*Pharmaceutical Research, Vol. 8, No. 1, 1991* Report

# Role of Complements C3 and C5 in the Phagocytosis of Liposomes by Human Neutrophils

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*Received April 23, 1990; accepted July 2, 1990*

During the course of a previous investigation, we noticed that the uptake of liposomes by human polymorphonuclear neutrophils (PMNs) was significantly lower in the presence of heat-inactivated serum compared to that in intact whole serum (Scieszka *et al., Pharm. Res.* 5:352, 1988). This observation suggested the participation of heat-labile complement components in the phagocytic process. In this report we conclude that complement C3bi is the component responsible for opsonization of the liposome surface. Phagocytosis was not supported by C3-deficient serum, and phagocytosis in whole serum was blocked by the antibody to the receptor for C3bi (CR3) but not by the antibody to the receptor for C3b (CR1). We also found that with C5-deficient serum the level of uptake was minimal but slightly higher than without any serum. When exogenous C5a was added along with C5-deficient serum, uptake levels similar in magnitude to those observed with intact serum were obtained. We conclude that C5a enhances phagocytosis of opsonized liposomes by activating the phagocytic capacity of CR3 on the PMN.

**KEY WORDS:** liposome; opsonin; C3; C5; phagocytosis.

## INTRODUCTION

Polymorphonuclear neutrophils (PMNs)[5] continuously emigrate from the blood stream to extravascular tissues in response to chemotactic signals. At sites of inflammation, chemotaxis is even more enhanced. We have attempted to utilize the phagocytic and migratory activities of PMNs as a model for potential drug delivery to inflammatory loci. The uptake by human PMNs *in vitro* of the fluid-phase marker lucifer yellow CH (LY) entrapped in liposomes was approximately 100× faster than when provided in plain solutions (1). In addition, it was recently found in an *in vitro* model that PMNs migrating across a confluent Madin Darby canine kidney cell monolayer can carry phagocytosed liposomal contents (2). This observation suggests the possibility that PMNs could serve as carriers of liposomes for targeted drug delivery to an extravascular site in various inflammatory and infectious diseases.

Materials incorporated in the aqueous compartment of liposomes can be taken up by cells via either endocytosis or fusion. Although the former process appears to be more likely for the liposome–phagocyte interactions and is thought to occur *in vitro* as well as *in vivo*, the exact mechanism by which liposomes are phagocytosed remains to be firmly established (3–5). The ligands and receptors that mediate endocytosis of liposomes are also not established. Since liposome uptake by human leukocytes in the presence of serum was somewhat reduced when the incubation was carried out in the presence of IgG aggregates, it was suggested that the uptake is mediated via Fc receptors on the cell surface (6). That the liposome uptake by human PMNs might be complement-mediated was suggested by the observation that in the presence of heat-inactivated serum the uptake was significantly lower than in the presence of intact serum (1). In this report, we propose that complement component C3bi is the opsonin responsible for the liposome recognition by CR3 on the PMNs and that PMNs are activated for enhanced phagocytosis primarily by complement component C5a.

## MATERIALS AND METHODS

### Materials

Lipids used to prepare reverse-phase evaporation vesicles (REVs) were egg L-α-lecithin, phosphatidylglycerol (Avanti Polar Lipids, Birmingham, AL), and cholesterol (ICN Biochemicals, Costa Mesa, CA). Hanks' balanced salt solution (HBSS) with or without $Ca^{2+}$ and $Mg^{2+}$ (Gibco, Grand Island, NY) was slightly modified to accommodate 20 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES; U.S. Biochemicals, Cleveland, Ohio). It was prepared by dissolving, in a final volume of 1.0 liter of glass-distilled water, 4.766 g of HEPES and 95.0 ml of 10 × HBSS and adjusting the final pH to 7.30 with 1.0 *N* NaOH. The osmotic pressure of the modified buffer (HBSS/HEPES) was generally in the range of 290 to 310 mOsmol/kg $H_2O$. [$^{14}$C]Sucrose (New England Nuclear) with a specific activity of $1.5 \times 10^{12}$ dpm/mmol and LY (Molecular Probes, Eugene, OR) were used without further purification. Plastic rather than glassware was exclusively used in handling LY solutions to avoid adsorption onto glass surfaces (7). Complement component C5-deficient human serum, C3-deficient serum, and recombinant C5a human complement were all obtained from Sigma (St. Louis, MO). Monoclonal anti-human C3b-receptor (CR1) and C3bi-receptor (CR3) antibodies were obtained from Dakopatts (Carpintaria, CA). Concentrations of IgG reported here were based on what the suppliers indicated.

