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# Analogous Recognition of Phospholipids by Insect Phagocytes and Mammalian Macrophages (42339)

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*Abstract.* Phagocytic cells from larvae of the moth *Heliothis virescens* and peritoneal macrophages of mice were observed to preferentially bind negatively charged phospholipid vesicles containing phosphatidylglycerol or phosphatidylserine as compared to neutral or positively charged vesicles. Since phagocytes have retained their primitive function of endocytosis throughout evolution, the recognition of negatively charged phospholipids may be a primitive mechanism for the identification of potential targets by macrophages. 

The recognition by phagocytes of "self" versus "nonself" occurs by a variety of mechanisms. In vertebrates, for example, target cell recognition can be mediated by antibody (Fc receptor) or complement (c′ receptor) (1–3). Both vertebrate macrophages and invertebrate phagocytes can, however, recognize many types of target cells without the aid of opsonizing or cytophilic antibodies; indeed such antibodies are absent in invertebrates (1–3). This nonimmunological recognition could be mediated by target cell surface oligosaccharides (1, 2, 4), electrostatic charge, or wettability (5–7). Recent studies have suggested that mammalian macrophages recognize phosphatidylserine (PS), a negatively charged phospholipid present in biological membranes (8). In addition, macrophage binding and uptake of RBC have been shown to be enhanced whenever PS is localized in the outer bilayer leaflet of red blood cells (9–11). In this study, we investigated whether mammalian macrophages and invertebrate phagocytes possess similar recognition systems involving negatively charged membrane phospholipids in a manner similar to the previously reported analogous vertebrate/invertebrate recognition of chemically modified erythrocytes (12) and cytotoxic response against vertebrate targets (13). Our results indicate that phagocytic cells from larvae of the moth *Heliothis virescens* and peritoneal macrophages of mice preferentially bind and endocytose negatively charged phospholipid vesicles as compared with neutral or positively charged vesicles. Since phagocytes have retained their primitive function of endocytosis throughout evolution, the recognition of negatively charged phospholipids may be a primitive mechanism for the identification of potential targets by macrophages.

**Materials and Methods.** *Materials and routine procedures.* Phosphatidylcholine (PC), phosphatidylglycerol (PG), PS, and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) were obtained from Avanti Polar Lipids (Birmingham, Ala.) and sterylamine (SA) was purchased from Sigma. *N*-[3-(3-[$^{125}$I]iodo-4-hydroxybenzyl)propionyl]phosphatidylethanolamine ($^{125}$I-PE) was synthesized from dipalmitoylphosphatidylethanolamine as described previously (14). All lipids were pure as assessed by thin-layer chromatography.

*Cells. H. virescens* hemocytes were obtained from the hemolymph of larvae reared to the fifth instar on artificial medium (5) by proleg amputation into Dulbecco's phosphate-buffered saline (pH 6.8) containing 8% saturated phenylthiourea, a phenol-oxidase inhibitor that prevents hemolymph melanization. Mouse macrophages were obtained from the peritoneal cavity of 8- to 10-week-old C57BL/6 mice 4 days after an intraperitoneal injection of 2 ml thioglycollate medium and resuspended in Hanks' balanced salt solution. Both cell types were washed with their appropriate media, and plated (in the same media) into 24-well plates (Costar, Cambridge, Mass.)



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containing sterile coverslips. The hemocytes and macrophages were then incubated overnight at 25 and 37°C, respectively.

*Lipid vesicles and phagocytosis.* Multilamellar vesicles (MLV) containing trace amounts of the nonexchangeable liposome markers, $^{125}$I-PE (14) and *N*-NBD-PE (15) were formed from appropriate lipid mixtures by vortexing in $Ca^{2+}$ $Mg^{2+}$-free PBS and sizing through 2-$\mu$m polycarbonate membranes as previously described (16). Neutral MLV were composed exclusively of PC, and the charged preparations were composed of PC admixed with the appropriate charged lipid at a 7/3 mol ratio, the optimum concentration of charged species for mouse macrophage uptake (17).

