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STIMULATION BY ENDOCYTOSIS OF THE SECRETION OF COLLAGENASE AND NEUTRAL PROTEINASE FROM RABBIT SYNOVIAL FIBROBLASTS*

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The catabolism of the macromolecules of connective tissues is largely mediated by enzymes synthesized by the cells within the matrix. It has been suggested that tissue proteinases acting at neutral pH values work synergistically with the lysosomal proteinases in matrix degradation (1), but the precise roles of the two groups of enzymes is unclear (1–4). A likely sequence of events is that the neutral enzymes initiate matrix degradation extracellularly and this is followed by endocytosis and further degradation of macromolecular material within the vacuolar system. In the case of collagen fibrils, an initial extracellular attack by collagenase (4, 5) could be followed by endocytosis of fragments, and digestion by cathepsin B1 within lysosomes (6).

The release of lysosomal enzymes from cells has been related to the endocytosis of materials (7–10), but little information is available on factors controlling the release of the neutral enzymes. We have recently shown that rabbit synovial fibroblasts in culture synthesize and secrete a specific collagenase (4, 11); these cells afford a model system for studying the synthesis and secretion of this neutral enzyme. Moreover, as reported in this paper, the fibroblasts secrete a neutral endopeptidase which may be important in the degradation of the noncollagenous proteins of the extracellular matrix. Consequently we have examined the effects of endocytosis on the secretion of these two neutral enzymes. In this paper we report that ingestion and subsequent intracellular storage of undigested materials such as latex particles greatly stimulates the secretion of the collagenase and neutral proteinase, but has little effect on the release of two lysosomal enzymes, cathepsin D and β-glucuronidase.

Materials and Methods

Materials. Reagents were obtained from the following sources: Dulbecco's modification of Eagle's medium (DMEM)[1] and fetal calf serum, Flow Laboratories Ltd., Irvine, Scotland; polystyrene latex

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[1] *Abbreviations used in this paper:* DMEM, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum.

particles, 1.10 μm in diameter, Micro-Bio Labs. Ltd., London, England; nontoxic India ink, G. T. Gurr, High Wycombe, England; Mycostatin ("Nystatin"), approximately 300 U/mg, E. R. Squibb & Sons, Twickenham, England; Azocoll, Calbiochem Ltd., London, England; methylumbelliferyl-β-glucuronide and methylumbelliferone, Koch-Light Laboratories, Colnbrook, England; and dextran sulfate 500, Pharmacia Fine Chemicals, Inc., London, England. Dextran sulfate 2000 was a gift of Pharmacia, G. B., Ltd., and purified rabbit liver cathepsin D was a gift of Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, England. All other reagents were of laboratory reagent grade.

Methods

CELL CULTURE. Fibroblast lines were established from rabbit synovium and cultured in plastic petri dishes (35-mm diameter, 2–4 × 10^5 cells; 50-mm, 5–10 × 10^5 cells) or glass medical flats (approximately 25 cm^2, 4–8 × 10^5 cells; approximately 100 cm^2, 2–5 × 10^6 cells) in DMEM supplemented with 10% (vol/vol) of fetal calf serum (FCS) and penicillin and streptomycin antibiotics as described previously (4). Cells were grown to confluence before use. For phagocytosis experiments, latex particles were washed in DMEM then resuspended in DMEM plus 10% FCS (usually at 500 μg/ml). After exposure to the latex the cultures were washed twice in DMEM. To study enzyme secretion the fibroblasts were then incubated in DMEM under the conditions described in the text. After incubation the decanted media were centrifuged at 10,000 *g* for 10 min to remove nonadherent cells and debris. Cell lysates were prepared using 0.1% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.5.

Mycostatin powder (initial particle size, about 3 μm) was suspended in sterile water then added to culture medium for phagocytosis. India ink (carbon particles) was sterilized by autoclaving before addition to cultures. Dextran sulfate was dissolved in medium immediately before use and sterilized by membrane filtration. For morphological studies cells were grown on cover slips as previously described (4).

ASSAYS. For assays of collagenase activity [^{14}C]glycine-labeled rat skin collagen was used (4, 12); radioactivity of the collagen was 10,100 dpm/mg. Collagenolytic activity was assayed with the radioactive substrate in the form of reconstituted fibrils (4), but 10 mM $CaCl_2$ was added to the reaction mixtures to ensure optimal enzymic activity. Usually 4-hydroxymercuribenzoate (1 mM) was used to activate the enzymic activity in crude culture medium. 1 U of collagenase activity is defined as the hydrolysis of 1 μg of reconstituted fibrils/min at 35°C (4). Incubations were usually for 18 h, and all assays included both appropriate blanks and trypsin controls to check that the collagen was not denatured.

For analysis of the reaction products of collagenase, assays with collagen in solution at 24°C were made as before (4). The reactions were stopped by the addition of EDTA to a final concentration of 30 mM; then samples were denatured and run on SDS-acrylamide gels (7% T, 0.9% C) using the discontinuous buffer system described by Neville (13), or on urea-acid gels as before (4). The gels were fixed and stained for 1 h at 55°C with Coomassie Brilliant Blue R (0.02%) using a solvent consisting of methanol (50%), glacial acetic acid (20%), and water (30%) by volume, then destained and stored in acetic acid (7%).