### Harvest of Human PMNs

The procedure used for isolation of human PMNs is a

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[5] Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; HBSS, Hanks' balanced salt solution; HBSS/HEPES, HBSS containing 20 m*M* HEPES; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; LY, lucifer yellow CH; PMN, polymorphonuclear leukocyte; REV, reverse-phase evaporation vesicle; CR1 and CR3, receptors to complement C3b and C3bi, respectively.



0724-8741/91/0100-0065$06.50/0 © 1991 Plenum Publishing Corporation

modification of Böyum (8) and was previously described in detail (1). Briefly, 500 ml of blood containing 40 ml of 3.8% sodium citrate was obtained from healthy volunteers, ages 18 to 55 years. The whole blood was divided up into 50-ml conical tubes and centrifuged at 300$g$ for 15 min. The plasma supernatant was removed, and each tube was made to 50 ml with saline followed by sedimentation with 6% dextran T-500 (Pharmacia, Piscataway, NJ) for approximately 60 min. The top layers of the sedimentation tubes were removed and centrifuged at 300$g$ for 15 min. The supernatant was removed and the cell pellet was dispersed in a 25% autologous platelet-poor plasma solution in saline. Each 8.0-ml cell suspension was further layered over 3.0-ml aliquots of Histopaque-1077 (Sigma, St. Louis, MO) and centrifuged at 750$g$ for 25 min. The supernatant containing the lymphocyte/monocyte band was suctioned off, and the PMN cell pellet was further purified by hypotonic lysis of residual red blood cells with distilled water for 20 sec. The cells were washed twice with 50 ml of $Ca^{2+}$- and $Mg^{2+}$-free HBSS/HEPES before being suspended at a concentration that yielded $10^7$ cells/ml in the final experimental medium. The procedure described above generally yielded approximately $5 \times 10^8$ cells from 400 ml of citrated blood with greater than 95% purity, as determined by Leukostat staining (Fisher Scientific, Orangeburg, NY).

### Preparation and Characterization of REVs

A literature procedure (9) was adopted with some modifications in preparing REVs using a 2:2:1 molar ratio of phosphatidylcholine, cholesterol, and phosphatidylglycerol. The procedure was previously described in detail (1). Briefly, a lipid mixture containing 20, 20, and 10 μmol/ml, and 10 μCi/ml of phosphatidylcholine, cholesterol, phosphatidylglycerol, and [$^3$H]dipalmitoylphosphatidylcholine (DPPC), respectively, was added to a 50-ml round-bottom flask and dried down. Ethyl ether was added to the lipids at a 3:1 ratio of ether:final aqueous volume. A LY:[$^{14}$C]sucrose solution at approximately 13 mg/ml:$10^8$ dpm/ml in HBSS was added to the lipid in ether mixture according to the above ratio. An emulsion was then produced from the mixture by cup horn sonication and mixing by Mixxor (Rainin, Woburn, MA). Controlled evaporation at 75-mm Hg vacuum for 30 min followed by 20 mm Hg for 10 min yielded the primary REV preparation. The REVs were then separated from unentrapped LY and [$^{14}$C]sucrose by size exclusion chromatography on a Sephacryl S-1000 (Pharmacia, Piscataway, NJ) column and eluted with HBSS/HEPES.

