Regulation of Human Monocyte/Macrophage Function by Extracellular Matrix

Adherence of Monocytes to Collagen Matrices Enhances Phagocytosis of Opsonized Bacteria by Activation of Complement Receptors and Enhancement of Fc Receptor Function

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Abstract

In inflammation monocytes emigrate from the peripheral circulation into an extravascular area rich in extracellular matrix proteins. In this milieu, phagocytes ingest and kill invading pathogens. In the present studies, we found that monocytes adhered to type I collagen gels phagocytized 2.5–12-fold more opsonized *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* than plastic-adherent monocytes. The rate of phagocytosis and the number of bacteria ingested by collagen-adherent monocytes was equal to, or greater than, the number of bacteria ingested by 7-d cultured macrophages (Mϕ). Although both collagen- and plastic-adherent monocytes were bactericidal for *E. coli* and *S. aureus*, more bacteria were killed by collagen-adherent monocytes by virtue of their enhanced phagocytic capacity. Cultured Mϕ only were bacteriostatic.

Adherence of monocytes to collagen gels activated C receptors (CR) types 1 and 3 for phagocytosis, and enhanced Fc receptor (FcR)-mediated phagocytosis. Collagen- and plastic-adherent monocytes produced equivalent amounts of superoxide anion in response to phorbol myristate acetate and opsonized zymosan. Thus, the enhanced phagocytosis and killing of opsonized bacteria by collagen-adherent monocytes appear to be by regulation of the function of membrane CR and FcR, without apparent enhancement of the respiratory burst. These data suggest that adherence of monocytes to the extracellular matrix during inflammation may rapidly activate these cells for enhanced phagocytic bactericidal activity. (*J. Clin. Invest.* 1990. **86**:703–714.) Key words: collagen • monocyte • macrophage • complement receptors • phagocytosis • Fc receptors

Introduction

Gram-positive and gram-negative pathogenic bacteria require opsonization by serum proteins to be recognized, phagocytized, and killed by "professional" phagocytes (neutrophils,

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1. *Abbreviations used in this paper:* BAP, blood agar plate; CR, complement receptor; E, sheep erythrocyte(s); FcR, Fc receptor; HBSA, Hanks'-Hepes buffer containing 0.25% BSA; Mϕ, macrophage(s); PHS, pooled human serum; PI, phagocytic index; VLA, "very late antigen(s)."

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monocytes, and macrophages [Mϕ])[1] (1–3). Initiation of the inflammatory response by these microbes activates the complement (C) system, and the serum opsonins C3b, iC3b, and IgG become bound to the microbial surface (4–10). The specific plasma membrane receptors to which these ligand-coated bacteria bind, C receptor (CR) type 1 (CR$_1$) (11), CR type 3 (CR$_3$) (12, 13), and Fc receptors (FcR) (14), respectively, have been identified and structurally characterized. The importance of these opsonins and receptors in host defense is illustrated by the fact that individuals with genetic deficiencies of C3 (15), IgG (16), or CR$_3$ (17) suffer recurrent life-threatening infections.

In vitro studies have shown that phagocytosis via FcR is a constitutive property of phagocytes and that CR-mediated phagocytosis is developmentally regulated (18–24). Thus, binding of C3b- or iC3b-coated particles to human neutrophils (PMN) (18–20) or monocytes (21–23) is not followed by ingestion, whereas IgG-coated particles always are ingested (18–23). CR may be activated for phagocytosis by several agents including phorbol esters (23, 25), cytokines (26), and extracellular matrix proteins (27–31). These agents also may enhance FcR-mediated phagocytosis (25, 28, 29, 31–33).

Type I collagen is the most abundant of the fiber forming collagens, and is the major collagen of skin, tendon, bone, and other tissues. Culture of monocytes on type I collagen for 72 h has been shown to induce the production of PGE$_2$ and IL-1 (34), and tumor necrosis factor-α (TNFα) mRNA was expressed as early as 4 h after adherence of monocytes to collagen (35). Monocytes cultured on collagen rather than glass phenotypically resemble resident tissue Mϕ and are not cytotoxic (36). In addition, monocytes cultured on collagen gels for 48 h exhibited CR-mediated phagocytosis and enhanced FcR-mediated phagocytosis compared with monocytes cultured on glass (27).

In the present experiments, the phagocytic and bactericidal activity of monocytes adhered to type I collagen gels was quantified and compared with the phagocytic and bactericidal activity of plastic-adherent monocytes and 7-d adherently cultured Mϕ. The data show that the rate of phagocytosis and the number of opsonized bacteria phagocytosed by collagen-adherent monocytes was severalfold greater than the phagocytic capacity of plastic-adherent monocytes, and similar to the phagocytic capacity of 7-d cultured Mϕ. Collagen- and plastic-adherent monocytes mediated significant bactericidal activity for opsonized *Escherichia coli* and *Staphylococcus aureus*, but more bacteria were killed by collagen-adherent monocytes by virtue of their increased phagocytic capacity. Cultured Mϕ only were bacteriostatic. The enhanced phagocytic bactericidal activity of collagen-adherent monocytes appeared to be mediated by activation of CR$_1$ and CR$_3$ for phagocytosis, and enhancement of FcR-mediated phagocytosis, without apparent enhancement of the respiratory burst.

Methods

Bacteria

Bacteria used in these experiments were as follows: *E. coli*, ATCC 25922; hemolytic *S. aureus*, ATCC 25923; a clinical throat isolate of group A, β-hemolytic *Streptococcus pyogenes;* and a clinical blood isolate of *Streptococcus pneumoniae* type III (4). Cultures were maintained on sheep blood agar plates (BAPs) (BBL Microbiology Systems, Cockeysville, MD) and subcultured every 2 wk. *E. coli* and *S. aureus* were grown in trypticase soy broth (Difco Laboratories, Detroit, MI), and *S. pyogenes* and *S. pneumoniae* were grown in Todd-Hewitt broth (BBL Microbiology Systems).

Preparation of ³H-labeled bacteria

Bacteria were labeled by adding 0.1–7 ml (depending on the species) of overnight culture to 200 ml of medium containing 400 μCi of [³H]-thymidine (ICN Biomedicals, Costa Mesa, CA; 60 Ci/mmol), and by incubating the bacteria to mid–log phase in a shaking water bath at 37°C. At the end of the incubation, 0.5 ml of bacteria was removed, serially diluted, and plated on BAPs. The remaining bacteria were centrifuged at 4°C, combined in sterile 0.15 M saline, washed three times, and resuspended to 30 ml in sterile saline. Bacteria then were heat-killed at 65°C for 1 h (*S. pneumoniae* were heat-killed in 0.4% formalin), washed twice in saline, and stored in 30 ml of saline containing 0.05% azide. A 50-μl aliquot was solubilized in 1 ml of BTS-450 (Beckman Instruments Inc., Fullerton, CA) for 1.5 h at 60°C. Scintillation cocktail was added, and the suspension was counted in a liquid scintillation counter. The number of bacteria/cpm averaged between 600 and 1,000.

Serum

Pooled human serum (PHS) was prepared from six donors by allowing blood to clot for 45 min at 25°C and 45 min at 4°C. After centrifugation, serum was pooled, aliquoted, and stored frozen at −80°C to preserve C activity. Aliquots of serum were thawed just before use, and used only once. In some experiments serum was treated with 5 mM Mg-EGTA to chelate calcium so that only the alternate C pathway was activated (37).

Monocyte preparation and culture

Citrate anticoagulated human blood was centrifuged at 300 *g* for 20 min at 25°C, and platelet-rich plasma was removed. Mononuclear cells then were isolated from buffy coats by dextran sedimentation and Ficoll-Hypaque centrifugation (22). Monocytes were adhered to various substrates for 1 h at 37°C in 5% CO_2-95% air in 24-well tissue culture plates (Costar Data Packaging Corp., Cambridge, MA). The adherent monocytes were washed, and then were studied immediately or cultured in M199 (Gibco Laboratories, Grand Island, NY) containing 10% autologous serum and 10 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO). Medium was replaced on day 3 when Mϕ were cultured for 7 d (22).

