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STIMULATION OF ENDOGENOUS PROTEOLYSIS IN MACROPHAGES EXPOSED TO SPORULATING BACTERIA *

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Summary

The relationship between degradation of endogenous macrophage protein and exposure to bacteria at various stages of sporulation was studied. Rabbit pulmonary macrophages were obtained by lavage, attached to plastic tissue culture dishes, and prelabeled with [^{14}C]- or [^{3}H]-phenylalanine for 1 or 20 h, respectively. *Bacillus licheniformis* (ATCC 14580) was grown in Bacto Marine Broth until at least 50% of the bacilli contained refractile spores (19 h), washed and lyophilized. The lyophilized bacilli were washed, counted and applied for 1.5 h to the washed prelabeled macrophages at a ratio of 300 bacteria/macrophage. Rates of degradation of both rapidly and slowly turning over proteins were increased. The effect was not mediated by soluble components of the incubation medium. Free spores, vegetative cells and autoclaved sporulating bacilli did not affect rates of proteolysis. We propose that proteases from the lyophilized sporulating bacilli participate in endogenous macrophage protein degradation following engulfment.

Introduction

Overall protein degradation is known to be regulated by a variety of agents in many organisms and tissues [1,2]. In reticuloendothelial tissues, however, little is known about the regulation of endogenous protein degradation. In rabbit pulmonary alveolar macrophages, phagocytosis of inert latex particles results in slower rates of proteolysis [3]. The effect was shown to be dose-

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dependent and it affected both rapidly and slowly turning over proteins. However, since these effects were obtained using inert particles, we asked whether similar effects would be seen using degradable proteinaceous particles. As examples of degradable particles, lyophilized preparations of various bacteria were presented to attached rabbit alveolar macrophages. We found that bacilli lyophilized during sporulation (with refractile spores observed within sporangia) caused significant increases in rates of macrophage protein degradation.

Methods

Animals. White male New Zealand rabbits were given Purina rabbit chow (Ralston-Purina Co., St. Louis, MO.) and water ad-libitum. Lighting was provided from 7 am to 7 pm daily. The rabbits were killed using 0.45 ml sodium pentobarbital (390 mg/ml).

Harvesting pulmonary macrophages. The chest was opened, the trachea was clamped, and the lungs, heart and thymus were excised and externally rinsed with 0.15 M NaCl. The trachea was cannulated and the lungs were lavaged 10 times at 37°C with 50—60 ml 0.15 M NaCl containing 20 U/ml porcine intestinal heparin. The lavage returns were centrifuged over 2 ml Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) at $400 \times g$ for 10 min. Cells were collected from the interface between the saline of the lavage returns and the more dense Ficoll-Paque. A 50-μl aliquot was mixed with an equal volume of 0.1% Trypan Blue in 0.15 M NaCl and used to fill a hemocytometer. Both cell counting and viability were performed 5 min at 24°C after mixing cells with Trypan Blue. Viability was above 90% in all preparations. Smears, prepared after mixing a sample of cell suspension with an equal volume of 10% bovine serum albumin, and stained with Wright's stain, showed that more than 95% of the cells were pulmonary macrophages.

Incubation of macrophages. Cells were allowed to attach to Falcon 3001 tissue culture dishes (35-mm diameter) during 30 min incubation in 2 ml Krebs-Ringer-bicarbonate [4] containing normal rabbit plasma amino acids [5]/10 mM glucose/100 μg/ml streptomycin and 100 U/ml penicillin (hereinafter called medium A). Approx. 10^6 cells were applied per dish, and incubated at 37°C in an incubator containing a humidified atmosphere of 5% CO_2 in air. No cells were observed by light microscopy in the medium poured from the dishes after 30-min attachment.

Prelabeling of macrophages. After attachment, the medium was replaced with 1 ml medium A containing 10 μCi/ml L-[2,3-^{3}H]phenylalanine. Phenylalanine specific activity in this medium was 0.156 Ci/mol. Incubation was continued for 19 h, and 60 μl neutral solution of 25 μCi/ml, L-[U-^{14}C]phenylalanine were added, bringing the [^{14}C]phenylalanine specific activity to 0.0234 Ci/mol. Incubation was continued for 1 h in the presence of both isotopes. Thus, the cells were labeled for 20 h with [^{3}H]phenylalanine and 1 h with [^{14}C]phenylalanine. In some experiments, cells were labeled with ^{14}C for 1 h only. No difference was noted in the control rates of proteolysis in 1 h prelabeled cells by either method of prelabeling.

