Fibronectin-mediated Uptake of Gelatin-coated Latex Particles by Peritoneal Macrophages

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ABSTRACT The present study demonstrates the ability of plasma fibronectin or cold-insoluble globulin (CIg) to promote the uptake of 125I-labeled, gelatin-coated latex beads (g-Ltx*) by monolayers of peritoneal macrophages (PM). The uptake of g-Ltx* by PM was enhanced by CIg in a concentration-dependent fashion and required the presence of heparin (10 U/ml) as an obligatory cofactor for maximal particle uptake. Treatment of PM monolayers with trypsin (1 mg/ml) for 15 min at 37°C after particle uptake removed <15% of the radioactivity incorporated by the monolayers. However, a similar trypsin treatment of the monolayers before the addition of latex particles depressed CIg-dependent uptake by >75%. Pretreatment of PM monolayers with inhibitors of glycolysis effectively reduced the CIg-dependent uptake of latex. Similarly, pretreatment of monolayers with either inhibitors of protein synthesis or agents that disrupt cytoskeletal elements also significantly depressed CIg-dependent particle uptake. Phagocytosis of g-Ltx* by PM in the presence of CIg and heparin was confirmed by electron microscopy. Finally, g-Ltx* could also be effectively opsonized with CIg at 37°C before their addition to the monolayers. These studies suggest that the recognition of g-Ltx* in the presence of CIg required cell surface protein(s) and that subsequent phagocytosis of these particles by PM was energy dependent and required intact intracellular cytoskeleton elements. Thus, PM monolayers provide a suitable system for further studies on the function of CIg in the recognition and phagocytosis of gelatin-coated particles by phagocytic cells.

The process of phagocytosis by vertebrate phagocytes is markedly enhanced by humoral recognition factors called opsonins. Immune opsonins are antibody or complement proteins that interact with foreign antigens and with receptors on the surface of phagocytic cells (26, 36). There also exist opsonins that enhance the uptake of colloidal material by macrophages of the reticuloendothelial system that are not immune proteins (34). One such nonimmune opsonin has been purified from rat (1, 2, 6, 22) and human serum (4) and was originally designated α-2-macroglobulin on account of its electrophoretic mobility and size (2, 22). More recent studies have demonstrated that this nonimmune opsonin isolated from human serum is identical to cold-insoluble globulin (CIg) or fibronectin (5), a high molecular weight adhesive glycoprotein, a form of which is also found on the surface of many cell types (10, 38).

Up until now, quantitation of the opsonic activity of CIg has also been measured by the agglutination of gelatin-coated colloids (7, 8, 28). More recently, an electroimmunoassay has been developed for measuring the concentration of CIg in rat or human serum (3); however, this method cannot distinguish between biologically inactive and active CIg preparations (14). Although it has been proposed that CIg promotes the uptake of gelatin-coated particles by liver slices via a phagocytic process, the mechanism of CIg-dependent particle uptake by phagocytic cells has not been clearly defined.

The present study described an assay utilizing monolayers of elicited peritoneal macrophages (PM) for analyzing the mechanism of CIg-dependent uptake of gelatin-coated latex particles (g-Ltx*). Evidence will be presented establishing that PM can be employed to quantitate the opsonic activity of CIg present in serum or in purified preparations of CIg.
more, these studies will demonstrate that CIg promotes uptake of gelatin-coated particles primarily by phagocytosis. Preliminary reports of these results have appeared elsewhere (17, 25).