Preparation of coverslips coated with extracellular matrix proteins

Collagen gels. Collagen gels were prepared as described by Kaplan and Gaudernack (27). Type I collagen from rat tails (Sigma Chemical Co.) was dissolved in ×1:10 M199, pH 3.0 at a concentration of 1 mg/ml, and aliquots of 0.25 ml were dispensed into the wells of 24-well tissue culture plates. To form gels, 25 μl of ×10 M199 and 12.5 μl of 0.142N NaOH were added simultaneously to each well, the contents were mixed, and the plates were incubated for 2 h at 37°C. Collagen gels and collagen-coated coverslips were washed four times with HBSS containing 20 mM Hepes (Hanks'-Hepes), and monocytes were adhered for 1 h at 37°C.

On SDS-PAGE, collagen from Sigma Chemical Co. showed characteristic $\alpha1(I)$ and $\alpha2(I)$ chains as well as some β chains, which were identical to those observed with purified type I collagen (kindly pro-

vided by Dr. Winston Kao, Department of Ophthalmology, University of Cincinnati College of Medicine). No bands were observed that co-migrated with purified fibronectin (Collaborative Research, Bedford, MA), and no other contaminating proteins were observed (data not shown).

Vitronectin. Purified vitronectin was a gift of Dr. Charles Parker, VA Medical Center, Salt Lake City, UT, and was used as described (33). Coverslips were covered with 0.3 ml of vitronectin (50 μg/ml) and incubated for 2 h at 25°C. Coverslips were washed twice in Hanks'-Hepes, and monocytes were adhered for 1 h at 37°C.

Gelatin-coated fibronectin. Fibronectin-coated coverslips were prepared by the method of Bevilaqua et al. (38). Coverslips were incubated with gelatin (2 mg/ml) for 1 h at 37°C, excess gelatin was aspirated, and plates were incubated overnight at 50°C. Plates were irradiated under UV light for 1 h, 0.3 ml of platelet-free plasma was added, and the plates were incubated for 30 min at 25°C, and then for 60 min at 4°C. Excess plasma was removed by aspiration, the wells were washed twice in Hanks'-Hepes, and monocytes were adhered for 1 h at 37°C.

Phagocytosis of ³H-labeled bacteria

Heat-killed, radiolabeled bacteria were suspended to 6 × 10⁸/ml in Hanks'-Hepes containing 10% PHS and incubated for 20 min at 37°C. Opsonized bacteria were studied as they were, or were washed twice in Hanks'-Hepes containing 0.25% BSA (HBSA) and then resuspended to 6 × 10⁸/ml. 0.1 ml of opsonized bacteria was added to adherent mono-cyte/Mϕ and incubated for varying periods of time at 25°C. At the end of the incubation period, monolayers were washed six times with HBSA to remove nonadherent bacteria. Monolayers were lysed with 0.1 ml of 0.05% Triton X-100 for 15 min at 37°C. Lysed cells and bacteria were transferred to a 17 × 100-mm polypropylene tube, the wells were washed twice with 0.2 ml of sterile water, and the wash supernates were combined with the lysate. The bacteria were solubilized, scintillation cocktail was added, and the mixture was poured into scintillation vials for counting. Control wells contained bacteria only, and the counts per minute obtained were subtracted as background to account for nonspecific adherence of the bacteria to the plastic (< 100 cpm) or the collagen gels (< 500 cpm). Removal of the bacteria also was confirmed by visual inspection on an inverted microscope. Monocytes that underwent all manipulations except incubation with bacteria were treated with ZAP-OGLOBIN II (Coulter Diagnostics, Hialeah, FL) to lyse the external membrane of the cells. Nuclei then were quantified on a Sysmex Microcell CC-110 counter (American Scientific Products, Columbus, OH). Results are expressed as the mean±SEM of the number of bacteria ingested per phagocyte.

Monocyte/Mϕ bactericidal assay

The bactericidal assay described by Leijh et al. (39) for quantifying killing in the absence of continuing phagocytosis was modified for study of adherent phagocytes. Except where noted, buffers did not contain antibiotic. Mid–log-phase bacteria were centrifuged, washed once in Hanks'-Hepes, and resuspended to 5 ml in HBSA. Bacteria were diluted 1:100, and 0.6-ml aliquots were further diluted to 1.2 ml in HBSA containing 10% PHS. Bacteria were opsonized for 20 min at 37°C, washed twice with HBSA, and then resuspended to 1.2 ml. Opsonized bacteria (0.1 ml) were added to each well and incubated for 10 min at 25°C to allow for phagocytosis. The inoculum was aspirated, and the monolayers were washed three times with HBSA, and once with HBSA containing 10 μg/ml of gentamicin. The gentamicin wash was added to kill the few extracellular bacteria that were observed to adhere to the collagen gels. Viable bacteria, unlike heat-killed bacteria, were more prone to stick to the collagen gels, particularly *S. aureus*. (Incubation of collagen-adherent monocytes with gentamicin reduced the number of viable bacteria recovered compared to untreated collagen-adherent monocytes. No viable bacteria were recovered from collagen gels incubated with bacteria alone and then washed with gentamicin; bacteria were recovered from collagen gels incubated with bacteria alone and then washed without gentamicin. Evidence that

gentamicin did not effect the number of intracellular bacteria came from control experiments with plastic-adherent monocytes. Thus, identical numbers of viable intracellular bacteria were recovered from plastic-adherent monocytes incubated with or without gentamicin.) Monolayers were covered with 0.2 ml of HBSA or 0.2 ml of HBSA containing 50% PHS, and incubated for 0, 30, 60, or 120 min at 37°C in 5% CO_2-95% air. At the end of each time period, the cells were washed twice with cold HBSA, and monolayers lysed in 1 ml of sterile water. After vigorous agitation, the lysate was transferred to a tube containing 9 ml of water, and the bacteria were serially diluted and plated on BAPs. Plates were incubated overnight at 37°C, and viable colonies enumerated. Results are expressed as the number of viable bacteria remaining at each time point. In preliminary experiments we found that significant killing occurred during the 10 min of phagocytosis, particularly of *S. aureus* by monocytes. To obtain a more accurate quantitation of the number of microorganisms initially phagocytosed, opsonized bacteria were incubated with adherent monocytes in the presence of 3.2 mM phenylbutazone to prevent the killing of ingested bacteria (39, 40). The viable bacteria count obtained from these wells was used as a measure of the number of microorganisms initially ingested.

Preparation of C3- and IgG-coated sheep erythrocytes (E)

Sheep E bearing C3b and iC3b (EC3b and EC3bi) were prepared with purified components of the alternate C pathway as previously described (24). EC3b(i) used in these experiments had 4–5 × 10⁴ C3 molecules bound per E (24). To prepare IgG-coated E (EIgG), E (1 × 10⁹/ml) were mixed with an equal volume of a 1:250 dilution of rabbit IgG anti-E (Cordis Laboratories, Miami, FL), and incubated for 30 min at 37°C and 30 min at 4°C (22). EIgG prepared in this manner were bound and ingested by 50–60% of freshly isolated monocytes (22).

Phagocytosis of ligand-coated E

Binding and ingestion of EC3b(i) and EIgG by adherent monocyte/Mϕ were quantified as described previously (22, 24). After incubation of ligand-coated E (2 × 10⁷) with monocyte/Mϕ for 1 h at 37°C in 5% CO_2-95% air, unbound E were removed by washing with HBSA, and bound but uningested E were lysed with an NH_4Cl lysing buffer. The monolayers then were fixed in 2% glutaraldehyde-1% sucrose in 0.01 M phosphate buffer, pH 7.4. Coverslips were sealed cell-side-down on microscope slides, and binding and ingestion were quantified by counting 100–200 cells per coverslip via phase-contrast microscopy at ×1,000. Results are expressed as percent rosettes (the percentage of cells that bound three or more E), percent ingestion (the percentage of cells that ingested one or more E), and the phagocytic index (PI, the total number of E ingested per 100 cells).

