Intracellular collagen degradation by fibroblasts is an important but poorly understood pathway for the physiological remodeling of mature connective tissues. The objective of this study was to determine whether gingival fibroblasts that express endogenous αβ1 integrin, the collagen receptor, would exhibit the cellular machinery required for phagosomal maturation and collagen degradation. There was a time-dependent increase of collagen bead internalization and a time-dependent decrease of bead-associated αβ1 integrin after initial bead binding. β-Actin and gelsolin associated transiently with beads (0–30 min) followed by LAMP-2 (60–240 min) and cathepsin B (30–240 min). Cytochalasin D prevented phagosome formation and also prevented the sequential fusion of early endosomes with lysosomes. Collagen bead-associated pH was progressively reduced from 7.25 to 5.4, which was contemporaneous with progressive increases in degradation of bead-associated collagen (30–120 min). Concanamycin blocked acidification of phagolysosomes and collagen degradation but not phagosome maturation. Phagosomal acidification was partly dependent on elevated intracellular calcium. These studies demonstrate that the cellular machinery required for intracellular collagen degradation in fibroblasts closely resembles the vacuolar system in macrophages.

Phagocytosis is central to the uptake and degradation of microorganisms as well as damaged or senescent cells and is therefore an essential process in host defense, tissue remodeling, and inflammation. Although "professional" phagocytic cells such as macrophages and neutrophils have been studied in considerable depth (1), several types of nonphagocytic cells such as epithelial cells and fibroblasts can internalize particles and matrix proteins in vivo and in culture. Collagen fibril phagocytosis is thought to be an important pathway for physiological degradation of extracellular matrix in mature connective tissues (2). Uterine, wounded dermal, and periodontal connective tissues exhibit rapid physiological turnover of matrix proteins, processes that are mediated by the intracellular degradation pathway (3). While in inflamed periodontal sites, extracellular matrix metalloproteinases are believed to be responsible for the bulk degradation of connective tissues (4), in normal turnover, fibroblasts are thought to use solely the intracellular vacuolar system for focal proteolysis of collagen (3, 5). However, at present, the vacuolar system that mediates intracellular collagen degradation is incompletely characterized.

In professional phagocytes, the phagocytic process is initiated by binding of particles to receptors on the plasma membrane, an event that subsequently generates a phagocytic signal (6). In fibroblasts, the initial internalization of intracellular collagen degradation is a specific process that is mediated by the adhesive interactions between ligand and collagen receptors (i.e. αβ1 integrins; Ref. 7). However, little is known about the regulation of the downstream events and the kinetics of the intracellular phagocytic pathway following the initial binding of collagen to the cell surface (8).

Following particle internalization in macrophages, the resulting intracellular vacuole (the phagosome) is subsequently transformed into an acidic, hydrolase-rich phagolysosome. The transition from early phagosome to phagolysosome involves the sequential fusion of endosomes and lysosomes with phagosomes (9–11). While there are extensive data on the phagocytic process and the regulation of phagosome formation/maturation in macrophages (6), similar studies in the context of intracellular collagen degradation in fibroblasts are limited. Electron microscopic studies on the digestion of phagocytosed exogenous collagen have shown cross-banded collagen profiles in the phagolysosomes of fibroblasts (12, 13), and there is an apparent requirement for lysosomal serine proteinases for collagen degradation (13, 14). A confounding factor in fibroblasts is that not all nascent collagen is secreted (15–17); a significant fraction is degraded intracellularly in the endoplasmic reticulum, in the Golgi network, and in the endosome/lysosome system, thereby complicating the distinction between exogenous (i.e. phagocytosed) and newly synthesized endogenous collagen.

To begin to address these questions, we have developed, characterized, and validated a model system that uses periodontal fibroblasts in which exogenous collagen binding is dependent on αβ1 integrin expression (7). Biotinylated collagen-coated magnetic beads were used to perform quantitative biochemical analyses of bead-associated proteins on relatively pure populations of phagosomes at different stages of formation/maturation. A major advantage of the new model system is that the bead-bound biotinylated collagen permits study of fusion processes and intracellular degradation of exogenous collagen in fibroblasts and simultaneously enables clear cut discrimination from nascent collagen that is undergoing degradation.
**EXPERIMENTAL PROCEDURES**

