Collagenase from corneal cell cultures and its modulation by phagocytosis

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The uptake of latex by fibroblasts in confluent primary culture results in the secretion of collagenase at a linear rate for a prolonged period. Phagocytosis might therefore constitute an important level of collagenase regulation in corneal ulceration. The collagenase in cell cultures is present in a latent form (40,000 MW) like that obtained from organ cultures of ulcerating corneas and can be activated proteolytically. Production of the latent collagenase in cell culture depends upon the presence of serum and diminishes greatly when serum is removed from the medium. Collagenase activity can be demonstrated after the latent collagenase has been separated from serum antiproteases in the media. Alternatively, careful titration of the crude media with trypsin to saturate serum antiproteases, to release collagenase from the complex with α2-macroglobulin, and to activate latent collagenase also results in measurable collagenase activity. The collagenase that is secreted cleaves fibrillar type I collagen and cleaves soluble type I collagen into the typical 5% and 6% length fragments, as demonstrated by SDS-gel electrophoresis and electron microscopy.

Key words: ulceration, cornea, collagenase, phagocytosis, fibroblasts, secretion

Minced ulcerating rabbit corneas consisting of different cell types and extracellular matrix have been used in organ culture in previous studies of the pharmacologic regulation of collagenase and as a source of the enzyme itself. Although such studies have provided important data on enzyme regulation in culture and the cultures have provided enzyme for other studies, organ cultures do not lend themselves to ready quantitative assessment of cell activities and are short-term sources of enzyme. For those reasons, we have set out to develop methods of cell culture that will permit more quantitative studies of the roles of various cell types in the synthesis, secretion, and activation of collagenase and that will also produce high yields of enzyme. The methods that we have developed are based upon the studies of Werb and colleagues. In brief, the continued secretion of collagenase from fibroblasts appears to depend upon serum and phagocytosis.

Materials and methods

Cell dissociation and culture. Corneas were excised from albino rabbits approximately 2 weeks after having been burned with 4N NaOH, at which time most corneas were actively ulcerating. Epithelium, Descemet's membrane, and retrocorneal membrane were removed by scraping.
under a dissecting microscope with a Bard-Parker blade. Eight to 10 stromas were minced and incubated, with stirring, in a water bath at 37°C for 1 to 2 hr in clostridial collagenase, E.C. 3.4.4.19, 4 mg/ml (CLS, Worthington), dissolved in 7.2 ml of mammalian Tyrode’s (TC Tyrode’s solution without calcium and magnesium, Difco) and 0.8 ml of chicken serum (Gibco), and also containing 4.2 mM CaCl<sub>2</sub> and 100 U/ml penicillin and 100 μg/ml streptomycin (penicillin-streptomycin mixture, Gibco). Following incubation, 2 ml of fetal calf serum (FCS, BBL) were added to the cell suspension to inhibit any trypsinlike protease activity in the collagenase preparation, as well as to inhibit any proteases liberated during mincing and cell dissociation. A 10 ml volume of Tyrode’s containing antibiotics was also added; the suspension was gently vortexed and then centrifuged at 6°C at 350 × g for 10 min to pellet the cells. The supernatant was discarded, the pellet was dispersed in 10 ml of Tyrode’s with gentle vortexing, and the cells were again centrifuged. This cycle was repeated once more. The final pellet was taken up in Dulbecco’s Modified Eagle’s Medium/Tyrode’s (D/AMT) (1:1, v/v) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (Fungizone, Squibb); the mixture was vortexed and then triturated by propipet to promote cell dissociation. The resulting cell suspension was pipetted to plastic culture flasks (25 cm<sup>2</sup>, Falcon), FCS was added to a final concentration of 10% by volume, and the cells were allowed to attach and grow out to confluence at 37°C under a gas phase of 5% CO<sub>2</sub>/95% air. After cell attachment, cultures were changed at 1- to 4-day intervals.

**Latex experiments.** Latex (polystyrene latex beads, PLB-8, 0.79 μm average particle size; Sigma) for use in phagocytosis studies was prepared in the following manner.