**MATERIALS AND METHODS**

**Preparation of PM Monolayers**

Inflammatory exudates were induced in male Sprague-Dawley rats weighing 200-250 g, by the intraperitoneal injection of 30 ml of 1% sodium caseinate (Eastman Kodak Co., Rochester, N. Y.) in 0.2 M phosphate-buffered saline (PBS), pH 7.4 (15, 16). Cells were harvested from the peritoneal exudate, 9 h after injection, by rinsing the peritoneal cavity with 50 ml of ice-cold PBS containing 5 U/ml of heparin. The cells were washed twice in Hanks‘ balanced salt solution (HBSS), pH 7.4, at 4°C, and erythrocytes were removed by hypotonic lysis. Cell counts were performed by routine hemocytometry, and viability was assessed by the exclusion of trypan blue. The cells were finally suspended in Dulbecco‘s modified Eagle‘s medium (DMEM) (Grand Island Biological Co., Grand Island, N. Y.) containing 20% fetal calf serum, 50 U/ml of penicillin, and 50 μg/ml of streptomycin to obtain a cell concentration of 2 × 10^6 cells/ml. 2-ml aliquots of the cell suspension were distributed into 35 mm plastic culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) and incubated in a humidified incubator at 37°C in an atmosphere of 5% CO\textsubscript{2}. The pretreated monolayers were incubated for 2 h at 37°C in an atmosphere of 5% CO\textsubscript{2}. The medium was discarded and the monolayers were washed twice with fresh DMEM without serum. Usually 50-60% of the added cells remained adherent.

**Isolation of CIg or Plasma Fibronectin**

This protein was prepared as a by-product of purifying the fibrin-stabilizing factor (coagulation factor XIII) from 10 liters of fresh or outdated human plasma (11, 18). The purity and opsonic properties of these CIg preparations have been recently described (24).

**Conditions of CIg-dependent Uptake by PM**

Phagocytosis was initiated by adding 100 μg of g-Ltx*, (100 μg dry weight corresponding to ~5 × 10^6 particles/dish, 10,000-30,000 cpm) to triplicate culture dishes containing 10 U/ml of heparin and either fresh rat serum or human CIg (isolated from plasma). The cells were incubated for 2 h at 37°C in an atmosphere of 5% CO\textsubscript{2}. The incubation was terminated by aspiration of the incubation medium, followed by three washes of the monolayers with ice-cold PBS. The monolayers were then solubilized with 1.0 ml of 0.1 N NaOH for a period of 30 min at 37°C (transferred to counting tubes, and the radioactivity was measured in a Packard 5130 Gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Protein content of the monolayers was determined by the method of Lowry et al. (19). Phagocytic activity by PM was expressed as cpm of g-Ltx*/2 h per 100 μg of cell protein. All results are given as the mean ± SEM of at least triplicate samples, and nonpaired assays were analyzed by the Student’s t-Test.

**Preopsonization of g-Ltx**

Preopsonization of latex particles was performed by incubating 100 μg of g-Ltx*, 10 U/ml of heparin, and specified amounts of CIg in a 1.0 ml volume of Krebs Ringer‘s bicarbonate buffer (KRB) for 15 min at 37°C. The pretreated latex particles were then centrifuged at 12,000 rpm for 10 min (S54B rotor, DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), and the resulting supernate was discarded and the latex pellet was resuspended to its original volume in KRB. The washed latex suspension was sonicated 30 s, and the centrifugation procedure was repeated. The preopsonized g-Ltx* was resuspended to its original volume in DMEM, sonicated, and 1.0 ml of the preopsonized particles was added to each monolayer. After a 2-h incubation period, the medium was removed, the monolayers were washed twice in PBS, and 1.0 ml of trypsin (100 μg/ml) in DMEM was added to the plates. After an additional 30-min incubation at 37°C, the trypsin-containing medium was removed, monolayers were washed twice more with PBS, and the cells were solubilized and counted as described above.

**RESULTS**

Table I demonstrates the ability of whole rat serum or a purified preparation of CIg, isolated from human plasma, to promote the uptake of g-Ltx* by PM monolayers. The addition of fresh rat serum (10% vol/vol) to the medium increased particle uptake by PM >200%. Latex uptake was further enhanced when heparin (10 U/ml) was added to the serum-containing medium, but was ineffective when added alone. The addition of purified CIg (200 μg/ml) alone to the PM monolayers promoted particle uptake >50%; however, when heparin was also added, the uptake of g-Ltx* was further increased to the level found with whole serum.

