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Multiple Systems for Recognition of Apoptotic Lymphocytes by Macrophages

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In vivo, apoptotic lymphocytes are recognized and phagocytosed by macrophages well before the final stages of DNA degradation and cell lysis. The recognition process is apparently triggered by the exposure of phosphatidylserine (PS) on the cell surface, an event which precedes cell lysis by several hours. However, multiple receptors appear to respond to this event. We demonstrate here that both activated and unactivated macrophages recognize PS, but with different receptor systems. Phagocytosis of apoptotic lymphocytes by activated (but not by unactivated) macrophages is inhibited by pure PS vesicles as well as by *N*-acetylglucosamine, implicating involvement of a lectin-like receptor in this case. Conversely, uptake of apoptotic lymphocytes by unactivated (but not by activated) macrophages is inhibited by PS on the surface of erythrocytes as well as by the tetrapeptide RGDS and cationic amino acids and sugars, implicating involvement of the vitronectin receptor in this case. Recognition by both classes of macrophages is blocked by the monocyte-specific monoclonal antibody 61D3. The signal recognized by activated macrophages appears to develop on the lymphocyte prior to assembly of the signal recognized by unactivated macrophages. Collectively, these results suggest that PS exposure on the surface of apoptotic lymphocytes generates a complex and evolving signal recognized by different receptor complexes on activated and unactivated macrophages.

INTRODUCTION

During primary lymphocyte differentiation in the thymus, a large fraction of immature T cells are removed by apoptosis (reviewed in Cohen *et al.*, 1992). One hallmark of this process is its tidiness: degenerating cells are observed only with difficulty in the thymus, even upon massive induction of apoptosis by corticosteroids (Schlegel *et al.*, 1993). This tidiness, which obviates the inflammation or tissue damage typical of necrosis, is a consequence of recognition and phagocytosis of apoptotic cells prior to cell lysis. Recognition implies that the surface of apoptotic cells must differ in some respect from that of their normal counterparts. To date, the only qualitative difference which has been documented is the exposure of phosphatidylserine (PS) on the apoptotic cell surface (Fadok *et al.*, 1992b; Koopman *et al.*, 1994; Martin *et al.*, 1995). Indeed, PS can inhibit phagocytosis of apoptotic lymphocytes by macrophages (Fadok *et al.*, 1992b), implying the existence of a macrophage receptor for PS. However, several other agents have also been shown to be effective inhibitors, implying that the apoptotic cell is recognized by multiple macrophage receptors: inhibition by antibody to the vitronectin receptor and by the tetrapeptide RGDS has implicated the vitronectin receptor (Fadok *et al.*, 1992a), and inhibition by certain sugars suggests lectin-like interactions (Duvall *et al.*, 1985).

The basis of this apparent multiplicity of recognition systems is clarified to some extent by the observation that certain of the systems are restricted to certain classes of macrophages (Fadok *et al.*, 1992a): the

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phagocytosis of apoptotic lymphocytes by unactivated macrophages (such as human monocyte-derived or mouse bone marrow macrophages) is inhibited by RGDS but not by PS, whereas phagocytosis of the same cells by activated macrophages (such as elicited mouse peritoneal macrophages) is inhibited by PS but not by RGDS. Remarkably, this difference is not due to the deployment of different recognition machinery: activated macrophages retain the vitronectin receptor (Fadok *et al.*, 1993), whereas PS vesicles are specifically recognized and phagocytosed by unactivated macrophages (Allen *et al.*, 1991; Lee *et al.*, 1993).

N-acetylglucosamine inhibits binding of apoptotic thymocytes to mouse peritoneal macrophages at 4°C, suggesting that surface carbohydrates on apoptotic thymocytes are recognized by lectin-like molecules on the macrophage surface (Duvall *et al.*, 1985). Confirmation of this observation or its extension to phagocytosis at 37°C has not appeared in the literature. On the other hand, cationic sugars and amino acids were reported to inhibit the phagocytosis of apoptotic neutrophils by monocyte-derived macrophages (Savill *et al.*, 1989), an effect which was attributed to blocking of the vitronectin receptor (Savill *et al.*, 1990). We demonstrate here that these inhibitors are also effective at blocking phagocytosis of apoptotic thymocytes, but more importantly, their effects too are restricted to one class of macrophage or the other: inhibition by cationic sugars and amino acids, as well as RGDS, segregates to unactivated macrophages, whereas inhibition by *N*-acetylglucosamine segregates to activated macrophages.

Among a panel of monoclonal antibodies directed against a range of human monocyte/macrophage surface antigens, only the 61D3 antibody (Ugolini *et al.*, 1980) was found to inhibit recognition of apoptotic lymphocytes by unactivated human monocyte-derived macrophages (Flora and Gregory, 1994). Blocking occurred at the macrophage surface because 61D3 antibody did not bind to the apoptotic cells, and pretreatment of macrophages, but not their targets, inhibited recognition. In contrast to the other agents, we find that the 61D3 antibody blocks uptake of apoptotic cells by both unactivated and activated macrophages, implying that its antigen is an element which is common to the recognition mechanisms of both classes of macrophages.

Recognition of apoptotic lymphocytes by both activated and unactivated macrophages implies that signals recognized by each type are simultaneously displayed on the target cell surface. However, we show here that competent signals are assembled on the apoptotic cell at different times during the apoptotic program: sensitivity of uptake to inhibition by PS vesicles precedes the development of sensitivity to inhibitors of integrins.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), dexamethasone, RGDS, sugars, and amino acids were purchased from Sigma Chemical Co.; brain PS was purchased from Avanti Polar Lipids, Inc.

Animals

Male CBA/J mice, 5–8 wk of age, were maintained on food and water ad libitum in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Isolation and Induction of Apoptosis in Thymocytes

Thymuses were removed from 4- to 6-wk old mice, dissociated in phosphate-buffered saline (PBS, 7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO4, 137 mM NaCl, 10 mM KCl) containing 5% calf serum, collected by centrifugation, resuspended in 17 mM Tris, 140 mM NH_4Cl, pH 7.2 (5 ml/thymus) to lyse erythrocytes, centrifuged, and resuspended at $\sim 5 \times 10^7$ cells/ml in DMEM containing 10% fetal bovine serum. Apoptosis was induced by addition of 10^{-6} M dexamethasone and incubation at 37°C in 5% CO_2. To monitor apoptosis, 10^6 cells were washed with PBS and fixed by resuspending in equal volumes of PBS and 70% ethanol. Fixed cells were then centrifuged and resuspended in 200 μl of PBS, stained with propidium iodide at a final concentration of 18 μg/ml, and analyzed by flow cytometry using a Coulter EPICS 753 flow cytometer equipped with an argon laser tuned to 488 nm and a 488-nm laser blocking filter. Propidium iodide fluorescence was reflected from a 590-nm shortpass dichroic filter and monitored through a 610-nm longpass filter.

Lipid-symmetric and Lipid-asymmetric Erythrocytes

Cells were prepared as described earlier (Schlegel *et al.*, 1987) using erythrocytes from fresh human venous blood obtained from volunteers according to institutional guidelines.