Neutral proteinase was assayed with azocoll as substrate. Reaction mixtures (usually 2.4 ml) contained 50 mM Tris-HCl buffer, pH 7.6, 10 mM $CaCl_2$, 200 mM NaCl, and 2 mg of azocoll; the incubations were for 14 h at 37°C with gentle agitation. At the end of the incubation period sodium acetate buffer, pH 5, (2 M; 0.1 ml) was added to stop the reaction and to reduce the interference due to the phenol red in the culture medium samples; the reaction mixtures were centrifuged and the OD_{520} of the supernatant solutions measured. OD_{520} was related to the degree of hydrolysis of azocoll by the use of trypsin standard curves. Activities were expressed as units: 1 U of neutral proteinase hydrolyzes 1 mg of azocoll/h under the experimental conditions. Assays for neutral proteinase were also made using gelatin and azocasein as described previously (4, 14). Cathepsin D activity was measured using [^{3}H]acetyl-hemoglobin as substrate (15), and units of enzymic activity were determined with purified rabbit cathepsin D as a standard.

β-glucuronidase was measured fluorometrically with methylumbelliferyl-β-glucuronide as substrate (15). 1 U of enzymic activity liberates 1 μmol of methylumbelliferone/min at 37°C. Lactate dehydrogenase was determined spectrophotometrically in freshly decanted media by measuring the

reduction of nicotinamide adenine dinucleotide at 25°C (16); 1 U of activity measures the reduction of 1 μmol of cofactor/min. All enzyme assays were performed under conditions giving linear release of products with respect to incubation time and enzyme concentration.

Protein was determined by the method of Lowry et al. (17), with crystalline bovine serum albumin as standard. Latex was measured by the method of Weisman and Korn (18). Results are usually shown as ± SEM, and statistical tests of significance were made with Student's *t*-test.

Results

General Considerations. Five cell strains were grown out from synovia from different rabbits under as near identical conditions as possible. These lines differed in their intrinsic levels of collagenase secretion: two lines had high secretory rates (>2 U of collagenase secreted/24 h/10^6 cells), two had moderate secretory rates (approximately 1 U/24 h/10^6 cells), and one had a low secretory rate (<0.5 U/24 h/10^6 cells). The lines had no obvious differences in rates of proliferation, morphology, or levels of lysosomal enzymes, but cultured cells often have clonal differences in enzyme levels (19).

Latex particles were readily ingested by all the cell strains. When cells were exposed to 500 μg of latex/ml in DMEM plus 10% FCS, ingestion did not plateau even after 48 h. Many of the cells ingested more than 100 particles and more than 99% of the cells were phagocytic after 24 h of exposure. At earlier times there was considerable heterogeneity in phagocytic response in confluent cultures; areas in which the cell density was high were often more phagocytic than areas in which the cells were barely confluent. A phase micrograph of a fibroblast which had ingested latex particles is shown in Fig. 1; contrast Fig. 1 *a*, the phagocytic cell, with a control cell, Fig. 1 *b*. The refractile latex particles assumed an ordered perinuclear distribution after phagocytosis, and electron microscopy showed that each latex particle was surrounded by a unit membrane. The fibroblasts which had ingested latex particles could be maintained in culture for at least 4 wk, although most of the latex-containing cells did not divide. For long-term survival in serum-free medium a recovery period of 24–48 h in serum-containing medium was required after the phagocytic bout. This may reflect a requirement for serum factors for replenishing membrane after phagocytosis (20). After this recovery period the latex-containing cells survived in the absence of serum for up to 2 wk, although the monolayers became easily dislodged from the dishes after about 6 days.

Fibroblast Neutral Proteinase. A neutral endopeptidase which hydrolyzes azocoll was found in the serum-free culture medium from confluent monolayers of rabbit synovial fibroblasts. The proteinase was stable at 37°C for at leat 96 h, because activities measured in media were similar whether measured as the cumulative total for daily medium changes for days, or at the end of a single 4-day incubation period. Media could be frozen and thawed for two cycles without a decrease in enzymic activity.

The activity of the proteinase was stimulated (+51%) by the presence of 4-chloromercuribenzoate (1 mM) in the reaction mixture; it was inhibited by 1,10-phenanthroline, (−90% at 1 mM) by EDTA-trisodium salt (−100% at 10 mM), and by serum (−100% at 2% by volume). Diisopropyl phosphorofluoridate