In some experiments, phagocytosis was quantified using ⁵¹Cr-labeled E as described previously (22). Phagocytosis experiments were performed as described for unlabeled E, except that after lysis of unbound E, monocytes were lysed with 1 ml of 0.05% Triton X-100 (Fisher Scientific Co., Pittsburgh, PA). The wells were washed once with HBSA, the lysate and wash supernate were pooled, and radioactivity was quantified in a Packard AUTO-GAMMA 500C (Packard Instruments Co., Inc., Downers Grove, IL). Adherent monocytes were quantified as described above, and results were expressed as the mean±SEM of the number of E ingested per monocyte. All experiments were performed in triplicate.

Superoxide anion production

Superoxide anion (O_2^-) generation by adherent monocytes was quantified as the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (type III, Sigma Chemical Co.) (41). Zymosan was opsonized in 10% PHS at 2 × 10⁸ particles/ml for 20 min at 37°C. After washing twice in HBSA, opsonized zymosan was resuspended in Krebs-Ringer phosphate buffer with 0.2% dextrose containing 80 μM cytochrome c. Adherent monocytes were washed, and then incubated for 1 h at 37°C with opsonized zymosan (2 × 10⁷ particles/ml) or 100

ng/ml of phorbol myristate acetate (PMA) (Sigma Chemical Co.) in the Krebs-Ringer phosphate dextrose buffer containing cytochrome c. Control wells contained cytochrome c without a stimulus (resting cells), or the stimulus plus 40 μg/ml of SOD (Sigma Chemical Co.). At the end of the incubation, the absorbance of the supernates at 550 nm was compared to a blank that underwent all manipulations but contained only cytochrome c. Results are expressed as nanomoles of cytochrome c reduced per 1 × 10⁵ monocytes per hour.

Results

Phagocytosis of opsonized bacteria by collagen- vs. plastic-adherent monocytes. Phagocytosis of ³H-labeled serum-opsonized *E. coli* by monocytes adhered to collagen gels or to the plastic surface of 24-well tissue culture plates was quantified after varying periods of time. Both the rate of phagocytosis and the number of *E. coli* ingested by collagen-adherent monocytes was greater than that of plastic-adherent monocytes (Fig. 1). After 30 min of incubation, collagen-adherent monocytes ingested 3.5-fold more *E. coli* than plastic-adherent cells. The enhanced rate and phagocytic capacity of collagen-adherent vs. plastic-adherent monocytes were maintained during 24 and 48 h. of culture. After 24 and 48 h, collagen-adherent Mϕ ingested 4.7- and 3.5- fold more *E. coli* per Mϕ, respectively, than Mϕ cultured on plastic (data not shown).

To determine whether the enhanced phagocytosis by collagen-adherent monocytes was applicable to other bacteria, we quantified the phagocytosis of other species of bacteria by collagen- vs. plastic-adherent monocytes. Collagen-adherent monocytes ingested 2.5–12-fold more opsonized *E. coli*, *S. aureus*, *S. pyogenes*, and *S. pneumoniae* than plastic-adherent monocytes (Fig. 2).

Fig. 3 shows that opsonization in fresh PHS was required for phagocytosis of *E. coli* and *S. pneumoniae* by collagen-ad-
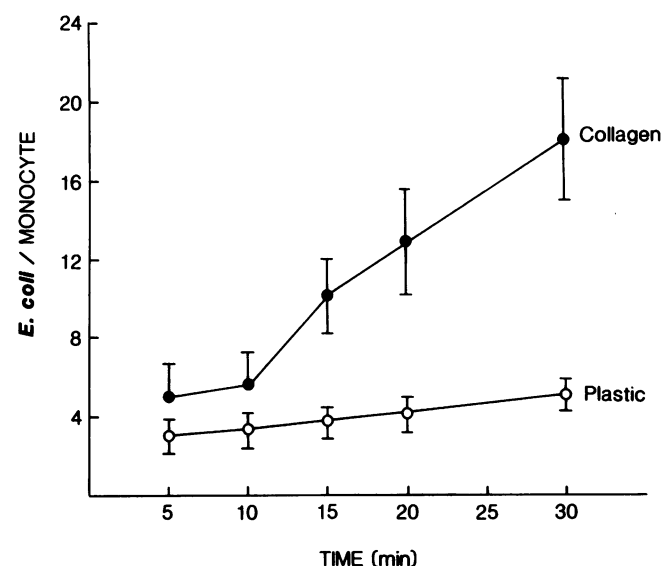

Figure 1. Kinetics of collagen- vs. plastic-adherent monocyte phagocytosis of ³H-labeled *E. coli*. [³H]Thymidine-labeled *E. coli* (6 × 10⁸/ml) were opsonized in 10% PHS for 20 min at 37°C. After opsonization, bacteria were washed twice in HBSA, and resuspended to 6 × 10⁸/ml in HBSA. Aliquots of 6 × 10⁷ bacteria then were incubated with collagen- or plastic-adherent monocytes and phagocytosis was quantified after 5–30 min at 25°C. Results are presented as the mean±SEM (n = 5) of the number of *E. coli* ingested per monocyte.
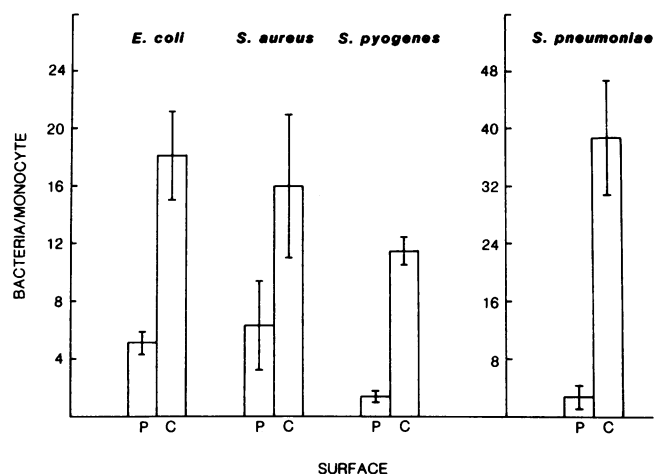
Figure 2. Phagocytosis of ^{3}H-labeled bacteria by collagen- vs. plastic-adherent monocytes. Collagen- and plastic-adherent monocytes were incubated with 6 × 10^7 opsonized [^{3}H]thymidine labeled *E. coli, S. aureus, S. pyogenes,* or *S. pneumoniae* and phagocytosis was quantified after 30 min at 25°C. Results are presented as the mean±SEM (*n* = 3–4) of the number of bacteria ingested per monocyte. P, plastic; C, collagen.

herent monocytes. Similar results were obtained with *S. aureus* and *S. pyogenes* (data not shown). Phagocytosis of bacteria opsonized in serum chelated with 5 mM Mg-EGTA (37) was equivalent to that obtained with untreated serum, demonstrating that opsonization of these bacteria via the alternate C pathway was sufficient for optimum phagocytosis. Opsonization of bacteria in heat-inactivated serum resulted in intermediate levels of ingestion, presumably due to antibody present in the PHS (Fig. 3).
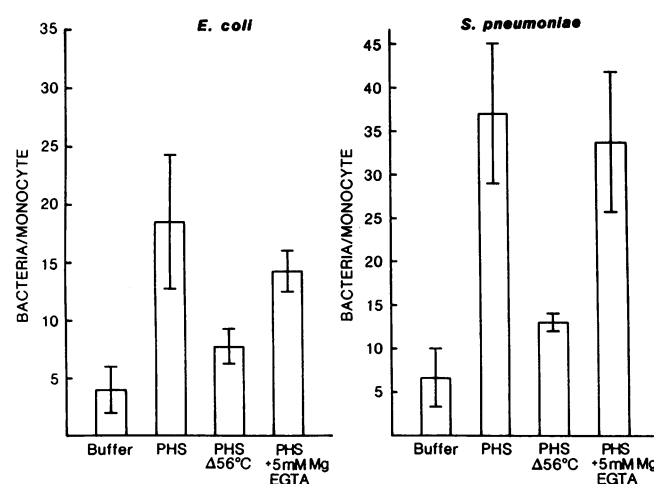

Figure 3. Requirement for opsonization of ^{3}H-labeled *E. coli* and *S. pneumoniae* for phagocytosis by collagen-adherent monocytes. [^{3}H]-Thymidine-labeled bacteria were opsonized for 20 min at 37°C in 10% PHS, 10% PHS heated at 56°C for 60 min, 10% PHS containing 5 mM Mg-EGTA, or buffer. Aliquots of 6 × 10^7 bacteria were incubated with collagen-adherent monocytes and phagocytosis was quantified after 30 min at 25°C. Results are presented as the mean±SEM (*n* = 3) of the number of bacteria per monocyte.