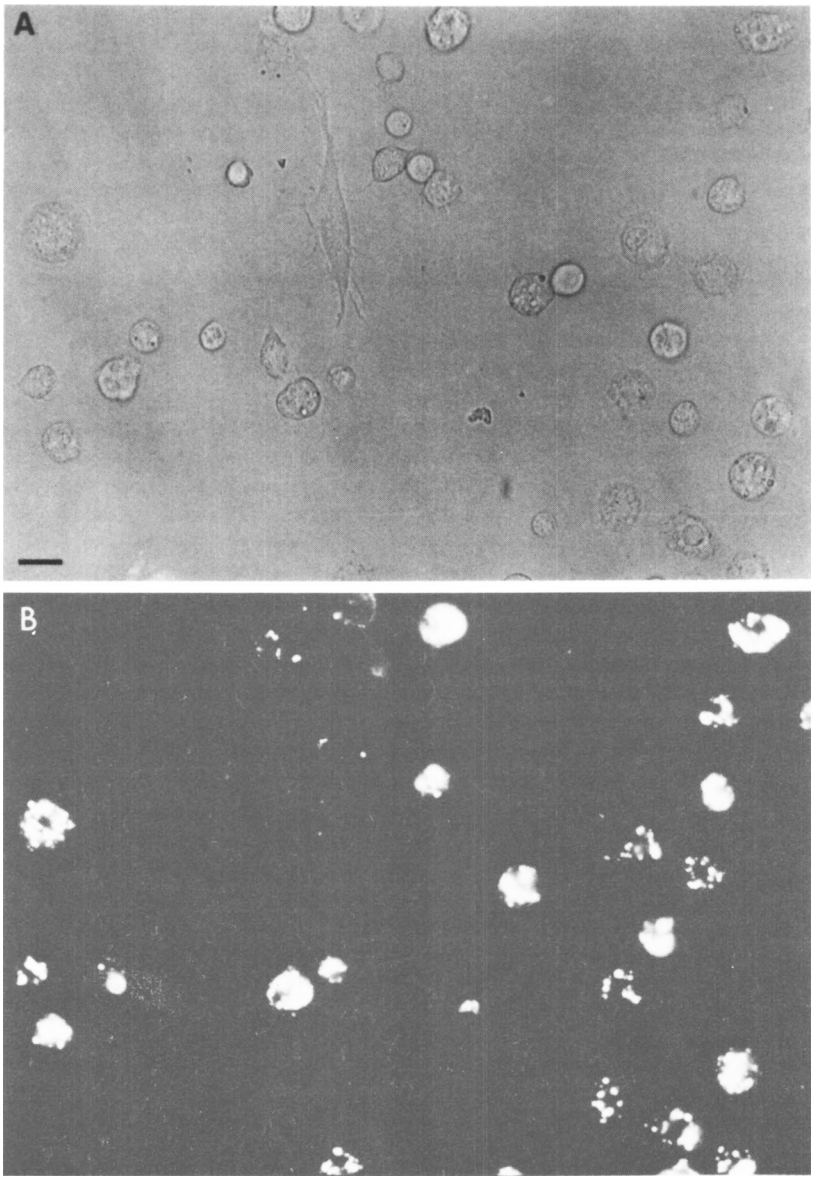


FIG. 1. Phagocytosis of MLV by glass-adherent hemocytes of *H. virescens*. Hemocytes were incubated for 3 hr with MLV composed of PC:PS (7:3) containing 0.1% of the fluorescent lipid *N*-NBD-PE. Light (A) and fluorescence (B) photomicrograph showing internalization of lipid and its accumulation in vacuoles (bar = 10 $\mu$m). Experimental conditions as described in Fig. 2.

The extent of endocytosis by the phagocytes was quantified by adding the various MLV to the hemocytes and macrophages (0.65 μmole lipid/ml/well) at 25 and 37°C, respectively. After the indicated time intervals, the coverslips were removed, washed with PBS, and the amount of cell associated $^{125}I$ was determined by scintillation counting. After washing, the number of adherent cells was determined by direct counting of randomized fields using a scored ocular lens.