Fig. 1 illustrates the effect of varying CIg concentration of g-Ltx* uptake by PM monolayers. As can be seen, a linear relationship between radioactivity incorporated by PM and the CIg present in the medium was achieved over the dose range tested. The effect of incubation time on CIg-dependent uptake of g-Ltx* by PM monolayers is presented in Fig. 2. The results showed that uptake of particles was linear for 60 min and reached a plateau by 120 min of incubation, indicating that saturation of the phagocytic process had occurred. The 2-h

**Table I**

| Additions* | Uptake by Macrophages (cpm at 100 μg protein) | Increase from control % |
|-----------|---------------------------------------------|------------------------|
| None      | 473 ± 43                                   | —                      |
| Heparin (10 U/ml) | 480 ± 17 | 202 |
| Rat serum (10% vol/vol) | 1,433 ± 35 | 298 |
| Rat serum + heparin | 1,885 ± 40 | 42 |
| Human CIg (200 μg/ml) | 675 ± 33 | 289 |

* Monolayers of PM (2 × 10^6 cells/dish) were incubated in 1.0 ml of DMEM containing the above-specified additions, and g-Ltx* (1.2 × 10^6 cpm) was added for 2 h at 37°C. After incubation, monolayers were washed twice with ice-cold PBS before solubilization and counting.

**Transmission Electron Microscopy Studies**

Peritoneal macrophage preparations were also examined by electron microscopy, after a 2-h incubation of monolayers in the presence or absence of CIg. Cells were released from the culture dishes by treatment of the washed monolayers with 3.0 ml of 12 mM lidocaine in DMEM for 1 h at 37°C, followed by gentle scraping with a rubber policeman. The cells were sedimented by centrifugation at 750 g for 20 min and suspended in a 1-ml solution containing 1.25% glutaraldehyde and 2% paraformaldehyde buffered with 0.1 M phosphate (pH 7.4). The fixed cells were pelleted again and postfixed with 1% osmium tetroxide, dehydrated in graded concentrations of ethanol, and embedded in Epon 812. Thin sections were cut with a diamond knife and double-stained with uranyl acetate and lead citrate (32), and examined with a Hitachi 300 electron microscope.

**Reagents**

HBSS, DMEM, fetal calf serum, and penicillin and streptomycin sulfate solution were obtained from Grand Island Biological Co. Colchicine, cycloheximide, and puromycin dihydrochloride were purchased from Nutritional Biochemical Corp. (Cleveland, Ohio). Carboxylated latex beads (450 nm in diameter) were obtained from Dow Chemical Co. (Indianapolis, Ind.). The [3H]-labeled lidocaine was purchased from Amersham Corp. (Arlington Heights, Ill.) Trypsin (3x crystallized), was purchased from Sigma Chemical Co. (St. Louis, Mo.). Heparin was obtained from the Upjohn Co., Agricultural Prods. MKT. (Kalamazoo, Mich.). Lidocaine was purchased from Astra Pharmaceutical Products Inc., Worcester, Mass.)
incubation interval was chosen for all subsequent experiments as a measure of phagocytic capacity.

In an effort to determine whether surface binding of latex particles accounted for the uptake of radioactivity by the monolayers, PM were treated with trypsin before and after Clg-dependent uptake of g-Ltx*. Table II demonstrates that pretreatment of PM with trypsin at 37°C before adding g-Ltx* produced a 75% reduction in particle uptake, while exposing macrophages to a similar trypsintreatment after incubation with Clg and g-Ltx* diminished by only 15% the radioactivity associated with the monolayers.

Because phagocytosis, but not surface binding of particles, by macrophages is dependent on metabolic energy derived principally via glycolysis, experiments were performed to determine whether Clg-dependent uptake of g-Ltx* was sensitive to known inhibitors of phagocytosis. Table III examines the effects of glycolytic inhibitors (sodium fluoride and iodoacetate), glucose uptake (2-deoxyglucose), and respiration (potassium cyanide) on Clg-dependent uptake by PM. Inhibitors of glycolysis and glucose uptake were all effective in markedly depressing g-Ltx* uptake, while inhibition of the electron transport system with cyanide did not significantly alter latex uptake by PM.