The LY, [$^{14}$C]sucrose, and lipid concentrations of column-eluted REVs were analyzed as previously described (1). In essence, LY was determined by dissolving REVs in 75% isopropyl alcohol and analyzing on an SLM-Aminco 500-C spectrofluorometer (Urbana, IL) with excitation and emission wavelengths set at 430 and 540 nm, respectively. Corresponding bandpasses were 10 and 20 nm. Radioisotopes were determined in dpm on a Beckman Model LS5801 liquid scintillation counter (Irvine, CA). The LY, [$^{14}$C]sucrose, and lipid concentrations of the separated REVs were typically in the ranges of 0.4–0.6 mg/ml, 3.0–5.0 $\times 10^6$ dpm/ml, and 2.5–3.6 μmol total lipids/ml, respectively. REV size, determined by a Nicomp Model 200 laser particle sizer (Nicomp Instruments, Santa Barbara, CA), was typically 0.3–0.5 μm.

### Uptake of LY by Human PMNs from REVs

Uptake was initiated by mixing PMNs, serum, and an REV preparation in an HBSS/HEPES buffer system. These reaction mixtures contained final PMN, serum, and REV concentrations of $10^7$ cells/ml, 10%, and 2.0–3.0 μmol total lipids/ml, respectively. All reaction mixtures were contained in round-bottom polypropylene tubes, continuously stirred by a cell stirrer, and maintained at 37°C by means of a water-jacketed beaker under normal atmospheric conditions. All reaction mixtures, with the exception of reaction mixtures in antibody experiments, had a total volume of 5.0 ml. For studies involving anti-human CR1 and CR3 antibodies, 1.0-ml aliquots containing 10% serum, PMNs, and various concentrations of antibody were preincubated on ice for 30 min. At $t = 0$, 1.0-ml aliquots of REVs with 10% serum were added to the above 1.0-ml aliquots, making a 2.0-ml total reaction mixture volume. The serum used for all experiments was either normal serum or serum devoid of either the C3 or the C5 component of complement. After experimentally determining that the source of normal serum made no difference in REV uptake profiles (data not shown), we used one source of serum from a batch preparation from one donor. The serum was divided into 2.0-ml aliquots, kept at −90°C, and used as needed throughout the entire series of experiments. For experiments with recombinant C5a, a stock solution in 0.25% bovine serum albumin in HBSS/HEPES was used for addition to the reaction medium at $t = 0$ to achieve a 25 ng/ml ($2.3 \times 10^{-9}$ $M$) final concentration.

The procedure adopted for separating PMNs from free liposomes was essentially the same as the one described in the literature (6). At a given time, duplicate 0.5-ml aliquots from the mixture were gently layered over 0.5 ml of silicone oil at density 1.05 (General Electric Versilube F-50), which had been, in turn, placed over 0.25 ml of 25% sucrose at density 1.10 in a 1.5-ml microcentrifuge tube (Brinkmann, Westbury, NY). It was then centrifuged at 8200$g$ for 60 sec. After the liquid layer was suctioned off, the bottom of the plastic tube which contained PMNs was cut away and added to 1.0 ml of 0.02% sodium deoxycholate (Sigma) containing 0.02% sodium azide (Sigma) in a microfuge tube, which was then continuously agitated in a wrist shaker for 16 to 20 hr at 4°C. A brief cup horn sonication was given to each tube after agitating to ensure a homogeneous suspension of cell debris.

Lowry's protein assay was run for a 0.1-ml aliquot from the cell debris suspension. To monitor the uptake of [$^{14}$C]sucrose, a 0.45-ml aliquot from the cell debris was subjected to dpm determination by liquid scintillation counting after dilution in 10 ml of ACS (Amersham, Arlington Heights, IL). The remaining sample in the microfuge tube was centrifuged at 16,000$g$ for 2 min. The concentration of LY in the supernatant was determined from the fluorescence intensity measured after proper dilution in 75% isopropanol as described earlier (1).

The accumulation rate of LY was very similar to [$^{14}$C]sucrose in all present and previous experiments done in this laboratory. As a result, all discussions of LY uptake, if not directly stated, apply to [$^{14}$C]sucrose as well. Uptake of

LY and [$^{14}$C]sucrose is expressed as μg LY · mg protein$^{-1}$ and pmol · mg protein$^{-1}$, respectively.