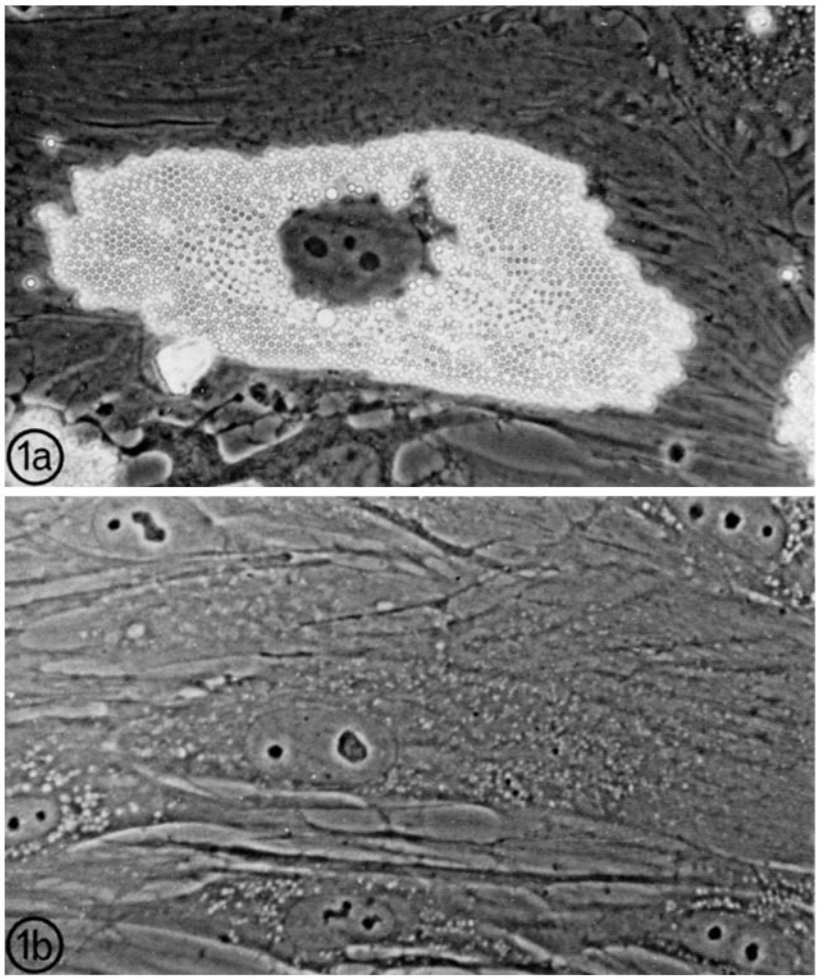

FIG. 1. Phase micrographs of rabbit fibroblasts. (*a*) A fibroblast which has been exposed to latex (500 μg/ml in DMEM plus 10% FCS) for 16 h, followed by a "chase" period of 24 h in DMEM plus 10% FCS. Note the refractile latex particles after phagocytosis. (*b*) A control cell which contains some phase-lucent pinocytic vacuoles. Magnification × 840.

had no effect on enzymic activity. All these characteristics are consistent with the classification of this enzyme as a metal proteinase (21).

The neutral proteinase could be separated from the fibroblast collagenase, also a metal proteinase (4, 14), by ion exchange chromatography. The neutral proteinase was not retained by Biorex-70, a weak cation exchanger, in Tris-acetate buffer (pH 6.5; 50 mM), whereas the collagenase was bound under these conditions and could be eluted by a salt gradient (at about 200 mM NaCl). The neutral proteinase isolated in this way had no collagenolytic activity when tested on reconstituted collagen fibrils. The neutral proteinase also hydrolyzed azocasein and gelatin although azocoll was used for routine assays.

Time-Course of Enzyme Secretion after Ingestion of Latex Particles. The

time-courses of secretion of collagenase and neutral proteinase from a moderately secreting fibroblast line are shown in Fig. 2 A and B. Increased secretion of these enzymes (more than fourfold) into the serum-free culture medium occurred 24 h after the phagocytic bout and continued at the increased rate for the duration of the experiment. Cathepsin D and β-glucuronidase activities in the cell-free medium of these same cultures were measured between the 4th and 9th days in serum-free DMEM. The values found were 0.045 ± 0.003 U/day and 0.022 ± 0.001 U/day for cathepsin D in latex-treated cells and controls, respectively; for β-glucuronidase the enzymic activities were 0.019 ± 0.001 U/day and 0.012 ±

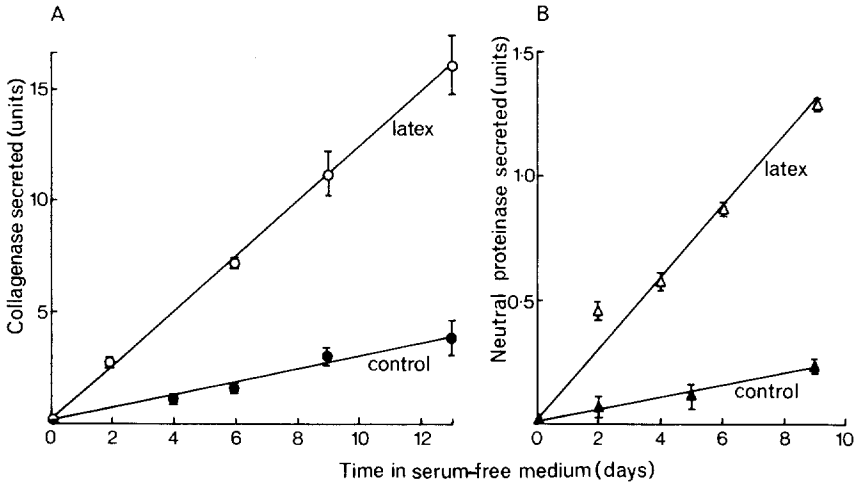

FIG. 2. Time-course of secretion of collagenase (*a*) and neutral proteinase (*b*) from rabbit fibroblasts after ingestion of latex particles. Confluent monolayers of fibroblasts (in 50-mm diameter dishes) were exposed to latex particles (500 μg/ml in DMEM plus 10% FCS) for 20 h. The cells were then cultured in DMEM plus 10% FCS for 24 h, then changed to 2.5 ml of DMEM. Media were changed at intervals as indicated. Values are the mean of determinations on three dishes and error bars represent standard errors. The straight lines were determined by the method of least squares and they were all significant ($P < 0.01$). Closed symbols, controls; and open symbols, latex-filled cells.

