

RECEPTORS FOR COLD-INSOLUBLE GLOBULIN (PLASMA FIBRONECTIN) ON HUMAN MONOCYTES*

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Blood monocytes, macrophages, and other cells of the mononuclear phagocytic system perform critical functions in inflammation and immunity. A large body of data has accumulated in recent years indicating that certain plasma proteins or their cleavage fragments can regulate mononuclear phagocytic cell function (1). For example, macrophages are involved in phagocytic recognition via plasma membrane receptors for the Fc region of IgG and for cleaved C3 (2); they show oriented migration when chemotactic agents such as C5a and fibrinopeptides are generated (3), and they respond with rapid spreading and inhibition of migration when exposed to the Bb fragment derived from factor B of the alternative pathway of complement fixation, or when the contact phase of blood coagulation is activated (4). These proteins probably participate directly in *in vivo* events triggered by tissue injury, particularly those involving accumulation of macrophages at the injury site and the clearance of debris that occurs before tissue reconstruction (1).

Considerable interest has developed in another plasma protein, cold-insoluble globulin (CIg, plasma fibronectin)¹ and the nature of its interaction with phagocytic cells. This glycoprotein is present in substantial amounts ($300 \pm 100 \mu\text{g/ml}$, [5]) in human plasma. The most common form in blood is a dimer (mol wt 450,000) composed of two disulfide linked chains of approximately equal size (6). Structurally and immunologically related forms of fibronectin are found on cell surfaces, in basement membranes, in intercellular matrices, and in extravascular fluids (7-10). Although certain differences among them have been described (7, 8, 11), all forms of fibronectin have binding sites for collagen (12), fibrin(ogen) (13, 14), heparin (15), and fibroblasts (7-10, 16). The avidity of binding between CIg and collagen is enhanced by denaturation of the collagen (17, 18). In addition, CIg binds fibrin with higher avidity than it binds fibrinogen (14).

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¹ *Abbreviations used in this paper:* BHK, baby hamster kidney; CIg, cold-insoluble globulin; CIgR, receptor for cold-insoluble globulin; Dulbecco's MEM, Dulbecco's modified Eagle's medium; EIgG, sheep erythrocytes coated with the IgG fraction of a rabbit antiserum to sheep erythrocytes; EIgMC, sheep erythrocytes coated with the IgM fraction of a rabbit antiserum to sheep erythrocytes and mouse complement; PBS, phosphate-buffered saline; TEG, gelatin-coated tanned sheep erythrocytes.

The present investigation focuses on the role that CIg plays in regulating monocyte function. The ability of CIg to bind substances that are likely to be present at sites of tissue injury (e.g., collagen and fibrin) led to the hypothesis that this protein is directly involved in regulation of cellular behavior in the inflammatory response. This hypothesis is supported by the investigations of Blumenstock et al., who have demonstrated the identity between CIg and a serum protein termed α -2-opsonic glycoprotein (19). This protein had previously been shown to promote the uptake of a gelatin-coated emulsion by rat liver slices, suggesting an interaction with macrophages (20, 21).

In this report we provide evidence that human peripheral blood monocytes possess a trypsin-sensitive plasma membrane receptor for surface-bound CIg. Binding of monocytes to CIg leads to enhanced functional expression of their plasma membrane receptors for the Fc portion of IgG and for the third component of complement. Based upon these results we hypothesize that CIg acts as a circulating probe for fibrin or for exposed or altered collagen at sites of tissue damage. Binding of CIg at sites of injury via fibrin or collagen affinity promotes monocyte retention and subsequent enhancement of their phagocytic capacity.

Materials and Methods

Preparation of Plasma and Serum. Human blood was collected by venipuncture into sodium heparin (131 USP U/mg, grade II; Sigma Chemical Co., St. Louis, Mo.). The final blood concentration of heparin was 5 USP U/ml. Platelet-poor plasma was prepared from this blood by centrifugation for 5 min at 8,700 *g* (Microfuge; Beckman Instruments, Inc., Fullerton, Calif.). Human serum was obtained from whole blood, which had been incubated at 37°C for 1 h (a temperature that minimizes the incorporation of CIg into the fibrin clot [13]) and then centrifuged at 8,700 *g* for 5 min. Plasma or serum was used fresh or stored at -20°C. CIg-depleted plasma or serum was prepared by twice incubating the plasma or serum for a 30-min period with gelatin-Sepharose beads (12) (10 ml plasma or serum per 2.5-ml beads) in an ice bath, after which the beads were pelleted by centrifugation. CIg levels were determined by electroimmunoassay using a rabbit antiserum to human CIg (5). For certain experiments serum or CIg-depleted serum were heated at 56°C for 1 h to inactivate the complement system. Sterilization of serum or plasma specimens by filtration through 0.45- μ m Nalgene filters (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) was carried out for those experiments that involved long-term culture periods.

Serum from a patient (M.W.) with acquired severe hypogammaglobulinemia had the following levels of immunoglobulins: IgG, 0.24 mg/ml (normal, 8-18 mg/ml); IgM, 0.4 mg/ml (normal, 0.6-2.5 mg/ml); IgA, 0.1 mg/ml (normal, 0.9-4.5 mg/ml). The complement level (CH50) was within normal limits, as was the immunoassayable level of CIg (0.226 mg/ml).

CIg Preparations. Purified human CIg was prepared by one of the following two methods: (a) a modification (22) of the glycine precipitation method of Mosesson and Umfleet (5). Among the steps in this procedure are included ethanol fractionation and DEAE-cellulose chromatography. (b) A more recent preparative procedure (D. L. Armani and M. W. Mosesson. Manuscript in preparation.) takes advantage of the fact that in the cold, heparin induces precipitation of CIg (14). CIg in the heparin precipitate was separated from fibrinogen, the only major protein contaminant, by stepwise elution from DEAE-cellulose with chaotropic salts (i.e., 0.3 M KSCN in 0.1 M Tris-HCl buffer, pH 7.5).