**Reagents—**Paramagnetic, carboxylated (1-μm diameter) beads were purchased from Bangs Laboratories Inc. (Fishers, IN). Antibodies to gelsolin (clone GL-1), cytochalasin D, colchicine, 4-μm-diamidino-2-phenylindole, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody, cathepsin B, trypsin, and tetrathymethyl rhodamine isothiocyanate rhodamine were from Sigma. Antibody to degraded collagen was provided by Osteometer (A7 clone; Denmark). Rabbit polyclonal antibody to human cathepsin B was from Vital Products Inc. (St. Louis, MO). Antibody to LAMP-2 (clone H4B4) was from Developmental Studies Hybridoma Bank (Iowa City, IA). Mouse monoclonal anti-human integrin antibodies were obtained to the β1 subunit (clone 4A31 (Transduction Laboratories Inc., Becton-Dickinson); clone 6B4 (Couler); and to the αv subunit (clone P1E6 (Calbiochem), clone 3S3 (Srotec)). Horseradish peroxidase (HRP)-streptavidin, antibody to FITC, low density lipoprotein-1,1-di-octadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (LDL-Dil), nigericin, and acridine orange were from Molecular Probes, Inc. (Eugene, OR). FITC-labeled streptavidin was from Roche Molecular Biochemicals. ImmunoPure sulfosuccinimidyl 6-(bis-oxa- and water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimidine hydrochloride) were from Bio lynx (Brockville, Ontario, Canada; Pierce).

**Bead Preparations—**Biotinylated collagen was prepared by dissolving 10 mg of lyophilized porcine type I collagen in 0.01% HCl overnight in the cold with constant stirring. Thereafter, it was adjusted to pH 8.5. Biotinylated collagen (1 mg/ml) was added to the collagen in two stages and incubated for 20 min at each stage with constant stirring at 4 °C. Biotinylated collagen was coupled to carboxylated magnetic 1-μm-diameter beads using a water-soluble carbodiimidine (1-ethyl-3-(3-dimethylaminopropyl)carbodiimidine hydrochloride. Beads were washed with MES buffer (pH 6.0, 50 mM) three times at room temperature. 1-Ethyl-3-(3-dimethylaminopropyl)hydrochloride (10 mg) was added slowly after bead sonication, and the mixture was incubated for 30 min at room temperature and subsequently washed in phosphate buffer (pH 8.2) before the addition of biotinylated collagen. The reaction was stopped by incubating for 30 min with 10 mM ethanolamine. Washed beads were stored in phosphate-buffered saline containing 0.1% BSA and NaCl.

**Cell Culture—**Human gingival fibroblasts were obtained from biopsies of normal gingiva in patients aged between 10 and 16 years as described (10). These cells constitutively express abundant αβ3, (18), and phagocytosis of exogenous collagen requires this integrin (7, 19). Cells between passages 3 and 12 were used for all experiments.

**Bead Incubation and Phagosome Isolation—**Cells were cooled to 4 °C for 10 min before and after the addition of beads (cell/bead ratio = 1:10) to allow bead binding but prevent internalization. Cells were warmed to 37 °C to allow phagocytosis and were collected at different time points. Cells were washed three times with phosphate-buffered saline to remove any excess unbound beads. Collagen-coated magnetic beads were isolated by cell lysis in homogenization buffer (1% Triton X-100, 50 mM NaCl, 30 mM sucrose, 3 mM MgCl2, 0.5 mM EGTA, 20 μg/ml aprotinin, 1 μg/ml Pefabloc, and 10 mM Pipes (pH 6.8) by passage through a Dounce homogenizer (30 times) over ice and washed (four times) after magnetic separation in a Dynal MPC apparatus.

**Fusion Assay—**Fusion of phagosomes with endosomes/lysosomes was assessed by the addition of HRP-streptavidin (120 mg, 106 cells) to cells for 30 min followed by a 1-h chase in α-MEM after washing four times in α-MEM (no serum). Biotinylated collagen beads were added to cells and incubated for 60, 120, and 240 min. The phagosomes were prepared as described above and dot-blotted onto nitrocellulose paper, and HRP enzymatic activity due to HRP-streptavidin-biotinylated collagen interaction was developed with ECL reagents and quantified by densitometry. To study localization of collagen-coated beads with respect to phagosomes and endosomes/lysosomes, FITC-streptavidin (40 μg/ml) was used instead of HRP-streptavidin as described above.