Phagocytoses of opsonized bacteria by plastic-adherent monocytes vs. cultured monocyte-derived Mϕ. Phagocytosis of opsonized *E. coli* and *S. aureus* by plastic-adherent monocytes and adherently cultured Mϕ was quantified after varying periods of time. Fig. 4 shows that the rate of phagocytosis and the number of bacteria ingested by Mϕ was greater than that of freshly isolated monocytes. After 30 min, Mϕ had ingested 3.7-fold more *E. coli* and 2.4-fold more *S. aureus* than monocytes.

Fig. 5 shows that opsonization with fresh PHS was required for phagocytosis of *E. coli* and *S. aureus* by plastic-adherent monocytes and cultured Mϕ. Opsonization in heat-inactivated PHS markedly reduced the phagocytosis of *E. coli* by monocytes and Mϕ. In contrast, monocyte/Mϕ phagocytosis of *S. aureus* opsonized in heat-inactivated serum was 56% and 63%, respectively, of that obtained with unheated serum, indicating that the PHS contained a considerable amount of antibody to this strain of *S. aureus*. Opsonization via the alternate C pathway was sufficient for optimum phagocytosis by both plastic-adherent monocytes and cultured Mϕ.

These data show that collagen-adherent monocytes ingested as many or more opsonized bacteria as cultured Mϕ (Figs. 1, 2, and 4). Thus, collagen-adherent monocytes ingested a mean±SEM of 18.6±5.8 (*n* = 3) *E. coli* per cell, and 16.0±10.5 (*n* = 3) *S. aureus* per cell. By comparison, cultured macrophages ingested 8.9±1.9 (*n* = 4) and 15.9±1.8 (*n* = 3) opsonized *E. coli* and *S. aureus* per Mϕ, respectively. Therefore, adherence of freshly isolated monocytes to collagen gels abrogated the requirement for long-term in vitro culture to achieve the same phagocytic capacity observed for 7-d cultured Mϕ. However, adherence of monocytes to collagen gels did not abrogate the requirement for serum opsonization.

Bactericidal activity of collagen- vs. plastic-adherent monocytes. We next sought to quantify and compare the bactericidal activity of collagen-vs. plastic-adherent monocytes. To quantify bactericidal activity, monocytes were incubated for 10 min with opsonized *E. coli* or *S. aureus,* and uningested organisms were removed by washing. Fresh buffer or buffer containing 50% PHS was added to the monolayers, and killing was quantified after incubation for 30, 60, and 120 min at 37°C. Identical numbers of monocytes adhered to both substrates, and, therefore, the results are presented as the amount of killing observed in each monolayer. For eight individual donors, the mean±SEM of the number of monocytes that adhered to collagen gels or plastic was 3.5±0.2 × 10^5 and 3.6±0.1 × 10^5, respectively. After 24 and 48 h of culture, the number of collagen-adherent Mϕ was 3.3±0.1 × 10^5 and 3.1±0.2 × 10^5 cells, respectively. The number of Mϕ adherent to plastic at 24 and 48 h was 3.3±0.2 × 10^5 and 3.0±0.2 × 10^5 cells, respectively. Thus, few Mϕ were lost after 24 and 48 h of culture on either substrate.

After 10 min of phagocytosis collagen-adherent monocytes ingested almost twice as many *E. coli* and fourfold more *S. aureus* than plastic-adherent cells. Despite the extra bacterial load, collagen-adherent monocytes killed 94% of ingested *E. coli* and 97% of ingested *S. aureus* when serum was present during the bactericidal assay. In the absence of extracellular serum, 80% of *E. coli* and 94% of *S. aureus* were killed (Fig. 6). Plastic-adherent monocytes killed 92% of ingested *E. coli* and 65% of ingested *S. aureus* in the presence of extracellular serum (Fig. 6).
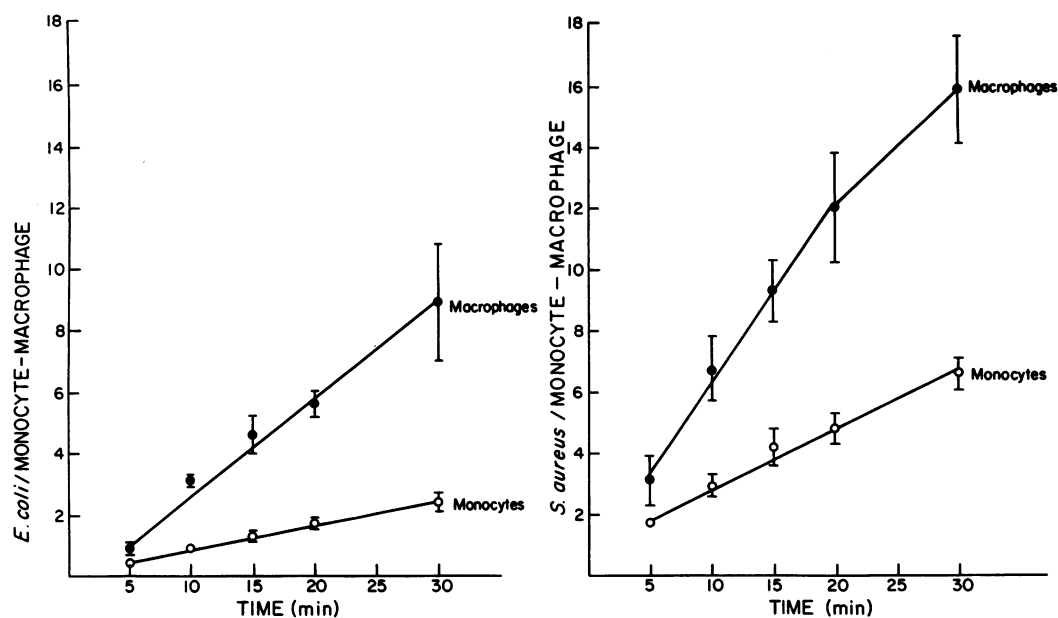
Figure 4. Kinetics of monocyte/Mφ phagocytosis of ^{3}H-labeled *E. coli* and *S. aureus.* Opsonized [^{3}H]thymidine-labeled bacteria (6×10^7) were incubated with plastic-adherent monocytes or 7-d cultured Mφ, and phagocytosis was quantified after 5–30 min at 25°C. Results are presented as the mean±SEM (*n* = 3–4) of the number of bacteria ingested per phagocyte.

The bactericidal activity of monocytes for *E. coli* and *S. aureus* was maintained over 24–48 h of culture on both collagen gels and plastic. After 10 min of phagocytosis, collagen-adherent Mφ ingested 2.4- and 3-fold more *E. coli* at 24 and 48 h, respectively; and 2- and 6-fold more *S. aureus* at 24 and 48 h, respectively. As was observed for freshly adherent monocytes, cultured collagen-adherent Mφ killed > 90% of ingested *E. coli* and *S. aureus* after 2 h. Again, optimum killing of *E. coli,* but not killing of *S. aureus,* required the presence of extracellular serum. Cultured plastic-adherent Mφ killed 83%

and 91% of ingested *E. coli* at 24 and 48 h, respectively; and killed 68% and 83% of ingested *S. aureus* at 24 and 48 h, respectively.