For separation of species of CIg differing in size (i.e., dimeric, zone I vs. monomeric, zones II and III) (23; D. L. Armani and M. W. Mosesson. Manuscript in preparation.), CIg prepared by either method was further fractionated on Sepharose 6B-CL, using recycling chromatography in the presence of 0.01 M Tris-HCl buffer, pH 8, containing 0.5 M KSCN. All relevant fractions were ultimately dialyzed against a 0.25 M Tris-PO₄ buffer, pH 7.0, and stored at -20°C. For use in tissue culture experiments, these materials were thawed, dialyzed against phosphate-

buffered saline (PBS), (obtained as a 10 times stock concentrate from Grand Island Biological Co., Grand Island, N. Y.), and maintained at 4°C.

Blood Cell Preparations. Normal human blood was collected by venipuncture into solutions containing EDTA (Sigma Chemical Co.) at a final concentration of 10 mM. The pH of the EDTA solution had been adjusted to 7.4 with NaOH. The blood mononuclear cell fraction was isolated from this material by centrifugation in a Hypaque (Hypaque-M, 75% solution; Winthrop Laboratories, New York)-Ficoll (Ficoll 400; Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) solution (density 1.078 g/ml) as described by Boyum (24). The mononuclear cell layer accumulating at the interface of the plasma and Hypaque-Ficoll solution was washed several times by centrifugation at 200 *g* for 10 min after resuspension in EDTA (5 mM)-Hanks' balanced salt solution (once), with Hanks' balanced salt solution alone (twice), and finally with Dulbecco's modified Eagle's medium (MEM). Frequently, the mononuclear cell fraction suspended in Dulbecco's MEM was centrifuged at low speed (100 *g* for 10 min) to remove platelets. Hanks' balanced salt solution without calcium or magnesium and Dulbecco's MEM were obtained from Grand Island Biological Co., as a 10 times liquid concentrate and in powdered form, respectively. For some experiments leukocyte-rich plasma, also containing a substantial number of erythrocytes, was prepared by sedimentation at 1 *g*. Polymorphonuclear leukocyte-rich cell fractions were prepared as described by Cramer et al. (25) using human venous blood anticoagulated with EDTA.

Cell Binding to Gelatin- or Collagen-coated Surfaces (the Monocyte Binding Assay)

The assay to evaluate and to quantify attachment of cells to gelatin- or collagen-coated surfaces involved several distinct steps: (a) preparation of gelatin- or collagen-coated surfaces; (b) incubation of these surfaces with plasma or serum proteins; (c) incubation of surfaces with blood cells; (d) determination of the type and number of attached cells. Details are given below.

PREPARATION OF GELATIN- OR COLLAGEN-COATED SURFACES. Acid-soluble collagen surfaces were prepared essentially as described by Klebe (26) using acid-soluble bovine tendon collagen (catalogue number 3511; Sigma Chemical Co.), which had been dissolved in 2% acetic acid by stirring overnight at 4°C. The bottom of each well (16 mm Diam) of a plastic tissue culture plate (24 wells/plate; Linbro Chemical Co., Hamden, Conn.) was coated with approximately 0.05 ml of the collagen solution. These solutions were exposed to NH₄OH fumes for 10 min to allow gelation of collagen and then dried overnight at room temperature. The dried surfaces were then washed with 0.1% NH₄OH followed by several washes with distilled H₂O. Subsequently, they were exposed to 5 M urea for 15 min at room temperature, washed several times with distilled H₂O, and air-dried.

Gelatin-coated surfaces were prepared by adding 0.5 ml of a solution of 30 mg/ml gelatin (catalogue number 6-2625, Sigma Chemical Co.) in water to each well of a Linbro tissue culture plate followed by incubation at 37°C for 2 h. The gelatin solution was removed by aspiration, and the plate was dried for 2 h at 40°C. These plates were stored at room temperature. The uniformity of the acid-soluble collagen surfaces or the gelatin surfaces was assessed by staining randomly selected sample wells with Coomassie brilliant blue (0.2 mg/ml in methanol-acetic acid solution).

INCUBATION OF COLLAGEN- OR GELATIN-COATED SURFACES WITH PLASMA OR SERUM PROTEINS. Heparinized plasma, serum, C1g-depleted plasma or serum, or purified human plasma proteins, viz., C1g, gamma globulin (Cutter Laboratories, Inc., Berkeley, Calif.) or albumin (Sigma Chemical Co.) were diluted to the desired concentration with PBS and added in 0.5-ml portions to each well of the gelatin- or collagen-coated plates. After an incubation period of 1 h at room temperature, the solutions were

removed by aspiration, and the wells were washed three times with PBS. Controls included incubation of wells with PBS alone.

INCUBATION OF SURFACES WITH BLOOD CELLS. Mononuclear-rich cell fractions or polymorphonuclear-rich cell fractions in Dulbecco's MEM were adjusted to cell concentrations of 2×10^6 /ml or 2×10^5 /ml, respectively, and 0.5 ml of the cell suspension was added to each well of the culture plate. The gelatin-coated plates were then incubated for 1 h at room temperature whereas the collagen-coated plates were incubated at 37°C in a 5% CO₂ atmosphere. At the end of the incubation period nonattached cells were removed by washing the wells three times with Dulbecco's MEM.

DETERMINATION OF THE TYPE AND NUMBER OF ATTACHED CELLS. One of two methods was used in counting attached cells. The first involved release of the adherent cells by incubation for 15–20 min at room temperature in the presence of 1 ml of Hanks' balanced salt solution containing 10 mM EDTA. A bulbed pasteur pipette was used to agitate the fluid in completing the release of cells, and the cell suspension was subsequently counted in an automatic cell counter (Cytograph; Ortho Pharmaceutical Corp., Raritan, N. J.).

The second method of counting entailed glutaraldehyde fixation (1.25% glutaraldehyde vol:vol, in PBS for 10 min) of attached cells followed by direct microscopic counting of five randomly selected high-power fields ($\times 500$) per well. The cell number was reported as a function of the surface area of the well (cells per square centimeter). Comparison of these two methods indicated that they yielded similar values.

The cell type attaching to surfaces was determined by standard morphologic criteria using phase-contrast microscopy of live or glutaraldehyde-fixed cells, or by direct light microscopy of fixed cells stained with McNeal tetrachrome (Harleco, American Hospital Supply Corp., Gibbstown, N. J.).