**Acidification of Phagosomes and Collagen Degradation—**Since fluorescence of FITC is sensitive to pH, this dye was used to monitor the acidicity of intracellular vesicles (20). First, we examined the FITC excitation spectra due to changes in the pH surrounding fluorescent-streptavidin conjugated to biotinylated collagen-coated beads. Calibration of the fluorescence ratio versus pH was performed for each experiment by equilibrating the cells in the presence of the K+/H+-ionophore nigericin (5 μM) at varying pH values. Calibration curves were constructed by plotting the extracellular pH (assumed to be identical to the cytosolic pH under these conditions) against the corresponding fluorescence ratio. We validated the ability of A7 antibody to recognize degraded collagen (21) by incubating native collagen-coated beads with purified cathepsin B in the presence of 1 mM EDTA and 1 mM cysteine for 4 h in phosphate buffer, pH 5.5, at 37 °C. The amount of beads on beads was determined by a Bio-Rad assay. Cathepsin-B-treated and native collagen-coated beads were incubated with A7 antibody followed by secondary antibody (FITC-goat anti-mouse antibody). Beads were assessed by fluorescence microscopy or flow cytometry.

**Electron Microscopy and Flow Cytometry—**Bead internalization was conducted as described above, and fibroblasts were fixed in 1% glutaraldehyde for 1 h. Samples were embedded in Epon 812, and thin sections were placed on nickel grids. Sections were stained with uranyl acetate and lead citrate and observed under an electron microscope (Hitachi-60). Counts of immunogold labeling for LAMP-2 or background counts of immunogold particles were obtained from samples incubated with secondary antibody only or with an irrelevant isotype control.

**RESULTS**

**Human Gingival Fibroblasts Phagocytose Collagen-Coated Beads through the αβ3 Integrin—**Human gingival fibroblasts were presented with collagen-coated beads, and the temporal relationship between bead binding and bead internalization was studied by conducting bead binding at 4 °C with subsequent warming to 37 °C for synchronization. To differentiate between internalized and cell surface-bound beads, collagen-coated beads were incubated with cells and were later stained with antibodies for immunofluorescence localization of collagen. Since cells were neither fixed or permeabilized, viable cells with intact cell membranes excluded antibody staining of collagen beads. This approach used microscopy to distinguish surface-bound (fluorescent) beads from internalized nonfluorescent beads. With these methods, we found a progressive increase in the number of internalized beads per cell over 30 min of incubation (Fig. 1A).

Phagosomal compartments containing the paramagnetic particles were recovered from whole cell homogenates using a magnet (23). This procedure yielded pure preparations of phagosomes and showed little contamination by cell debris or by unbroken whole cells (Fig. 1B). Immunoblotting of bead-associated proteins at 30 min showed no significant lysosome-associated protein (LAMP-2; Fig. 2A), a marker of late endosomes and lysosomes that appeared only at later times following bead incubation. Thus, the phagosome preparations were free of lysosomal membrane contamination at the outset of the experiments.

The association of plasma membrane proteins with beads prepared by magnetic pull-offs was examined by immunohistochemistry with an anti-annexin antibody. There was an initially high level of annexin that decreased to nearly zero by 30 min (Fig. 1C). This observation suggested an early engagement of surface membrane with the particle surface as the pseudopod enveloped the bead, a contention that was consistent with electron microscopic studies at initial binding times demonstrating pseudopod extensions around the beads (Fig. 1D). In gingival fibroblasts, the αβ3 integrin is required for
phagocytosis of collagen-coated beads (7, 19). We studied the specificity of this pathway with collagen and BSA-coated beads and with blocking antibodies to the α5β1 integrin. After 1-h incubations, there were >5-fold higher percentages of cells binding collagen beads than BSA beads (collagen = 29.7 ± 1.5%; BSA = 5.7 ± 0.3%). Pretreatment with blocking antibodies showed a 10-fold reduction of binding with collagen beads but only a small reduction with BSA beads (collagen plus antibody = 2.9 ± 1.7%; p < 0.01; BSA plus antibody = 4.7 ± 0.4%; p > 0.1). Immunostaining for surface α5 by flow cytometry analyses showed a time-dependent reduction after bead incubation (t1/2 = 53 ± 3 fluorescence units; t100 = 25 ± 6 fluorescence units). Immunoblots of bead-associated α5 and β1 integrin subunits showed an initially high level that was reduced to very low levels by 60 min, suggesting that after bead internalization there was a loss (or recycling) of integrins from the bead.

Fig. 1. Bead internalization. A, to distinguish internalized from cell surface-bound beads, collagen-coated beads were incubated with unfixed cells followed by immunostaining for collagen with anti-collagen antibody and FITC-conjugated secondary antibody. Surface-bound (i.e. noninternalized) beads exhibited green fluorescence, while internalized beads were excluded from the antibodies and were nonfluorescent. Total bead counts and FITC-fluorescent bead counts were made by microscopy. The number of non-FITC fluorescent beads per cell (i.e. internalized beads) increased from 0 to 30 min and stabilized between 30 and 60 min. B, phagosomes were prepared as described under “Experimental Procedures.” Paramagnetic beads used for the assays were synthesized from polystyrene microspheres infused with colloidal carbon. Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). 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Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows). Latex beads were used for this assay and observed by electron microscopy. Note the cytoplasmic extensions around the bead (arrows).