Bactericidal activity of monocytes vs. cultured Mφ. Next we quantified and compared the bactericidal activity of plastic-adherent monocytes vs. 7-d cultured Mφ against *E. coli* and *S. aureus.* Monocytes killed 98% and 91% of ingested *E. coli* and *S. aureus,* respectively, after 2 h (Fig. 7, *upper panels*). In contrast, 7-d cultured Mφ only were bacteriostatic. As was found for collagen-adherent monocytes, the presence of extra-
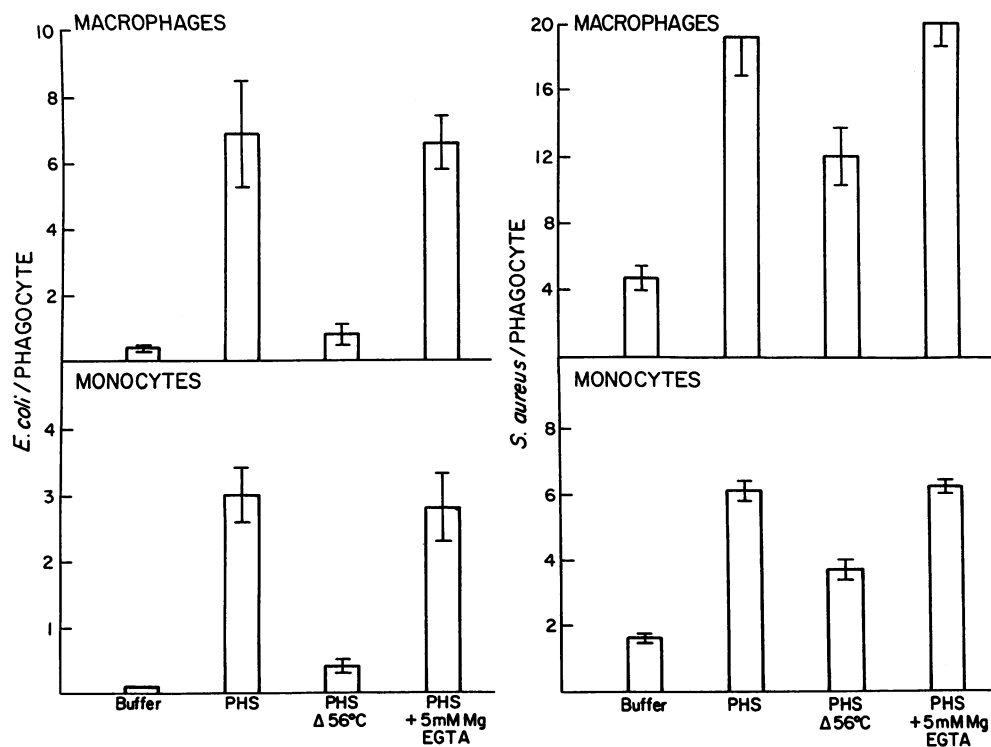

Figure 5. Requirement for opsonization of ^{3}H-labeled *E. coli* and *S. aureus* for phagocytosis by plastic-adherent monocytes and cultured Mφ. [^{3}H]Thymidine-labeled bacteria were opsonized for 20 min at 37°C in 10% PHS, 10% PHS heated at 56°C for 60 min, 10% PHS containing 5 mM Mg-EGTA, or buffer. After opsonization, bacteria were washed twice in HBSA, and resuspended to 6 × 10^8/ml in HBSA. Aliquots of 6×10^7 bacteria were incubated with monocyte/Mφ and phagocytosis was quantified after 30 min at 25°C. Results are presented as the mean±SEM (*n* = 4–5) of the number of bacteria ingested per phagocyte.
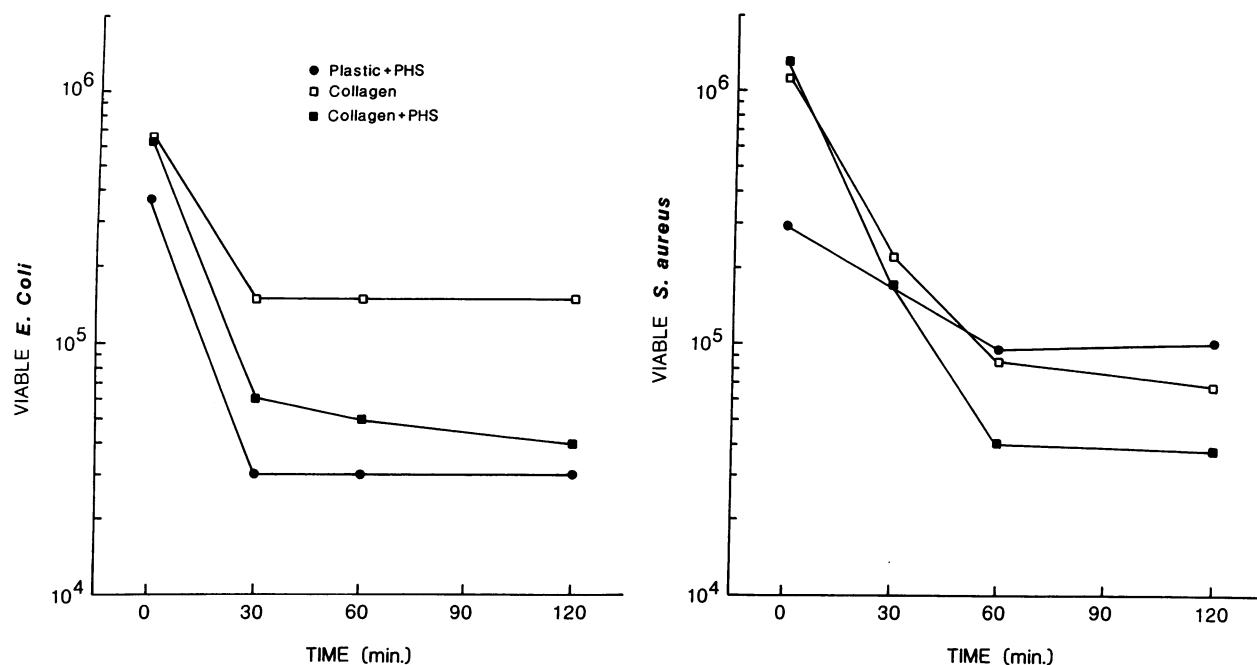
Figure 6. Collagen- and plastic-adherent monocyte bactericidal activity against *E. coli* and *S. aureus.* Mid–log-phase bacteria were opsonized in 10% PHS and washed twice in HBSA, and 0.1-ml aliquots were incubated with monocytes for 10 min at 37°C to allow for phagocytosis. After washing off the noningested bacteria, plastic-adherent monocytes were covered with HBSA (●), and collagen-adherent monocytes were covered with HBSA (□) or HBSA containing 50% PHS (■). Bactericidal activity was quantified after 30, 60, and 120 min at 37°C. The results show the number of remaining viable bacteria at each time point and are the means from four separate experiments.

cellular serum was required for optimum killing of *E. coli* by plastic-adherent monocytes, but was not required for killing *S. aureus.*

Activation of CR₁ and CR₃ for phagocytosis by adherence of *monocytes to collagen gels.* The increased phagocytosis of opsonized bacteria demonstrated by collagen-adherent monocytes might be explained by an overall increase in the phagocytic activity of monocytes, or by activation of individual membrane receptors for enhanced phagocytosis. Since opsonization of these bacteria with C3b(i)/IgG is required for recognition and ingestion, we sought to determine if adherence of monocytes to collagen gels might have a specific regulatory effect on CR and FcR function. Monocytes were adhered to collagen gels or glass coverslips, and attachment and ingestion of EC3b and EC3bi quantified immediately after adherence, or after culture of monocytes for 24 and 48 h. The results of these experiments are shown in Fig. 8. Zero (0) time in culture refers to monocytes studied immediately after the 1 h of adherence.

As has been shown by us (22, 24) and others (21, 23), glass-adherent monocytes avidly bind EC3b and EC3bi (Fig. 8 *A*), but do not ingest them (Fig. 8, *B* and *C*). In contrast, collagen-adherent monocytes not only bound EC3b and EC3bi (Fig. 8 *D*), but ingested them as well (Fig. 8, *E* and *F*). After adherence to collagen gels, 34±4% of monocytes ingested an average of 2.5 EC3b per monocyte, and 44±5% of cells ingested 2.9 EC3bi per monocyte. As reported previously (22), culture of monocytes for 24–48 h on coverslips resulted in a decrease in the capacity of these cells to bind EC3b and EC3bi (Fig. 8 *A*). In contrast, monocytes cultured for 24–48 h on collagen gels maintained their ability to form rosettes with

EC3b(i) (Fig. 8 *D*), and the number of particles ingested increased with time in culture (Fig. 8 *F*). These data confirm the report of Kaplan and Gaudernack (27) that monocyte CR were activated for phagocytosis by 48 h of culture on collagen gels.