0.001 U/day, respectively. These increases were much smaller than those for the two neutral enzymes.

In another experiment a line of fibroblasts with a high intrinsic level of collagenase secretion was used. Fibroblasts were grown to confluence in six glass culture flasks (approximately 5×10^5 cells). Three flasks were exposed to 5 ml of 200 μg latex/ml of DMEM plus 10% FCS for 16 h and the other flasks used as controls; the free latex particles were then removed from the cultures, the cells placed in DMEM plus 10% FCS for 24 h, and the secretion of collagenase into DMEM was then measured at intervals of 4 days for a total of 12 days. For the control cultures, 6.2 ± 0.55, 8.7 ± 1.58, and 10.7 ± 0.78 U of collagenase were found in the media for the consecutive 4-day periods; the average rate of collagenase secretion from the controls was 2.56 U/24 h. The phagocytosing cultures secreted 23.7 ± 2.3, 23.8 ± 4.9, and 18.1 ± 3.3 U of collagenase for the 4-day conditioning periods, giving an average secretory rate of 5.3 U/24 h. In

contrast, the mean values for cathepsin D activity in the media were 80 mU/24 h and 58 mU/24 h for the control and latex-containing cultures, respectively. The higher values for the controls probably were due in part to the increased number of cells (approximately threefold increase over the 12-day period). The collagenase in the medium represented secretion since the amount of enzyme measurable in the cells was very small. At the end of the experiment the control cells contained an average of 0.18 U/culture, and the latex-containing cultures, 0.33 U. These figures represent less than 8% of the collagenase secreted per day into the medium, and were low enough to indicate that reuptake of enzyme from the medium by pinocytosis could contribute significantly to these values.

Comparison of the Cellular and Medium Enzymic Activities. Additional experiments were made to demonstrate that collagenase and neutral proteinase were secreted specifically in response to the ingestion of latex particles by the fibroblasts, whereas lysosomal enzymes were not. As shown in Table I the secretions of the neutral proteinase and collagenase were related to the phagocytosis of latex particles. There were very small intracellular pools of active enzyme for these two neutral enzymes, whereas the intracellular levels of the lysosomal enzymes, cathepsin D and β-glucuronidase, were very large compared to their extracellular release. The low extracellular levels of lactate dehydrogenase indicated that cell death did not contribute significantly to release of the neutral enzymes, but could account for at least part of the small release of lysosomal enzymes into the medium.

The sharp contrast between cell-bound and medium activities for a lysosomal enzyme, cathepsin D, and a neutral enzyme, collagenase, was clearly demonstrated in a further experiment (Table II). Whereas the maximum activity of

TABLE I

*The Effect of Phagocytosis of Latex Particles on Enzyme Release from Rabbit Fibroblasts**

Enzyme	Activity in cells		Activity in medium secreted in 24 h	
	Control	Latex	Control	Latex
Collagenase (U)	<0.07‡	<0.07	0.24 ± 0.04	1.08 ± 0.09
Neutral proteinase (mU)	<3	<6	13 ± 2	89 ± 15
Cathepsin D (mU)	185 ± 9	198 ± 26	22 ± 1	41 ± 7
β-Glucuronidase (mU)	350 ± 50	410 ± 70	12 ± 0.7	19 ± 1.2
Lactate dehydrogenase (U)	0.87 ± 0.27	1.12 ± 0.31	0.06 ± 0.03	0.09 ± 0.02
Protein (μg)	186 ± 21	175 ± 31	ND§	ND

* Triplicate 35-mm dishes containing approximately 2×10^5 cells were used. The cells were exposed to either 200 μg of latex/ml of DMEM plus 10% FCS for 24 h, or serum-medium alone, followed by a recovery period of 24 h in DMEM plus 10% FCS. Serum-free DMEM (1.5 ml) was added to each culture and changed every day for 4 days. Medium activities are expressed as the average release per day. Cellular activities were determined at the end of the experiment.

‡ These are maximum values since the counts released in the assay with radioactive collagen fibrils were less than those for the trypsin controls.

§ ND, not determined.

TABLE II

*Comparison of Cellular and Medium Activities of Collagenase and Cathepsin D after Phagocytosis of Latex**

Exp.	Enzyme	Activity in cells (per 10^6 cells)		Activity in medium (total for days 0–4)
		day 0	day 4	
Control	Collagenase (U)	<0.11‡	<0.15	1.03
	Cathepsin D (U)	0.80	0.71	0.14
Latex	Collagenase (U)	<0.22	<0.16	6.68
	Cathepsin D (U)	0.82	0.84	0.27

* Fibroblasts from a line with a low secretory rate for collagenase were grown to confluence in 35-mm dishes, then exposed to either 500 μg of latex/ml of DMEM plus 10% FCS or DMEM plus 10% FCS alone for 20 h. Triplicate dishes were taken for cell counts and enzymic activity measurements (day 0), and DMEM was added to the remaining cultures with daily changes for 4 days and cellular activities were determined in these cultures (day 4). Values are normalized to 10^6 cells at day 0.

‡ These are maximum values since at no time did release of counts in assay with radioactive collagen fibrils exceed the trypsin blank.

cathepsin D found in the medium of either phagocytosing or control fibroblasts over 4 days was about one-third of the cellular activity, the collagenase activity of the medium was more than 6-fold greater than the cell-bound activity for controls, and 30-fold greater for the cells containing latex.