Culture of Mouse Peritoneal, Bone Marrow, and J774A.1 Macrophages

Inflammatory macrophages were elicited in the peritoneal cavity of 6- to 8-wk old mice by i.p. injection of 1 ml of 3% Brewer's thioglycollate. Cells were harvested 5–7 days later by peritoneal lavage using 10 ml of PBS containing 10 units/ml heparin. The exudate was centrifuged at 4°C for 10 min and washed in RPMI 1640. The cells were finally suspended in 5% calf serum in RPMI 1640 (4×10^6/ml), and 150 μl (6×10^5 cells) were pipetted onto 18-mm bicarbonate-treated glass coverslips pretreated for 2 h with calf serum. Triplicate coverslips were placed in 60-mm Petri dishes. After a 2-h incubation at 37°C, nonadherent cells were removed by aspiration, the media were replaced with 150 μl/coverslip of fresh RPMI 1640 containing 5% calf serum, and cells were cultured overnight at 37°C in 5% humidified CO_2. Mouse bone marrow macrophages were harvested as described by Stewart (1981) and cultured for 5 to 7 days as described by Fadok *et al.* (1993), except that conditioned medium from human giant cell tumor cells (product M7657, Sigma Chemical Co.) was used as a source of macrophage colony-stimulating factor. Cells of the J774A.1 mouse monocyte-derived macrophage cell line (American Type Culture Collection) were grown in 10% fetal bovine serum in DMEM at 37°C in 5% CO_2. Twenty-four hours prior to phagocytosis assays, triplicate coverslip cultures of 3×10^5 bone marrow or J774 macrophages were prepared on 18-mm bicarbonate-treated glass coverslips kept in 60-mm Petri dishes.

Phagocytosis Assays

Thymocytes (10^6) or erythrocytes (15×10^6), in 150 μl of DMEM, were overlayed onto macrophages, with or without inhibitors, and

coverslips were incubated at 37°C in 5% CO_2. At various times, coverslips were washed three times with ice-cold PBS, fixed either with 1.8% formaldehyde or ice-cold acidic methanol, and stained with Diff-Quik (Baxter). In experiments with erythrocytes, coverslips were treated with 17 mM Tris 140 mM NH_4Cl (pH 7.2) prior to fixation to lyse uningested erythrocytes. To distinguish erythrocytes from thymocytes, coverslips were stained for hemoglobin with benzidine prior to staining with Diff-Quik. Using this procedure, erythrocytes stained brown; thymocytes stained uniformly intensely blue; only the nuclei of macrophages stained intensely blue, with the cytoplasm staining less intensely, and the cell periphery being discernible by its increased staining relative to the cytoplasm. Phagocytosed cells were enumerated by microscopy, selecting random fields and counting 400 macrophages/coverslip.

Thymocytes were counted as phagocytosed if they were entirely within the outline of the macrophage and in the same focal plane as the macrophage. Thymocytes not entirely within the outline of the macrophage but in the same focal plane and obviously being engulfed by a distended macrophage were also counted as phagocytosed; they represented no more than 10% of the cells counted as phagocytosed. Thymocytes not contained within the outline of the macrophage, but which appeared to touch it, were considered adherent, as were thymocytes within or partially within the outline of the macrophage but in a different focal plane from the macrophage; the latter cells were rare, representing less than 5% of thymocytes. Thus, cells within or partially within the outline of the macrophage which were in the process of being phagocytosed were not counted as phagocytosed cells if not in the same focal plane as the macrophages. Although this underestimates the number of phagocytosed cells, the error is small because of the small number of thymocytes which fell in this category.

The ratio of adherent to phagocytosed cells was roughly 3:1. In some experiments, identical coverslips were treated with 0.25% trypsin for 5 min, after washing with ice-cold PBS. Although the numbers of adherent thymocytes were reduced about 5-fold, not all adherent cells were removed by the treatment. On the other hand, the number of thymocytes counted as phagocytosed was no different from that of untreated identical coverslips. For this reason, a trypsin treatment did not remove the requirement for applying the criteria presented above and was therefore not routinely performed.

Inhibitors

PC and PS vesicles were prepared by first drying a film of the phospholipid under a stream of nitrogen, followed by 4 h under vacuum, and then hydrating the film in PBS to 150 μM lipid and sonicating in a bath-type sonicator (Pradhan *et al.*, 1994). Fifty microliters (7.5 nmol) of these vesicles were added to 10^6 thymocytes in 100 μl, and the mixture was overlayed onto the macrophages. Fifteen $\times$ 10^6 erythrocytes and 10^6 thymocytes were resuspended in a total of 150 μl of DMEM which was overlayed onto macrophage monolayers. For RGDS, sugars, and amino acids, either thymocytes were resuspended in 150 μl of DMEM containing the inhibitor at the desired concentration (1 mM for RGDS, 20 mM for sugars and amino acids) or a concentrated stock solution of the inhibitor was added to thymocytes suspended in 150 μl of DMEM to give the desired final concentration just prior to overlaying onto macrophages. 61D3 monoclonal antibody (1 mg/ml, purified by protein A affinity chromatography, a gift from Dr. Donald Capra, University of Texas Southwestern Medical School at Dallas, Dallas, TX) or antihuman CD14 monoclonal antibody (clone UCHM-1, Sigma Chemical Co.) was added to 10^6 thymocytes or 15 $\times$ 10^6 erythrocytes in 150 μl of DMEM to the concentration indicated just prior to overlaying onto macrophages.

RESULTS

To determine whether cells of the J774 line of mouse macrophages, like primary macrophages, can specifically recognize apoptotic lymphocytes, they were presented with primary thymocytes that had been treated with dexamethasone to induce apoptosis. After 6 h of treatment, 48% of the target thymocytes were apoptotic as judged by fragmentation of chromatin monitored by propidium iodide staining of DNA. In control cultures incubated in the absence of dexamethasone, 9% of the cells spontaneously became apoptotic. Viability of the cultures at this time, as judged by the ability to exclude propidium iodide before fixation, was >95% with or without dexamethasone. As shown in Figure 1A, after 30 min of exposure to thymocytes, J774 macrophages had taken up over five times as many cells from induced versus uninduced cultures. Because this ratio did not increase thereafter, even though absolute numbers of thymocytes phagocytosed did, subsequent phagocytosis assays were conducted for 30 min. This result indicates that J774 macrophages are capable of specific recognition and phagocytosis of apoptotic thymocytes.

As presented in the INTRODUCTION, apoptotic cell recognition by unactivated macrophages is insensitive to inhibition by PS vesicles. Because J774 macrophages behave in other respects like unactivated macrophages (Pinson *et al.*, 1991), the ability of PS vesicles to block recognition of apoptotic thymocytes by J774 cells was tested. As shown in Figure 1B, PS vesicles had no significant effect on phagocytosis of apoptotic thymocytes by J774 cells. Similarly, uptake by unactivated macrophages is dependent on the vitronectin receptor and is thus sensitive to the RGDS peptide (Fadok *et al.*, 1992a). As also shown in Figure 1B, RGDS substantially reduced phagocytosis of apoptotic thymocytes by J774 macrophages to an extent similar to that reported for human monocyte-derived macrophages (Fadok *et al.*, 1992a). Similar results using PS vesicles and RGDS as inhibitors were obtained in all of at least 10 other experiments, although the percentage of inhibition by RGDS varied from about 50% to nearly 100% from experiment to experiment; the source of this variability has not yet been established. Together, these data indicate that J774 macrophages behave as unactivated macrophages in the recognition of apoptotic thymocytes.