Enhancement of FcR-mediated phagocytosis by adherence of monocytes to collagen gels. In conjunction with the experiments on CR-mediated phagocytosis, FcR-mediated phagocytosis also was quantified. Freshly isolated collagen-adherent monocytes ingested more than twice as many EIgG as did glass-adherent monocytes (PI = 315±101 vs. 135±11, respectively) (Fig. 8, *C* and *F*), although the percentage of phagocytic monocytes on both surfaces was the same (Fig. 8, *B* and *E*). The PI of collagen-adherent monocytes remained unchanged through 24 and 48 h of culture, and was always greater than the PI of plastic-adherent monocytes (Fig. 8, *C* and *F*).

To determine whether the number of EIgG ingested by plastic- vs. collagen-adherent monocytes, was dependent on the concentration of antibody used to prepare the EIgG, sheep E were labeled with ⁵¹Cr and sensitized with varying concentrations of IgG anti-E, and phagocytosis of ⁵¹Cr-labeled EIgG was quantified. This assay procedure was used for these experiments because monocytes adherent to collagen gels did not spread well and remained rounded up, and ingestion was difficult to quantify accurately if more than five E were present in a single monocyte.

At all four concentrations of antibody tested, collagen-adherent monocytes ingested approximately twice as many EIgG/cell as plastic-adherent monocytes (Fig. 9). Thus, in-
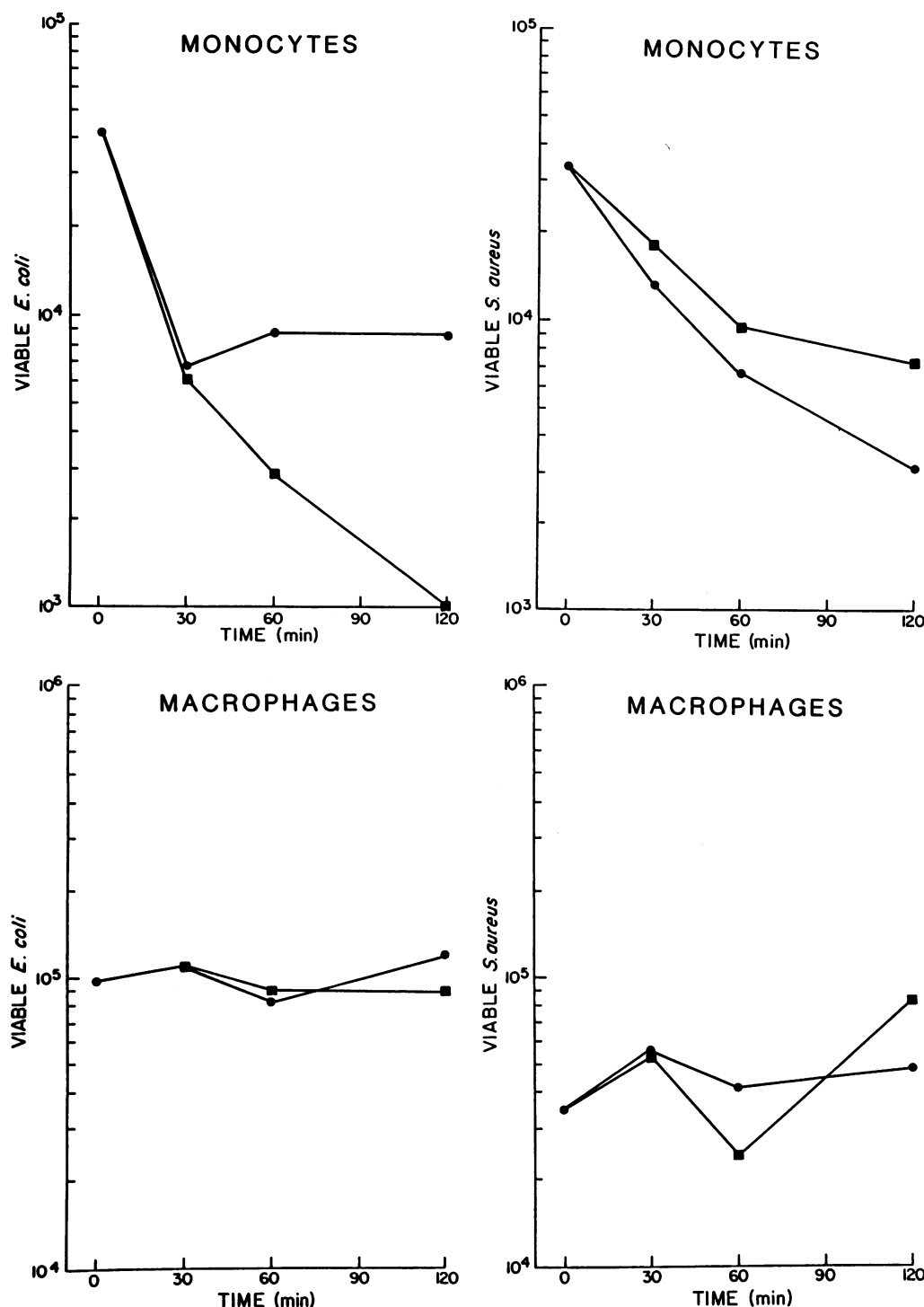
Figure 7. Monocyte/Mφ bactericidal activity against E. coli and S. aureus. Phagocytic bactericidal activity was quantified as described in Fig. 6. Monolayers were covered with HBSA (●) or HBSA containing 50% PHS (■) and bactericidal activity was quantified after 30, 60, and 120 min at 37°C. The results show the number of remaining viable bacteria at each time point, and are the means from two experiments.

creased FcR-mediated phagocytosis by collagen-adherent monocytes was observed independent of the amount of antibody used to prepare the EIgG.

Effect of collagen vs. collagen gels on monocyte CR- and FcR-mediated phagocytosis. To determine whether augmentation of monocyte receptor function required that the collagen be in the form of a hydrated collagen lattice (which is the form of collagen in extracellular connective tissue matrices