Functional differentiation of attached cells types (i.e., monocytes vs. lymphocytes) was made by determining (a) their ability to spread on surfaces (27) and (b) their capacity to ingest IgG-coated erythrocytes (28).

Particle-binding Assays

Assays for attachment and/or ingestion of indicator particles by monocytes were performed in the following stages: (a) preparation of monocyte cultures; (b) preparation of indicator particles; (c) incubation of indicator particles with cultured monocytes; and (d) determination of the number and location of cell-associated particles. Details are given below.

PREPARATION OF MONOCYTE CULTURES. Monocytes were established in culture essentially following the procedure of Johnson et al. (29). In general, the mononuclear cell layer obtained from the Hypaque-Ficoll procedure was suspended in Dulbecco's MEM (2.0×10^6 cells/ml) containing either 7% heat-inactivated human serum or heat-inactivated CIg-depleted human serum. The cells were incubated in the wells of plastic tissue culture plates for 1 h at 37°C in a 5% CO₂ atmosphere, and then the wells were washed with Dulbecco's MEM to remove nonadherent cells. Adherent cells were maintained in Dulbecco's MEM or in the serumless medium of Newman Tytell (Grand Island Biological Co.) to which we added penicillin, streptomycin, fungizone, and human serum (2%, final concentration) or CIg-depleted serum (2%, final concen-

tration). Alternatively, monocyte cultures were established and maintained on plastic surfaces, which had been preincubated with CIg, or gelatin- or acid-soluble collagen-coated surfaces, which had been preincubated with CIg or with 50% human serum. Modifications of the above procedures are specified in the text.

PREPARATION OF INDICATOR PARTICLES. Gelatin-coated beads were prepared from polyvinyltoluene latex beads (2 μm Diam, Dow Chemical Co., Midland, Mich.), which had been washed in 70% alcohol, followed by three washes in distilled H_2O . Approximately 10^{10} washed beads were suspended to 10 ml in PBS containing heat-liquefied gelatin (10 mg/ml). The suspension was incubated at room temperature for 2 h and then stored at 4°C . Before use, the gelatin was liquefied at 37°C , and the beads were washed four times by centrifugation in PBS. The final pellet was suspended in Dulbecco's MEM to the desired particle concentration, typically 3.0×10^7 beads/ml.

Gelatin-coated tanned erythrocytes (TEG) were prepared from fresh sheep blood, which had been diluted 1:1 with Alsever's solution (30) and then stored at 4°C for periods of up to 2 wk. These erythrocytes were washed three times by centrifugation (250 g) in PBS. Tannic acid (Fisher Scientific Co., Pittsburgh, Pa.) treatment was carried out as described by Rabinovitch (31), by incubation for 30 min at 37°C at an erythrocyte and tannic acid concentration of $1.3 \times 10^8/\text{ml}$ and 25 $\mu\text{g}/\text{ml}$, respectively. The tanned cells were then washed twice with PBS and suspended (2.5×10^8 cells/ml) in a gelatin(10 mg/ml)-PBS solution. The suspension was incubated at room temperature for 3 h and then at 4°C for at least another 24 h. Before use, the gelatin-cell suspension was further processed as described above for the latex beads and suspended in Dulbecco's MEM to a final concentration of 2.0×10^7 cells/ml.

CIg treatment of gelatin-coated latex beads and TEG was as follows. Washed particle suspensions were mixed with CIg solutions in plastic tubes at a final volume of 1 ml. The tubes were placed on a rotary shaker and incubated for 30 min at room temperature in a 5% CO_2 atmosphere. At the end of the incubation period, 0.5 ml of each suspension was added to duplicate wells of the culture plates.

IgG-coated sheep erythrocytes (EIgG) or IgM and complement-coated erythrocytes (EIgMC) were prepared as previously described (28) using mouse serum as a source of complement plus the IgG or the IgM fraction from an antiserum to sheep erythrocyte (Cordis Laboratories Inc., Miami, Fla.). These indicator erythrocytes were prepared on the day of use and suspended at a concentration of 1×10^8 cells/ml in Dulbecco's MEM.

INCUBATION OF INDICATOR PARTICLES WITH CULTURED MONOCYTES. Before the addition of test particle suspensions to the wells (0.5 ml/well), the monocyte cultures were washed twice with Dulbecco's MEM. The particles were then added, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for periods varying from 30 min to 3 h. At the end of this incubation the wells were washed three times with Dulbecco's MEM to remove nonadherent material.

DETERMINATION OF THE NUMBER AND LOCATION OF CELL-ASSOCIATED PARTICLES. Differentiation of surface-attached from ingested particles was accomplished in the following ways. In the case of erythrocytes, surface-bound particles were lysed by exposure of the culture to a hypotonic solution (PBS diluted 1:4 in H_2O) for 10 s, the culture fluid aspirated and fixative then added (1.25% glutaraldehyde, vol:vol in PBS). In the case of gelatin-coated latex beads, particles bound to cells cultured on a

plastic surface were released by exposure to a solution of trypsin (100 $\mu\text{g}/\text{ml}$) and EDTA (10 mM) in Hanks' balanced salt solution for 20 min at 37°C, and then washed three times with Dulbecco's MEM. The cells were subsequently fixed with glutaraldehyde.

Particle counting was done in duplicate wells using an inverted phase-contrast microscope. At least 200 monocytes from three or more randomly selected fields were counted per well. The data were reported as the number of particles attached to or ingested by 100 monocytes (attachment or ingestion index).

Results

Monocyte Binding to Surfaces Coated with Collagen and CIg. Human peripheral blood monocytes adhere rapidly to plastic or glass surfaces. Coating such surfaces with gelatin or acid-soluble collagen abrogates this adherence activity. However, prior incubation of collagen-coated surface with a source of CIg, at levels of 2.5 $\mu\text{g}/\text{cm}^2$ surface or higher, results in a concentration-dependent increase in the number of monocytes that adhere (Fig. 1). Other serum proteins such as IgG or albumin, do not promote such binding.