Fig. 2. Phagosome-associated proteins. A, phagosomes were collected as described under “Experimental Procedures,” and bead-associated proteins were assessed by immunoblotting. Note the transient increase in actin and gelsolin (an actin-binding and -severing protein). Cathepsin B, an enzyme involved in collagen degradation, starts to appear at 30 min and increases up to 240 min, suggesting that phagosomes and lysosomes begin to fuse at 30 min. Lysosome-associated membrane protein (LAMP-2) is detected from 60 to 240 min. B, immuno- fluorescence staining of 15.8-μm diameter latex beads shows localization of actin (C) and gelsolin (D) around the bead periphery at 10 min.

Protein Composition of Phagosomes during Maturation—Immunoblot analysis of phagosomes at various stages after bead binding showed an initial increase in the amount of β-actin (0–5 min) that declined thereafter by 60 min (Fig. 2A). These increased levels of β-actin were accompanied by a parallel increase in the amount of the actin-binding protein gelsolin. For all analyses of bead-associated proteins, equal amounts of beads were assessed at each time point. Immunolabeling of intact cells with antibodies to β-actin and gelsolin demonstrated that these two proteins colocalized with internalized beads and formed a ring of staining around the bead perimeter (Fig. 2B). Cytochalasin D, a toxin that depolymerizes actin and reduces collagen phagocytosis in vitro (8, 24), prevented the recruitment of actin and gelsolin to beads (data not shown), consistent with an involvement of actin and gelsolin in phagosome formation. We measured the percentage of cells that bound collagen beads in the presence of cytochalasin D (1 μM). There was no significant difference between bead binding in treated cells (23.9 ± 4.2%) and untreated cells (24.8 ± 3.2%; p > 0.2).

Immunoblotting of bead preparations for cathepsin B showed a progressive increase between 30 and 240 min, while the level
of LAMP-2 increased from 60 to 240 min. Thus, the enzymes involved in collagen degradation start to appear in the phagosomes in parallel with the maturation of phagosomes to phagolysosomes. Quantitative analysis of LAMP-2 localization around the periphery of beads by immunoelectron microscopy demonstrated an increase from 26 gold particles/100 μm² after 10 min to 290 gold particles/100 μm² after 120 min. Background counts of gold particles per 100 μm² (no primary antibody) were subtracted in both cases, and these background counts with no primary antibody were very similar to those obtained with an irrelevant isotype control antibody.

**Fusion of Phagosomes with Endosomes/Lysosomes**—We assessed vacuolar membrane proteins associated with internalized collagen-coated beads. Cells were incubated with soluble FITC-streptavidin, washed, and chased followed by incubation with biotinylated-collagen-coated beads. This assay distinguished internalized beads from external beads, since only internalized beads exhibited FITC fluorescence. We used LDL-DiI and LAMP-2 immunostaining to study the spatial association of endosomes with lysosomes. For study of endosome-phagosome fusion, cells were loaded with FITC-streptavidin and biotinylated beads as described above, and internalized beads were distinguished by the same criteria as in A. Fixed and permeabilized cells were immunostained for LAMP-2. Optical sections of cells by confocal microscopy show overall distribution and co-localization of phagolysosomes labeled for LAMP-2 after a 240-min chase. a, pattern of LAMP-2 staining in untreated fibroblasts. b, internalized beads. c, LAMP-2 distribution in a phagocytic cell. d, yellow staining shows discrete sites of co-localization of phagosomes with LAMP-2 in a cell process.

**FIG. 3.** A, colocalization of the endosomal marker (LDL) and internalized beads. a, cells not engaged in phagocytosis were incubated with LDL-DiI (red) for 30 min, chased for 10 min. b and f, cells exposed to FITC-streptavidin for 30 min, chased for 10 min followed by incubation with biotinylated collagen-coated beads for 30 and 90 min, respectively. Only internalized beads appear green because of fusion with FITC-streptavidin. c and g, pattern of LDL staining in a phagocytic cell (same as in b) after 30 and 90 min of chase, respectively. d and h, colocalization of phagocytosed internalized particles and LDL-DiI at 30 and 90 min (yellow particles). B, colocalization of internalized beads and LAMP-2. Cells were loaded with FITC-streptavidin and biotinylated beads as described above, and internalized beads were distinguished by the same criteria as in A. Fixed and permeabilized cells were immunostained for LAMP-2. Optical sections of cells by confocal microscopy show overall distribution and co-localization of phagolysosomes labeled for LAMP-2 after a 240-min chase. a, pattern of LAMP-2 staining in untreated fibroblasts. b, internalized beads. c, LAMP-2 distribution in a phagocytic cell. d, yellow staining shows discrete sites of co-localization of phagosomes with LAMP-2 in a cell process.
Collagen Phagocytosis and Degradation in Fibroblasts