Table IV demonstrates the effects of protein synthesis inhibitors and agents which disrupt the cytoskeletal architecture on g-Ltx* uptake by PM. A 1-h preincubation with either cycloheximide or puromycin caused a significant reduction in particle uptake. Pretreatment of PM with colchicine or cytochalasin B depressed particle uptake, although cytochalasin B was a far more potent inhibitor than colchicine at the doses tested.

Macrophages were examined by electron microscopy to confirm that Clg enhanced the uptake of g-Ltx* by a phagocytic

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**TABLE II**

| Additions* | Uptake by macrophages (cpm of 125I/100 μg protein) |
|------------|---------------------------------------------------|
| None       | 3,132 ± 616                                       |
| Prephagocytic trypsintreatment, 1 mg/ml | 753 ± 128                                         |
| Postphagocytic trypsintreatment, 1 mg/ml | 2,676 ± 250                                       |

* Monolayers of PM (2 x 10^6 cells/dish) were treated with trypsin for 15 min at 37°C before or immediately after a 2-h incubation with 120 μg/ml of Clg, 10 U/ml of heparin, and 4 x 10^4 cpm of g-Ltx*. Data represent the mean ± SEM of triplicate samples.

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**TABLE III**

| Inhibitors* | Uptake by macrophages (cpm 125I/100Wg protein) | Inhibition |
|-------------|-------------------------------------------------|------------|
| None        | 6,957 ± 533                                     | -          |
| Sodium fluoride, 8 mM | 3,986 ± 475 (P < 0.05) | 43         |
| Iodoacetate, 0.8 mM | 2,469 ± 267 (P < 0.01) | 65         |
| 2-Deoxyglucose, 4 mM | 3,198 ± 642 (P < 0.05) | 55         |
| Potassium cyanide, 1 mM | 6,676 ± 1,176 | 5          |

* Monolayers of PM (2 x 10^6 cells/dish) were preincubated with the specified inhibitor for 1 h at 37°C before measuring uptake of g-Ltx* (4 x 10^4 cpm/dish) in the medium containing 136 μg/ml of Clg and 10 U/ml of heparin. Data represent the mean ± SEM of triplicate samples.

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**TABLE IV**

| Inhibition* | Uptake by macrophages (cpm 125I/100Wg protein) | Inhibition |
|-------------|-------------------------------------------------|------------|
| None        | 2,588 ± 132                                     | -          |
| Cycloheximide, 5 μg/ml | 457 ± 89 (P < 0.01) | 83         |
| Puromycin, 10 μg/ml | 1,006 ± 28 (P < 0.01) | 62         |
| Colchicine, 10 μg/ml | 1,943 ± 157 (P < 0.05) | 25         |
| Cytochalasin B, 5 μg/ml | 4,47 ± 110 (P < 0.01) | 83         |

* Monolayers of PM (2 x 10^6 cells/dish) were pretreated with or without the above inhibitors for 1 h at 37°C before the addition of 100 μg/ml of Clg, 10 U/ml of heparin, and 1.2 x 10^9 cpm of g-Ltx* for 2 h at 37°C. Data represent the mean ± SEM of at least triplicate samples.
process. Fig. 3a illustrates a macrophage isolated from a monolayer that was incubated with gelatin-coated latex and heparin. As can be seen, the PM has not ingested any latex after the 2-h incubation period. Fig. 3b presents a macrophage from a monolayer incubated with gelatin-coated latex, heparin, and 100 μg/ml of Clg. In the presence of Clg, the internalization of latex particles is clearly observed.