We observed some variability in the background adherence of monocytes to the collagen-coated surfaces. Background monocyte adherence could be maintained at low levels (<10% of maximum) by the selection of culture plates that had been carefully monitored for completeness of gelatin coating of the surfaces and by avoiding platelet contamination in the mononuclear cell fraction.

The Hypaque-Ficoll system used in the preparation of mononuclear leukocytes for

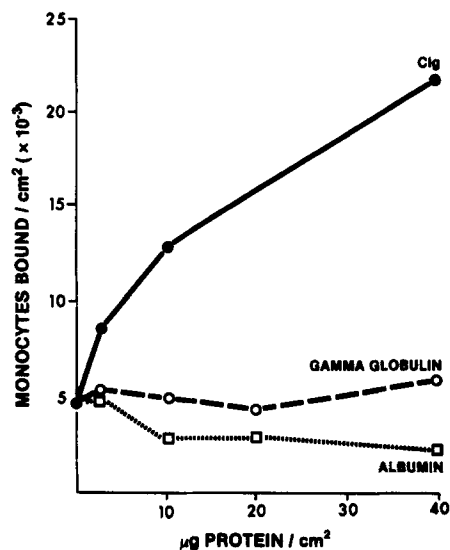


FIG. 1. An experiment showing binding of human peripheral blood monocytes to gelatin-coated surfaces, which had first been incubated with various human plasma proteins. The medium in all such experiments contained Ca^{++} and Mg^{++} . In this type of experiment, the maximum number of adherent monocytes was as high as $50 \times 10^3/\text{cm}^2$. The number of monocytes bound is indicated on the ordinate; the levels of protein preincubated in the wells are expressed on the abscissa as micrograms per square centimeter. Determination of the amount of CIg bound to coated surfaces as assessed with ^{125}I -labeled CIg indicated that only a small fraction of the added CIg (<1%) had become surface bound.

these experiments yields a fraction consisting of about 20% monocytes and 80% lymphocytes. When this fraction is incubated with CIg-collagen surfaces, only monocytes adhere. The evidence for this is the following: (a) within 30 min virtually all attached mononuclear cells demonstrated typical monocyte spreading and membrane ruffling, as assessed by phase-contrast microscopy; furthermore, typical monocyte morphology was observed in fixed and stained preparations; (b) 96% of the attached cells ingested two or more IgG-coated erythrocytes, thus demonstrating their viability and capacity for immune-mediated phagocytosis.

Polymorphonuclear leukocytes from polymorphonuclear leukocyte-rich fractions (three experiments) showed no tendency to adhere to CIg-gelatin surfaces. In addition, in similar experiments with leukocyte-rich plasma, fewer than 5% of the cells that bound to gelatin-CIg surfaces were neutrophils, and more than 95% were monocytes.

The mechanism of attachment of monocytes to CIg-collagen surfaces differs in several respects from that to plastic in that (a) as pointed out above, attachment of monocytes to plastic surfaces does not require CIg; (b) vigorous washing of cell cultures with a pasteur pipette will readily release monocytes from a CIg-gelatin surface but not from a plastic surface; (c) trypsin treatment releases nearly 100% of the cells from the CIg-gelatin surface whereas the same treatment of monocytes bound to plastic does not cause release but instead leads to enhanced spreading.

The presence of heparin at levels as high as 100 $\mu\text{g}/\text{ml}$ or β -mercaptoethanol at levels as high as 1 mM, or both, did not modify the characteristics of attachment of monocytes to CIg-gelatin surfaces.

Divalent Cation Requirements for Monocyte Binding. The following experiments were designed to investigate divalent cation requirements for monocyte binding to CIg-gelatin-coated surfaces (Fig. 2). Although gelatin surfaces themselves could be coated with CIg in the absence of divalent cations, monocytes did not adhere to surfaces

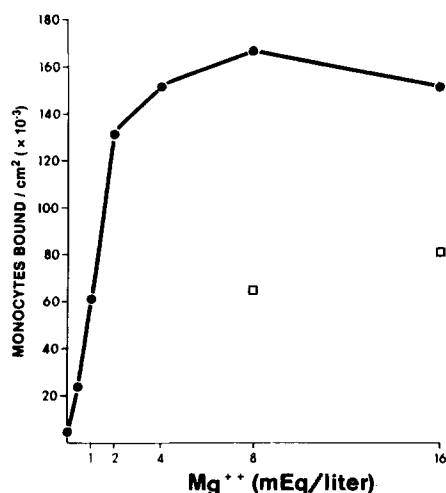


FIG. 2. The magnesium requirement for monocyte binding to CIg-gelatin surfaces. In this experiment gelatin-coated surfaces were preincubated with CIg ($50 \mu\text{g}/\text{cm}^2$ surface) and then washed with PBS. A mononuclear cell-rich fraction from human peripheral blood was then suspended in Hanks' solution containing added Mg^{2+} (abscissa), and the cells were allowed to become adherent to the CIg-gelatin surface for 1 h at room temperature (closed circles). Some cell suspensions contained Ca^{2+} , 1 mEq/liter, in addition to Mg^{2+} (open squares).

without divalent cations being present in the medium. (This was not true of monocyte attachment to plastic surfaces.) The presence of Mg^{++} in the suspension resulted in a concentration-dependent increase in the number of monocytes that attached to CIg-gelatin surfaces. Comparative experiments with Ca^{++} at relatively low concentration (i.e., up to 2 mEq/liter) resulted in modest augmentation of the number of cells attached (less than one third of that observed with Mg^{++}); at Ca^{++} concentrations about 2 mEq/liter toxic effects on the cell population were observed. Mixtures of Mg^{++} and Ca^{++} resulted in significantly reduced binding relative to Mg^{++} alone.

The cation (i.e., Mg^{++}) dependence of monocyte attachment was also inferred from the effect of chelating agents. EDTA (5 mM) promoted quantitative release of previously bound monocytes. EGTA (5 mM), a chelating agent with much higher affinity for Ca^{++} than for Mg^{++} , had little effect on cell attachment to CIg-gelatin surfaces.