## RESULTS AND DISCUSSION

In defense against invading pathogenic microorganisms, the human complement system in serum can enhance leukocyte phagocytosis (opsonization) via two pathways of activation; in the classical pathway, antigen–antibody complexes trigger the activation cascade. The sequence of events in the alternative pathway does not involve antibody but begins with the third component of complement, C3. Since most of the proteins involved in the complement activation are heat-labile (56°C × 30 min) and since phagocytosis of REVs by human PMNs with heat-inactivated serum was minimal, we speculated that the uptake of REVs by PMNs in the presence of intact serum entails complement activation (1). Since there is no reason to believe a priori that normal human serum contains antibodies against any lipid component of REVs used in our studies, we have further believed that the alternative activation of complement induces the PMN uptake of REVs. In the present study, we present data supporting that complement C3bi is responsible for REV opsonization and that complement C5a activates PMNs for phagocytosis.

As shown by the accumulation of the aqueous phase markers, LY and [$^{14}$C]sucrose (Fig. 1), PMNs avidly phagocytose REVs in the presence of 10% serum. Fluorescence microscopy images using various planes of focus as well as 4°C experiments revealed LY to be within the PMNs, not merely attached to the cell surface (1,10). Uptake of REVs by PMNs was completely dependent on serum components since no uptake could be observed in its absence. To determine the role of complement proteins in the phagocytosis of REVs, we compared uptake of REVs in the presence of whole serum to C3-deficient serum. In the absence of C3, accumulation of LY during 60-min incubation was reduced from 2.1 to 0.16 μg LY · mg protein$^{-1}$ (92% reduction), and uptake of [$^{14}$C]sucrose was reduced from 7.6 to 0.42 pmol · mg protein$^{-1}$ (94% reduction). Data presented in Fig. 1 are from a single experiment. However, repeated experiments with many different PMN donors yielded essentially the same profile. These results indicate that C3 plays a critical role in the interaction of REVs with PMNs.

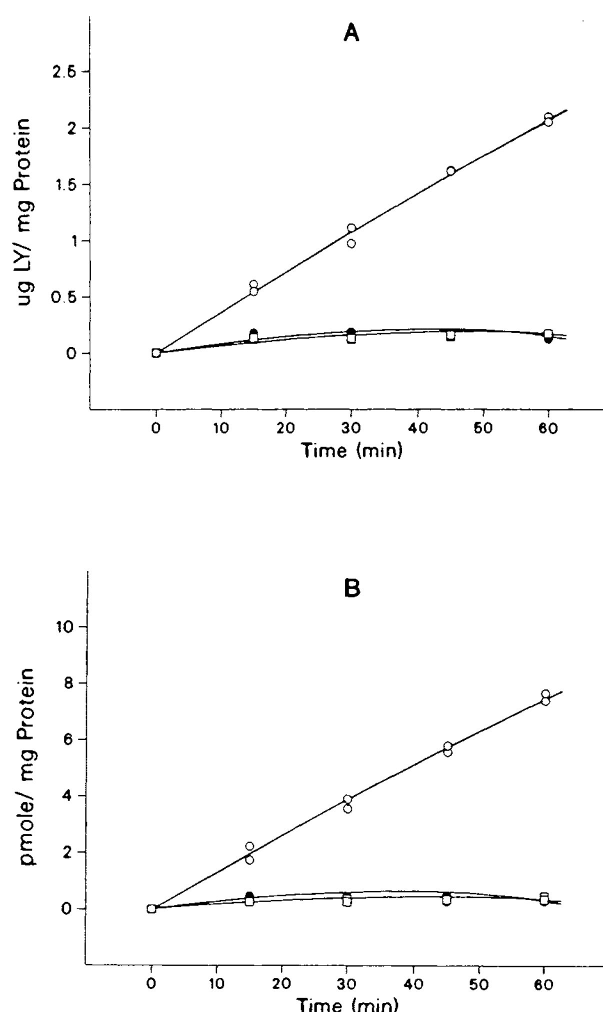


**Fig. 1.** Accumulation of LY (A), and [$^{14}$C]sucrose (B) in human PMNs at 37°C in the presence of either 10% normal serum (open circles), 10% complement C3-deficient serum (open squares), or no serum (filled circles). REVs at initial LY, [$^{14}$C]sucrose, and total lipid concentrations of 0.27 mg/ml, 0.50 μ*M*, and 2.88 m*M* were added to PMNs at $10^7$ cells/ml and accumulation was followed over 60 min.

