Insertion of Fluorescent Phosphatidylserine into the Plasma Membrane of Red Blood Cells

RECOGNITION BY AUTOLOGOUS MACROPHAGES*

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The interaction of macrophages with red blood cells (RBC) displaying phosphatidylserine (PS) in their surface membranes was investigated after the transfer of an exogenously supplied fluorescent lipid analog to the RBC. Nonfluorescent (quenched) lipid vesicles were formed by ultrasonication from 1-acyl-2-[(N-4-nitrobenezoyl-2-oxa-1,3-diazole)aminocaproyl]phosphatidylserine (NBD-PS) or 1-acyl-2-[(N-4-nitrobenezoyl-2-oxa-1,3-diazole)aminocaproyl]phosphatidylcholine (NBD-PC). The interaction of these vesicles with RBC was monitored as a function of vesicle concentration by assessment of the degree to which cell-associated lipid fluorescence was quenched after vesicle treatment. When vesicle concentrations of <100 ng/ml were used, lipid fluorescence was largely quenched, indicating that lipid transfer was the predominant mechanism of both NBD-PS and NBD-PC uptake; however, when vesicle concentrations were increased to >100 ng/ml, a concentration-dependent increase in the fraction of quenched cell-associated lipid was observed, indicating that another mechanism, possibly vesicle-cell adhesion, also occurred.

Using NBD-PS at concentrations at which dilution of all the phospholipid analog in the recipient cell membrane could be unequivocally confirmed, we observed that the uptake of NBD-PS-treated RBC by macrophages was increased 5-fold over that of controls, whereas the uptake of RBC containing an equivalent amount of exogenously supplied NBD-PC was unaltered. Furthermore, preincubation of macrophage monolayers with vesicles containing PS resulted in a ~60% inhibition in the uptake of NBD-PS-treated RBC, whereas no inhibition in the uptake of control, opsonized, or NBD-PC-treated RBC was observed. These findings suggest that PS in the outer leaflet of RBC might serve as a signal for triggering their recognition by macrophages.

The ability of mononuclear phagocytes to selectively remove particulate material from the circulation is essential for the maintenance of homeostasis (1, 2). This phenomenon is dependent on the ability of the phagocytes to recognize and destroy invading microorganisms and to distinguish between “self” and “senescent self.” Thus, mononuclear phagocytes can phagocytose and eliminate from the blood stream cells that are no longer functional, while leaving mature viable cells unharmed.

Clearly, effete cells must acquire a new and specific membrane determinant that provides the signal for their removal by phagocytic host cells; however, the exact mechanism(s) by which macrophages recognize and remove effete cells has remained controversial. Initially, the removal of senescent RBC1 by macrophages was thought to result from a decrease in the net surface charge of aged cells (3) associated with dense RBC populations (4, 5). Later, other investigators suggested that the decrease in surface charge was the result of sialic acid removal (6, 7). However, in more recent studies, net surface charge was found to be the same for RBC of different densities (and presumably of different ages) (8, 9); hence, density per se does not appear to play a role in recognition of RBC by macrophages.

An alternative and attractive proposed mechanism for the recognition of aged RBC by macrophages also involves the removal of sialic acid; however, instead of directly affecting the surface charge, its removal is believed to initiate the binding of autologous antibodies to the RBC surface via newly exposed antigens (10), probably asialoglycophorin (11). After binding a sufficient number of antibody molecules to opsonize them, the RBC bind to macrophages via Fc receptors (10–12).

We and other investigators have observed that incorporation of negatively charged lipids into artificial model membranes (liposomes) greatly enhances the interaction of the liposomes with macrophages. Specifically, negatively charged vesicles composed of PS and PC bind to and are phagocytosed by macrophages at rates significantly higher than those obtained with neutral vesicles composed exclusively of PC (13, 14). In line with these observations, we noted the finding that of the four major phospholipids comprising the RBC membrane, only PS is found exclusively in the inner bilayer leaflet (15, 16). This apparent complete absence of PS on the outer membrane of normal RBC, together with the observation that PS enhances recognition of liposomes by macrophages, prompted us to investigate whether PS provides a stimulus for the recognition of RBC by macrophages. For this purpose, experimental procedures were developed to define those conditions under which exogenously supplied fluorescent phosphatidylserine (PS) binds to and is recognized by macrophages.