**FIG. 4.** Association of endosomal and lysosomal markers with phagosomes. Cells were incubated with LDL-DiI for 30 min, chased for 10 min with MEM, exposed to FITC-streptavidin for 30 min, and then chased for 10 min with MEM followed by incubation with biotinylated collagen-coated beads. The cells were fixed at 30, 60, 120, and 240 min, and the percentage of beads that colocalized with LDL-DiI was computed. To assess the temporal association of lysosomes with phagosomes, cells were loaded with FITC-streptavidin and beads as described above; fixed at 30, 60, 120, and 240 min; permeabilized; and immunostained for LAMP-2. Internalized beads were identified by FITC fluorescence on the beads as described in Fig. 3 (n = 50 cells for each group; data are means and S.E.).

co-localization of internalized collagen-coated beads with LDL-DiI, which decreased to nearly 0% at 240 min (Fig. 4). Immunostaining for LAMP-2 showed punctate staining in untreated cells (Fig. 3B, a). The spatial distribution of internalized beads (Fig. 3B, b) colocalizing with LAMP-2 immunostaining (Fig. 3B, d) also permitted quantitative analysis of LAMP-2-phagosome association (Fig. 4). There was a progressive increase in the spatial association of LAMP-2 with phagosomes from 30 to 240 min.

We distinguished surface-bound from internalized probe with an anti-FITC antibody to quench extracellular FITC fluorescence. The quenching efficiency of anti-FITC antibody was determined by adding antibody to FITC-streptavidin-loaded cells followed by cell permeabilization and fluorometric quantification. The mean fluorescence intensity of FITC-streptavidin-loaded cells was 37.2 ± 3.6 (n = 10 cells) and 36.1 ± 4.2 (n = 10 cells) after the addition of anti-FITC quenching antibody to unpermeabilized cells. In contrast, the mean fluorescence intensity of permeabilized cells decreased to 15.5 ± 2.3 units (n = 10 cells) after the addition of anti-FITC quenching antibody, indicating that the FITC-streptavidin was indeed internalized. We assayed the extracellular medium from fusion assays with biotin-HRP and found that the amount of exocytosed probe was negligible. Similarly, we studied FITC-streptavidin fluorescence at different time points (0–240 min) and found that lysosomal enzymes did not reduce FITC fluorescence.

Biochemical analysis of the fusion product of endocytosed HRP-streptavidin and phagocytosed biotinylated collagen-coated beads also showed increasing amounts of fusion over 30–240 min (Fig. 5). Since endosome/lysosome trafficking is thought to require cycles of assembly and disassembly of microtubules and actin (25, 26), phagosomal maturation in fibroblasts may also require the coordinated interaction of the actin-based and tubulin-based cytoskeletons. Accordingly, in blasts may also require the coordinated interaction of the actin-based and tubulin-based cytoskeletons. Accordingly, in blasts may also require the coordinated interaction of the actin-based and tubulin-based cytoskeletons. Accordingly, in blasts may also require the coordinated interaction of the actin-based and tubulin-based cytoskeletons.

We assayed the extracellular medium from fusion assays with biotin-HRP-streptavidin and phagocytosed biotinylated collagen-coated beads. The cells were fixed at 30, 60, 120, and 240 min. Internalized beads were identified by FITC fluorescence on the beads as described in Fig. 3 (n = 50 cells for each group; data are means and S.E.).

**FIG. 5.** Biochemical fusion assay for endocytic and phagocytic pathways. HRP-streptavidin (120 mg, 10⁶ cells; endocytic probe) was added to cells for 30 min followed by 1 h of chase in MEM after washing four times in MEM (no serum). Biotinylated collagen beads were added to cells and incubated for 30, 60, 120, and 240 min. Phagosomes/phagolysosomes were prepared as described under “Experimental Procedures” and dot-blotted, and peroxidase activity due to streptavidin-biotin interaction was detected by ECL and quantitated by densitometry. As shown in control, there is increasing fusion of the HRP-streptavidin with the biotinylated collagen beads over time. Pretreatment and the continuous presence of cytochalasin D (1 μM) during the experiment reduce fusion to low levels between 30 and 240 min. To a lesser extent, colchicine (10 μM) inhibited the fusion process.