Monocytes released by EDTA (5–10 mM) were viable in that they, like previously unattached monocytes, readily adhered to a plastic surface and could be reattached to CIg-gelatin surfaces in the presence of Mg^{++} . Furthermore, these monocytes were capable of ingesting IgG-coated sheep erythrocytes. Thus, reattachment of EDTA-released monocytes to a CIg-gelatin surface demonstrates the reversibility of the cell-CIg interaction. This manipulation provides a simple method for preparing a pure suspension of monocytes (M. P. Bevilacqua, M. W. Mosesson, and C. Bianco. Manuscript in preparation.).

Evaluation of Various Preparations of CIg. We evaluated highly purified CIg, which had been prepared in the presence of ethanol and glycine (5) or which had been prepared using heparin precipitation (D. L. Amrani and M. W. Mosesson. Manuscript in preparation.). Both types of CIg preparations were comprised of ~80% dimeric species (zone I, mol wt 450,000) and ~20% smaller molecular weight forms (zone II, mol wt 190,000–235,000) (22, 23). Some preparations were further subfractionated using molecular exclusion chromatography to separate the larger dimeric species from those of smaller molecular weight.

All unsubfractionated CIg preparations (viz., containing both zones I and II species) were equally effective in promoting monocyte attachment to gelatin surfaces. CIg zone II also promoted concentration-dependent attachment of monocytes to a collagen surface, but this material was less effective than unsubfractionated material over the entire concentration range tested (Fig. 3). This difference was somewhat more marked at the lower concentrations (i.e., $12.5 \mu\text{g}/\text{cm}^2$ surface or less).

Serum-mediated Monocyte Attachment to Gelatin Surfaces. Initial results demonstrated that human plasma or serum promoted concentration-dependent attachment of monocytes to gelatin surfaces. The role of CIg in this phenomenon was investigated by depletion-reconstitution experiments (Fig. 4). Serum that had been depleted of CIg did not support monocyte attachment to gelatin surfaces. Reconstitution of this serum with CIg restored the monocyte attachment activity to a level that was equivalent to that of the untreated serum or to that of purified CIg itself. Similar quantitative results were obtained with heparinized plasma which had been processed in the same way as the serum.

Several additional experiments were performed to exclude participation of other serum proteins known to interact with monocytes. Heat inactivation of serum complement components did not affect monocyte attachment activity, thus eliminating

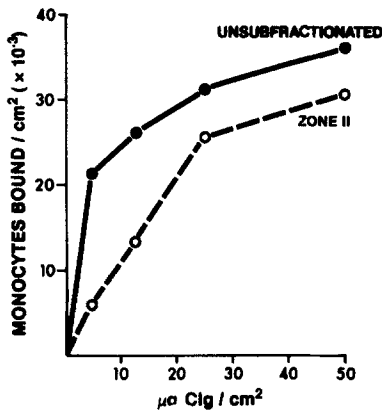


FIG. 3. Binding of human peripheral blood monocytes to gelatin surfaces preincubated with unsubfractionated plasma CIg obtained by heparin precipitation (●) or with zone II CIg (○).

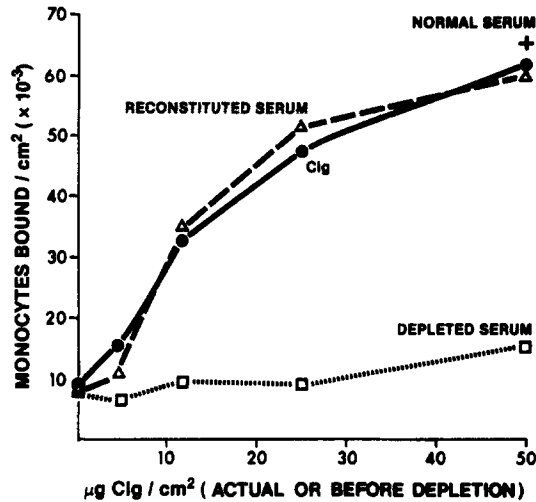


FIG. 4. Results of an experiment showing CIg dependence of serum-mediated binding of human monocytes to gelatin surfaces. Gelatin surfaces were incubated with the following: normal human serum (+), 272 μg CIg/ml; the same serum after CIg depletion (□); CIg-depleted serum after reconstitution with purified CIg (Δ); purified CIg (●). Data relating to CIg-depleted serum are plotted in terms of the original CIg concentration.

the possibility that either the classical or the alternative pathways of complement fixation had participated in this reaction. The possibility that serum-mediated attachment of monocytes could be a result of adsorption of IgG molecules to the gelatin surface was excluded by the demonstration that serum from a patient with severe hypogammaglobulinemia promoted monocyte attachment to gelatin in a manner that corresponded with its level of CIg.

Attachment of CIg-coated Particles to Monocytes. The capacity of monocytes to recognize collagen-bound CIg was also studied using gelatin-coated particles. Several types of particles were evaluated, but gelatin-coated latex beads and TEG proved to be the most useful and convenient. When such gelatin-coated particles were incubated with monocytes that had been plated and maintained on plastic surfaces under serum-free

conditions for periods of 2 h to 8 d, only small numbers of particles became attached (<50/100 monocytes in the case of latex particles, or <10/100 monocytes in the case of tanned erythrocytes).

Preincubation of gelatin-coated particles with CIg promoted a concentration-dependent increase in the number of particles binding to such monocytes (Fig. 5). As had been the case with monocyte binding to CIg-gelatin surfaces (see Fig. 2), divalent cations were required for CIg-dependent particle attachment. Similar results were obtained (single experiment) when gelatin-coated latex beads were offered to monocytes that had first been plated on a CIg-gelatin surface.

On the other hand, preincubation of CIg with monocyte monolayers (rather than with gelatin-coated particles) followed by washing with medium to remove unbound material, did not result in increased gelatin-coated particle binding (Fig. 5). Furthermore, in other experiments, preincubation of mononuclear cell suspensions with CIg followed by washing to remove unbound material, also did not result in augmented attachment of the cells to gelatin-coated surfaces.