Partial inhibition could be the result of heterogeneity within the apoptotic thymocyte population or heterogeneity within the macrophage population, i.e., the inhibitor might block uptake of only some apoptotic thymocytes or by only some macrophages. Alternatively, it might simply represent a reduction in the rate of recognition and phagocytosis. To examine these possibilities, the effect of RGDS on uptake was measured as a function of time. As shown in Figure 2, the inhibitor slowed uptake, so that the most dramatic inhibition was seen at the shortest time. Uptake in the presence of inhibitor eventually reached the same plateau as in the absence of inhibitor, although more

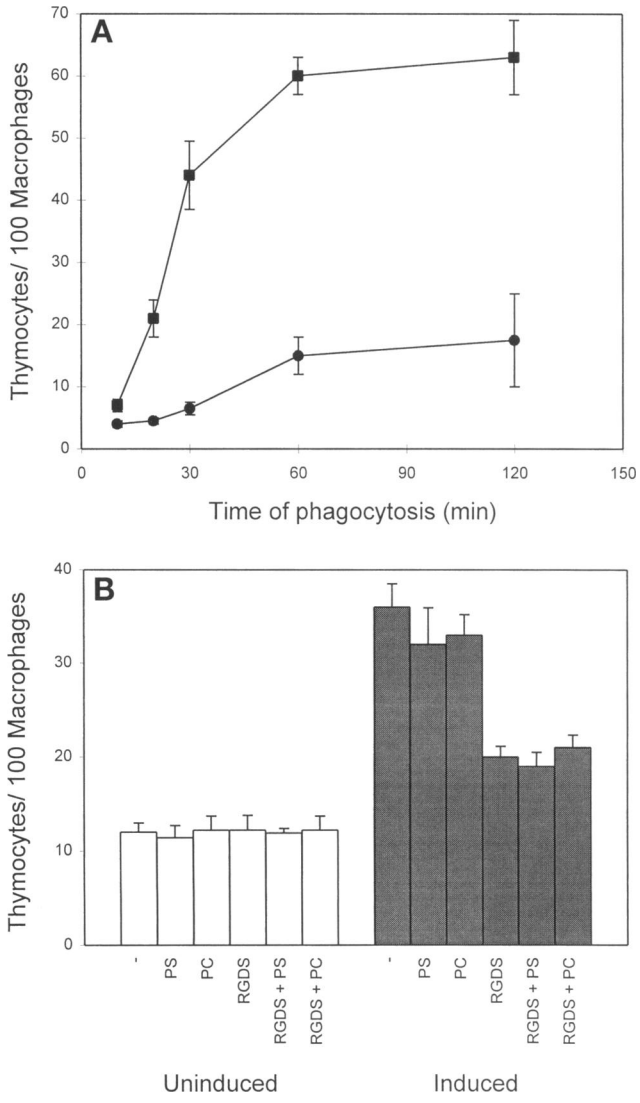

Figure 1. Phagocytosis of thymocytes by J774 macrophages. (A) Thymocytes were incubated for 6 h in the presence (■) or absence (●) of 10^{-6} M dexamethasone, added to monolayer cultures of macrophages, and at various times the number of thymocytes phagocytosed were counted. (B) Thymocytes were incubated for 6 h in the presence (induced) or absence (uninduced) of 10^{-6} M dexamethasone and then mixed with PS vesicles, PC vesicles, RGDS, or combinations. The mixtures were added to monolayer cultures of macrophages, and the number of thymocytes phagocytosed in 30 min was counted.

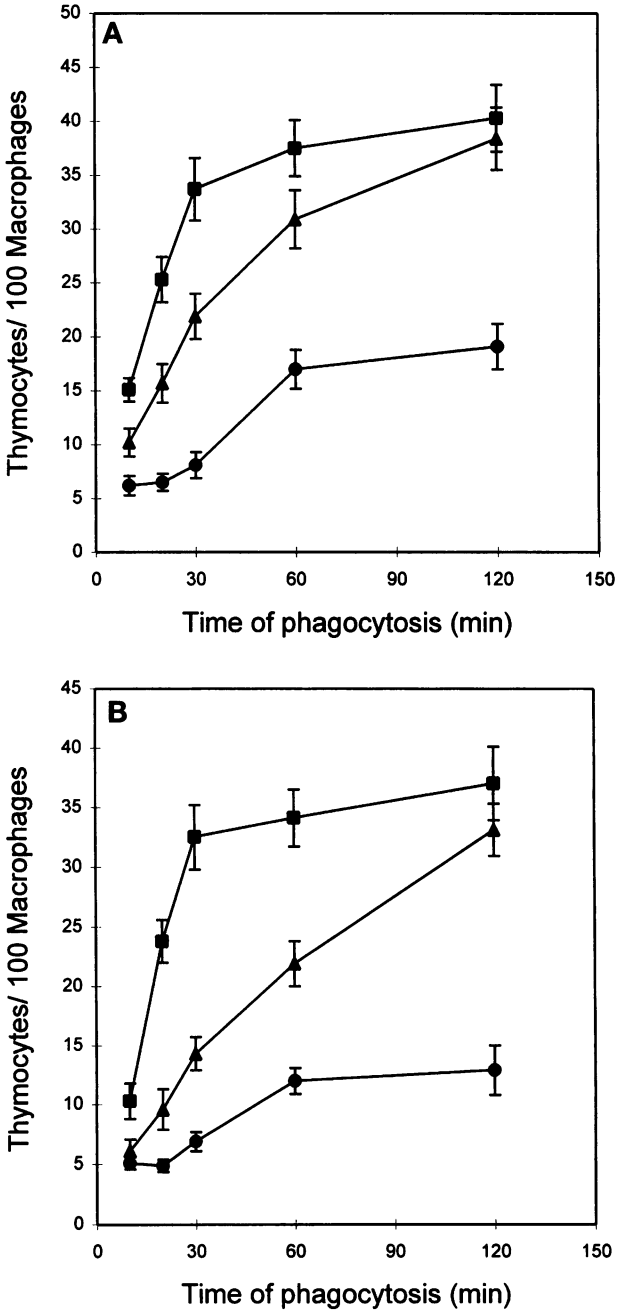

Figure 2. Time course of phagocytosis of thymocytes by macrophages in the presence of inhibitor. Thymocytes were incubated for 6 h in the presence or absence of 10^{-6} M dexamethasone, added to monolayer cultures of macrophages in the presence or absence of inhibitor, and at various times the number of thymocytes phagocytosed were counted. (A) J774 macrophages; RGDS. (B) mouse peritoneal macrophages; *N*-acetylglucosamine. ●, uninduced; ■, induced; ▲, induced in the presence of inhibitor.

slowly. These results indicate that the inhibitor affects the rate of apoptotic cell recognition and phagocytosis, without affecting the capacity of the macrophages to recognize and engulf apoptotic cells.

As indicated in the INTRODUCTION, a variety of sugars and amino acids have been tested for their ability to inhibit binding/phagocytosis of apoptotic lymphocytes/neutrophils by activated/unactivated macrophages (Duvall *et al.*, 1985; Savill *et al.*, 1989). To clarify the relationship of these agents to each other and to the action of other inhibitors, their ability to inhibit phagocytosis of apoptotic thymocytes by both

activated and unactivated macrophages was examined. As shown in Figure 3, the agents fall into two groups, distinguished by the type of macrophage to which their effects are restricted: glucosamine, galactosamine, and arginine (as well as lysine, but not glucose or galactose) were effective at inhibiting uptake by J774 macrophages (Figure 3A) but had no effect on recognition by activated mouse peritoneal macrophages (Figure 3B). In contrast, *N*-acetylglucosamine inhibited phagocytosis by mouse peritoneal macrophages (Figure 3B) but was ineffective against J774 macrophages (Figure 3A). As in the case of inhibition of unactivated macrophages by RGDS, *N*-acetylglucosamine slowed the rate of uptake by activated macrophages (Figure 2B).