* This research was supported by the National Cancer Institute, Department of Health and Human Services, under Contract N01-C0-23909 with Litton Bionetics, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: RBC, red blood cells; NBD-PC, 1-acyl-2-[(N-4-nitrobenezoyl-2-oxa-1,3-diazole)aminocaproyl]phosphatidylcholine; NBD-PS, 1-acyl-2-[(N-4-nitrobenezoyl-2-oxa-1,3-diazole)aminocaproyl]phosphatidylserine; PBS, 0.15 M NaCl, 0.01 M Na phosphate, pH 7.2; PC, phosphatidylcholine; PS, phosphatidylserine.

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phospholipid analogs (18-21) would transfer to and become incorporated in the plasma membrane of RBC as opposed to other mechanisms of vesicle-cell interaction (20, 22, 23).

In this study, we used vesicles composed exclusively of NBD-PS, which can unequivocally be demonstrated to integrate into the plasma membrane bilayer, to determine whether RBC expressing this lipid are recognized by autologous macrophages.

**EXPERIMENTAL PROCEDURES**

**Materials and Routine Procedures**—Beef brain PS, egg yolk PC, and NBD-PC were purchased from Avanti Polar Lipids (Birmingham, AL). NBD-PS was prepared from NBD-PC by phospholipase D-catalyzed base exchange in the presence of L-serine (24) and purified by thin layer chromatography on activitated silica gel 60 thin layer plates (Merck) in CHCl3/methanol/acetic acid/H2O (5:1:2:1:0.5). Analysis of the purified product by thin layer chromatography in basic, acidic, and neutral systems revealed a single fluorescent phosphate- and ninhydrin-positive spot. Steady state fluorescence was quantified with a Farrand MK spectrophotofluorometer at 20 °C. The fluorescence was excited at 470 nm and monitored at 525 nm using narrow band-pass slits to minimize light scatter.

**Cells**—Macrophages were obtained from the peritoneal cavity of 8- to 10-week-old C57BL/6 mice 4 days after an intraperitoneal injection of thioglycolate. The cells were washed and suspended in serum-free Eagle’s minimum essential medium and added to each well (10⁵ cells/well) of a Microtest plate (Falcon Plastics, Oxnard, CA). After a 60-min incubation at 37 °C in a 5% CO₂ humidified incubator, nonadherent cells were removed by washing and fresh medium was added.

**Lipid Vesicles**—Small unilamellar vesicles (verified by their characteristic chromatographic properties on Bio-Gel A-15m columns) were prepared by sonication of NBD-PC or NBD-PS (10 µg of lipid/ml of PBS) at 10 °C under nitrogen and used within several hours. Vesicle-Binding Assays and Quantification of Lipid Uptake—Washed (and in some experiments, ¹⁰⁹Cr-labeled) RBC were resuspended in the indicated vesicle suspensions (2 × 10⁷ vesicles/ml) for 30 min at 37 °C. The cells were then washed three times with warm PBS and divided into aliquots for the various experiments. Extraction of membranes from cells and thin layer chromatographic analysis of the cell-associated fluorescent lipids revealed a single fluorescent spot which co-chromatographed with the original fluorescent lipid standards.

**RESULTS**

**Incorporation of NBD-PS and NBD-PC into Erythrocyte Membranes**—The fluorescence of NBD-labeled phospholipid analogs in vesicles is proportional to the amount of analog present; however, as the concentration of analog is increased above ~0.1%, self-quenching results in the rapid decrease of the fluorescence yield/molecule (25, 26). Therefore, suspensions of vesicles composed exclusively of NBD-labeled phospholipids are essentially nonfluorescent (quenched), whereas dilution of the lipid in cell membranes or in detergent micelles catalyzes self-quenching and re-expression of fluorescence. Self-quenching thus allows lipid remaining in vesicles to be distinguished from that which is released and subsequently diluted in cell membranes (19, 20).