First, 0.1 ml of suspension was treated with 20 ml of a sterile solution of penicillin (200 U/ml) and streptomycin (200 μg/ml) for sterilization, followed by centrifugation at 6°C at 200 × g for 1 hr to pellet the latex. The supernatant was discarded, the latex adherent to the sides of the glass centrifuge tube (in the presence of salt) was scraped down with a rubber policeman, and the latex was resuspended in 20 ml of sterile H<sub>2</sub>O and centrifuged for 1 hr. After discarding of the supernatant (all the latex is pelleted in the absence of salt), the latex was resuspended in 10 ml of sterile D/AMT by vortexing and trituration by propipet. The resulting latex suspension was distributed in 2.5 ml aliquots to culture flasks, after which first 2 ml of D/AMT and then 0.5 ml of FCS were added per flask to present cells with latex in D/AMT plus 10% FCS. Incubation in latex was allowed to proceed undisturbed for 2 days, after which the medium was exchanged for fresh medium, with or without 10% FCS.

**Characterization of collagenase.** Trypsin-treated cell culture medium protein was incubated with soluble collagen, after which collagen degradation fragments were characterized by electrophoresis in 10% acrylamide gel containing 0.1% sodium dodecylsulfate (SDS) or by segment long-spacing (SLS) crystallites, as described previously.

**Collagenase assays.** Assays of collagenase activity in cell culture media and in column effluents were made in triplicate in a capillary “heat gel” system containing calf tendon collagen as substrate. Data are expressed for cell cultures as "cu-
Fig. 2. Reversal of collagenase inhibition and activation of latent collagenase in the presence of 10% FCS. A preparation of collagenase from organ culture containing both active and latent enzyme was incubated with 10% FCS to inhibit completely the active enzyme. Subsequently, trypsin was added to aliquots of the mixture, followed by soybean trypsin inhibitor, and collagenase activity was determined. One-hundred percent control is equivalent to the active collagenase in the uninhibited preparation. Trypsin treatment (140 to 300 μg/ml) allowed recovery of activity equivalent to the original level of active collagenase as well as activity equivalent to that resulting from the activation of latent collagenase.

Cumulative collagenase activity," in millimeters of lysis per hour per milliliter of medium. Data for column effluents are expressed as millimeters of lysis per hour. One millimeter of lysis per hour in the capillary gel assay corresponds to 0.15 μg of collagen hydrolyzed per minute at 37° C for semipurified corneal collagenase (Bio-Gel P-150). Collagenase profiles from both assays have been published previously.13

As discussed in the text, culture medium containing serum protein was treated with sufficient trypsin (T, 2× recrystallized, Nutritional Biochemical Corp.) to saturate serum antiproteases, to release collagenase from complexes with antiproteases,12, 13 and to activate latent (40,000 molecular weight (MW)) collagenase.13 A standard activation mixture contained 20 μl of dialyzed culture harvest and 10 μl of 280 μg/ml trypsin. After 15 min at 20° C, 10 μl of 2.8 mg/ml soybean trypsin inhibitor (STI, 5× crystallized, ICN) was added to inhibit any free trypsin; collagenase determinations were then made in the capillary gel system.

Cell culture media without serum and column effluent fractions were assayed in buffer alone ("active (manifest) collagenase") and after having been activated by trypsin (10 μg/ml) for 15 min at 20° C ("total collagenase"); after this, STI (112 μg/ml, final concentration) was added to inhibit trypsic activity, and collagenase determinations were made. As reported previously,13 latent collagenase activity is the difference between total collagenase and active collagenase in crude media without serum or in column effluent fractions. Trypsin itself, under the conditions used, does not solubilize the gel substrate, and STI does not affect collagenase activity.

Chromatography of collagenase from culture and molecular weight determinations. Cell culture media were dialyzed against buffer containing 0.025M Tris-HCl, 0.005M CaCl₂, and 0.1M NaCl, pH 7.9 (6°C), lyophilized, and redissolved in distilled, deionized H₂O. Subsequently media protein was chromatographed directly upon Bio-Gel P-150 (see below) or was first treated with trypsin at the same ratio of trypsin/serum protein as was used to activate harvests for measurement of collagenase activity. Activation with trypsin was followed by treatment with STI to inhibit free trypsin activity. Chromatography was performed on Bio-Gel P-150 (100 to 200 mesh), bed volume 170 ml. Elution was with buffer containing 0.05M Tris-HCl, 0.005M CaCl₂, and 0.1M NaCl, pH 7.9 (6° C), at a flow rate of 2.5 ml/hr. Effluent fractions of 1.0 ml were collected, protein concentration was estimated by absorbance at 280 nm; effluent fractions were lyophilized, redissolved in H₂O, treated with trypsin or buffer, and assayed for collagenase activity.