Both unactivated human monocyte-derived (McEvoy *et al.*, 1986) and J774 (Pradhan *et al.*, 1994) macrophages recognize erythrocytes with PS on their surface produced by abolishing the asymmetric transbilayer distribution of phospholipids in which PS is restricted to the inner leaflet of the membrane bilayer (Williamson *et al.*, 1985). Since uptake of these lipid-symmetric erythrocytes is completely inhibitable by PS vesicles (Pradhan *et al.*, 1994), unactivated macrophages recognize these erythrocytes by the PS exposed on their surface, and PS is the only signal they recognize. Given the differences in the systems used for recognition of apoptotic thymocytes by activated and unactivated macrophages revealed by various inhibitors, it was of interest to determine whether the systems for recognition of lipid-symmetric erythrocytes also differed between activated and unactivated macrophages. As shown in Figure 4A, only PS vesicles, and not *N*-acetylglucosamine, RGDS, or lysine, were able to inhibit uptake of lipid-symmetric erythrocytes by J774 macrophages, confirming that recognition is by PS alone. However, as shown in Figure 4B, activated mouse peritoneal macrophages could be inhibited in their uptake of lipid-symmetric erythrocytes by both PS vesicles and *N*-acetylglucosamine but not RGDS or lysine, mirroring what was seen in the uptake of apoptotic thymocytes. Because neither PS vesicles nor *N*-acetylglucosamine inhibited uptake completely, the two agents were tried in combination. As seen in Figure 5, when the two inhibitors were present simultaneously, they were no more effective than either inhibitor alone, implying that the two agents do not inhibit separate recognition pathways.

The inability of PS vesicles to affect recognition of apoptotic lymphocytes by unactivated macrophages, even though apoptotic thymocytes expose PS on their surface (Fadok *et al.*, 1992b; Koopman *et al.*, 1994; Martin *et al.*, 1995; Verhoven *et al.*, 1995), and the ability of J774 cells, as well as primary unactivated macrophages, to specifically recognize PS vesicles and lipid-symmetric erythrocytes with PS on their surface (Lee *et al.*, 1993; Pradhan *et al.*, 1994; McEvoy *et al.*, 1986) suggest that either unactivated macrophages cannot recognize PS on thymocytes or PS vesicles are ineffective competitors. Evidence for the latter possibility was obtained by testing the inhibitory effect of PS presented on lipid-symmetric erythrocytes rather than in the form of vesicles. Figure 6A compares the uptake of apoptotic thymocytes by J774 cells in the presence of RGDS or in the presence of lipid-symmetric erythrocytes or lipid-asymmetric erythrocytes as

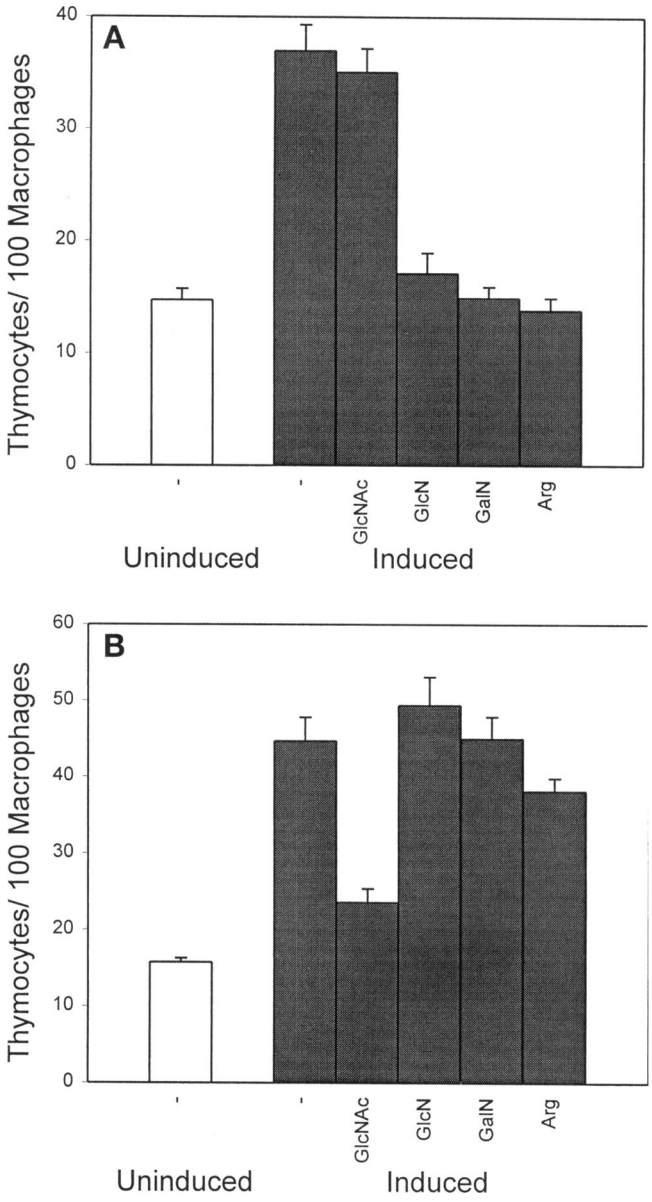

Figure 3. Phagocytosis of thymocytes by macrophages in the presence of sugars and amino acids. Induced thymocytes were suspended in 20 mM *N*-acetylglucosamine (GlcNAc), glucosamine (GlcN), galactosamine (GalN), or arginine (Arg), added to J774 (A) and mouse peritoneal (B) macrophages, and the number of thymocytes phagocytosed in 30 min was counted.

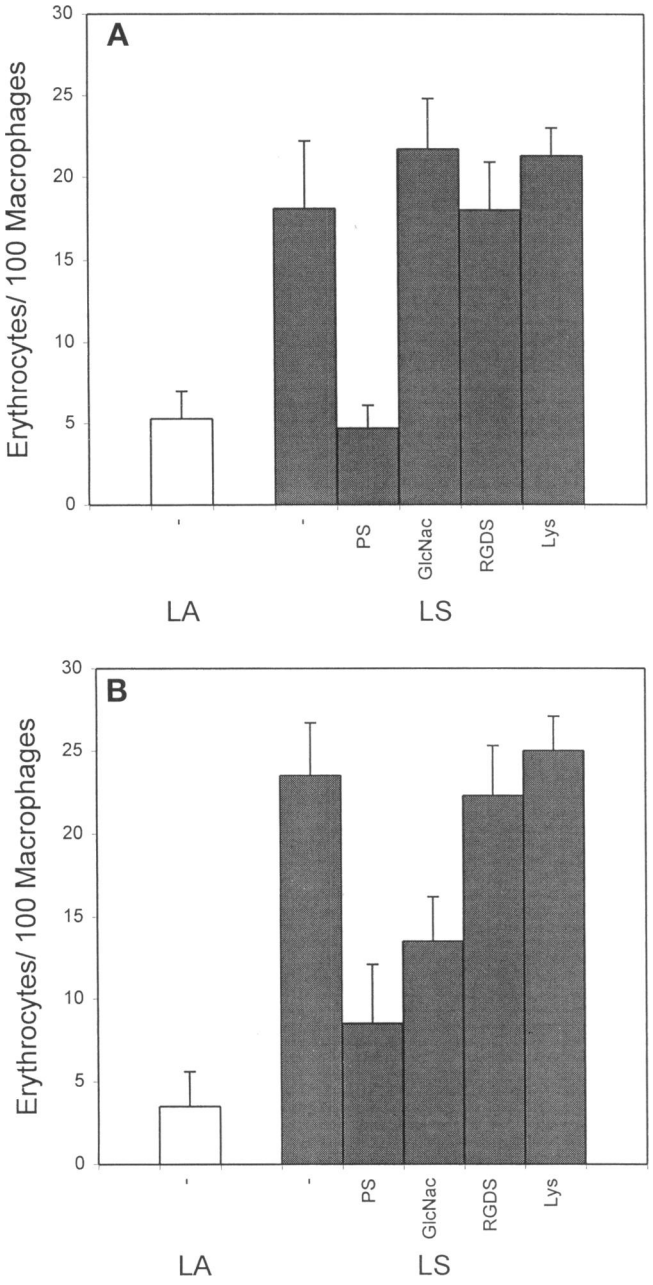

Figure 4. Phagocytosis of lipid-symmetric erythrocytes in the presence of various inhibitors. Lipid-symmetric erythrocytes were mixed with PS vesicles or suspended in RGDS, *N*-acetylglucosamine (GlcNAc), or lysine (Lys), added to J774 (A) or mouse peritoneal (B) macrophages, and the number of erythrocytes phagocytosed in 30 min was counted.