Collagen Degradation during Phagosome Maturation—We determined whether there was progressive degradation of the native collagen coating the beads with an antibody (A-7) that specifically recognizes degraded (but not native) collagen. To verify that the A-7 antibody recognizes degraded collagen, native collagen-coated beads were treated with cathepsin B (0.5 units) over varying times in the presence of 1 mM EDTA and 1 mM cysteine (in phosphate buffer, pH 5.5, at 37 °C). The beads were fixed and immunostained with A-7 antibody, and the fluorescence intensity was measured with a cooled CCD camera (Pentamax; Princeton Instruments) interfaced to a microscope. There was a gradual increase in fluorescence intensity over time with cathepsin B treatment (Fig. 6A). Since cathepsin B degrades collagen by an initial cleavage of telopeptides to release fibrils (27, 28), our results suggest that the A-7 antibody (21) recognizes an epitope exposed during the initial unfolding of collagen by cathepsin B. Accordingly, we used the A-7 antibody and flow cytometry to examine during phagocytosis the temporal degradation of native collagen coated on the beads. There was a steady increase in the amount of degraded collagen up to 120 min that decreased slightly at 240 min, possibly due to proteolytic epitope degradation (Fig. 6B).

Since nascent collagen can also be degraded intracellularly (15–17), we used a higher resolution confocal microscopy assay that measures only bead-associated collagen degradation in the cells and thus discriminates spatially from potential intracellular nascent collagen degradation elsewhere. Fluorescent collagen-coated beads were incubated with cells for different times and then sorted by flow cytometry on the basis of cells with and without beads. The collected cells were fixed and sedimented to slides using cytocentrifugation, and the presence of degraded collagen associated solely with beads was determined with the A-7 antibody and confocal microscopy. There was a nearly 2-fold increase in bead-associated fluorescence between 10 and 120 min after bead internalization of native collagen-coated beads (Table I).

Biochemical analysis of bead-associated collagen in the pha-
gosomes was detected on blots by probing with HRP-streptavidin followed by development with ECL reagents. We confirmed the integrity of the triple helical structure of biotinylated collagen (0.5 mg/ml) used in our studies by treating with trypsin (0.05 mg/ml; pH 7.5 for 18 h at 37 °C; Fig. 6C, a). Following incubation with cells, the biotinylated collagen attached to the beads showed degradation into smaller fragments, which appear below the α chains, indicating that the collagen was indeed undergoing degradation in the phagolysosome over time (Fig. 6C, lanes c–f). These results are consistent with the collagen degradation mediated by cathepsin B at pH 5.5 (measured with A-7 staining; Fig. 6A) and are similar to previous in vitro studies showing the effect of pH on cathepsin-mediated collagen degradation (27).

Phagosome Acidification Is Required for Collagen Degradation but Not Phagosomal Maturation—Since cathepsin B requires an acidic phagosomal compartment (28, 29) to effect collagen degradation (27), we examined the pH of the bead-associated compartment. To calibrate the intracellular response of FITC-streptavidin-biotinylated collagen beads to changes in pH, we used nigericin-KCl (30), which enabled us to equilibrate the pH in the cell with that of the medium (Fig. 7A). The pH dependence of FITC fluorescence (20) allowed us to measure the intraphagosomal pH of bead-associated collagen.
We established a calibration curve that showed the relationship between excitation fluorescence ratios of 440/490 nm and the pH of the solution surrounding the collagen bead (Fig. 7A). Since not all of the particles that bind to cells become internalized, we verified the intracellular location of beads by abruptly acidifying the extracellular pH (pH 6). This intervention greatly reduced the fluorescence ratio of FITC-labeled extracellular particles (pH 6.0) but had little effect on the fluorescence ratio of intracellular and presumably intraphagosomal particles, which indicated a relatively constant pH, ~5.4–5.6. Conversely, after the addition of NH₄Cl to the medium, the extracellular pH was hardly affected (pH 7.45), while the intracellular bead-associated pH increased to pH 7.1. Of the 50 presumptive phagosomal particles that were studied in these experiments, 45 (90%) were confirmed to be in an intracellular compartment by this method. Results shown in Fig. 7B were determined from beads shown to be internalized. After the addition of labeled beads, the pH of the compartment (phagosome) containing the collagen bead decreased from 7.4 to 5.4 over 120 min, and by 240 min the mean pH had reached 5.6 (Fig. 7B).