The apparent MWs of "latent" and "active" collagenase were estimated from their elution positions from the P-150 column calibrated with the molecular weight markers blue dextran (average MW 2,000,000; Sigma), egg albumin (45,000, 5x crystallized, chicken; Pentex), and myoglobin (17,800; 2x crystallized, horse; Pentex).

Results

Preliminary experiments (data not shown) demonstrated that stromal dissociation with clostridial collagenase produced the same end-point of cell separation as did dissociation by Coon's mixture,14 which also contains trypsin. Moreover, cells obtained by collagenase alone showed higher plating efficiency and denser growth at apparent confluence.
than cells obtained by using Coon's mixture. Data reported in this paper therefore are from primary cultures obtained by using clostridial collagenase as the dissociating enzyme.

In initial studies, no active collagenase was detected in harvests containing 10% FCS from cultures of fibroblasts (keratocytes), with or without identifiable islands of epithelium. This result was not surprising, since serum is known to contain inhibitors of collagenase, especially α-macroglobulins. Indeed, incubation of active collagenase from organ culture with FCS demonstrated that 10% FCS and even 1.25% FCS was completely inhibitory. Prior acid treatment (to pH 2.5 and back to 7.5) of the FCS, which is reported to destroy the functional activity of the antiprotease, human α₂-macroglobulin (α₂-m), did reduce the collagenase inhibitory activity of the serum but only at concentrations below 10%. Thus either the α₂-m in FCS is acid stable, with regard to collagenase inhibition, or other acid-stable collagenase inhibitors are present in the serum.

On the basis of previous organ culture and serum inhibition studies, it was expected that collagenase was present in the medium as the latent 40,000 MW enzyme as well as in complex with α₂-m. Thus, to detect collagenase directly in serum-containing medium, it was necessary to treat the medium with a protease that would saturate free α₂-m, would cleave collagenase/antiprotease complexes to release collagenase, and would also activate latent collagenase. We have shown previously that trypsin activates the latent 40,000 MW enzyme; it is known, from...
the work of others, that trypsin releases collagenase from α2-m, presumably by degrading the α2-m and not simply by exchange of protease. These events are shown diagrammatically in Fig. 1.

To determine the conditions for measuring collagenase in serum-containing medium, a corneal collagenase preparation from organ culture containing both active and latent collagenase (activities of 0.283 and 0.142 mm lysis/hr, respectively) was first inhibited completely by incubation for 30 min at 20° C in 10% FCS. Aliquots of trypsin of increasing concentration were then incubated with aliquots of the FCS-collagenase mixture for 30 min at 20° C. The trypsin incubations were followed by incubations for 15 min at 20° C with added STI at a ratio of STI/ trypsin of 10:1 (w/w) to inactivate any excess trypsin. Collagenase activity was then measured (Fig. 2). With this procedure, 70 μg/ml trypsin were found to be able to saturate or degrade serum inhibitors and to cause the recovery of activity equivalent to that of the original active collagenase; 140 to 300 μg/ml trypsin were able to cause recovery of activity equivalent to the latent collagenase as well as the active collagenase in the mixture (Fig. 2). Since reversal by trypsin of collagenase inhibition and activation of latent collagenase (40,000 MW form) were not determined independently, it is not possible to know the
Fig. 6. The effects of latex and frequency of medium change on the production of collagenase in cell culture. Cultures A1, A2, A3, and A4 were initiated as replica cultures at the same time from the same suspension of fibroblasts from alkali-burned corneas. Latex caused collagenase secretion to continue at a linear rate in cultures (A1, A2) whose media were changed at 2- and 4-day intervals. In cultures without latex (A3, A4) enzyme production decreased over the corresponding 4-day interval. Changing the media at daily intervals increased the rate of enzyme secretion in culture with latex (A1, A2) as well as in cultures without latex (A3, A4).

contribution of each process to the collagenase activity observed after treatment with a given level of trypsin.

Having established the conditions for the complete recovery of once-active collagenase and activation of latent collagenase in the presence of 10% FCS, we treated harvests of cell culture media with trypsin (Fig. 3). After such treatment, but not without it, collagenase activity was demonstrable in the dialyzed culture media containing the 10% FCS (Fig. 3). When expressed as cumulative collagenase activity, it is seen (Fig. 3) that collagenase is secreted at a constant rate for about 2½ weeks after confluence. Significantly (see below), after that time collagenase was not detectable in later harvests of serum-containing media that were trypsin-treated.