Characterization of Collagenase. All five lines of synovial fibroblasts showed increases in collagenase secretion after ingestion of latex particles which were qualitatively similar. Continued secretion of collagenase and neutral proteinase at the higher rates after phagocytosis did not require continuous culture in the absence of serum. Cells transferred from DMEM to DMEM plus FCS, then back to DMEM, continued to secrete these proteinases at the stimulated level.

Assays with collagen in solution at 24°C showed that the collagenase in the conditioned media after the ingestion of latex particles gave the same reaction products as the enzyme from unstimulated cells. The reactions were allowed to proceed nearly to completion as shown in Fig. 3. The products represented the cleavage of collagen into two fragments, a one-quarter length "B" fragment arising from the C-terminal portion of the collagen molecule, and the three-quarter length "A" fragment from the N-terminal portion, and were characteristic of a specific collagenase (5).

Dose-Response Relationship between Phagocytosis of Latex and Enzyme Secretion. The amount of collagenase secreted from fibroblasts was related to amount of latex ingested (Fig. 4). In a parallel experiment it was seen that neutral proteinase secretion was also related to the amount of latex ingested (Fig. 5 A), whereas there was little stimulation of the secretion of cathepsin D (Fig. 5 B). These correlations can be more clearly illustrated when the data from Figs. 4 and 5 are replotted to show that the average rate of enzyme release is a function of the amount of latex ingested (Fig. 6).

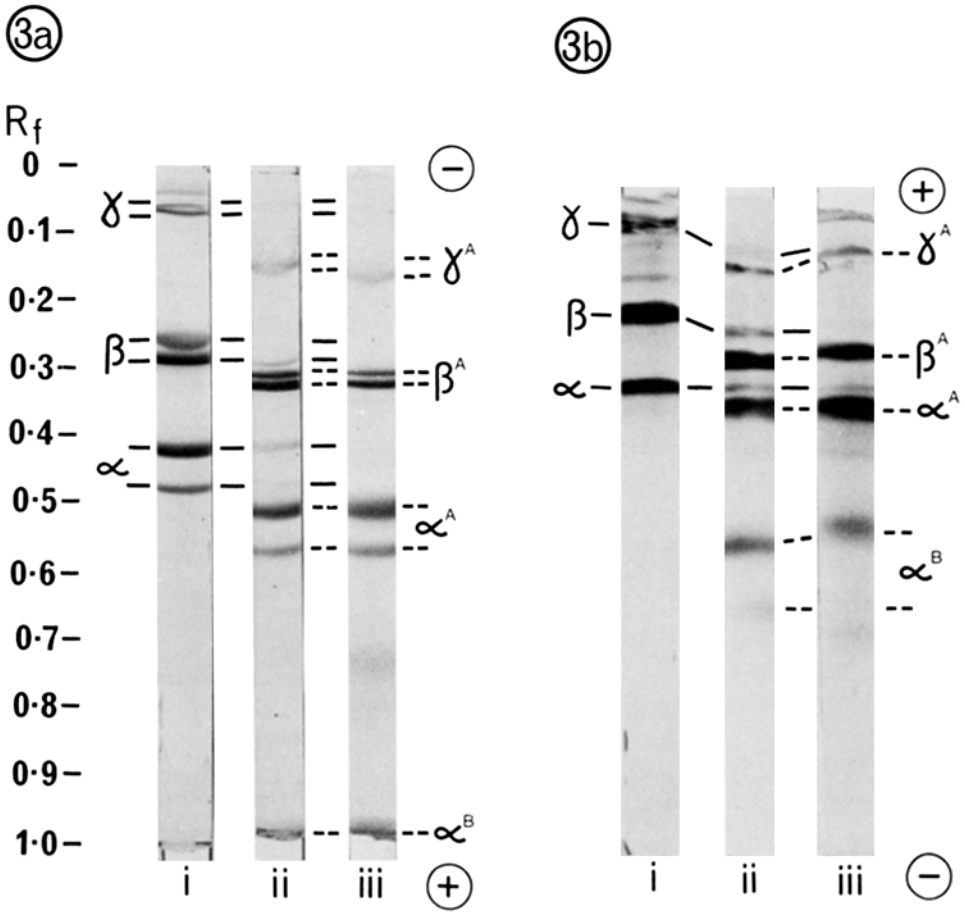

FIG. 3. Gel electrophoretic patterns of the denatured products of reaction of collagenases with collagen in solution at 24°C. (*a*) Products run on SDS gels with migration downwards towards the anode; and (*b*) products on urea-acrylamide gels at pH 3.5, with migration downwards towards the cathode. Doublets from the intact single subunits, α, the cross-linked dimers of the chains, β, and higher-order cross-linked aggregates, γ (4, 5) are degraded by collagenase to specific cleavage products. The three-quarter fragments, β^A and α^A, are seen below the β and α bands, respectively, and the one-quarter fragments, α^B, run near the buffer front. (i) Control collagen, no enzyme; (ii) collagen digested with collagenase from control (nonphagocytosing) cultures; and (iii) collagen digested with collagenase from fibroblasts which had ingested latex particles.