We used concanamycin A to prevent vacuolar acidification. From initial experiments with concanamycin A and acridine orange (1 μg/ml), we determined the optimal concentrations and time of incubations required (31). Because of the high protein/volume ratio in the assays, we used 10 mM concanamycin A for 1 h to obtain complete inhibition of phagosomal acidification. After concanamycin treatment, the pH for FITC-labeled extracellular particle-associated pH increased to pH 7.1. Of the 50 particles, which indicated a relatively constant pH, ~5.4–5.6. Conversely, after the addition of NH₄Cl to the medium, the extracellular pH was hardly affected (pH 7.45), while the intracellular bead-associated pH increased to pH 7.1. Of the 50 presumptive phagosomal particles that were studied in these experiments, 45 (90%) were confirmed to be in an intracellular compartment by this method. Results shown in Fig. 7B were determined from beads shown to be internalized. After the addition of labeled beads, the pH of the compartment (phagosome) containing the collagen bead decreased from 7.4 to 5.4 over 120 min, and by 240 min the mean pH had reached 5.6 (Fig. 7B).
greatly reduced fluorescence at intracellular bead sites in the concanamycin-treated cells (Fig. 8, A and B). In comparison, vehicle-treated cells (Me₂SO only) exhibited bright fluorescence staining (Fig. 8, C and D). These findings indicated that inhibition of acidification prevents degradation of collagen. This notion was also confirmed by analyzing bead-associated protein after 240 min in the presence of concanamycin (Fig. 6C). Lane b shows undegraded α, β, and γ collagen chains in phagosome preparations from cells preincubated with concanamycin before bead incubation.

To determine if phagosomal maturation is affected by treatment with concanamycin, we performed phagocytosis assays with FITC-streptavidin and biotinylated collagen beads as described for the fusion assay above. After 240 min of chase, fixed and permeabilized cells were immunostained with cathepsin B antibody. Fig. 9A (c) shows close spatial colocalization of cathepsin B with beads in cells treated with concanamycin. Further, treatment with concanamycin did not effect the ability of cells to internalize beads; there was 24 ± 2% collagen bead phagocytosis in untreated cells compared with 23 ± 3% in concanamycin-treated cells (1 h pretreatment; 60-min bead incubation). Similarly, fusion of phagosomes with endosomes/lysosomes was not affected by concanamycin when used in experiments as described in Fig. 5 (Fig. 9B), suggesting that dissipation of the pH gradient had no effect on the fusion process. Further, treatment with concanamycin did not affect the association of LAMP-2 or the vacuolar enzyme cathepsin B with phagosomes (Fig. 9C).

**Role of Calcium in Phagosomal Acidification**—We assessed the requirement for calcium in phagosome formation and maturation (32). The addition of collagen beads to cells induced a robust increase of \([Ca^{2+}]_{i}\) (Fig. 10A). Depletion of intracellular stores with thapsigargin (5 \(\mu\)M) reduced the \([Ca^{2+}]_{i}\) response to the beads (Fig. 10B). Similar results were seen by preloading cells with the intracellular calcium-chelating agent BAPTA/AM (3 \(\mu\)M; Fig. 10C). In parallel experiments to assess regulation of phagosomal acidification, intracellular calcium

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**Fig. 8.** Acidification and collagen degradation. Concanamycin-treated cells (A and B) and vehicle-treated cells (C and D) were incubated with collagen-coated sulfated UV beads. After 120 min, cells were immunostained with A-7 antibody to show degraded collagen on beads. B and D show identical fields of A and C. A and C show UV fluorescence of beads and 4′,6-diamidino-2-phenylindole-stained nuclei. The circled beads in A and C represent beads that were not internalized and therefore were unstained by A-7. The arrowheads in D show internalized beads that stained with A-7 antibody.

**Fig. 9.** Effect of concanamycin on bead internalization and fusion process. A, cells were pretreated for 1 h with 10 \(\mu\)M concanamycin (conA), incubated with collagen beads for 2 h, fixed, permeabilized, and immunostained for cathepsin B. a, FITC-streptavidin staining of biotinylated beads; b, cathepsin B-immunostained cells; c, colocalization of FITC-streptavidin and cathepsin B. Immunostaining for cathepsin B colocalizing with internalized beads indicates that concanamycin did not affect the maturation of the phagosomes. B, cells were pretreated with concanamycin as described above. HRP-streptavidin (120 mg, 10⁶ cells) was added to cells for 30 min followed by a 1-h chase in MEM after washing four times in MEM (no serum). Biotinylated collagen beads were added to cells and incubated for varying times. Bead-associated proteins were prepared as described under “Experimental Procedures” and dot-blotted, and peroxidase activity due to streptavidin-biotin binding was developed by ECL and quantitated by densitometry. Fusion was not affected by pretreatment and the continuous presence of concanamycin (n = 4). C, immunoblots of bead-associated proteins in cells pretreated with concanamycin.
store depletion or chelation of intracellular Ca\(^{2+}\) prevented phagosomal acidification (Fig. 10D). Jointly, these experiments indicate that \(\alpha_\beta_1\) receptor-mediated phagosomal maturation is partly dependent on elevated [Ca\(^{2+}\)]\(_i\).