Effect of phagocytosis on collagenase secretion. Following the reports by Werb and colleagues that phagocytic stimuli increase the rate of collagenase secretion by rabbit synovial fibroblasts, we have investigated the role of phagocytosis in collagenase secretion from corneal cells. In our studies, the uptake of latex granules, as phagocytosable material, has been found not to enhance the secretory rate of collagenase compared to cultures with serum but without latex when secretion is at a linear rate but found only to prolong the time during which secretion occurs at a linear rate. Thus, as shown in Fig. 3, a culture initiated from a different suspension of cells was given latex and secreted collagenase at the same linear rate as the previous culture without latex, except that secretion continued for 25 days, at which time the culture was terminated. These initial results suggested that latex prolonged the period of linear secretion of enzyme. Significantly (Fig. 3), a culture exposed to latex and then switched to serum-free medium did produce both active and latent collagenase but at much lower rates than the production of collagenase in the presence of serum, with or without latex.

Normal corneal fibroblasts, with no history of alkali burn, also respond to phagocytosed latex, as demonstrated in Fig. 4. After ingestion of latex, collagenase was secreted at the same linear rate as in cultures of fibroblasts from ulcerating corneas. In this study, no measurements were made of collagenase in
Fig. 7. The effects of repeated exposure to latex on the secretion of collagenase in culture. Cultures A₁, A₂, A₃, and A₄ were initiated as replica cultures at the same time from the same suspension of fibroblasts from alkali-burned corneas. Giving latex to cultures A₁ and A₂ at a time when their rates of collagenase secretion were decreasing caused them to resume their initial linear secretory rates. A₁ subsequently went into plateau, was activated by latex treatment, and went into plateau again but could be reactivated by another latex treatment. A₂, on the other hand, has continued to produce collagenase, without plateau, after a single reactivation with latex. Giving latex to A₃ and A₄, after they had gone into plateau, caused them also to resume their original secretory rates, Culture A₃ again went into plateau, could be reactivated by added latex even 2 months later, but subsequently went into plateau again. Culture A₄ has continued secreting collagenase at a linear rate for over 3 months after a single activation by latex. Letter and number and subscripts and arrows indicate when 2-day exposures to latex were initiated.

cultures of normal fibroblasts immediately after confluence. Therefore we do not know whether such fibroblasts had secreted collagenase but had entered plateau prior to treatment with latex or if they had secreted collagenase at any time prior to treatment with latex.

The apparent uptake of latex by ulcer or normal fibroblasts can be seen in phase micrographs (Fig. 5, A) in which the cytoplasm is quite dark due to the refractile latex granules. The intracellular location of the latex is more obvious in a higher power micrograph (Fig. 5, B). That latex is, indeed, taken up internally is confirmed by an electron micrograph which shows two membrane-enclosed granules near the nucleus (Fig. 5, C).
Fig. 8. Characterization of collagenase from cell cultures containing serum and latex. A, SDS-gel electrophoresis. Trypsin-activated media have cleaved intact collagen to %-length dimers ($\beta^\alpha$) and %-length $\alpha$ chains ($\alpha_1^\beta$, $\alpha_2^\beta$) in addition to $\frac{1}{2}$-length, $\alpha_1^\beta$ and $\alpha_2^\beta$ fragments. $\beta$ = intact dimers, $\alpha$ = intact $\alpha$ chains. Patterns 1, 2, 3, and 4 are from successive harvests of culture medium. B, SLS crystallites. The reaction products demonstrate that trypsin-activated collagenase from cell culture has cleaved tropocollagen $\frac{1}{4}$ the distance from the carboxyl terminus of the molecule. $A$ = amino terminus, $B$ = carboxyl terminus. Arrow is at site of cleavage.

Additional studies indicated that the frequency of medium changes as well as phagocytosis influenced the secretory rate of collagenase. Thus (Fig. 6) giving latex to cultures A$_1$ and A$_2$, which were secreting enzyme at a linear rate, caused the secretion to remain linear as compared to diminished secretory rates in two cultures, A$_3$ and A$_4$, which did not receive latex. Increasing the frequency of medium changes to daily affected the secretory rates in the cultures with latex (A$_1$, A$_2$) as well as the rates in cultures without latex (A$_3$, A$_4$). In such a last-named culture (A$_2$), however, the increased frequency of changes did not prevent the culture from eventually ceasing to secrete enzyme.