It was possible that the fibroblasts were induced to increase the secretion of neutral enzymes only after a critical number of particles had been ingested. In all the preceding experiments the cells were incubated with latex particles for increasing lengths of time in order to vary the amount of latex ingested: at the short time intervals there was considerable heterogeneity in the number of particles per cell. Accordingly in an additional experiment cultures were exposed to latex particles at concentrations of 100, 250, 500, and 1,000 μg/ml for 24 h with gentle rocking of the cultures at intervals; this resulted in a more even

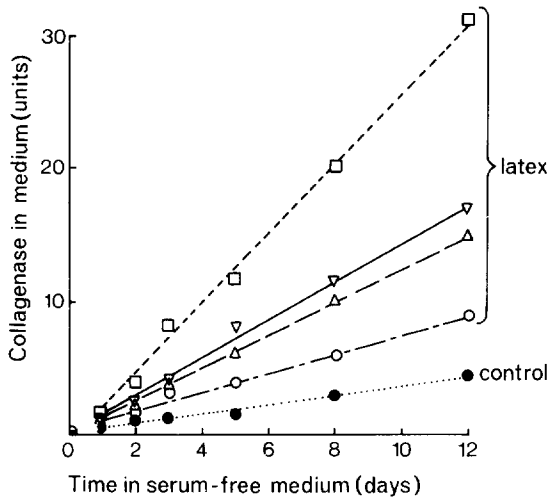

FIG. 4. Dose-response relationship for collagenase secretion after ingestion of various doses of latex. So that cells ingested varying amounts of latex, fibroblast cultures (50-mm dishes) were incubated with 4 ml of 500 μg of latex/ml in DMEM plus 10% FCS for varying time periods. The cells were then placed in DMEM plus 10% FCS for 24 h, followed by serum-free DMEM for up to 12 days. Exposure time to latex and the amount of cell-bound latex at the end of the experiment were: ●, zero time, control no latex; ○, 1 h (0.26 mg of latex culture); △, 6 h (0.71 mg of latex); ▽, 25 h (1.05 mg of latex); and □, 48 h (1.29 mg of latex). The lines were plotted by the method of least squares and were all significant ($P < 0.01$). Error bars are omitted for clarity.

distribution of latex particles among the cells. The collagenase activities secreted into the serum-free media between 2 and 4 days after phagocytosis were respectively 179, 268, 583, and 706% of the nonphagocytosing controls. We therefore conclude that there is probably no threshold effect.

Endocytosis of Other Materials. Increased secretion of collagenase and neutral proteinase was not a unique response to the phagocytosis of latex particles, and a variety of other materials were tested. Pinocytosis of dextran sulfate resulted in the accumulation of a large number of phase-lucent vacuoles in the cytoplasm of the fibroblasts. Increased secretion of collagenase was observed in the conditioned media of these cultures after the endocytic stimulus. Mycostatin, a particulate fungicide, was readily ingested by the fibroblasts. After ingestion the levels of collagenase and neutral proteinase secreted was increased up to 12-fold. Carbon particles ingested by the fibroblasts also increased secretion of collagenase from the fibroblasts. The results obtained with these substances are summarized in Table III.

The question of whether the continued intracellular storage of indigestible materials was required for the secretion of collagenase activity to continue at the stimulated level was examined. In one experiment with cells which had pinocytosed and stored dextran sulfate 500 in their cytoplasm it was observed that the number and size of the phase-lucent vacuoles in the cells began to decrease after about 7 days. During the subsequent 6 days the rate of secretion of collagenase decreased from the stimulated level to the rate seen in the control

cells. Placing these cells in medium containing dextran sulfate for 48 h restored the number and size of the vacuoles and increased the rate of collagenase secretion. The reason for the slow disappearance of the material from the lysosomes could have been due to slow digestion of the dextran, or by desulfation

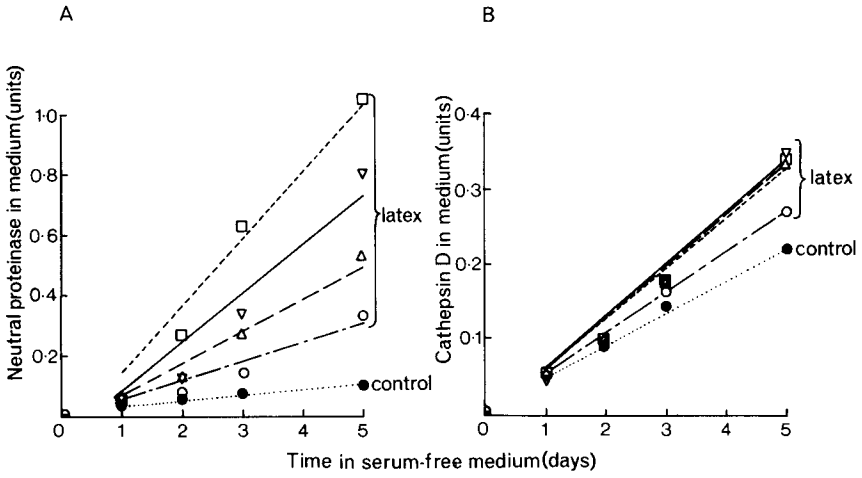

FIG. 5. Neutral proteinase and cathepsin D release after latex ingestion: dose-response relationship. This figure shows data from a parallel experiment to that in Fig. 4. (See legend of Fig. 4 for experimental details and description of symbols). (A) Neutral proteinase. Lines were dotted by the method of least squares and were all significant ($P < 0.01$). (B) Cathepsin D. Straight lines were plotted by the method of least squares and were all significant ($P < 0.01$).