**Results and Discussion.** Negatively charged MLV were readily taken up by *H. virescens* phagocytes. Fluorescence microscopy revealed that the uptake of NBD-labeled liposomes was confined mainly to the rounded, vacuolated mononuclear cells with preferential perinuclear localization of the particles, strongly suggesting true endocytosis (Fig. 1). Initially, no significant differences in uptake were observed among the two types of negatively charged MLV (Fig. 2). By 3 hr, however, PC:PG MLV were taken up to a significantly greater extent than the other MLV ($P < 0.05$). Uptake of the negatively charged MLV at 3 hr ranged from 13.8 ± 5.0 nmole for PC:PS to 20.2 ± 3.4 nmole for PC:PG. Uptake of neutral or positively charged MLV, on the other hand, never exceeded 1.3 nmole, a value significantly less ($P < 0.05$) than that of negatively charged MLV, even at 1 hr (Fig. 2). The observed pattern of phospholipid vesicle recognition and uptake was very similar to that observed for mammalian macrophages (8, 17), which also bind and endocytose negatively charged MLV more readily than neutral or positively charged MLV (Fig. 2).

To investigate the possibility that hemocyte binding of the negatively charged MLV might represent binding to the same surface structures, *H. virescens* phagocytes were incubated with labeled PC:PS or PC:PG MLV alone or admixed with a fourfold excess of unlabeled MLV composed of PC, PC:PS, or PC:PG. After 3 hr of incubation, the cultures were washed and uptake of the labeled MLV was determined. The results presented in Table I show that the uptake of PC:PS MLV was significantly inhibited by the addition of PC:PS and PC:PG MLV (57 and 89% inhibition, respectively). In contrast, the uptake of PC:PG MLV was only inhibited by the addition of PC:PG MLV. When similar competition assays were carried out with murine macrophages, PC:PS and PC:PG MLV did not differ in their ability to inhibit uptake of either liposome population. Liposomes composed of

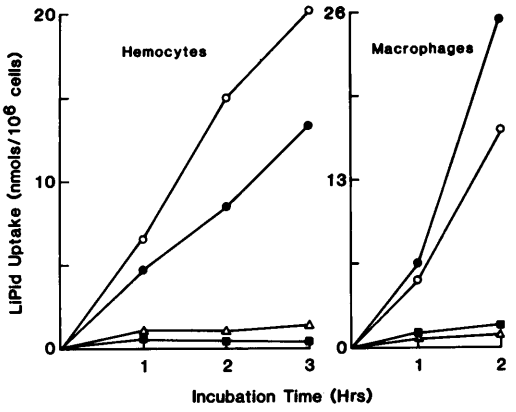


FIG. 2. Phagocytosis of liposomes by *H. virescens* hemocytes and mouse macrophages. Larvae were reared to the fifth instar on artificial medium[5] then bled by proleg amputation into a large volume of Dulbecco's phosphate-buffered saline (PBS, GIBCO, Grand Island, N.Y.) (pH 6.8). All buffers and media contained 8% saturated, filtered phenylthiourea (Kodak, Rochester, N.Y.), a phenol-oxidase inhibitor that prevents hemolymph melanization. The hemocytes were washed and resuspended in PBS at $10^6$ cells/ml. A 1-ml cell suspension was added to each well of a 24-well plate (Costar, Cambridge, Mass.); each well contained a 12-mm sterile glass coverslip. After 1-hr incubation at 25°C in humidified ambient air, the glass coverslips were washed free of nonadherent cells and placed into culture wells containing 1 ml of serum-free Grace's insect tissue culture medium, pH 6.8 (GIBCO). After an overnight incubation, approximately $2 \times 10^5$ cells adhered to each coverslip. The medium was removed and replaced with 1 ml PBS containing MLV labeled with trace amounts of the nonexchangeable liposome markers *N*-NBD-PE (*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine)[12] and $^{125}I$-PE (*N*-[3-(3-[$^{125}I$]iodo-4-hydroxybenzyl)propionyl]phosphatidylethanolamine)[13] prepared by vortexing appropriate lipid mixtures and extrusion through 2 μm polycarbonate membranes[21] (0.65 μmole lipid/ml/well). After 1, 2, or 3 hr, the glass coverslips were removed and washed in PBS to remove unbound liposomes. Thioglycollate-elicited mouse peritoneal macrophages were processed similarly, except Hanks' balanced salt solution was substituted for the specialized insect medium, and incubations were done at 37°C. Coverslips were then monitored for radiation by scintillation counting. The uptake of MLV was determined as the percentage of added cpm taken up by the individual cultures normalized to nmole lipid per $10^6$ cells. The results presented represent the average of three individual experiments. (■—■) PC; (△—△) PC:SA; (●—●) PC:PS; (○—○) PC:PG. The statistical significance of differences in uptake of liposomes was determined by a two-tailed Student's *t* test (see text).