After determination of the conditions that permit linear secretion of collagenase, further studies demonstrated that cultures with serum alone could be caused to resume their original secretory rate if latex was given at a time when collagenase secretion was just beginning to diminish (Fig. 7). Thus post-confluence cultures A$_1$, A$_2$, A$_3$, and A$_4$ produced collagenase at a linear rate for about 1 week, after which the rate decreased. Giving latex to A$_1$ and A$_2$ caused them to resume their linear rate while A$_3$ and A$_4$, without latex, went into plateau. Giving latex to A$_3$ subsequently caused renewed secretion for several weeks before the collagenase secretion again ceased. Even so, A$_5$ could be reactivated by latex treatment 2 months later to cause collagenase secretion. Some cultures reactivated once by latex, e.g., A$_4$, have continued to secrete collagenase at a linear rate for more than 3 months.

**Characterization of collagenase.** That the collagenolytic activity produced in cell culture containing serum and latex is due to tissue collagenase was confirmed by examination of the reaction products with soluble collagen. After SDS-gel electrophoresis (Fig. 8, A) collagen is seen to have been cleaved to the typical $\frac{3}{4}$-, $\frac{1}{2}$-length fragments as compared to the intact, control collagen.$^9$ $^{16}$ $^{19}$ As
Fig. 9. Chromatography of collagenase from cell cultures containing latex and serum. Cultures A, B, C, and D were initiated as replica cultures at the same time from the same suspension of fibroblasts from alkali-burned corneas. A, The difference in collagenase activities between total collagenase (●) and active collagenase (○) indicates that most collagenase eluted as a latent form of 40,000 MW. The small peak of active enzyme (○) at the 40,000 position is thought to represent collagenase that has "autoactivated" or has been activated after separation from serum inhibitors. Collagenase was also detected in the void volume (V₀) after treating fractions (in brackets) with 100 or 1,000 μg/ml trypsin. This collagenase is thought to have been released from α₂-m. B, Treatment of cell culture media with trypsin before chromatography resulted in collagenase of mostly 23,000 MW. A small peak of collagenase also eluted at the 40,000 MW position.

seen also in an electron micrograph (Fig. 8, B) of SLS crystallites, the collagenase from cell culture has cleaved collagen at the typical site ¼ the distance from the carboxyl end of the molecule.⁹, ¹⁶, ¹⁹

Chromatography of collagenase from cell culture. Chromatography upon Bio-Gel P-150 (Fig. 9, A) demonstrated that collagenase from cell cultures containing latex and serum is, indeed, present in the latent, trypsin-activatable 40,000 MW form obtained previously from organ cultures of ulcerating rabbit corneas.¹¹ A small peak of 40,000 MW collagenase that was also detected (Fig. 9, B) is thought to represent enzyme that has "autoactivated" or has been activated after chromatographic separation from serum inhibitors. In addition, collagenase could also be recovered by treatment of the α₂-m-containing void volume fractions with high levels of trypsin (Fig. 9, A). This observation suggests that some latent collagenase is activated (or activates) in culture but that, as expected, it is complexed by the α₂-m.¹⁶

Trypsin activation of crude culture medium, under the same conditions used (Fig. 3) to activate collagenase in harvests for measurement of total activity, resulted in a distribution of collagenase (Fig. 9, B) that elutes almost entirely as the 23,000 MW species obtained previously by trypsin treatment of media from organ cultures of ulcerating rabbit corneas.¹¹ For reasons not understood, however, media pretreated with trypsin demonstrated the recovery of much more total collagenase in the effluent (45% recovery) than equal aliquots of media whose effluent fractions were treated with trypsin after chromatography (14% recovery).
Discussion

Organ cultures of minced, ulcerating rabbit corneas have been used to study the regulation of secretion of collagenase and as a source of collagenase for other studies. Such cultures have the virtue of being more representative of the in vivo situation than cultures of a particular cell type in that they contain various cell types that might participate in ulceration and also contain the stromal extracellular matrix. In such cultures also, the degradation of stromal collagen can be related to drug effects on collagenase levels. The fact that organ cultures do contain diverse cell types (epithelium, fibroblasts, polymorphonuclear leukocytes (PMNs), macrophages) makes it difficult, however, to understand important cellular interactions in ulceration and also to interpret the locus of drug effects. Moreover, the presence of the extracellular matrix makes it difficult to obtain cells in the explant for biochemical analyses. For those reasons, cell culture methods have been developed which permit a study of the regulation of the synthesis, secretion, and activation of collagenase.