Fate of Particles Attached to Monocytes. It was important to determine whether attached CIg-coated particles were subsequently ingested by the cells. In the case of latex beads, even after periods of incubation with monocyte monolayers of up to 3 h, the addition of trypsin (100 $\mu\text{g}/\text{well}$) and EDTA (5 mM) for 20 min reduced the number of bound particles to background levels, an indication that interiorization had not taken place (data not shown).

In the case of TEG, which had been incubated with surface-attached monocytes for periods of up to 3 h, hypotonic lysis also permitted a distinction between interiorized erythrocytes (protected from lysis, Fig. 6) from those bound to the cell surface (lysed). This type of experiment showed that there had been no CIg-dependent ingestion regardless of the degree of particle attachment.

Although CIg does not itself promote ingestion of gelatin-coated particles, it was

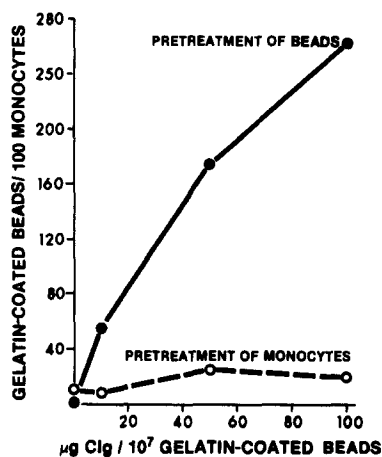


FIG. 5. Results of an experiment in which CIg was preincubated with either monocytes attached to plastic surfaces or with gelatin-coated latex beads before contact with one another. Each culture well ultimately received 10^7 beads. The number of latex beads attaching per 100 monocytes (ordinate) was plotted against the amount of CIg expressed as microgram per CIg 10^7 beads (abscissa). The amount of CIg used for preincubation of monocytes is normalized to this scale. Background attachment (50 beads/100 monocytes) was subtracted.

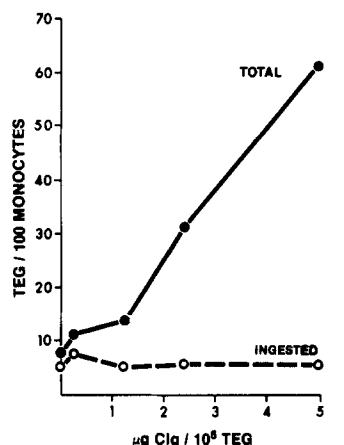


FIG. 6. The number of TEG associated with monocytes before and after hypotonic lysis. In this experiment TEG were incubated with various concentrations of CIg (abscissa) for 30 min and then incubated with monocyte monolayers for 3 h at 37°C. Nonadherent TEG were removed by washing, and all cell-associated TEG were counted (Total). The cultures were then subjected to hypotonic lysis for 10 s, and the TEG internalized by monocytes were scored (Ingested).

possible to demonstrate that CIg-mediated binding of particles to monocytes does not impede subsequent particle ingestion induced by appropriate opsonic signals. CIg-coated TEG were first incubated with monocytes for 3 h, then nonadherent TEG were gently washed away, and the IgG fraction of an antiserum to sheep erythrocytes was added to some wells. After 30 min, all wells were exposed to hypotonic lysis for 10 s, and the number of internalized TEG was then determined. In sharp contrast with control wells, almost all previously attached erythrocytes had been ingested by monocyte cultures to which the IgG had been added.

Adding heparin at levels of 100 $\mu\text{g}/\text{ml}$ and/or β -mercaptoethanol at levels of 1 mM did not modify the fate of the CIg-coated particles in this experimental system.

Protease Susceptibility of Monocyte Binding of CIg. The foregoing experiments indicate that monocytes are capable of recognizing CIg bound to collagen, presumably via a receptor. Proteolytic degradation of monocyte surface proteins provided information concerning the nature and location of the putative receptor. Preincubation of monocyte monolayers with trypsin completely prevented the subsequent binding of latex beads coated with gelatin and CIg (Fig. 7) and, in addition, caused the cells to exhibit enhanced spreading. Chymotrypsin was only weakly effective in preventing particle binding. After tryptic or chymotryptic digestion the monocytes remained attached to the plastic surfaces, thus providing one indication of their viability. Further evidence of cell viability was obtained by the demonstration that trypsin- or chymotrypsin-treated monocytes retained their capacity to ingest EIgG. Because trypsin does not normally penetrate the surface of viable cells (32), these data suggest that at least a portion of the structure representing the CIg receptor faces the cell exterior and is sensitive to tryptic hydrolysis.

Effect of CIg on the Fc and C3 Receptor Activity of Macrophages. Several experiments were carried out to investigate the relationship between the surface on which a monocyte is plated and the expression of its Fc and C3 receptors (Figs. 8 and 9). Mononuclear leukocytes that had been suspended in 7% heat-inactivated, CIg-de-

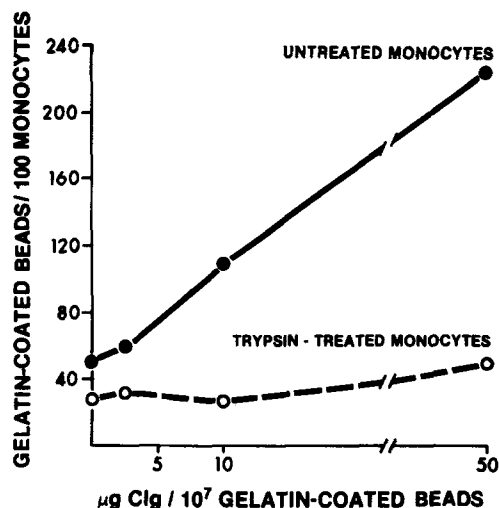


FIG. 7. The attachment of CIg-gelatin-coated latex beads to untreated or trypsin-treated monocyte cultures ($200 \mu\text{g}/\text{well}$ for 15 min at room temperature). Tryptic digestion was terminated by the addition of soybean trypsin inhibitor ($200 \mu\text{g}/\text{well}$) followed by several washes with Dulbecco's MEM. Control wells were washed in the same way. The gelatin-coated beads had been incubated with CIg (abscissa) for 30 min, and subsequently were incubated with the monocyte cultures for 1 h at 37°C . Cell-associated beads are indicated on the ordinate.