When NBD-PC or NBD-PS vesicles were added to a suspension of RBC, immediate dequenching of fluorescence was observed, with maximum fluorescence intensity developing within 2 and ~5 min, respectively (Fig. 1). No enhancement of fluorescence occurred in the absence of cells nor was there an increase in baseline vesicle fluorescence after cells were removed by centrifugation, suggesting that the observed increase in intensity was exclusively caused by lipid molecules that had transferred to and were comitantly diluted in the erythrocyte membranes (Fig. 1). Indeed, fluorescence microscopy of erythrocytes incubated with NBD-PS or NBD-PC vesicles at 20 °C revealed the development of bright, uniform rings of fluorescence within seconds after mixing (Fig. 2), corroborating the rapid transfer observed by fluorometry (Fig. 1).

Since fluorescence quenching of NBD-labeled phospholipid analogs is proportional to the density of the analog in bilayer membranes (25, 26), the phenomenon of dequenching alone does not rule out the possibility that significant amounts of intact vesicles (quenched lipid) might be cell-associated. In order to determine the fraction of the total cell-associated lipid diluted in the membrane, fluorescence intensity was assessed in the absence (a measure of lipid inserted in the membrane) and presence (a measure of total cell-associated lipid) of Triton X-100, which effectively reduces lipid density. This approach is relatively straightforward; however, in order to calculate the fraction of dequenched cell-associated lipid, the intensity of fluorescence/molecule must be normalized for possible changes in quantum yield caused by differences in the probe environment, whether the analog be in an RBC membrane, phospholipid vesicle, or detergent micelle.

To accomplish this, we assessed the fluorescent yield/ng of lipid at infinite dilution for each of the experimental systems and normalized this figure to the yield of fluorescence in appropriate standard curves generated from known amounts of lipid. We assumed that at infinite probe dilution, all self-quenching constraints caused by energy transfer between adjacent fluorophores are reduced to an insignificant value; therefore, by extrapolation to zero lipid concentration, the inherent quenching properties of the system in question could be estimated relative to a standardized assay system.
As shown in Fig. 3, the intensity of NBD-PS fluorescence in vesicles is a function of the concentration of NBD-PS in the membrane. Maximum fluorescence was obtained at NBD-PS concentrations ≤0.1%, whereas a dramatic decrease in fluorescence intensity was observed at higher concentrations. Upon the addition of Triton X-100 to the vesicles, the effective lipid density was reduced, which resulted in either enhancement or quenching of fluorescence depending on the initial NBD-PS concentration in the phospholipid vesicles. Since the emission and excitation maxima of NBD fluorescence were not altered in the vesicles or in the detergent-lipid micelles (results not shown), we concluded that the reduction in fluorescence intensity at low concentrations of NBD-PS reflects a change in quantum yield. Fig. 3 shows that in order to normalize the quantum yield of fluorescence in the detergent micelles to the yield of dequenched vesicle fluorescence, a Triton X-100 quenching correction factor (TQ) of 1.6 must be introduced.

The quantum yield of the NBD-labeled phospholipid analog in RBC membranes relative to the yield of fluorescence in Triton X-100 cell lysates was determined in an analogous manner but required the assessment of different factors. Since the assumption that all self-quenching is reduced to zero is valid only at infinite probe dilution, we determined the inherent quenching characteristics of intact RBC by extrapolation. This was performed by calculation of the ratio of fluorescence in the presence and absence of Triton X-100 in cells contain-
ing decreasing, but known, quantities of NBD-PS (calculated from a standard curve generated from known amounts of lipid in a Triton X-100 RBC lysate; see "Experimental Procedures").

The dependence of $TQ_{10}^{-1}$ (which is the fluorescent intensity of x ng of NBD-PS in intact cell membranes relative to the intensity of x of ng NBD-PS in a Triton X-100 lysate) on the amount of cell-associated NBD-PS is shown in Fig. 4. $TQ_{10}^{-1}$ decreased from 1.77 to 0.87 with an ~10-fold decrease in the amount of cell-associated NBD-PS. By extrapolation to $TQ_{10}^{-1}(x=0)$ using an exponential curve fit ($y = ae^{bx}$), we found that $TQ_{10}^{-1}$ at infinite probe dilution is 0.833. Simply stated, this number shows that the fluorescent yield of NBD-PS in RBC membranes at concentrations where self-quenching is essentially eliminated is 1.2-fold higher than in the Triton X-100.