**DISCUSSION**

We have developed, characterized, and validated a model system in which biotinylated collagen-coated magnetic beads were used to perform quantitative biochemical analyses of bead-associated proteins on relatively pure populations of phagosomes at different stages of formation and maturation. A major advantage of the new model system is that the bead-bound biotinylated collagen permits simultaneous study of fusion processes and intracellular degradation of exogenous collagen in fibroblasts. This system enables clear cut discrimination of the exogenous collagen from nascent collagen (15) that also undergoes intracellular degradation (16).

Currently there are no data on the protein composition of maturing phagosomes or on the regulation of the lysosomal maturation process that leads to intracellular collagen degradation. Our experiments were conducted so that initial bead binding at 4 °C was followed by subsequent warming to 37 °C, an approach that enhanced the entry of a cohort of internalized beads into the intracellular trafficking pathways. Data from these experiments demonstrated that high levels of collagen receptors and the cell membrane protein annexin were associated with phagocytosed beads at early time points and declined shortly thereafter. These data, the antibody-blocking experiments, and previous findings (7, 8, 19) show that the collagen protein associated with the collagen-coated beads in parallel with actin. This association was contemporaneous with the initial rearrangement of the cell membrane in collagen bead phagocytosis, a process that involved extension and wrapping of cell processes around the bead. The initial bead internalization process is thought to require rearrangements of cortical actin filaments (24), a contention that is supported by Desjardins et al. (11), who showed temporal variations of the amounts of actin and actin-binding proteins associated with maturing phagosomes in macrophages.

The presence of LAMP-2 (a late endocytic and lysosomal marker) in phagosomes was negligible at early stages of bead internalization but quickly increased to maximal levels from 60 to 240 min. These results are similar to previous observations in macrophages (9, 34) showing that LAMP-1 increases in abundance from the transition of endosomes to lysosomes. Notably, the levels of bead-associated cathepsin B increased slowly from 30 to 240 min as would be expected if collagen degradation were to be effected in vacuolar compartments.

**Fusion and Collagen Degradation**—As assessed by bead-associated HRP activity or FITC fluorescence, vesicle fusion was increased from 30 to 240 min. These data indicate that when phagosomes form, they begin fusing with early endosomes. In the endosome maturation model (35, 36), early endosome-endosome exchange is proposed to lead to formation of lysosomal compartments. Similarly in fibroblasts, fusion of phagosomes appears to occur at later stages of endosome maturation. Indeed, maximal fusion between phagosomes and ly-
sosomes occurred after 120 min of bead internalization, since the lysosomal protein LAMP-2 was most abundant on beads from 60 to 240 min.

In fibroblasts, the digestion of internalized fibrillar collagen depends to a large extent on the activity of cysteine proteinases (e.g. cathepsin B; Ref. 3). Our data demonstrate association of cathepsin B with the collagen bead-containing phagosomes starting at 30 min and steadily increasing up to a maximum at 240 min. Since the exo- and endopeptidase activities of cathepsin B are dependent on an optimal pH (27), we measured the pH of the collagen bead compartment. Our data illustrate that there is a substantial increase in the hydrogen ion concentration in collagen bead-containing phagosomes over time. We used concanamycin A, a relatively specific inhibitor of the V-ATPase (31), to reduce formation of a pH gradient. In our system, inhibiting acidification reduced collagen degradation but did not affect fusion events leading to phagosomal maturation. This is in contrast to results showing that the V-ATPase inhibitor bafilomycin A1 affects transit from early to late endosomes (39) and late endosomes to lysosomes (40) and suggests that phagosomal maturation is a distinct pathway. Experiments in which depletion of intracellular calcium stores reduced phagosomal acidification in fibroblasts indicate that processes that lead to acidification of phagosomes are, at least in part, calcium-dependent. In this context, there have been conflicting reports on the requirement of calcium transients in neutrophils (41) and macrophages (42) for phagocytic function.

Perturbation of intracellular digestion of collagen is crucial in the pathogenesis of a wide variety of drug-induced fibrotic lesions of connective tissues (42). To characterize the basic regulatory processes in the collagen degradative pathway of fibroblasts.

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REFERENCES