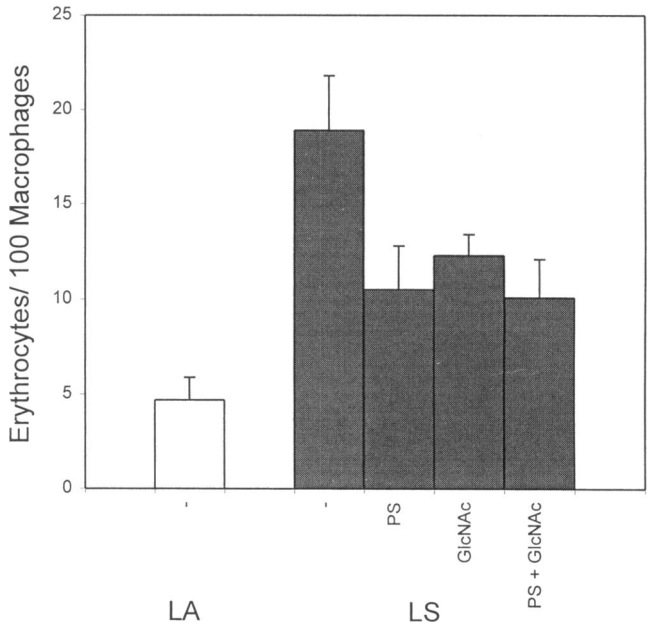

Figure 5. Phagocytosis of lipid-symmetric erythrocytes by mouse peritoneal macrophages in the presence of a combination of PS vesicles and *N*-acetylglucosamine. Lipid-symmetric erythrocytes were added to mouse peritoneal macrophages in the presence of PS vesicles, *N*-acetylglucosamine (GlcNAc), or a combination of the two inhibitors, and the number of erythrocytes phagocytosed in 30 min was counted.

controls, added at a ratio of erythrocytes to lymphocytes of 15:1. As in the experiment shown in Figure 1B, RGDS was effective at inhibiting uptake of apoptotic thymocytes. Addition of lipid-symmetric erythrocytes also inhibited phagocytosis of apoptotic cells (Figure 6A). However, lipid-asymmetric erythrocytes, which do not have PS exposed on their surface, had little effect. This inhibition is not peculiar to cultured J774 cells. As shown in Figure 7, primary mouse bone marrow macrophages behave similarly: their uptake of apoptotic thymocytes is also inhibited in the presence of lipid-symmetric but not lipid-asymmetric erythrocytes.

Although phagocytosis of lipid-symmetric erythrocytes is not affected by RGDS, addition of both RGDS and lipid-symmetric erythrocytes did not result in more inhibition than that observed with either agent alone (Figure 6A), implying that they do not inhibit separate recognition pathways. As expected from the excess of erythrocytes to thymocytes present, inhibition of the uptake of apoptotic cells by lipid-symmetric erythrocytes was not reciprocal: counts of phagocytosed erythrocytes, distinguishable from thymocytes by differential staining, indicated that lipid-symmetric erythrocytes were phagocytosed equivalently whether or not thymocytes were also present (25.2 ± 3.4, compared with 20–25 in numerous published experiments, Pradhan *et al.*, 1994). If PS-dependent recognition of lipid-symmetric erythrocytes is responsible for their inhibitory effect, the addition of PS vesicles (which block this recognition, Figure 4A) should restore phagocytosis of apoptotic thymocytes to normal levels. Such was the case (Figure 8). Taken together, these results suggest that the failure of PS vesicles

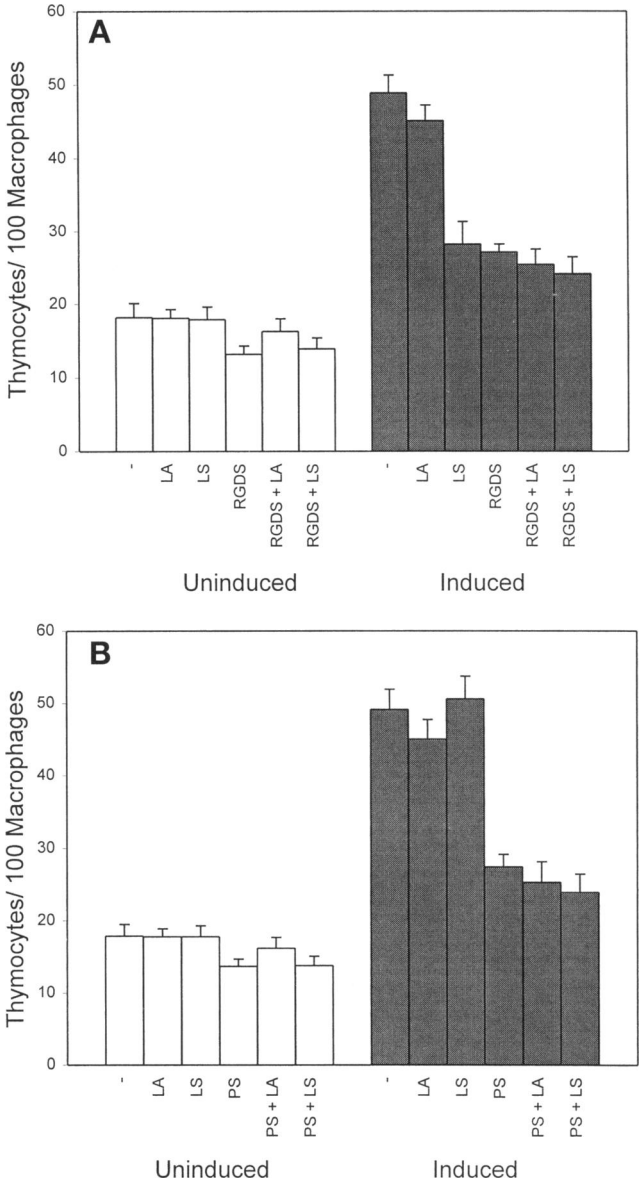

Figure 6. Phagocytosis of thymocytes by macrophages in the presence of lipid-symmetric erythrocytes, lipid-asymmetric erythrocytes, or RGDS. Induced or uninduced thymocytes were mixed with lipid-symmetric erythrocytes (LS), lipid-asymmetric erythrocytes (LA), RGDS, or combinations. The mixtures were added to monolayer cultures of J774 macrophages (A) or mouse peritoneal macrophages (B), and the number of thymocytes phagocytosed in 30 min was counted.