TABLE I. INHIBITION OF LIPOSOME UPTAKE BY MLV

| Competitive MLV composition | % Inhibition of uptake | | | |
|---|---|---|---|---|
| | Hemocytes | | Macrophages | |
| | PC/PS | PC/PG | PC/PS | PC/PG |
| PC | 18 ± 0.5 | −10 ± 0.3 | 15 ± 1.4 | 6 ± 1.0 |
| PC/PS | 57 ± 1.3 | −7 ± 1.0 | 52 ± 12.2 | 37 ± 6.8 |
| PC/PG | 89 ± 4.8 | 59 ± 0.2 | 49 ± 14.4 | 45 ± 8.2 |

*Note.* Hemocyte and macrophage cultures prepared as described in Fig. 2 were incubated with $^{125}$I-PE-labeled test MLV (0.6 μmole) admixed with unlabeled competitor MLV. After 1 hr of incubation at 25 and 37°C for the hemocytes and macrophages, respectively, the cultures were washed and the amount of cell-associated $^{125}$I-labeled PE was determined.

PC alone did not significantly inhibit uptake in either system. The results imply that both *H. virescens* phagocytes and murine macrophages possess a surface structure that binds both PC:PS and PC:PG MLV. The phospholipid binding structures of the two species are apparently not identical, however, since the *H. virescens* phagocyte exhibits a greater affinity for PC:PG than for PC:PS, while the murine macrophage does not.

Our data on the preferential binding of negatively charged lipids to *H. virescens* phagocytes are remarkably similar to the observed phospholipid recognition pattern of mouse macrophages and human monocytes (8). The mechanism of this recognition would not seem to be due to simple nonspecific electrostatic attraction, since if it were, the negatively charged cell surface would be expected to more avidly bind positively charged PC:SA MLV. In addition, the uptake of PC:PG (net charge −1) would not have been expected to be greater than PC:PS (net charge −1). Due to technical difficulties with the hemocytes, however, we could not rule out the participation of calcium (by using EDTA) in this process, although it should be noted that the media employed for the uptake experiments was $Ca^{2+}$–$mg^{2+}$ free. Furthermore, if the process were due to $Ca^{2+}$-mediated cell-liposome bridging, uptake of the negatively charged MLV should have been independent of the negatively charged species used. In this context, we also stress that it is unlikely that MLV recognition involves the prophenyloxidase system known to operate in certain arthropodan nonself responses (17), since all culture media and buffers contained the phenyl-oxidase inhibitor, phenylthiourea. It must be determined whether the cell surface binding structures are specific for negatively charged compounds in the manner of the "scavenger reception" (19, 20).

The similar patterns of liposome uptake by phagocytes of an insect and a mammal may reflect analogous phospholipid recognition mechanisms that arose independently, perhaps in response to similar selective pressures. One such pressure might have been bacterial infection, since bacteria display appreciable amounts of negatively charged PG on their surfaces (21). Indeed, the inability of PS to inhibit PG uptake by the insect hemocytes suggests greater PG avidity to these cells. It is, however, difficult to speculate about other common selective pressures since, except for specialized instances of PS display in certain pathological states such as in RBC of sickle cell anemia (22) and chronic myeloid leukemia patients (23), the display of PS on other macrophage targets has not been determined. Alternatively, phospholipid recognition in insects and mammals could be homologous, having arisen in a common ancestor of the two groups and having been conserved through an enormous span of evolution. This latter issue can be investigated further by studying the phagocytes of such invertebrates as the echinoderms, which diverged more recently than insects from the ancestral line leading to the vertebrates.

Whatever their evolutionary origins, phagocyte phospholipid recognition systems must be involved in more than the speculated elimination of pathological erythrocytes (8–11) since it is does appear from this study that such a system operates in the phagocytes of an arthropod, a phylum that far predates the

emergence of erythrocyte-bearing animals. It is possible that the "phylogenetic preservation" of this type of recognition system represent yet another important component in host defense mechanisms, as proposed for antigen-independent cytotoxicity (13) and phagocytosis of chemically modified red cells (12) by other invertebrate phagocytes.