The measured values of relative fluorescence were appropriately corrected for volume dilution and fluorescent yield, and these corrected values were used to estimate the fraction

![Fig. 4. Fluorescence yield of NBD-PS in RBC. RBC were incubated with increasing amounts of NBD-PS vesicles for 30 min at 37 °C. Cells were washed, and fluorescence intensity was measured in the absence and presence of Triton X-100 (2% final concentration). After correction for sample dilution caused by the addition of detergent, the ratio of fluorescence with and without Triton X-100 ($TQ_{10}^{-1}$) was determined and plotted against the total amount of cell-associated lipid determined from an appropriately generated standard curve (see "Experimental Procedures").](image)

![Fig. 5. Uptake of NBD-PS and NBD-PC by RBC. RBC were incubated with increasing amounts of vesicles for 30 min at 37 °C. Cells were washed, and the total amount of cell-associated lipid was determined from an appropriately generated standard curve (see "Experimental Procedures"). The fraction of dequenched lipid (O-* - - - - O) to total lipid (● - - - - - O) was assessed by using the values of DQ from Table I for NBD-PS. The data for NBD-PC was calculated in a manner identical with that for NBD-PS, using independently generated correction factors where $TQ_{c} = 2.03$, $F_{c} = 0.01$, and $TQ_{c} = 1.66$.](image)

| Initial Vesicles | Vesicles | Cells | $F_{c}$ | $F_{c}$ | DQ
|-----------------|----------|-------|---------|---------|-----|
| 32 ng lipid/ml   | 0.004    | 0.273 | 0.015   | 0.015   | 96.9 |
| 125 ng lipid/ml  | 0.015    | 0.787 | 0.019   | 0.015   | 91.7 |
| 500 ng lipid/ml  | 0.048    | 3.177 | 0.015   | 0.015   | 80.6 |
| 32               | 0.063    | 0.065 | 0.969 ± 0.014 | 96.9 |
| 63               | 0.124    | 0.135 | 0.919 ± 0.015 | 91.7 |
| 125              | 0.207    | 0.256 | 0.809 ± 0.005 | 80.6 |
| 250              | 0.289    | 0.481 | 0.601 ± 0.016 | 59.5 |
| 375              | 0.328    | 0.638 | 0.514 ± 0.007 | 50.7 |
| 500              | 0.348    | 0.736 | 0.473 ± 0.018 | 46.5 |

* $2 \times 10^7$ RBC were incubated with the indicated vesicle preparations for 30 min at 37 °C. The cells were then washed and fluorescence was measured in the absence and presence of Triton X-100 as described under "Experimental Procedures."

* Values presented are corrected for sample dilution and appropriate correction factors of 1.60 and 1.20 obtained from Figs. 3 and 4 for Triton X-100-mediated quenching of vesicle and cell fluorescence, respectively. Triplicate samples measured individually.

* $F_{c} = 0.0146$ was used for the calculation of dequenched lipid (DQ) which was the average of eight determinations throughout the range of 32–500 ng of vesicle lipid/ml.

* DQ was calculated from Equation 1.
of dequenched lipid (DQ) in relation to the total amount of cell-associated lipid by the following relationship (19, 20).

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DQ = \left(\frac{(F_c - F_t)}{(1 - F_t)}\right) \times 100
\]  

(1)

where \(F_c\) and \(F_t\) are the ratios of corrected fluorescence in the absence and presence of detergent of the washed, vesicle-treated cells and the initial vesicle population, respectively.