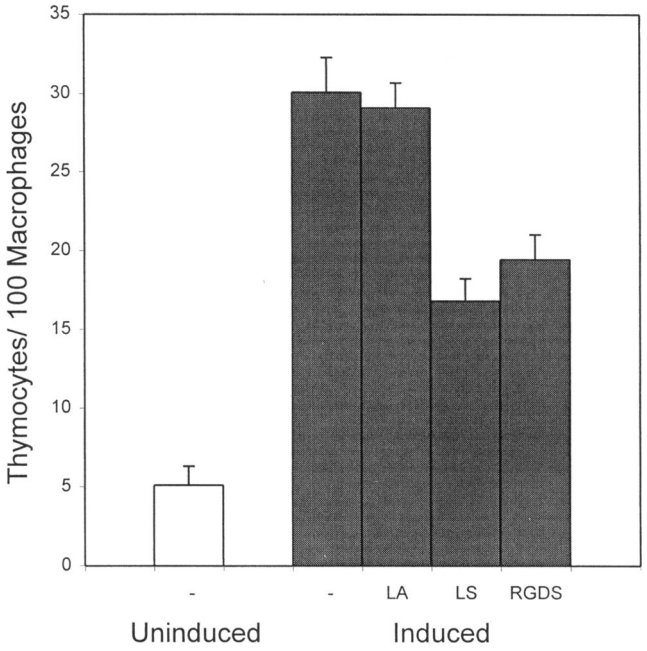

Figure 7. Phagocytosis of thymocytes by mouse bone marrow macrophages in the presence of lipid-symmetric erythrocytes. Induced thymocytes were mixed with lipid-symmetric erythrocytes (LS), lipid-asymmetric erythrocytes (LA), or RGDS. The mixtures were added to monoloayer cultures of mouse bone marrow macrophages, and the number of thymocytes phagocytosed in 30 min was counted.

to inhibit thymocyte uptake is not because unactivated macrophages do not recognize the PS on the apoptotic cell surface, but rather stems from the inefficiency of PS as a competitor when presented in the form of a pure lipid vesicle.

Activated macrophages recognize apoptotic thymocytes by a mechanism which is sensitive to PS vesicles (Fadok *et al.*, 1992b); this effect is illustrated in Figure 6B, which shows that phagocytosis of apoptotic thymocytes by mouse peritoneal macrophages was reduced approximately 50% by the presence of PS vesicles. Since uptake of lipid-symmetric erythrocytes by these cells was also sensitive to the presence of PS vesicles (Figures 4 and 5), it might be expected that lipid-symmetric erythrocytes, with exposed PS on their surface, should block the uptake of apoptotic thymocytes by activated macrophages as they do in the case of unactivated macrophages. However, this expectation was not confirmed by direct test: uptake of apoptotic thymocytes by activated macrophages was not inhibited by the addition of lipid-symmetric (or lipid-asymmetric) erythrocytes (Figure 6B). Importantly, when lipid-symmetric erythrocytes and thymocytes were presented together, both were taken up in numbers equivalent to those observed when each was presented separately (for erythrocytes, 18.2 ± 2.1 versus 14.5 ± 3.2).

The mouse monoclonal antibody 61D3, specific for a M_r 75,000 protein on the surface of human monocytes (Nunez *et al.*, 1982), has been shown to inhibit the uptake of apoptotic lymphocytes and neutrophils by human monocyte-derived macrophages, although it did not appear to interfere with phagocytosis in general or with vitronectin-based recognition (Flora and Gregory, 1994). When this antibody was used in as-

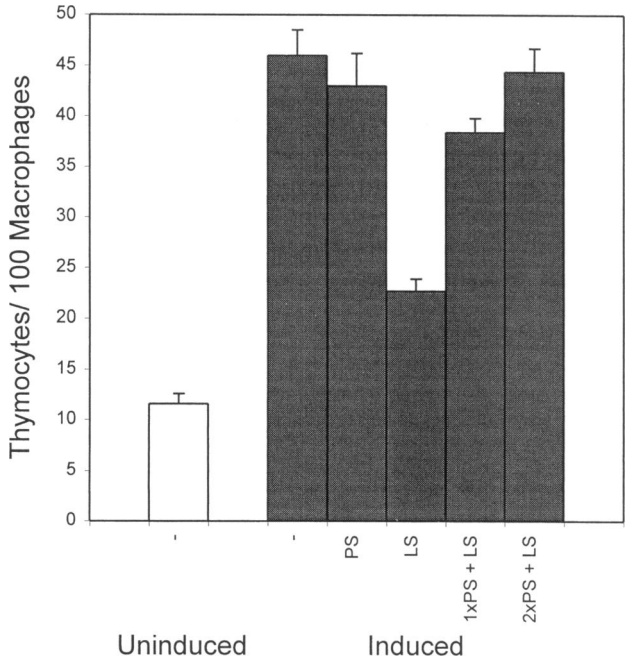

Figure 8. Phagocytosis of thymocytes by J774 macrophages in the presence of both lipid-symmetric erythrocytes and PS vesicles. Induced and uninduced thymocytes were mixed with either PS vesicles, lipid-symmetric erythrocytes, or a combination of lipid-symmetric erythrocytes and 7.5 nmol (1×) or 15 nmol (2×) of PS vesicles. The mixtures were added to monolayer cultures of J774 macrophages, and the number of thymocytes phagocytosed in 30 min was counted.

says with mouse J774 macrophages, the same inhibition was observed (Figure 9A), implying that the effective epitope is conserved between these two species. When the antibody was tested with activated mouse peritoneal macrophages, the same result was observed (Figure 9B), suggesting that the recognition element against which the antibody is directed participates in recognition by both classes of macrophages. Uptake of lipid-symmetric erythrocytes by both classes of macrophages is also sensitive to the antibody (Figure 9, C and D), indicating that participation of the element is not limited to recognition of apoptotic cells.

The existence of disparate recognition systems in the two classes of macrophages raises the question of whether the two systems recognize different aspects of the same signal on the lymphocyte surface. This issue was addressed by examining the effectiveness of inhibitors at blocking recognition of lymphocytes at various times after induction of apoptosis. As shown in Figure 10, recognition of apoptotic thymocytes by activated macrophages is uniformly sensitive to inhibition by PS vesicles; in contrast, the sensitivity of uptake by unactivated macrophages becomes progressively more sensitive to RGDS with time. This result suggests that the signals recognized by the different macrophage types are distinct, and that the vitronectin-dependent signal develops somewhat later in the apoptotic program.

DISCUSSION

The experiments reported here imply that PS-dependent recognition by macrophages is not a simple process. To facilitate comparisons, the effects of all of the inhibitors used in the current study on phagocytosis of lipid-symmetric erythrocytes and apoptotic thymocytes by J774 and activated mouse peritoneal macrophages are summarized in Table 1. The mechanism by which unactivated macrophages recognize erythrocytes with PS exposed on their surface appears to be the simplest recognition system examined in this study. Because only PS vesicles inhibit uptake and inhibition is complete, it appears that uptake is mediated by recognition of PS on the erythrocyte surface by a PS receptor on unactivated macrophages. The fact that the 61D3 monoclonal antibody blocks the macrophage side of the couple (Flora and Gregory, 1994) suggests that the epitope being recognized by the antibody resides on a PS receptor. However, these results raise the conundrum of why PS vesicles fail to inhibit the uptake of apoptotic thymocytes by unactivated macrophages. PS appears on the surface of lymphocytes undergoing apoptosis (Fadok *et al.*, 1992b; Koopman *et al.*, 1994; Martin *et al.*, 1995) as a consequence of up-regulation of a scrambling activity which randomizes the normal asymmetric transbilayer lipid distribution (Verhoven *et al.*, 1995), bringing PS to the surface, combined with down-regulation of the enzyme responsible for clearing PS from the outer leaflet of the membrane bilayer (Verhoven *et al.*, 1995). Since both primary unactivated macrophages and J774 cells can specifically recognize PS in the form of vesicles (Allen *et al.*, 1991; Lee *et al.*, 1993; Pradhan *et al.*, 1994) and also recognize erythrocytes solely by the PS exposed on their surface, why is recognition of apoptotic thymocytes not PS sensitive?