Fig. 4 demonstrates the presence of single and multiple latex particles within phagocytic vacuoles of a macrophage treated with Clg. Furthermore, the formation of a phagosome partially surrounding a single latex particle can also be seen on the cell surface.

Table V illustrates the effect of precoating g-Ltx* with heparin and/or Clg before their addition to the PM mono-

![Image](https://example.com/image.png)
FIGURE 4 Electron micrograph of a peritoneal macrophage incubated with Clg and gelatinized latex. Incubation conditions identical as in Fig. 3 b. Arrows indicate single and multiple latex particles in phagocytic vacuoles. Bar, 1 μm. × 17,000.

TABLE V

| Preincubation additions* | Uptake by macrophages$ |
|-------------------------|------------------------|
|                         | cpm of $^{125}$I/100 μg protein |
| None                    | 420 ± 50               |
| Heparin, 10 U/ml        | 422 ± 51               |
| Clg, 22 μg/ml           | 846 ± 43               |
| Clg + heparin           | 1,049 ± 108            |

* g-Ltx* (100 μg) was incubated with the specified additions for 15 min at 37°C in 1.0 ml of KRB. The preopsonized latex was centrifuged, washed twice, and resuspended to a 1.0-ml volume in DMEM before adding pre-coated particles to the monolayers.

$ 1.0 ml of preopsonized g-Ltx* in DMEM was added to PM monolayers (2 × 10⁶ cells/dish) containing 10 U/ml of heparin and incubated for 2 h at 37°C. After incubation, monolayers were washed twice with PBS and treated with trypsin (100 μg/ml) for 30 min at 37°C to minimize cell surface binding of latex particles.

DISCUSSION

The present study examined the cellular mechanism by which Clg promotes the uptake of a gelatin-coated colloid by peritoneal macrophages. Although the presence of nonimmune opsonins in plasma has been well documented utilizing a variety of in vivo and in vitro test systems (1–5, 9, 13, 28, 33), isolated phagocytes have not been previously used as a test system for Clg-dependent particle uptake. In the past, a variety of test colloids have been used to measure nonimmune opsonin-dependent uptake by macrophages of the reticuloendothelial system in normal and diseased states (27, 30, 35). However, the present study utilized latex particles to which gelatin was covalently attached and labeled with $^{125}$I, because these particles offer the advantages of longer half-life, minimal manipulation after preparation, and better stability than any other test colloids previously described. The phagocytosis-promoting function of this opsonin, which promotes the uptake of gelatin-coated colloids, has been characterized primarily by the use of a liver slice assay (33). The opsonin has been recently purified from human and rat sources (1, 2, 4, 6, 22) and has been shown to be identical to fibronectin or its soluble derivative, Clg (10, 38). Although the specificity of Clg for collagen and gelatin-coated surfaces is now well established (10, 13, 22, 24, 28), the mechanism and recognition of such colloids by phagocytic cells remain poorly understood.

The present data demonstrated that incubation of macrophages in the presence of whole serum or purified Clg, isolated from human plasma promoted the uptake of gelatin-coated latex particles. In the monolayer assay system, heparin was required for maximum particle uptake which confirmed earlier observations using the liver slice system and lipid emulsion as test particle (8, 13, 23, 24). Whole serum alone stimulated gelatinized latex uptake by PM monolayers, probably because of the endogenous heparin present in serum. However, adding exogenous heparin to serum-containing monolayers further augmented particle uptake, demonstrating that serum-stimulated uptake of gelatinized latex is enhanced, if not dependent upon, heparin as a cofactor. Furthermore, a previous study has shown that serum absorbed with gelatinized lipid emulsion in the presence of heparin did not promote the uptake of gelatinized colloids (22). To clarify whether the Clg-mediated uptake of latex particles observed was a measure of phagocytosis or surface adherence by macrophages, cells were treated with trypsin to release surface bound particles. Treatment of macrophages with trypsin before, but not after, incubation with latex particles resulted in a large reduction of radioactivity incorporated into the cells. The finding that trypsin treatment...
after incubation with particles and Clg did not significantly reduce incorporation suggested that the uptake process was via phagocytosis and not surface binding.