Although epithelium, PMNs, and fibroblasts have all been considered as sources of collagenase in ulceration, the actual biologic roles of those cell types have not been well understood. The clinical observation that stromal ulceration is correlated with epithelial defects and the observation that explants of epithelium lyse collagen gels suggested that the corneal epithelium is one source of collagenase in corneal ulceration. Epithelium might also release chemotactic factors for PMNs and stimulate fibroblasts to secrete collagenase and cause the activation of latent collagenase derived from fibroblasts.

Stromal ulceration is also associated invariably with PMNs that demonstrate phagolysosomal activity. It is known that PMNs release some hydrolases extracellularly in the course of phagocytosis and PMNs might thus contribute collagenase and possibly also elastase to degrade the extracellular matrix.

Fibroblasts from various sources, including the cornea, have been shown to make collagenase in cell culture. Rabbit synovial fibroblasts and human skin fibroblasts secrete latent collagenase of about 40,000 MW into serum-free medium. The enzymes from synovium and skin can be activated by trypsin and mercurial, in both respects like the latent 40,000 MW collagenase obtained previously from organ cultures of ulcerating rabbit corneas. In the present study, the latent collagenase from cell cultures of corneal fibroblasts was found to be activated by trypsin and plasmin. Activation by mercurial has not yet been attempted. All three enzymes appear to "autoactivate" after chromatographic separation from other components of the culture medium. Also like collagenase from ulcerating corneas in organ culture, trypsin-activated collagenase from cell culture has an apparent MW of 23,000 and appears to be capable of being complexed by $\alpha_2$-m. The collagenase from corneal fibroblasts also cleaves collagen in the fashion typical of tissue collagenase, into $\frac{3}{4}$- and $\frac{1}{2}$-length fragments.

In the current work, fibroblast cell cultures, whether initiated from the same or different suspensions of cells, produced collagenase in the presence of serum at approximately the same initial linear rate after reaching confluence. Typically, also, cultures initiated from the same cell suspension went into plateau at the same time (Fig. 7) but at a different time from cultures initiated from a different cell suspension (Fig. 3). The basis for the variability in the duration of the plateau of the initial linear secretion of collagenase is not as yet understood.

Although attempts were made to remove all corneal epithelium in certain experiments, islands of epithelium containing a few dozen to a few hundred cells were still observed in some cultures. In fact, the mincing and collagenase-dissociating procedures did not separate epithelial cells, and small masses of epithelium could sometimes be seen sticking to the culture plate surface soon after plating. Cells identified by phase microscopy as epithelium were identical in shape and mosaic arrangement to those grown out to
confluence from scrapings of superficial cornea in other studies (data not shown), and islands of such cells could also gradually displace adjacent fibroblasts, as observed by others.30 The fact that the initial secretory rate and the times at which cultures went into plateau were independent of the number of observed epithelial cells and the fact that the same rate of collagenase secretion occurred in cultures with some epithelial cells as in those which contained no detectable epithelial cells argue strongly, however, for fibroblasts being the major source of collagenase in the current studies. These observations, plus the fact that the renewed secretion of collagenase from plateau cultures was correlated with the ingestion of latex by fibroblasts and that the epithelial cells did not ingest latex, also support the interpretation that the fibroblasts are the major source of the enzyme in cell culture.

The continued secretion of collagenase at a high rate appears to depend on the ingestion of particulate material (latex) as well as the presence of serum in the culture medium. Although culture in the presence of serum, without exposure to latex, results in the secretion of enzyme at the same linear rate as after exposure to latex (Fig. 3), prior ingestion of latex appears to prolong the period of linear secretion, and fibroblasts on serum alone that are no longer secreting detectable collagenase can be "reactivated" by giving latex to the cells (Fig. 7). Although cultures that cease to secrete enzyme can be reactivated at least twice, and it is clear that the capacity for secreting enzyme has not been lost during the plateau period (Fig. 7). It would also appear (Fig. 6) that the rate of secretion of collagenase can be increased by more frequent changes of serum-containing medium. Even so, such cultures still eventually cease secreting enzyme.