The dependence of DQ on the concentration of vesicles incubated with the cells is shown in Table I. At very low vesicle concentrations (<100 ng of lipid/ml), virtually all of the cell-associated lipid was apparently dequenched (DQ = 100%), strongly suggesting complete dilution of the probe in the membrane. On the other hand, when higher vesicle concentrations were employed (>100–500 ng of lipid/ml), a proportional decrease in DQ was observed, reaching values as low as 46%, even though proportionally more lipid became cell-associated. The total amount of completely dequenched NBD-labeled phospholipid analog (calculated from DQ values shown in Table I) in relation to the absolute amount of lipid uptake by the cells is shown in Fig. 5. As vesicle concentrations decreased, proportionally less lipid became cell-associated; however, the absolute amount of dequenched lipid remained relatively constant. Therefore, this finding suggests that the additional cell-associated lipid at initial vesicle concentrations >100 ng/ml (quenched lipid) may have become cell-associated via a mechanism other than lipid transfer, although a combination of processes cannot be excluded (see “Discussion”). Analogous experimental procedures were performed...
formed with NBD-PC exactly as described in detail for NBD-PS. These data are summarized in Fig. 5 and show that, similar to the findings with NBD-PS, the use of low initial vesicle concentrations resulted in apparent complete dilution of the NBD-PC in the recipient cell membrane.

Additional evidence that the mode of vesicle lipid-cell association is dependent on the concentration of the donor vesicle population was obtained by the use of nontreated RBC to dequench cells that contained large amounts of quenched lipid. This experiment was based on the assumption that unlabeled cells should be able to remove, possibly by lipid transfer, a substantial proportion of those vesicles that associate with the cells via adsorption and thereby cause enhancement of fluorescence. RBC were incubated with vesicles at both high (2 x 10^7 RBC/500 ng of lipid/ml) and low (2 x 10^7 RBC/125 ng of lipid/ml) lipid concentrations for 30 min at 37 °C. Both cell populations were washed and mixed with an aliquot of untreated erythrocytes (2 x 10^7) for ~10 min. As shown in Table II, the percentage of quenched lipid (100 - DQ from Equation 1) that associated with vesicle-treated RBC at high and low concentrations of vesicles was 54 and 0%, respectively. After the addition of untreated RBC, the cells treated with high concentrations of vesicles were partially dequenched (showed increased fluorescence); however, the fluorescence of cells treated with low concentrations of vesicles did not increase, suggesting strongly that all of the lipid analog was initially fully diluted in the cell membrane. Examination of the mixed RBC populations by fluorescence

FIG. 8. Phase (a) and fluorescent (b) photomicrograph of NBD-PS-treated RBC-macrophage rosettes. The macrophage monolayer was incubated with NBD-PS-treated RBC (80 ng of NBD-PS/2 x 10^7 RBC) for 30 min at 37 °C. The culture was then washed and photographed. Bar = 20 μm.
Microscopy revealed that only RBC treated with high concentrations of vesicles transferred NBD-PS to untreated RBC (Fig. 6). This is evident from the observation that virtually all the cells seen in Fig. 6a are fluorescent (Fig. 6b), whereas Fig. 6d shows only about half as many cells as does Fig. 6c. Based on these observations, we used low concentrations of NBD-PS vesicles for the treatment of RBC in the binding experiments presented below. Under these conditions, all of the NBD-PS should be properly inserted in the RBC membrane.

Uptake of PS-enriched RBC by Macrophages—A 51Cr-labeled RBC treated with the NBD-PS or NBD-PC vesicles (80 ng of lipid/ml) were washed and incubated with macrophages at 37°C. Fig. 7 shows that the uptake of NBD-PS-treated RBC by macrophages was 4–5-fold greater than that of either NBD-PC-treated or untreated control RBC. These results strongly suggest that the addition of PS to RBC enhances their recognition by macrophages and results in more avid binding and phagocytosis. Indeed, fluorescence microscopy of macrophages incubated for 30 min at 37°C with NBD-PS-treated RBC revealed fluorescent RBC-macrophage rosettes (Fig. 8). Control experiments indicated that NBD-PS-treated RBC did not exhibit enhanced binding to other types of adherent cells, such as endothelial cells (results not shown).