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1. Nappi AJ, Silvers M. Cell surface changes associated with cellular immune reactions in *Drosophila*. Science **225**:1166–1168, 1984.
2. Sharon N. Surface carbohydrates and surface lectins are recognition determinants in phagocytes. Immunol Today **5**:143–147, 1984.
3. Pollara B, Finstad J, Good RA. In: Smith RT, Mecher PA, Good RA eds. Phylogeny of Immunity. Univ of Florida Press, Gainsville, pp88–97, 1966.
4. Lackie AM, Lackie JM. Invertebrate immunity. Parasitology **80**:393–412, 1980.
5. Lackie AM, Lackie JM. Evasion of insect immune response by *Maniliformis dubius* (Acanthocephala): Further observations on the origins of the envelope. Parasitology **79**:297–301, 1979.
6. Lackie AM. Effect of substratum wettability and charge on adhesion *in vitro* and encapsulation *in vivo* by insect haemocytes. J Cell Sci **63**:181–190, 1983.
7. Ryter A, Dechastellier C. Phagocyte–pathogenic microbe interactions. Int Rev Cytol **85**:287–325, 1983.
8. Schroit AJ, Tanaka Y, Madsen J, Fidler IJ. The recognition of red blood cells by macrophages: Role of phosphatidylserine and possible implications of membrane phospholipid asymmetry. Biol Cell **51**: 227–238, 1984.
9. Tanaka Y, Schroit AJ. Insertion of fluorescent phosphatidylserine into the plasma membrane of red blood cells. J Biol Chem **258**:11,335–11,343, 1983.
10. Schroit AJ, Madsen J, Tanaka Y. *In vivo* recognition and clearance of red blood cells containing phosphatidylserine in their plasma membrane. J Biol Chem **260**:5131–5138, 1985.
11. Schwartz RS, Tanaka Y, Fidler IJ, Chiu D, Lubin B, Schroit AJ. Increased adherence of sickled and phosphatidylserine enriched human erythrocytes to cultured human peripheral blood monocytes. J Clin Invest **75**:1965–1972, 1985.
12. Rabinovitch M, De Stefano H-J. Interaction of red cells with phagocytes of the was-moth (*Galleria Mellonella, L.*) and Mouse. Exp Cell Res **59**:272–282, 1970.
13. Decker JM, Elmholt A, Muchmore AV. Spontaneous cytotoxicity mediated by invertebrate mononuclear cells toward normal and malignant vertebrate targets: Inhibition by defined mono- and disaccharides. Cell Immunol **59**:161–170, 1980.
14. Schroit AJ. Synthesis and properties of a nonexchangeable radioiodinated phospholipid. Biochemistry **21**:5323–5328, 1982.
15. Struck DK, Pagano RE. Insertion of fluorescent phospholipids into the plasma membrane of a mammalian cell. J Biol Chem **255**:5404–5410, 1980.
16. Olson F, Hunt CA, Szoka FC, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta **557**:9–23, 1979.
17. Fidler IJ, Raz A, Rogler WE, Kirsh R, Bugelski P, Poste G. Design of liposomes to improve delivery of macrophage-augmenting agents to alveolar macrophages. Cancer Res **40**:4460–4466, 1980.
18. Ratcliffe NA, Leonard C, Rowley AF. Prophenoloxidase activation: Nonself recognition and cell cooperation in insect immunity. Science **226**:559–560, 1984.
19. Van Berkel TJC, Nagelkerke JF, Kruijt JK. The effect of $Ca^{2+}$ and trifluoroperazine on the processing of human acetylated low density lipoprotein by nonparenchymal liver cells. FEBS Lett **132**:61–66, 1981.
20. Goldstein JL, Ho YK, Basu JK, Brown HS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci **76**:333–337, 1979.
21. Lehninger AL. Biochemistry: The Molecular Basis of Cell Structure and Function. Worth New York, pp195, 1970.
22. Lubin B, Chiu D, Bastacky J, Roelofsen B, van Deeman LLN. Abnormalities in membrane phospholipid organization in sickled erythrocytes. J Cin Invest **67**: 1643–1649, 1981.
23. Kumar A, Gupta CM. Red cell membrane abnormality in chronic myeloid leukemia. Nature (London) **303**:632–633, 1983.

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