Complement C3 has been well recognized as the principal opsonic component in the complement cascade. A small amount of C3b generated from C3 in the fluid phase first deposits on the surface of foreign particulate matters such as microorganisms, cells, or liposomes as in the present case. On the surface, more C3 is cleaved to C3b by serum factors. This fragment of C3 is specifically recognized by receptor CR1 on the PMN for subsequent binding and internalization. Alternatively, foreign particles can be also bound via receptor CR3 on the PMN which recognizes C3bi, a fragment derived from further cleavage of C3b on the particle surface (11,12). In order to determine whether C3b or C3bi from C3 functions as the opsonin in the uptake of REVs, we measured the accumulation of LY and [$^{14}$C]sucrose in PMNs in the presence of monoclonal antibodies against the receptors CR1 and CR3.

As shown in Fig. 2, anti-CR3 antibody caused a concentration-dependent inhibition of uptake of REVs during the initial 45 min of incubation. For each experiment, the uptakes observed with antibodies were normalized to that observed without antibodies. At anti-CR3 IgG > 2 μg/ml, more than 70% inhibition was observed. Interestingly, however, at IgG = 0.25 μg/ml, the uptake was significantly enhanced, which we cannot explain properly with limited data available at present. In contrast to the observations made with anti-CR3 antibody, anti-CR1 antibody did not alter the uptake (open symbols in Fig. 2). The uptake was slightly, about 15%, greater than that observed without any antibodies. In the presence of both anti-CR1 and anti-CR3 antibodies, the effect was the same as with anti-CR3 antibody only (data not shown). These data suggest that C3bi functions as the opsonin for the binding of REVs to PMNs and that CR3 functions as the principal opsonic receptor independently of CR1. Conversion of C3b to C3bi on the surface of yeast (11) and on the surface of *S. aureus* and *E. coli* (14) has been

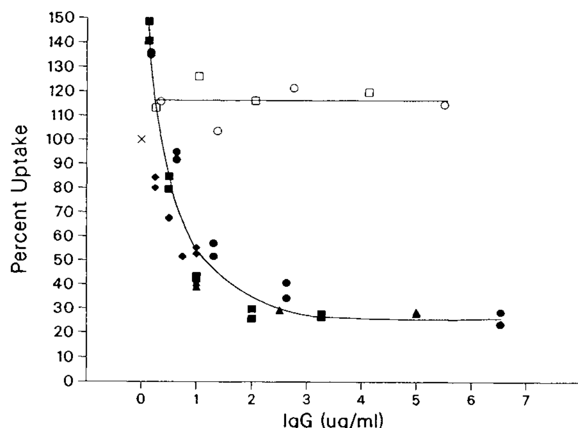


Fig. 2. Effect of anti-CR3 and anti-CR1 antibodies on accumulation of LY in human PMNs. PMNs were incubated on ice with antibodies for 30 min prior to addition of REVs at $t = 0$. REVs at an initial LY concentration of 0.174 mg/ml were incubated with PMNs for 45 min, all in 10% normal serum at 37°C in the presence of monoclonal anti-CR3, anti-CR1, and without antibody. Data are shown from four different experiments for a range of anti-CR3 (filled symbols) and anti-CR1 (open symbols) concentrations and, also, without antibody (cross). Percentage uptake for each antibody concentration was determined relative to uptake without antibody being set at 100% for each experiment. An average of duplicate samples was used for plotting anti-CR1 data for the sake of simplicity.

