	1.25 ± 0.08
4:1 erythrocyte-carriers (S)	2.8	4.6 ± 0.4	1.43	3.48 ± 0.12
Mg^{2+} erythrocyte-carriers (A)	0.8	1.3 ± 0.1	0.49	1.21 ± 0.07
Ca^{2+} erythrocyte-carriers (S)	3.5	5.4 ± 0.4	1.52	3.70 ± 0.14

*(A) denotes asymmetric and (S) denotes symmetric distribution of phospholipids.
†Average number of cells ingested per macrophage from one representative experiment, in which 200 macrophages in each of triplicate cultures were examined. Only one experiment is given since these absolute numbers varied (from 0.2 to 1.0 for intact erythrocytes, for example) depending on the population of macrophages used.
‡Mean ± SD of four separate experiments.
§Average percent of input ^{51}Cr ingested in triplicate wells from one representative experiment. These absolute numbers varied (from 0.23 to 0.51 for intact erythrocytes, for example) depending on the population of macrophages used.
¶Mean ± SD of four separate experiments.

Table 3. Partitioning of intact erythrocytes and erythrocyte-carriers between PEG and dextran

Cell type*	Partition coefficient†
Intact erythrocytes (A)	48.0 ± 0.9
1:1 erythrocyte-carriers (A)	57.6 ± 2.4
4:1 erythrocyte-carriers (S)	94.7 ± 2.8
Mg^{2+} erythrocyte-carriers (A)	43.9 ± 1.8
Ca^{2+} erythrocyte-carriers (S)	96.1 ± 3.8

*(A) denotes asymmetric and (S) denotes symmetric distribution of phospholipids.

†Percent of cells partitioned into the PEG phase; mean ± SD for four separate experiments.

been clearly demonstrated to be exponentially related to partitioning (18, 20), the natural logarithm of the partition coefficients of each population of erythrocytes was compared with the respective phagocytic index. Linear least-squares analysis of the data revealed a good correlation ($P < 0.01$).

DISCUSSION

The results of these *in vitro* studies indicate that the recognition of erythrocytes by macrophages can be regulated by manipulating the phospholipid asymmetry of the erythrocyte membrane. Erythrocyte-carriers with symmetric membranes are recognized more readily and ingested in numbers 4 times greater than asymmetric erythrocyte-carriers or intact erythrocytes. Two biophysical properties of erythrocytes have been identified that are altered when normal phospholipid asymmetry is disrupted, and these may therefore be directly or indirectly responsible for increased recognition by macrophages.

There are several possible ways in which a loss of phospholipid asymmetry might be translated into a signal of recognition by macrophages. Biochemically, the surface of the erythrocyte is altered by the translocation and appearance in the external leaflet of negatively charged lipids, previously restricted to the inner leaflet. The resulting increase in negative charge at the cell surface could affect electrostatic interactions with other cells. However, the absence of a correlation between the ζ potential, a measure of surface charge, of particles or cells and their ability to be phagocytosed has been thoroughly documented (21). It is also possible that the presence of external phosphatidylserine may be more directly responsible for the enhanced interaction of symmetric erythrocytes by serving as a specific recognition signal. Indeed, phosphatidylserine appears to be able to function in such a capacity in virus–host cell recognition (22). Such a mechanism has also been postulated to explain the increased binding to macrophages by erythrocytes whose surface has been altered by the incorporation of a phosphatidylserine analogue (23).

Besides this possible biochemical mechanism of recognition, the results presented here suggest that two global biophysical properties may be of relevance in affecting increased phagocytosis. A direct mechanism by which macrophages could detect the looser packing of the lipids in the external leaflet of symmetric membranes is not immediately obvious. However, a change in lipid packing might act indirectly by increasing the lateral mobility of membrane proteins, resulting in their collection into a functional receptor for recognition. Such clustering of surface molecules has been suggested to explain the enhanced interaction of sickle erythrocytes, whose phospholipid asymmetry has been marginally perturbed (24), with endothelial cells (25). Should loosely packed lipids somehow be directly responsible for recognition, this mechanism may not be restricted to erythrocytes but might also apply to other types of blood cells. Circulating, noninteractive monocytes and lymphocytes both fail to bind MC540, whereas adherent monocytes and many tissue-bound lymphocytes do bind the dye (L.M., C. Piecyk, and R.A.S., unpublished observation; ref. 26). Whether alterations in the transbilayer distribution of phospholipids are responsible for the altered packing in the plasma membranes of these adherent cells is not known.