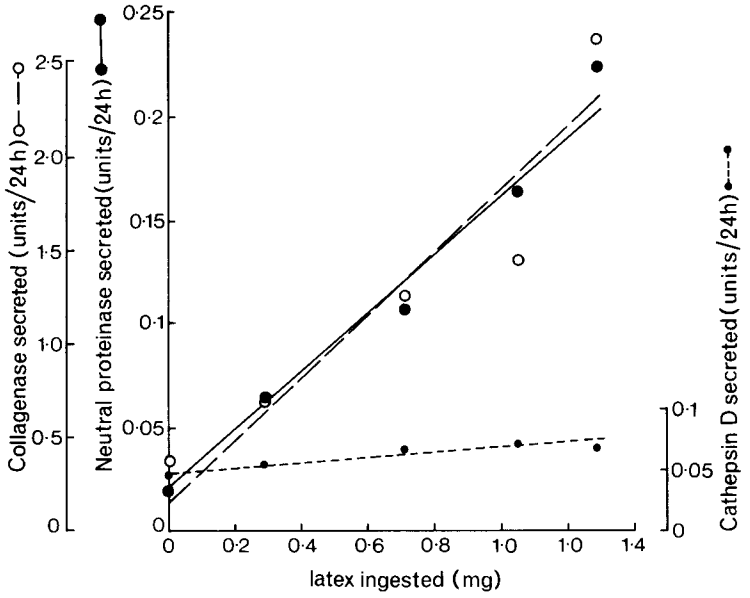

FIG. 6. Rates of enzyme release as a function of latex ingestion by fibroblasts. Rates of release of collagenase, neutral proteinase, and cathepsin D into the culture media were calculated from the slopes of the lines shown in Figs. 4 and 5. Straight lines in this graph were again plotted by the method of least squares and were all significant ($P < 0.025$).

TABLE III

Secretion of Collagenase from Rabbit Fibroblasts after Endocytosis of Various Materials

Exp.	Material ingested	Relative rate of collagenase secretion (treated/control)
1.	Mycostatin	5.5 ± 1.2
2.	Mycostatin	11.8 ± 2.1
3.	Dextran sulfate 500	10.9 ± 1.6
4.	Latex	2.3 ± 0.2
	Mycostatin	4.5 ± 1.1
	Dextran sulfate 2000	2.5 ± 0.4
	Carbon particles	1.7 ± 0.5

Experimental details: Exp. 1. 35-mm dishes containing approximately 160 μg of cell protein were exposed to 200 U of mycostatin/ml for 24 h in DMEM + 10% FCS or medium alone followed by a 24-h period in serum-containing medium. Collagenase secretion was determined daily, for 3 days. Average secretion from mycostatin-treated cells was 0.90 ± 0.23 U/24 h. Exp. 2. Same as exp. 1. Average secretion for cells with ingested mycostatin was 2.11 ± 0.38 U/day. Exp. 3. 5×10^6 cells in 100 cm^2 medical flats were exposed to 200 μg of dextran sulfate 500/ml for 72 h. Collagenase secretion was assessed during a 10-day period in serum-free medium. The average collagenase secretion from the fibroblasts containing the polymer was 3.0 ± 0.35 U/24 h. Exp. 4. 35-mm dishes containing approximately 2×10^5 cells (150 μg of protein) were exposed to: latex (100 μg/ml), 16 h; mycostatin (200 U/ml), 24 h; dextran sulfate 2000 (40 μg/ml), 48 h; and India ink (1:100 dilution), 24 h. Collagenase secretion was assessed daily, for 2 days. Control cultures secreted 0.15 U of collagenase/24 h.

by a lysosomal sulfatase (15). Although possible, it seems unlikely that "regurgitation" of the storage granules took place, since latex particles appeared to remain within the cells indefinitely. In another experiment the fibroblasts were exposed to heat-aggregated bovine gamma globulins (22) at 1 mg/ml for 24 h. Small aggregates were ingested slowly since the fibroblasts lack a specific IgG receptor (23), and these particles could be slowly digested by the cells. Increased secretion of collagenase and neutral proteinase was observed on the 2nd and 3rd days after ingestion, but this returned to control levels at longer times as the protein was digested. These observations suggest that continued presence of material within the secondary lysosomes is required for the continued stimulation of secretion of the two neutral proteinases, but clearly further studies are needed.

Discussion

The turnover of connective tissue macromolecules has been envisaged as a two-stage process (1–3). The initial extracellular phase may be mediated by the release of a group of enzymes acting at neutral pH values and could be followed by endocytosis and further

degradation at acid pH values by lysosomal enzymes. The two classes of enzymes are probably functional under different physiological conditions: the lysosomal enzymes are unlikely to be active in the extracellular milieu, except perhaps in a confined region close to the cell surface where metabolic waste products of the cell could lower the effective pH (24), and the neutral enzymes can have little or no activity at the functional lysosomal pH (4). The nature of the cells which produce all these enzymes has not been elucidated, but fibroblasts, chondrocytes, other connective tissue cells, and mononuclear phagocytes could all be involved (25).

In this paper we present evidence that the ingestion and continued intravacuolar storage of undigested materials increases the secretion of neutral enzymes, namely a collagenase (4) and a neutral metal proteinase, from cells derived from rabbit synovium (11). In contrast with these data there is little change after phagocytosis in the extracellular levels of lysosomal enzymes, such as cathepsin D and β-glucuronidase. Whereas the lysosomal enzymes are concentrated within cells, both of the neutral enzymes are not stored intracellularly to any appreciable extent in an active form, and these data support the putative roles of these two classes of enzymes, intracellularly and extracellularly, respectively.