The results presented here suggest that the solution to this riddle is that recognition of apoptotic target cells by unactivated macrophages is PS sensitive, as revealed by the ability of lipid-symmetric erythrocytes to inhibit phagocytosis. If correct, however, the problem becomes why lipid-symmetric erythrocytes are effective competitors in this case and PS vesicles are not. One simple explanation is that macrophages have a higher affinity for PS on the surface of erythrocytes than on vesicles, making them a better inhibitor. This argument is consistent with the fact that macrophages readily phagocytose lipid-symmetric erythrocytes where the concentration of PS on the surface does not exceed approximately 6 mol % of the total lipid, whereas uptake of pure phospholipid vesicles containing PS at this concentration is not markedly enhanced

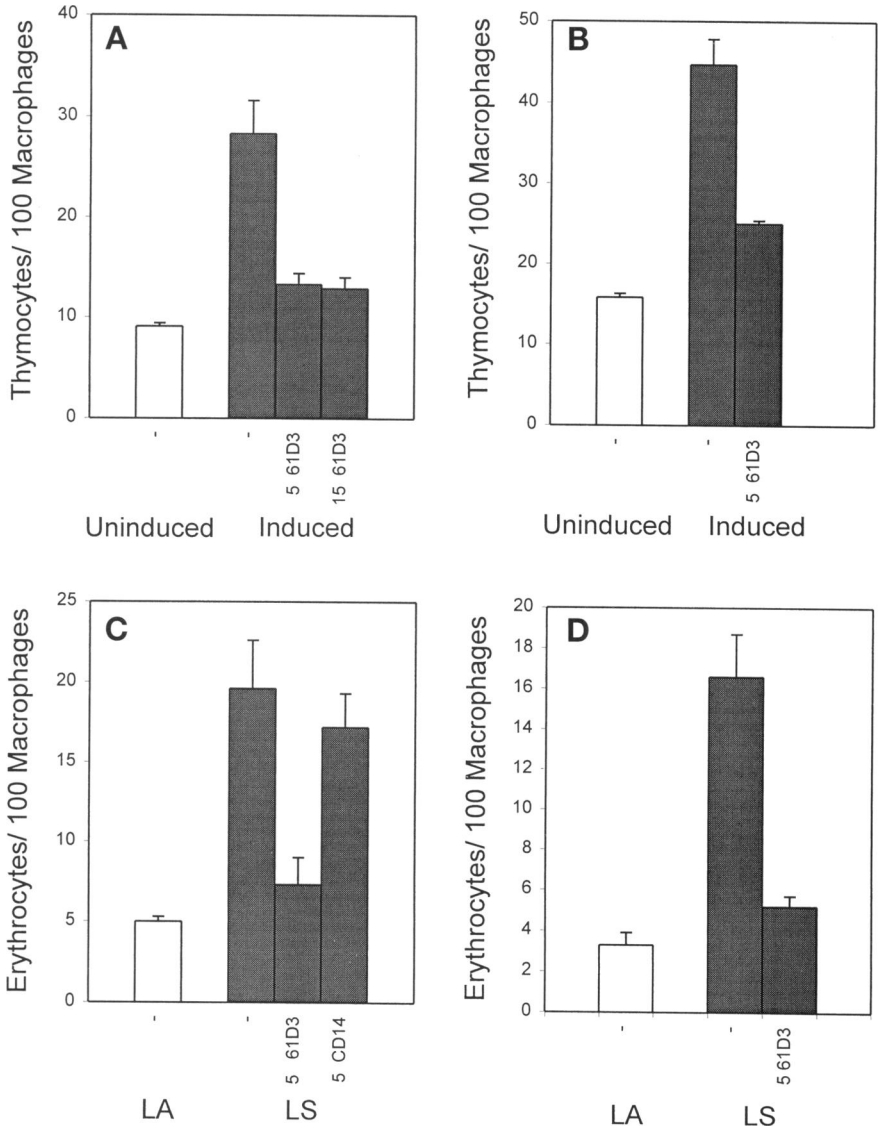

Figure 9. Phagocytosis of thymocytes or lipid-symmetric erythrocytes by macrophages in the presence of 61D3 monoclonal antibody. Either 5 or 15 μg of 61D3 monoclonal antibody (or anti-CD14 monoclonal antibody as a control in C) were added to 150 μl of a thymocyte (A and B) or erythrocyte (C and D) suspension to give final antibody concentrations of 33 or 66 μg/ml, and phagocytosis by J774 (A and C) or mouse peritoneal (B and D) macrophages was measured.

(Pradhan *et al.*, 1994). Importantly, phagocytosis of lipid-symmetric erythrocytes and apoptotic thymocytes by activated macrophages continues unabated in the presence of excesses of both cell types, suggesting that the active recognition and phagocytosis of lipid-symmetric erythrocytes does not simply sterically inhibit the association of unactivated macrophages and apoptotic cells.

Alternatively, the effectiveness of lipid-symmetric erythrocytes over PS vesicles in inhibiting uptake of apoptotic lymphocytes by unactivated macrophages may be related to the complexity of the signal assembled on the lymphocyte surface. The idea of a complex signal is supported by the observation that RGDS and lipid-symmetric erythrocytes in combination are no more effective than either agent alone in inhibiting uptake of apoptotic thymocytes by unactivated macrophages (Figure 3), suggesting that the ligand for the vitronectin receptor and PS are elements of a single complex recognition signal, recognized by a receptor complex. Cationic sugars and amino acids are thought to exert their inhibitory effects by masking anionic molecules on the apoptotic cell surface (Savill *et al.*, 1989). Their remarkable effectiveness in the present study may therefore reflect their ability to mask the entire complex signal rather than block individual elements of a receptor complex as PS and RGDS do.

The existence of another complex, but different, PS signal is implied by the studies with activated macrophages. Both PS vesicles and *N*-acetylglucosamine were able to partially inhibit uptake of both lipid-symmetric erythrocytes and apoptotic thymocytes. Yet the combination of the two agents was no more effective than either alone. This result suggests that PS and