A second biophysical property altered when lipid asymmetry is lost is cell surface hydrophobicity. Symmetric erythrocyte-carriers exhibit enhanced relative hydrophobicity compared to asymmetric erythrocyte-carriers and intact erythrocytes, as measured by partitioning in a dextran/PEG two-phase system. The role of a number of surface parameters in nonimmune phagocytosis of bacteria has been thoroughly investigated (21). While no correlation was found between surface charge and phagocytosis, a very good correlation between increased hydrophobicity (measured by the contact-angle method) and increased phagocytosis was reported. Only bacteria more hydrophobic than phagocytes were readily engulfed. If an analogous situation exists for erythrocytes, asymmetric, relatively hydrophilic erythrocytes might be expected to be relatively resistant to phagocytosis, whereas symmetric, more hydrophobic erythrocytes would be susceptible.

As might be expected from their mutual correlation with phagocytosis, the amount of MC540 bound by and the natural logarithm of the partition coefficient of each population of erythrocytes were found to be highly correlated ($P < 0.01$). It might, therefore, be reasonably asked whether these two biophysical properties are causally related rather than each being an independent consequence of loss of phospholipid asymmetry. It is conceivable that the looser lipid packing of symmetric erythrocyte-carriers might allow increased access to the hydrophobic core of the membrane by solvent molecules intercalating into the bilayer (27), thereby increasing the affinity of these cells for the upper, more hydrophobic phase. Since very little is known of the mechanistic basis of partitioning, perhaps differential intercalation of PEG is actually responsible for partitioning in this system. Such a causal link would be consistent with the correlation observed between the partition coefficients of erythrocytes of different species and their ratio of polyunsaturated to monounsaturated fatty acids (27).

Although our results were obtained using an *in vitro* system, there is evidence to suggest that loss of phospholipid asymmetry by erythrocytes also functions *in vivo* as a signal for recognition. Erythrocytes normally associate with macrophages only during two phases of their life cycle, at their inception and when they are removed from the circulation by splenic macrophages. The final step in mammalian erythroid differentiation in the bone marrow is the extrusion of nuclei from orthochromatic erythroblasts to yield enucleated reticulocytes which are released into the peripheral circulation. Interestingly, the portion of the membrane located directly over the nucleus in enucleating cells is stained by MC540 (28). In the bone marrow, the extruded nucleus is rapidly engulfed by macrophages, often before it is actually separated from the reticulocyte (29), suggesting that loss of phospholipid asymmetry in the discarded portion of the membrane could account for its specific recognition. Perhaps elimination of this adherent portion of the membrane actually releases the cell from retention within the marrow. Later, upon reaching the end of their normal 120-day lifespan, senescent erythrocytes have apparently regained a property responsible for specific recognition and interaction, since they are removed from the circulation by splenic macrophages (30). Analysis of aged erythrocytes by phospholipase digestion has shown an increase in the accessibility of phosphatidylethanolamine in the outer monolayer of the membrane (31), suggesting again that *in vivo*, maintenance of

normal phospholipid asymmetry is necessary to avoid clearance by macrophages.

The implications of these results in drug-delivery schemes utilizing erythrocyte-carriers may be far-reaching. Knowledge of a property of erythrocytes involved in their recognition by macrophages should permit more intelligent exploitation of the potential of these drug carriers. By controlling phospholipid asymmetry, it should be possible to engineer the sustained delivery of a systemic drug by using erythrocyte-carriers with asymmetric membranes loaded with an agent that slowly diffuses from the carrier into the circulation. Alternatively, should it be desirable to deliver agents directly to the cells of the reticuloendothelial system, erythrocyte-carriers in which phospholipid asymmetry has been disrupted should be efficacious. Severely perturbed cells may be targeted for nearly immediate removal by the Kupffer cells of the liver (2), whereas those only marginally altered may be removed in a more sustained manner by the normal elimination mechanisms of the spleen. The consequences of several popular loading procedures on maintenance of phospholipid asymmetry as assessed by MC540 staining have been reported (14). Using this information as a guideline, it should be possible to prepare erythrocytes whose surface properties are amenable to each of these modes of drug delivery.

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