[42]), monocytes were adhered to glass coverslips, or coverslips coated with collagen, collagen gels, fibronectin, or vitronectin, and attachment and ingestion of EC3b, EC3bi, and EIgG were quantified. Monocytes adherent to glass, vitronectin, or fibronectin, did not ingest EC3b or EC3bi, confirming our previous results (22) and the reports of others (21, 30, 33). Monocytes adherent to collagen demonstrated some ingestion of EC3b(i), but optimum phagocytosis of these particles required adher-
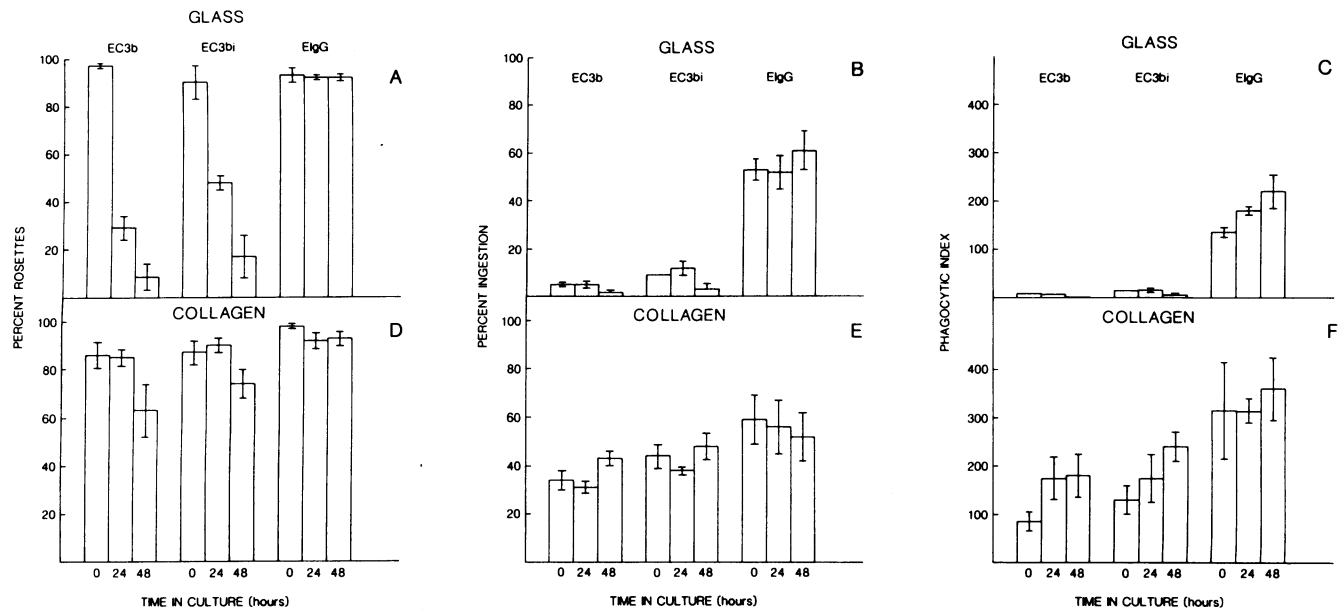
Figure 8. Binding and ingestion of EC3b(i) and EIgG by collagen- and plastic-adherent monocytes cultured for 0, 24, and 48 h. Ligand-coated E (2×10^7) were added to freshly isolated monocytes (0 time in culture) or monocytes cultured for 24 and 48 h on glass coverslips or collagen gels. Attachment and ingestion was quantified after 1 h at 37°C. Results are presented as the mean±SEM of four to seven experiments. The upper panels show the results obtained with glass-adherent monocytes, and the lower panels show the results obtained with collagen-adherent monocytes. (*A* and *D*) Percent rosettes; (*B* and *E*) percent ingestion; (*C* and *F*) PI.

ence to collagen gels (Fig. 10). There was no difference in the binding of ligand-coated E to monocytes adherent to the different substrates (data not shown).

Compared to glass-adherent monocytes, monocytes adherent to fibronectin and vitronectin ingested 34% and 46% more EIgG, respectively (Fig. 10). Adherence of monocytes to collagen had no effect on FcR-mediated ingestion, whereas monocytes adherent to collagen gels ingested twice as many EIgG as

Figure 9. Phagocytosis of ^{51}Cr-labeled EIgG. ^{51}Cr-labeled EIgG were prepared with varying concentrations of IgG anti–E and aliquots of 2×10^7 EIgG were added to monolayers of collagen- and plastic-adherent monocytes in triplicate. Phagocytosis was then quantified after 1 h at 37°C. Results are presented as the mean±SEM ($n = 3$) of the number of EIgG ingested per monocyte.

glass-adherent monocytes (Fig. 10). Also, under the conditions of assay, monocytes adherent to collagen gels ingested greater numbers of EIgG than monocytes adherent to either fibronectin or vitronectin.

Effect of adherence of monocytes to collagen gels on the respiratory burst. Finally, we sought to determine whether adherence of monocytes to collagen gels had a regulatory effect on the generation of the respiratory burst. Monocytes were adhered to plastic or to collagen gels, and O_2^- production quantified. The substrate to which monocytes were adhered did not effect the production of O_2^- when monocytes were incubated with either a particulate (opsonized zymosan) or a soluble (PMA) stimulus (Table I). In addition, no difference in O_2^- release by plastic- or collagen-adherent monocytes was observed with resting cells. Thus, generation of the respiratory burst was not modified by adherence of monocytes to collagen gels.

Discussion

We quantified and compared the phagocytic and bactericidal activity of collagen-adherent monocytes vs. plastic-adherent monocytes and 7-d adherently cultured monocyte-derived Mφ. All three cell preparations phagocytosed serum opsonized bacteria in a time-dependent manner. However, both the rate of phagocytosis and the total number of opsonized bacteria ingested by collagen-adherent monocytes was greater than that of plastic-adherent monocytes. Collagen-adherent monocytes ingested 2.5–12-fold more microorganisms than plastic-adherent monocytes after 30 min of incubation. The numbers of *E. coli* and *S. aureus* ingested by collagen-adherent monocytes was equal to or greater than the number of organisms ingested by 7-d cultured Mφ. Adherence of monocytes to collagen did

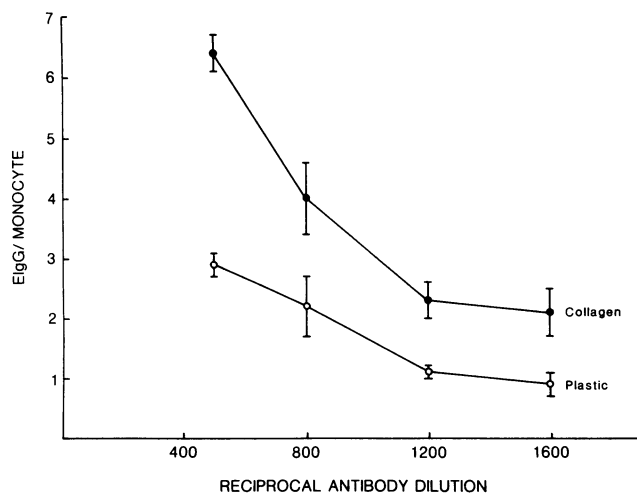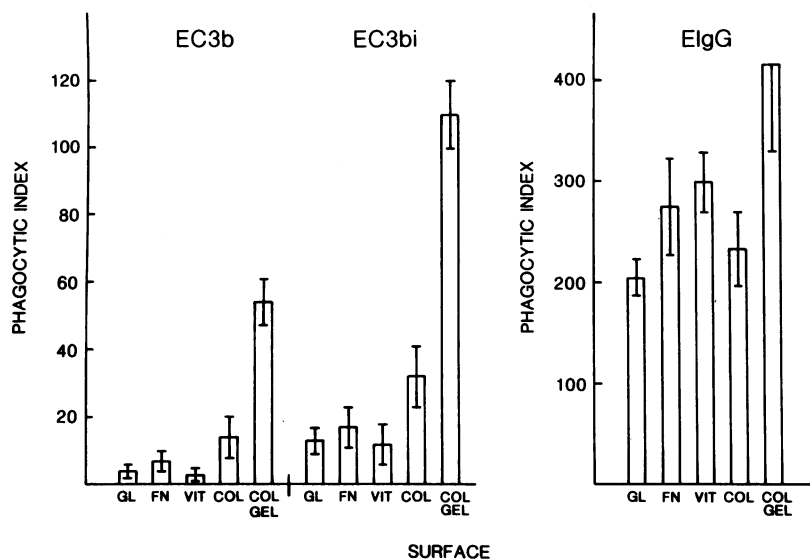*Figure 10.* Phagocytosis of EC3b(i) and EIgG by monocytes adherent to various substrates. Monocytes were adhered to glass coverslips (*GL*) or to coverslips coated with fibronectin (*FN*), vitronectin (*VIT*), collagen (*COL*), and collagen gels (*COL GEL*). Ligand-coated E (2×10^7) were added to the monolayers and phagocytosis was quantified after 1 h at 37°C. Results are presented as the mean±SEM ($n = 5$) of the phagocytic indices (the number of E ingested per 100 monocytes).

not abrogate the requirement for serum opsonization; opsonization of bacteria via the alternate C pathway was necessary and sufficient for optimum phagocytosis. Thus, adherence of monocytes to collagen gels can significantly increase the phagocytic capacity of monocytes without the need for long-term culture and differentiation into Mϕ.