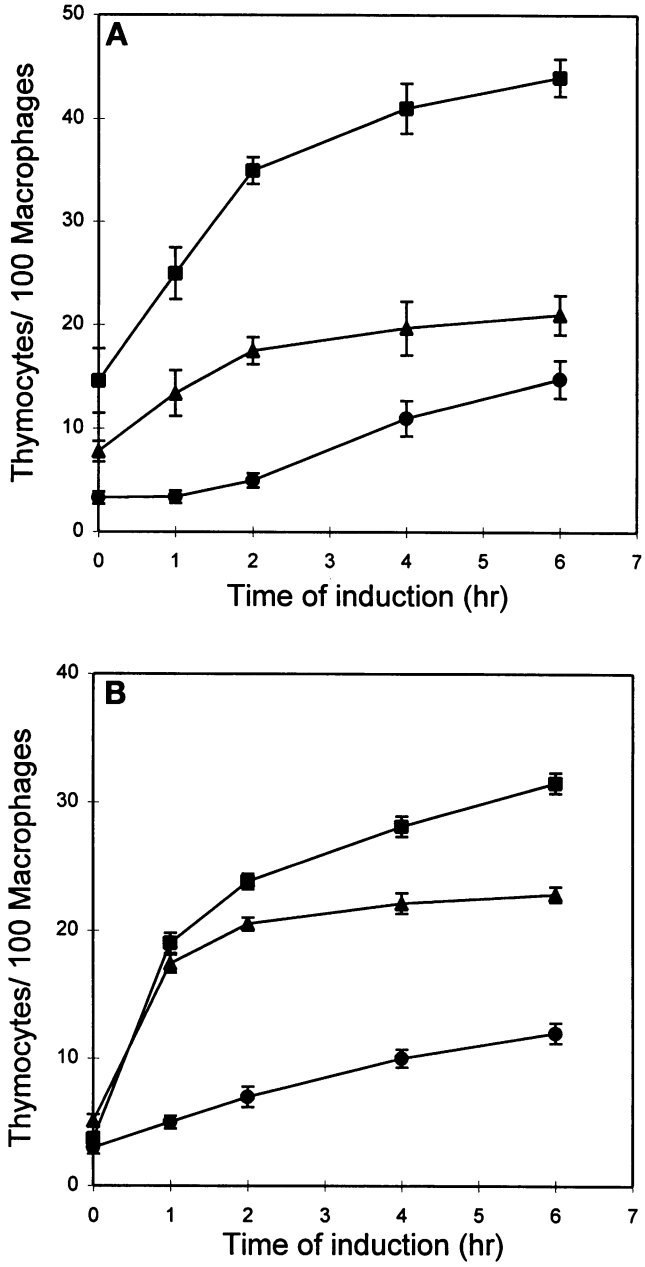

Figure 10. Inhibition of phagocytosis of thymocytes by PS vesicles or RGDS as a function of time of induction. Thymocytes were incubated in the presence or absence of 10^{-6} M dexamethasone, samples were taken at various times, and phagocytosis of uninduced thymocytes (●), induced thymocytes (■), or induced thymocytes in the presence of PS vesicles (▲) by mouse peritoneal macrophages (A) or RGDS (▲) by J774 macrophages (B) was measured.

the ligand for a lectin-like receptor are elements of a single complex recognition system, recognized by a receptor complex different from the one on unactivated macrophages. Interestingly, when labeled lectins were used to probe the surface of the thymocytes for carbohydrate ligands, no difference between apoptotic and nonapoptotic cells was seen (Morris *et al.*, 1984), suggesting that it is not the simple presence or absence of a carbohydrate ligand, but perhaps its association with PS, that defines a complex signal. The result that lipid-symmetric erythrocytes were ineffective inhibitors of the uptake of apoptotic thymocytes was unexpected, since uptake of each was inhibitable by both PS vesicles and *N*-acetylglucosamine. This finding makes it reasonable to suspect that, as in the case of unactivated macrophages, there are aspects of the signal on apoptotic thymocytes which are not present on lipid-symmetric erythrocytes. The data admit the possibility that activated macrophages possess one system for recognition of lipid-symmetric erythrocytes and another for recognition of apoptotic thymocytes, both of which are similarly inhibited by PS vesicles and *N*-acetylglucosamine.

There is evidence elucidating at least some of the components of one of these complex recognition systems, the dual PS/RGDS-sensitive recognition system. A variety of studies have suggested that RGDS-sensitive recognition of apoptotic neutrophils (Savill *et al.*, 1992, 1993) and virally infected apoptotic T lymphocytes (Akbar *et al.*, 1994) involves a complex of the vitronectin and CD36 receptors on the macrophage surface and thrombospondin which is recognized by both of these receptors. CD36 is a class B scavenger receptor which, along with another receptor of this class, SR-BI, has recently been shown to tightly bind artificial lipid vesicles containing PS or another anionic phospholipid, phosphatidylinositol (Rigotti *et al.*, 1995). This result suggests that these molecules might participate in the PS-specific arm of the recognition machinery. Whether CD36 is an element shared by and linking the PS- and RGDS-sensitive mechanisms is an intriguing possibility.

The monoclonal antibody 61D3 defines a recognition element which is shared by both classes of macrophages. Because recognition of PS appears to be a common feature of the recognition system of both classes, one possibility is that the epitope recognized by the antibody is on a receptor element which recognizes PS, consistent with the ability of 61D3 antibody to block uptake of lipid-symmetric erythrocytes by unactivated macrophages. The relationship between the 61D3 antigen and class B scavenger receptors is not clear. CD36 and SR-B1 have apparent molecular weights of 88,000 and 82,000, respectively (Acton *et al.*, 1996). The 61D3 antibody has been reported to recognize a M_r 75,000 polypeptide, though assigning the molecular weight was difficult because of the broadness of the reactive band (Nunez *et al.*, 1982). Whereas the native conformation of (class A) scavenger receptors is a trimer, converted to monomers by reduction (Krieger and Herz, 1994), the molecular weight of the polypeptide recognized by 61D3 antibody did not change upon reduction and alkylation, suggesting it

Table 1. Effects of various inhibitors on phagocytosis of lipid-symmetric erythrocytes and apoptotic thymocytes

Cells phagocytosed	Inhibitor	Macrophages	
		J774	Mouse peritoneal
Apoptotic thymocytes	PS vesicles	Not inhibited	Inhibited
	Lipid-symmetric erythrocytes	Inhibited	Not inhibited
	RGDS	Inhibited	Not inhibited
	Cationic sugars/amino acids	Inhibited	Not inhibited
	N-acetylglucosamine	Not inhibited	Inhibited
	61D3 monoclonal antibody	Inhibited	Inhibited
Lipid-symmetric erythrocytes			
	PS vesicles	Inhibited	Inhibited
	RGDS	Not inhibited	Not inhibited
	Cationic sugars/amino acids	Not inhibited	Not inhibited
	N-acetylglucosamine	Not inhibited	Inhibited
	61D3 monoclonal antibody	Inhibited	Inhibited

existed as a monomer in its native state (Nunez *et al.*, 1982). These considerations leave open the question of whether the protein recognized by the antibody and the scavenger receptors are one and the same.

Another protein, of a clearly different molecular weight and distinct from CD36 (Ottnad *et al.*, 1995), may also participate in PS recognition. Oxidized low-density lipoprotein has recently been shown to inhibit the binding of apoptotic thymocytes to mouse peritoneal macrophages, though not as well as PS vesicles (47 versus 84% inhibition; Sambrano and Steinberg, 1995). Using ligand blots of mouse peritoneal macrophage membranes, oxidized low-density lipoprotein and PS liposomes were shown to bind to a M_r 94,000–97,000 protein, and binding of PS liposomes was inhibited by oxidized low-density lipoprotein (Sambrano and Steinberg, 1995); this protein has recently been identified as macrosialin (Ramprasad *et al.*, 1995). Thus, there may be multiple PS receptor elements on macrophages which could serve as the basis for the multiplicity of recognition systems actually observed.

This report is the first to examine in one study the many different reagents reported to block recognition of apoptotic lymphocytes. The discovery that macrophages use one or the other of two different recognition systems, each inhibitable by a different set of reagents, brings order to the otherwise bewildering list of inhibitors. The two systems differ both in the nature of the signal and in the nature of the complex which recognizes the signal. Yet in both systems PS plays a central role. The data suggest that apoptotic lymphocytes may display on their surface at least two different complex signals of PS in association with other membrane molecules, interpreted by more than one receptor complex. The temporal differences in the development of sensitivity to PS and RGDS suggest that the signals evolve during the apoptotic program, thereby progressively engaging a larger repertoire of receptors and phagocytic cells to ensure that dying cells will be engulfed before lysis can occur.

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