Phagocytosis by macrophages is an energy-dependent process, and, in the case of peritoneal macrophages, utilizes primarily glycolysis as a source of ATP (29). To determine whether inhibition of macrophage energy metabolism would depress Clg-dependent particle uptake, cells were pretreated with inhibitors of either glycolysis or respiration before adding g-Ltx*. Inhibitors of glycolysis and glucose transport were all effective in markedly depressing particle uptake, while treating macrophages with cyanide, an inhibitor of cellular respiration, did not alter uptake. These metabolic data provided further evidence that Clg-mediated uptake represented true internalization of latex particles by macrophages. Phagocytosis of g-Ltx* was further substantiated by electron microscopy, which demonstrated that only Clg-treated macrophages contained a large number of latex particles (25 or more particles/cell) enclosed within phagocytic vacuoles; in the absence of Clg, none or only a few particles could be seen within cells. Because Clg causes aggregation of gelatinized colloids, it was of interest to determine whether single particles or aggregates of gelatinized latex were phagocytosed. Our data in Fig. 4 reveal the uptake of single gelatinized particles present within phagosomes. However, because some of the phagocytic vacuoles contained multiple particles, it cannot be ruled out at this time whether small aggregates of latex particles can also be internalized.

The possibility that Clg-promoted uptake of gelatinized colloids is mediated by surface receptors on macrophages has not been previously explored. The almost complete reduction in radioactivity incorporated by macrophages treated with trypsin before incubation with particles suggested that cell surface protein(s) acts as receptors for the g-Ltx*-heparin-CIg complex. Although it is not clear how protein synthesis is involved in the phagocytic process, our findings that low concentrations of cycloheximide and puromycin inhibited particle uptake strongly suggest that particle recognition and internalization is dependent upon replenishment of cell surface or possibly intracellular proteins by de novo synthesis. Recent evidence has established that a network of filament-like structures prominent near the membrane surface of phagocytic cells plays an important role in the internalization of the membrane during the phagocytic event (31, 36). Cytochalasin B and colchicine have become powerful probes in the investigation of the role of microfilaments and microtubules in the process of endocytosis. Cytochalasin B disrupts microfilaments in macrophages and leukocytes, thus inhibiting phagocytosis and cell motility (20, 39). Colchicine, by preventing the polymerization of tubulin to form microtubules, also impairs phagocytosis (21).

In the present study, both cytochalasin B and colchicine were effective in depressing Clg-dependent phagocytosis of latex particles by macrophages. However, as these agents have many complex effects on phagocytic cells, including alternations in membrane functions and substrate transport (37), further studies are required to clarify which cellular functions are primarily involved to account for the observed depression in Clg-mediated phagocytosis.

Finally, the interaction of Clg and heparin with g-Ltx* was examined by preincubating particles with either heparin and/or Clg to determine whether opsonization requires both Clg and heparin and whether such preopsonized particles are taken up by macrophages. Our studies demonstrated that preincubating g-Ltx* with both heparin and Clg was more effective in stimulating phagocytosis than Clg alone, indicating that Clg interacts directly with the gelatinized colloid and that heparin promotes the opsonization process. Thus, the results of these studies demonstrate an important function for Clg in the recognition and phagocytosis of denatured collagen-coated particles by macrophages. Furthermore, the macrophage monolayer system provides a suitable in vitro model for future studies on the regulation of Clg-mediated macrophages phagocytosis during the inflammatory process and wound repair.

This work was supported by a grant from the National Institutes of Health (9 RO1 CA25047) and grants from Campus Research Board and BRSG (7864) from the University of Illinois, and at Northwestern University by a U. S. Public Health Service Career Award (5 K06 HL03512) awarded to L. Lorand, and by a grant from the National Heart, Lung and Blood Institute (HL 02212).

Received for publication 6 March 1980, and in revised form 16 June 1980.

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