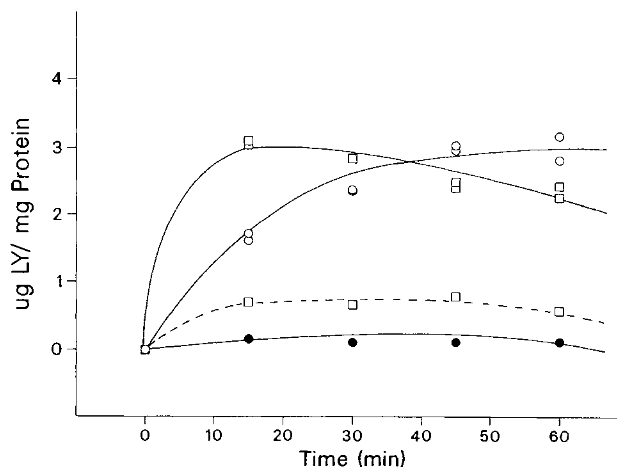


Fig. 3. Accumulation of LY in human PMNs in the presence of normal serum (open circle), complement C5-deficient serum (open squares/dashed line), complement C5-deficient serum with 25 ng/ml exogenously added recombinant C5a (open squares), and no serum (filled circles). REVs at an initial LY concentration of 0.666 mg/ml were incubated with PMNs at 37°C. The C5-deficient serum series is represented by a dashed line since this experiment was run independently of the others using another source of PMNs. Since the phagocytic activity of PMNs varies between donors, the data were normalized to the $y$ axis shown in Fig. 2.

reported to occur instantaneously and almost quantitatively. Such a facile conversion of C3b to C3bi on the REV surface may account for our observations.

The complement receptor CR3 on the unstimulated PMN surface is in an inactive or non-functional state (15). The avid function of CR3 in uptake of REVs was therefore surprising. However, it is known that stimulation of PMNs with PMA (15), TNF, or C5a (13) causes both an increase in the number of surface CR3 and a dramatic increase in the function of the receptor. We thus hypothesized that C5a liberated during complement activation may serve to activate PMNs for uptake of REVs via CR3. To test this hypothesis, uptake of REVs was measured with C5-deficient serum. In the absence of C5, uptake of LY during 60 min of incubation was reduced from 6.8 to 1.3 $\mu g$ LY · mg protein$^{-1}$ (80% reduction; Fig. 3), and uptake of [$^{14}$C]sucrose was reduced from 43.2 to 8.0 pmole · mg protein (82% reduction, data not shown). While uptake of REVs was strongly reduced in the absence of C5, it was not completely eliminated, possibly indicating that some other factors in the C5-deficient serum can also stimulate PMNs. Reconstitution of C5-deficient serum with 2 n$M$ C5a caused complete restoration of its phagocytosis-promoting capacity (Fig. 3). The initial rate of phagocytosis in the presence of exogenous C5a was markedly enhanced, suggesting that the production of C5a during complement activation could be a rate-limiting step. That C5a functions in the modulation of complement-dependent uptake is supported by the observation that the addition of C5a in the absence of serum caused no uptake of REVs (data not shown).

Numerous studies have shown that complement receptors on leukocytes of many species are subject to functional regulation (12). For instance, macrophages phagocytose complement-coated particles only after treatment with an ancillar stimulus (16) and PMNs do not even bind complement-coated particles without proper stimuli (11). Similarly, C5a is a good stimulus for PMNs (17) and in the present study addition of purified recombinant C5a to C5-deficient serum restored its ability to promote phagocytosis of REVs. We thus presume that the C5a liberated through the activation of the complement cascade in normal serum functions to activate PMNs and enable binding and uptake. While the regulated activity of complement receptors and their sensitivity to C5a have been known for many years, we believe that the data presented here are the first to show that the opsonic fragments of complement (C3b and C3bi) and the anaphylatoxic fragment (C5a) function synergistically to promote phagocytosis in whole serum. The synergism recognized here may play an important role in the complement-dependent clearance of microbes *in vivo* and, perhaps more importantly, in the field of targeted drug delivery to phagocytes by means of particulate drug carriers such as liposomes, microspheres, emulsions, and other colloidal carriers (18).

## ACKNOWLEDGMENTS

The authors wish to acknowledge valuable discussion with Dr. Tom J. Lobl, Immunetech Pharmaceuticals, San Diego, CA, at the early phase of the present study. S. D. Wright was supported by USPHS Grants AI22003 and AI24775. S. D. Wright is an established investigator of the American Heart Association.

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