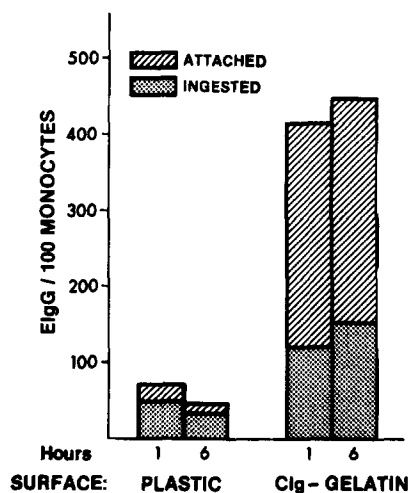


FIG. 8. Attachment and ingestion of IgG-coated erythrocytes (EIgG). Monocytes in certain wells were incubated for 1 h in the presence of 7% CIg-depleted serum (1 h). Some of these wells were incubated for an additional 5 h in 2% CIg-depleted serum (6 h). After each type of incubation the cells were offered EIgG. The results shown reflect data averaged from two separate experiments.

pleted serum were allowed to attach to plastic surfaces or to gelatin surfaces that had been pretreated with CIg ($50 \mu\text{g}/\text{cm}^2$). After 1 h incubation, nonadherent cells were removed, and the monocyte cultures were maintained on these surfaces for 1 or 6 h before addition of sensitized erythrocytes.

As compared with monocytes bound to plastic surfaces (Fig. 8), monocytes plated on CIg-gelatin surfaces bound and ingested five or more times the number of IgG-

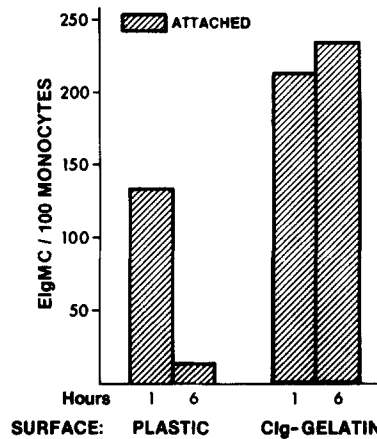


FIG. 9. Attachment and ingestion of IgM- and complement-coated erythrocytes (EIgMC) by monocytes on plastic or CIg-gelatin surfaces. Experimental design was the same as that described in the preceding legend, except for the type of particle introduced.

coated erythrocytes. Markedly enhanced Fc receptor activity was also observed when monocytes were plated on CIg-coated plastic surfaces (data not shown).

C3-mediated attachment of particles (EIgMC) to human monocytes is not followed by ingestion (Fig. 9). The C3 receptor activity that is evident after 1-h incubation on plastic surfaces was virtually lost (<10%) after 6 h. Monocytes plated on CIg-coated plastic surfaces showed relative enhancement of C3 receptor activity at the 6-h time period but not at 1 h (data not shown). Time-dependent loss of C3 receptor activity was not evident in the case of CIg-gelatin surfaces. Instead there was enhancement of this activity at both time periods.

Augmentation of Fc and C3 receptor activity was retained after the cells had been released from the surface by EDTA treatment and subsequently reattached to a plastic surface in the absence of CIg.

Discussion

Our present experiments have provided insights into the control of monocyte behavior by the plasma protein, CIg (plasma fibronectin). The data indicate that human peripheral blood monocytes have plasma membrane receptors (CIgR) for this protein, and these receptors are not expressed on other leukocytes. Generally accepted criteria supporting the notion of such a receptor include: specificity of the interaction, ligand concentration dependence, reversibility of binding, receptor location on the plasma membrane, and induction of cellular function after ligand-receptor interaction. Our evidence for CIgR is as follows: (a) among all plasma proteins, the interaction between monocytes and collagen is mediated specifically by CIg (Figs. 1 and 4); (b) attachment of monocytes to collagen-coated surfaces and to collagen-coated particles is mediated by CIg in a concentration-dependent manner (Figs. 1 and 5); (c) the receptor-ligand interaction can be reversed by manipulation of the concentration of extracellular Mg^{++} (Fig. 2); (d) trypsin treatment of monocytes ablates the CIg-monocyte interaction (Fig. 7); (e) the expression of other known monocyte membrane receptors, namely, those for the Fc portion of IgG and for C3b is enhanced as a result of the CIg-monocyte interaction (Figs. 8 and 9).

Other receptor characteristics such as saturability and displacement of ligand binding that have been applied in evaluating peptide hormone receptors (33) cannot yet be studied unambiguously in the case of CIgR because CIg monocyte interactions are promoted only by surface-bound molecules. A similar situation has been encountered in characterizing C3b receptors (34).

The direct correlation between the number of monocytes bound to a CIg-collagen surface and the amount of CIg added to the system (Figs. 1 and 3) suggests that CIgR may not be expressed uniformly in the monocyte population. The uneven expression can be accounted for by differences in the number of CIgR population per cell, by the relative binding affinities among the CIgR, and/or by CIgR membrane mobility. Whatever the explanation, monocyte surface attachment occurs *in vitro* at CIg levels that could be expected to occur *in vivo*. That is, the CIg concentration above which measurable cell attachment occurs is at about 3% of the normal plasma level CIg (Fig. 1). At higher concentrations, still well below circulating levels, virtually all monocytes bind to collagen surfaces.

The high avidity of monocytes for CIg bound to collagen compared with their lack of avidity for soluble CIg was evident in several types of experiments (Fig. 5 and see above). This behavior is analogous with that of leukocyte Fc receptors, which have a higher avidity for multimolecular antigen antibody complexes than they do for paucimolecular complexes or for soluble monomolecular IgG (35). Just as has been postulated for IgG, fibronectin monocyte interaction may either require allosteric changes of the fibronectin molecule or, alternatively, may be related to cooperation of multiple low affinity binding sites, or both.