The data presented above suggest that the mode of macrophage recognition of PS-treated RBC may involve a ligand-receptor type of interaction. In order to determine whether such a receptor exists on the macrophage membrane, we performed the following inhibition experiment. Macrophages were treated with increasing amounts of small unilamellar vesicles composed of PC and PS (7:3 mol ratio) for 30 min at 37°C. They were then washed and incubated with NBD-PS-treated RBC, NBD-PC-treated RBC, opsonized RBC, and control (untreated) RBC suspensions for 30 min. The uptake of the RBC preparations by the macrophages is shown in Fig. 9; pretreatment of macrophages with small unilamellar vesicles composed of PC and PS resulted in 60% inhibition in the uptake of NBD-PS-treated RBC, whereas the uptake efficiencies of control (untreated), opsonized, and NBD-PC-treated RBC were unaffected. Furthermore, no inhibition of uptake were observed in control experiments with identical concentrations of vesicles composed exclusively of PC (results not shown).

**DISCUSSION**

The mechanism by which mononuclear phagocytes distinguish between normal and effete cells has been a controversial subject for more than a decade. Various mechanisms by which macrophages recognize these cells have been proposed, ranging from direct alterations in surface charge (4–7) to the opsonization of senescent RBC by autologous antibodies (10–12). Our study, which suggests that a normal lipid constituent of the RBC membrane may also play a role in this process, was initiated on the basis of two unrelated observations: 1) normal RBC do not express PS on their external membrane (15–17) and 2) the inclusion of PS in artificial membranes (liposomes) significantly enhances the recognition of the liposomes by macrophages (13, 14).

On the basis of previous findings that certain NBD-labeled phospholipid analogs rapidly transfer to mammalian cell membranes (18–21), we developed a similar approach for the insertion of an NBD-PS analog into RBC. In order to verify transfer of the lipid to RBC and to rule out the possibility of vesicle-cell adhesion, we made use of the inherent self-quenching properties of these fluorescent lipids (19, 20, 25, 26). As our results indicate, the extent to which donor vesicle lipids mix with phospholipids in the RBC membrane can be monitored by dequenching, and the measured fluorescent intensities can be used to quantify the extent of lipid transfer.

The observed dequenching of NBD-PS and NBD-PC can be best explained by the rapid lateral diffusion of lipids in biological membranes (~10−8 cm2/sec (27)), which allows for the average distance between fluorophores to fall outside the limits of self-quenching. If lipid uptake was caused by adsorption of intact vesicles, then the fluorescence efficiency (the degree of Triton X-100-induced enhancement of fluorescence) of the vesicle-treated RBC would have been similar to that of the initial vesicle population, assuming that the degree of self-quenching in the adherent cell population was identical with that of the initial vesicle population. Although self-quenching of NBD-labeled phospholipid analogs is dependent on their density and concentration, it is independent of particle number (as shown in Table I), which implies that during the time course of our experiments, the lipids were primarily in the form of intact bilayer structures rather than soluble lipid monomers. This concept is supported by the observations that, in addition to the constant Ft values obtained throughout the range of 30–500 ng of lipid/ml (Table I), no fluorescence could be detected in the supernatants of washed NBD-PS-treated RBC after incubation in PBS (results not presented), nor was there any detectable transfer of NBD-PS from RBC to macrophages (Fig. 8).

The rapid dequenching of fluorescence that occurs when NBD-PS vesicles are mixed with RBC (Fig. 1; Table I) strongly suggests that the exogenously supplied lipid became diluted and therefore was probably inserted into the recipient cell membrane. These results combined with the data presented in Fig. 5 are consistent with the interpretation that, as the concentration of donor vesicles decreases, the proportion of NBD-PS and NBD-PC diluted in the RBC membrane increases. Thus, at low concentrations of vesicles, the percentage of integrated lipid approaches 100%, suggesting that intact vesicle-cell adhesion is unlikely to be the mechanism.