The mechanism by which latex stimulates the continued secretion of collagenase or activates cells that are not producing the enzyme is not known. Werb and Reynolds7 have reported that the rate of intracellular degradation of endocytosed material is related to the secretory rate of collagenase—cells that take up dextran sulfate, for example, show increased secretory rates until the dextran is degraded. Also, fibroblasts from patients with hereditary storage diseases show abnormally high secretion of collagenase in vitro until "corrective factors" are supplied which cause degradation of the stored materials.34 Thus it would seem that the turnover of phagolysosomes in fibroblasts is "coupled" somehow to the rate of secretion of collagenase. Latex, then, would act to trap cells in a secretory mode. Why it is, however, that cultures that have taken up latex go
eventually into plateau but will respond to subsequent bouts of latex is not understood. It would not seem that latex, the stimulus for collagenase secretion, is simply diluted out by cell replication. Replication in these confluent cultures, if latex-containing cells replicate at all, is probably limited to the replacement of dead cells. During the period of culture and collagenase determination, very few cells with or without latex were observed in the medium, and the latex first given appears to remain intracellular. Subsequent exposure to latex, which results in renewed secretion of collagenase, is correlated with more apparent uptake by the same cells, since the cells look more swollen with latex. Moreover, the rapidity with which cultures go from linear secretion of collagenase to no apparent secretion would not appear compatible with simple diluting out of latex.

It would seem that on the basis of our data, the presence of latex in intracellular vacuoles generates a "signal" that causes prolonged secretion of enzyme. The fact that collagenase secretion does eventually cease, however, even though the latex is still present intracellularly, suggests that the "signal" attenuates and that secretion is not simply coupled to turnover of the vacuolar system.

What is the biologic significance of the observations in the current work? Werb has speculated that the phagocytosis of extracellular matrix might constitute an important regulatory signal for the secretion of collagenase from fibroblasts. Indeed, fibroblasts in biologic situations of rapid remodeling, as in the turnover of the periodontal ligament in rats, have been reported to have phagolysosomes containing collagen. In such situations, there might also be enhanced secretion of collagenase. With regard to corneal ulceration after alkali burns, the apparent morphologic evidence would not support an important role of phagolysosomal activity of fibroblasts in the secretion of collagenase. In timed stages after alkali burns in rabbits, the predominant cell type in the ulcer region is the PMN; fibroblasts are mostly peripheral to the ulcer region and do not show obvious signs of phagolysosomal activity. Yet it should be noted that organ cultures of ulcerating rabbit corneas produce high levels of soluble, latent collagenase, which has the same chromatographic and activation properties as the enzyme detected in the media from cultures of fibroblasts. It would seem, then, that latent collagenase of fibroblast origin could diffuse into the more central stroma where it is activated, possibly involving proteases derived from PMNs and the corneal epithelium. The lack of observation of phagolysosomes in fibroblasts in sections from the ulcerating cornea might only reflect the rapid degradation of intraphagolysosomal material and the short existence of phagolysosomes in the cells. Alternatively, the fact that latex stimulates collagenase secretion by fibroblasts might have nothing to do with real biologic controls. That fibroblasts secrete collagenase without added phagocytic material, however, supports the possibility that they secrete the enzyme in corneal ulceration. Moreover, fibroblasts obtained from collagenase digestion of stromal matrix were seen to contain many phase-lucent vacuoles for a time after plating, which might contain degraded matrix. Perhaps the initial period of collagenase secretion observed prior to the addition of latex represents a response to ingested matrix materials which continues until those materials are completely degraded. Even if the effect of latex does not correspond to biologic "reality," the use of latex to promote enzyme secretion raises the yield of collagenase for other studies.

The cell culture system described in the current work will permit the study of the regulation of synthesis, secretion, and activation of corneal collagenase. It is possible, for example, to treat fibroblast cultures with extracts and media from epithelium and PMN to examine the possible roles of these cells in stimulating fibroblasts to produce collagenase and in activating the latent enzyme produced in culture. In addition, it is possible to test the ability of natural matrix components as endocytosable material, for example, collagen and proteoglycan, to promote the secretion of collagenase from fibroblasts. Moreover, the cell culture system...
should permit the study of the regulation of collagenase secretion by added drugs and by inflammatory mediators, thereby helping us in our attempts to understand the mechanisms of corneal ulceration.

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