There also are parallels between the characteristics of CIgR and the monocyte receptor for C3b. Both receptors require Mg^{++} for activity, both are sensitive to tryptic proteolysis (36), and both mediate particle attachment but not ingestion (37). Despite these similarities, differences in the cell types bearing C3 receptors (i.e., monocytes, B lymphocytes, neutrophils, erythrocytes) suggest that monocyte CIgR and C3b receptors are functionally distinct. For example, under our conditions, neutrophils, B lymphocytes, and erythrocytes were unable to recognize and to bind to CIg-collagen surfaces. Although recent preliminary reports suggest that neutrophils do bind to CIg (38, 39), we did not observe such an activity either in our present experiments or in other investigations (unpublished data) in which CIg coating of plastic surfaces actually inhibited adherence of neutrophils.

Fibronectin receptors have also been identified on fibroblasts (16). Although some of the features of fibroblast receptors are similar to those that we have described for monocyte CIgR (e.g., susceptibility to trypsin), the lack of requirement for Mg^{++} for receptor-ligand interaction constitutes a major difference.

We found that the reproducibility of data involving background cell binding in CIg-dependent systems required mononuclear cell preparations having minimal platelet contamination. This finding is not surprising because platelets are known to contain substantial amounts of fibronectin in their α -granules (40, 41). Upon stimulation, a portion of this CIg is released into the surrounding medium (40, 41) while some becomes inserted into the platelet membrane (41). Without further experimentation, it is difficult to assess the role that platelet fibronectin plays in modulating *in vivo* monocyte/macrophage function or for that matter the role that is played by fibronectin secreted by monocytes (42) or macrophages (43).

It will be informative to study the monocyte binding activity of matrix forms of fibronectin such as those produced by fibroblasts or those found in basement membranes (reviewed in references 7-10). One previous report (44) showed that substrate-bound microexudate produced by baby hamster kidney (BHK) cells in culture was an excellent surface for plating human monocytes. Furthermore, these attached cells could be released by adding EDTA to the system. Although the exact nature of the attachment was not investigated, the fact that fibronectin is a major component of the BHK microexudate is consistent with the idea that matrix forms of fibronectin can support monocyte adherence.

Interaction between CIg-collagen surfaces or particles and monocytes leads to the two following significant events: (a) attachment of monocytes to the collagen surfaces or particles, and (b) enhancement of the expression of cell receptors for Fc and for C3. These phenomena relating to CIg-mediated monocyte attachment may be physiologically significant. That is, CIg may behave as a circulating probe for tissue damage by virtue of its capacity for recognizing fibrin and denatured collagen. Such interactions may be a determinant of monocyte retention at sites of injury.

Our finding that CIg does not mediate particle ingestion is consistent with the observation of Molnar et al. (45) on particle release from trypsin-treated rat liver slices, and deserves special discussion. Most previous experimental systems used for assessing mononuclear phagocyte-CIg interactions did not address the question of attachment vs. ingestion (19-21, 45-47). Opsonins, as first defined by Wright and Douglas (48), promote phagocytosis, and this process involves both attachment and ingestion (49). Thus, although some investigators have classified CIg as an opsonic protein, our evidence indicates that CIg is not a complete opsonin. Nevertheless, it is possible that CIgR behave like C3b receptors on mouse macrophages, which themselves are able to mediate ingestion only at specific stages of cell differentiation (50). CIg, like C3b, may also participate in phagocytosis by increasing the contact between the cell and the phagocytic target.

As compared with the liver slice assay, neither heparin nor β -mercaptoethanol was required for functional activity in our system. Our speculation as to a possible role played by heparin in the liver slice assay is that in the absence of heparin, the assay is relatively insensitive, the presence of heparin promotes aggregation of CIg-coated beads or particles, which in turn lowers the threshold for detection of binding.

Another consequence of the interaction of monocyte CIgR with CIg-gelatin surfaces is the enhanced expression of Fc and C3 receptors (Figs. 8 and 9). It is tempting to suggest that this phenomenon reflects the ability of CIg to induce monocyte differentiation to stimulated macrophages such as are found in inflammatory sites. It will be interesting to investigate whether these monocytes develop the capability of secreting neutral proteases such as collagenase, elastase, and plasminogen activator, which are able to degrade the same substrates to which they are attached via CIg.

The observations that we have made may have relevance for understanding the different behavior of neutrophils and monocytes in the acute inflammatory response. During early phases of this reaction, both neutrophils and monocytes migrate to sites of injury as a consequence of their response to chemotactic peptides generated by activation of the complement and coagulation systems (1), and are retained at the injury site as a result of agents derived from factor B of the alternative pathway of complement or from the contact phase of blood coagulation (4). During later phases

of inflammation, macrophages predominate over neutrophils. This occurrence may reflect the disappearance of complement- and coagulation-derived peptides resulting in selective retention of those cells bearing CIg receptors.

Summary

This investigation focused on the role played by cold-insoluble globulin (CIg, plasma fibronectin) in monocyte function. Surface-bound CIg mediated a concentration-dependent attachment of human blood monocytes to gelatin-coated surfaces. CIg also mediated the binding of gelatin-coated particles such as latex beads or tanned erythrocytes to surface-bound human monocytes. However, CIg did not mediate particle ingestion.

Subfractionated CIg that was highly enriched in monomeric forms (zone II CIg, mol wt 190,000–235,000) was less effective than were fractions enriched in dimeric forms (zone I CIg, mol wt 450,000) in promoting monocyte attachment. Binding of CIg to a gelatin or plastic surface occurred in the absence of divalent cations, but monocyte attachment to CIg-coated surfaces required divalent cations, Mg^{++} being much more effective than Ca^{++} . Cation-dependent cell attachment was reversible in that bound cells could be released by treatment with EDTA. Serum-mediated binding of monocytes to gelatin-coated plastic dishes was a result of its content of CIg because the binding activity was abolished by removal of CIg from serum, and could be restored by readdition of purified CIg. Treatment of monocytes with trypsin abolished subsequent cell attachment to CIg-gelatin surfaces or particles. Expression of certain other known monocyte membrane receptors (Fc and C3b) was markedly enhanced as a result of CIg-monocyte interaction. These several observations indicate that monocytes bear membrane receptors (termed receptor cold-insoluble globulin) for surface-bound CIg.

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