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2 We cannot rule out the possibility that some degree of vesicle-cell fusion occurs in this system; however, even if this were to occur, our conclusion as to the insertion of NBD-PS in RBC membranes would be the same.
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involved in this process. Although the Triton X-100-induced enhancement of RBC-associated NBD-PS and NBD-PC fluorescence appears to be relatively high at the higher concentrations of vesicles (approaching 60% quenched lipid), it should be noted that the TQ factor employed (Fig. 4) in the calculation of our data is likely to be an underestimate if the curve is assumed to be biphasic like that obtained for TQ (Fig. 3). In any event, we stress that quenched cell-associated lipid is not absolutely indicative of adherent vesicles; at the higher concentrations used, properly inserted lipid may itself be partially self-quenched due to the formation of lipid domains or simply because of the relatively high density of the RBC-associated lipid. In this context, it is noteworthy that even at the initial low concentrations of vesicles (32 ng/ml), relatively large amounts of lipid were transferred to the cells (~1.2 x 10^12 mol/10^6 cells). If we assume that vesicle-cell lipid transfer is the predominant mechanism of lipid uptake by the RBC and that NBD-PS exhibits negligible rates of bilayer translocation, as has been shown for other NBD-labeled phospholipids (28, 29), then most of the lipid should reside in the outer phospholipid bilayer of the cells (15.2 x 10^{-11} mol of phospholipid/10^6 RBC, determined by F_1 analysis of extracted mouse RBC lipids (30)).

Transfer of NBD-PS to RBC resulted in a significant increase in the uptake of the cells by macrophages, whereas the transfer of similar amounts of NBD-PC was without effect (Fig. 4). Careful examination of macrophages that bound NBD-PS-containing RBC did not reveal any detectable transfer of the fluorescent probe to the macrophage membrane or any reorganization of the fluorescence, suggesting that the lipid is stable once inserted into RBC membranes (Fig. 5), which is unlike the rapid transfer properties of NBD-PC (18).

Inhibition experiments were performed to determine whether a receptor binding site capable of recognizing PS exists on the macrophage membrane. The finding that small unilamellar vesicles containing PS specifically inhibited the uptake of only NBD-PS-treated RBC suggests that such an entity might exist, although the presence of charge-specific binding sites cannot be ruled out. In this regard it is noteworthy that we were unable to inhibit phagocytosis of RBC by the inclusion of l-serine (10 mM) or a variety of other amino acids. The reason for the lack of inhibition is not known; although if the binding affinity of PS to this putative receptor is low, a multivalent ligand may be required for binding. Alternately, the binding may be specific for the phosphoserine moiety. Experiments are currently underway in an attempt to clarify some of these possibilities.

We are also studying whether the expression of PS, a normal lipid constituent of RBC, plays a physiological role in the maintenance of homeostasis. Our preliminary findings from experiments using established procedures for the separation of dense (presumably old) RBC and trinitrobenzene sulfonic acid-labeling techniques have not revealed any significant differences in the distribution of PS in RBC. This lack of alteration in PS asymmetry is not surprising if the exposure of PS results in the rapid removal of RBC by circulating phagocytic cells. Indeed, in this case, one would not expect to find such cells in peripheral blood. This hypothesis is, however, especially appealing when one considers the large body of evidence showing that the plasma membrane of sickle RBC contains a variety of moieties that confer unique properties upon these cells. Of particular interest are the greatly exaggerated alterations in lipid asymmetry caused by hypoxia-induced sickling, especially the appearance of PS on the outer bilayer (31–34). Preliminary evidence from our laboratory indicates that sickle cells avidly bind to human monocytes, in contrast to oxygenated s cells, which are morphologically normal in appearance and do not express PS on their outer surface.

In conclusion, the data presented here, together with independent observations of the effect of PS in artificial membrane systems and its expression in sickle RBC, suggest that PS can serve as a signal for recognition of RBC by macrophages. Our data do not rule out the possibility that the expression of age-related antigens on senescent RBC (10–12) plays a role in their removal from the circulation but do suggest that alternative mechanisms may also be operative. Indeed, given the extremely complex nature of homeostasis, the likelihood is that several mechanisms are required.

Acknowledgments—We thank Dr. I. J. Fidler for his continuous support and advice. The expert technical assistance of J. Madsen and A. Benton and the editorial services of V. Rooge are gratefully acknowledged.

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J. Biol. Chem. 1983, 258:11335-11343.

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