Although both collagen- and plastic-adherent monocytes were bactericidal for *E. coli* and *S. aureus,* more bacteria were killed by collagen-adherent monocytes by virtue of their enhanced phagocytic capacity. In contrast, cultured Mϕ only were bacteriostatic. Thus, collagen-adherent monocytes have the same phagocytic capacity as cultured Mϕ, but unlike cultured Mϕ, these cells retained their bactericidal activity. The loss of bactericidal activity by cultured Mϕ is consistent with previous reports which demonstrated a loss of antimicrobial activity against viral (43), fungal (44, 45), bacterial (46–48), and protozoan (49, 50) intracellular pathogens by monocytes cultured in vitro. The loss of antimicrobial activity by cultured Mϕ parallels the loss during culture of the enzyme myeloperoxidase (45), and the decline in the ability of cultured Mϕ to be stimulated to release O_2^- (45, 51), H_2O_2 (45, 49), and $\cdot$ OH (45), and again emphasizes the importance of the respiratory burst in phagocyte antimicrobial activity.

Table I. Superoxide Anion Generation by Collagen- vs. Plastic-adherent Monocytes

	O_2^-		
Substrate	Resting cells	Opsonized zymosan	PMA
	nmol/10^5 monocytes per h		
Plastic	1.4±0.4 (10)	5.0±1.1 (5)	3.9±0.3 (5)
Collagen	1.5±0.4 (10)	5.5±0.8 (5)	4.1±0.2 (5)

Adherent monocytes were incubated with opsonized zymosan (2 $\times 10^7$/ml) or PMA (100 μg/ml) for 1 h at 37°C, and superoxide anion production was quantified by the reduction of cytochrome *c.* Values are given as mean±SEM (*n*).

Optimum killing of *E. coli* but not killing of *S. aureus* by collagen- and plastic-adherent monocytes required the presence of extracellular serum, and significant killing of *E. coli* occurred in the absence of extracellular serum. These results differ from the experiments of Leijh et al. (39), who reported that monocytes absolutely required extracellular serum to kill these two microorganisms. No bactericidal activity was evident in the absence of extracellular serum (39). The reason(s) for the discrepancy between our results and the previous studies are unclear. One possible explanation is that the specific strains of *E. coli* and *S. aureus* used in these and the previous experiments have different requirements for intracellular killing. This is unlikely, since all strains of these species are probably killed by toxic oxygen metabolites. One important difference between the two studies is that Leijh et al. (39) studied the bactericidal activity of monocytes in suspension, whereas we studied the bactericidal activity of adherent monocytes. Adherence may, therefore, abrogate the requirement for extracellular serum. Since phagocytes in vivo are adherent to biological surfaces except when in the peripheral circulation, these data may more accurately reflect the in vivo situation.

The mechanism of enhanced phagocytosis of opsonized bacteria by collagen-adherent monocytes appeared to be through regulation of CR and FcR function. Adherence of monocytes to collagen gels effected an immediate activation of both CR$_1$ and CR$_3$ for phagocytosis, and FcR-mediated phagocytosis was enhanced two-fold. Activation of monocyte CR for phagocytosis and enhancement of FcR function was maintained during 24–48 h of culture on collagen gels. These results confirm the observations of Kaplan and Gaudernack (27) that monocytes cultured on collagen gels activated their CR for phagocytosis after 48 h of culture; culture times earlier than 48 h were not examined. In contrast to culture on collagen gels, culture of monocytes on glass coverslips results in down regulation of CR expression as we reported previously (22).

Previous studies of the effect of extracellular matrix proteins on monocyte/Mϕ receptor function have found that differentiation in vitro is required for activation of CR for phagocytosis. Monocytes required 3 d of culture in suspension in vitro before adherence of cells to fibronectin (30) or laminin

(31) activated CR for phagocytosis. Vitronectin, a 165-kD extracellular matrix protein, was reported to enhance FcR-mediated phagocytosis of freshly isolated and adherent monocytes, but did not activate CR for phagocytosis (33), a finding confirmed in the present report. In addition, adherence of monocytes to collagen gels increased FcR-mediated phagocytosis to a greater extent than adherence to vitronectin or fibronectin. Since the extracellular matrix contains a mixture of these, and other proteins, FcR-mediated phagocytosis may be significantly upregulated at sites of inflammation.

In contrast to the results obtained with monocytes, preliminary experiments have indicated that adherence of PMN to collagen gels does not increase the ingestion of opsonized bacteria, activate CR for phagocytosis, or increase FcR-mediated phagocytosis (data not shown). Differential regulation of the phagocytic capacity of monocyte and PMN CR and FcR also has been observed upon stimulation with PMA (23, 25) or soluble fibronectin (28, 29). Thus, PMA activated PMN CR for phagocytosis (25), but not monocyte CR (23). Likewise, it has been reported that monocytes incubated in suspension with soluble fibronectin were activated to ingest EC3b(i), and FcR-mediated phagocytosis was enhanced (28). However, soluble fibronectin activated PMN CR for phagocytosis only after pretreatment of the cells with a chemotactic factor such as FMLP or C5a (29). Soluble fibronectin enhanced FcR-mediated phagocytosis either after pretreatment of the cells with chemotactic factors (29), or in the presence of catalase (52).

Although adherence of monocytes to collagen gels had a profound effect upon phagocytosis, there was no apparent regulatory effect on the respiratory burst. O_2^- release by plastic- and collagen-adherent monocytes was equivalent in response to PMA and to opsonized zymosan. Adherence of monocytes to collagen vs. plastic also did not effect the amount of O_2^- released by unstimulated cells.

Overall, the data presented suggest that in vivo emigration of monocytes from the peripheral circulation into extravascular sites rich in extracellular matrix proteins may rapidly regulate the ability of monocytes responding to an inflammatory stimulus to phagocytose and kill pathogenic microorganisms. Although current dogma would suggest otherwise (53, 54), it remains to be determined whether monocytes adherent to collagen might phagocytose and kill microorganisms coated only with C3b(i). This possibility currently is under investigation.

The mechanism(s) by which adherence of monocytes to collagen gels activates CR for phagocytosis is unknown. Several potential mechanisms include increasing the number of cell surface receptors, changing the topology of the receptors, or effecting linkage of the receptors to the cellular cytoskeleton. Several studies suggest that increasing the number of CR is not sufficient to activate them for phagocytosis. Thus, treatment of human PMN with PMA (25) or the chemotactic peptide FMLP (55–57) increased the number of cell surface CR, but only treatment with PMA lead to activation of CR for phagocytosis (25). In fact, although FMLP caused an increase in CR number, ligand binding was not increased (57). In addition, PMA, but not FMLP, caused aggregation of PMN CR into distinct clusters on the cell surface, suggesting that clustering is important for enhanced receptor activity (57). However, fibronectin does not increase CR number (28, 58) or effect its cell surface distribution (58) on human Mϕ, but still activates CR for phagocytosis. Thus, neither an increase in CR

number nor a change in their topology is necessarily sufficient to lead to activation for phagocytosis.

The ability of extracellular matrix proteins to enhance ingestion of opsonized particles by monocyte/Mϕ (59, 60) does appear to be dependent on recognition of Arg-Gly-Asp (RGD) sequences within the matrix proteins. The RGD sequence is present in four different locations in the $\alpha2$ chain and two locations in the $\alpha1$ chain of the triple-helical region of type I collagen (61). Thus, recognition of one or more of these RGD sequences by specific receptors on monocytes may be required for enhanced CR and/or FcR function. The human osteosarcoma cell line, MG-63 possesses receptors that recognize RGD sequences in the triple helix conformation of type I collagen, but not in heat-denatured collagen, fibronectin, or vitronectin (62). Such receptors have not yet been identified on human monocytes.

Alternatively, monocyte collagen receptors that do not recognize the RGD sequence in collagen may be involved in regulation of receptor function. The "very late antigens" (VLA) are a receptor family of cell surface glycoproteins that promote cell-matrix adhesion, and belong to the integrin family of adhesion molecules (63). Of the six VLA molecules that have been described, VLA-2 has been shown to be specific for collagen, and VLA-3 has been shown to be specific for collagen, laminin, and fibronectin (64, 65). Of these, low amounts of only VLA-2 have been detected on human monocytes using α-chain specific mAbs. No VLA-2 has been found on human neutrophils (66). Although VLA-2 promotes adhesion to collagen, it does not recognize collagen within the RGD sequence (64). Studies are underway to determine if the regulation of CR and FcR function on collagen-adherent monocytes is via VLA-2, a type I collagen receptor that recognizes the RGD sequence within collagen, or both.

Acknowledgments

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