The specific collagenase described in this report is the same enzyme characterized previously as a secretory product of rabbit fibroblasts (4). The post-phagocytic stimulation of collagenase secretion is due to an increase in the amount of this enzyme. The newly reported neutral proteinase shares many of the characteristics of the endopeptidase released from tadpole tissues along with collagenase (26) and from rheumatoid synovium in culture (27), although the rabbit proteinase does not have appreciable activity on the chromogenic substrate, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg. The neutral proteinase could be important in the degradation of the noncollagenous proteins of the intercellular matrix, because it can hydrolyse proteoglycans.[2] Although the increased activity of the two neutral enzymes might be due to the conversion of precursors to active enzyme, it seems unlikely because the increased levels of secretion after phagocytosis are stable for weeks, and also there is no conclusive evidence that such precursors exist for these enzymes. It is probable that other proteins are secreted at increased levels in response to the phagocytic stimulations but these have yet to be investigated.

The observed phenomena may have general applicability. It has recently been shown that macrophages elicited with thioglycollate broth, which have ingested agar in vivo and stored this material in secondary lysosomes, secrete a neutral serine proteinase which activates plasminogen. As with the collagenase and neutral proteinase from rabbit fibroblasts, this plasminogen activator has only a small intracellular store (28). After ingestion of indigestible materials in vitro, mouse macrophages also secrete a specific collagenase.[3] In inflammatory conditions mononuclear phagocytes may well contribute enzymes to connective tissue turnover.

The finding that a polyanion, dextran sulfate, stimulates the secretion of

[2] Werb, Z., J. J. Reynolds, J. T. Dingle, and A. J. Barrett. Manuscript in preparation.

[3] Werb, Z. Unpublished observations.

collagenase and neutral proteinase may explain the observation that heparin stimulates collagenase secretion from bone explants in culture (29, 30). Proteoglycans are also polyanions and may have analogous effects in vivo.

Since the collagenase and neutral proteinase are secreted in parallel it seems likely that they may be secreted together in a secretory granule. In the polymorphonuclear leukocyte, collagenase is found in the specific or secondary granules (31). This granule does not contain lysosomal enzymes, yet like the primary granules empties either into the phagocytic vacuole, or extracellularly, during phagocytosis (10, 32). The fibroblast and the leukocytes are derived from the same primitive mesenchymal cell and there may be analogies.

It should be emphasized that our observations are not in disagreement with previous reports on the release of lysosomal enzymes. The release of lysosomal enzymes from fibroblasts, macrophages, and polymorphonuclear leukocytes in response to ingestion of immune complexes and other particles, occurs over a time span of minutes to hours after ingestion (8–10), whereas the stimulated secretion of neutral-acting enzymes does not occur until a day or more after phagocytosis and is then stable for days or weeks in the absence of any further phagocytic stimulation.

The mechanism by which the secretion of the nonlysosomal enzymes is related to the ingestion and intralysosomal storage of materials remains obscure. The magnitude of the stimulation is controlled by the amount of material stored, and it may be exerted at the level of the amount of membrane interiorized during ingestion. Certainly the resynthesis of membrane after phagocytosis is related to the amount of membrane interiorized (20), and endocytosis-exocytosis coupling has been demonstrated in a number of cases (33, 34). There may be direct feedback from the lysosome; cytoplasmic structures such as microtubules and microfilaments may be involved, or changes in the cell surface may occur.

In vivo, it is well established that cells which have phagocytosed undigested materials often become the center of inflammatory lesions. In carrageenin granulomas, fibroblasts surround the material and secrete collagenolytic enzymes (35) and in rheumatoid nodules fibroblasts surround the necrotic center (36). During rapid remodelling fibroblasts can contain intralysosomal collagen fibrils (37). In all these cases collagenase, an extracellular enzyme, is implicated. A coupling between endocytosis and the secretion of the neutrally acting enzymes constitutes a mechanism for the chronic and ever increasing destruction of extracellular macromolecules. The observations reported here have direct implications in our understanding of connective tissue disorders. The secretion of enzymes that can degrade connective tissue matrix in response to phagocytic stimulation may contribute to the cycle of events leading to joint destruction in rheumatoid arthritis. Treatment may depend on our ability to inhibit the secretion of these neutral enzymes, whereas attempts to inhibit the activity of enzymes within lysosomes could result in further intracellular storage of undigested materials (38), and could lead to stimulation of secretion of the nonlysosomal enzymes, and further tissue damage. Physiologically, the controlled secretion of these enzymes may mediate tissue turnover and remodelling.

Summary

Rabbit synovial fibroblasts in monolayer culture secrete a specific collagenase and a neutral endopeptidase into their serum-free culture medium. The rate of secretion of these two enzymes is increased after the ingestion and storage of latex particles within the vacuolar system of the cells. The increased rates of secretion of the neutral enzymes are stable for over 2 wk in the absence of a further phagocytic bout. In constrast there is little change in the extracellular levels of two lysosomal hydrolases, cathepsin D and β-glucuronidase. The increase in the secretory rates for the two neutral enzymes is related to the number of latex particles ingested by the cells, and increases of up to 12-fold over the nonphagocytosing cultures were observed. A variety of other materials including mycostatin particles and dextran sulfate also induced increases in the secretion of collagenase. These results are discussed in relation to the turnover of connective tissue matrix macromolecules.

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