Measurement of proteolysis. The method was adapted from that described earlier [3]. Prelabeled cells were washed 4 times by replacing medium A with 1 ml medium A containing 4 mM L-phenylalanine. This washing procedure

served to reduce the free labeled amino acid concentrations to low levels. The medium used for measurement of rates of proteolysis contained 4 mM L-phenylalanine, shown previously to block effectively the incorporation of free labeled phenylalanine into cellular protein [3]. 0.1-ml samples were removed at 0.5, 1 and 1.5 h following gentle but thorough swirling to mix the unstirred layers of incubation medium. Labeled phenylalanine was measured by a modification of a method described earlier for valine [6]. Samples of medium were applied to small columns of Bio-Rad AG 50W-X4 resin (200—400 mesh, H^+ form). After the columns were washed with water, the amino acids were eluted with 5 ml 5 M NH_4OH and dried at 60°C under a stream of nitrogen. The dried residue was dissolved in water and counted in Aquasol (New England Nuclear). The dishes were cooled to 4°C, and the remaining medium was removed. The dishes were washed with three 1-ml portions of cold 0.15 M NaCl. To the remaining medium and washes were added 1.5 ml 20% (w/v) trichloroacetic acid and 0.2 ml carrier protein [3] and left to stand at 4°C for 30 min. The protein was washed with 5% trichloroacetic acid and dissolved in 0.6 ml 1 M NaOH. The tissue culture dishes were treated with 1 ml 5% trichloroacetic acid at 4°C overnight to extract intracellular amino acids [7]. To 0.5 ml extract were added 0.5 ml carrier protein and 1.0 20% trichloroacetic acid, left to stand for 30 min at 4°C, centrifuged, and 1.0 ml was counted in 10 ml Aquasol. Cellular proteins attached to the tissue culture dish were washed with 5% trichloroacetic acid and dissolved in 1 M NaOH. Proteins dissolved in base were counted in 10 ml scintillation fluid (2 l Toluene/1 l Triton-X-100/16.5 g diphenyloxazole, and 333 ml water). This solution was neutralized with 1—2 drops of glacial acetic acid before counting to eliminate chemiluminescence. Thus from each tissue culture dish four types of labeled fractions were obtained: (1) free amino acids from the medium, arising from protein degradation; (2) free amino acids in the cells at the end of incubation; (3) labeled protein in the medium (usually quite small); and (4) labeled protein from the macrophages attached to the tissue culture dishes. These fractions were counted in a Beckman LS-100 liquid scintillation spectrometer in ^3H and ^{14}C energy channels. Counts were corrected for background, quenching, spillover, and volumes of sampling. Released labeled phenylalanine was expressed as a percent of total label present at the beginning of the measurement of proteolysis.

Growth of bacteria. Bacillus licheniformis, ATCC 14580, was grown in 100 ml of Difco Marine Broth at 37°C, with shaking at 250 cycles/min. The optimum time required for batches of bacteria to attain 50% or more cells containing refractile spores was found to be 19 h. Normal vegetative cells were produced by growth in Difco Brain Heart Infusion medium for 24 h at 37°C. Batches consisting entirely of spores were produced by growth for 24 h in Marine Broth at 37°C. Bacteria were harvested by centrifuging, washing with 0.15 M NaCl, resuspending the pellet with 20 vol. 0.15 M NaCl, and lyophilizing. Cells were counted in a Petroff-Hausser chamber (Scientific Products). Sufficient bacteria were added to the incubation medium for $1 \cdot 10^6$ macrophages to produce bacteria: macrophage ratios of 150 : 1, 300 : 1, or 600 : 1. After incubation for 90 min, viability of the macrophages was determined by adding an equal volume of 0.1% (w/v) Trypan Blue solution to the medium, and observing the cells with a Nikon inverted microscope after 5 min at 24°C.

Phenylalanine incorporation into protein. Macrophages attached to tissue culture dishes were incubated with medium A containing 1.55 μCi/ml L-[U-^{14}C]-phenylalanine, and 1 mM unlabeled L-phenylalanine. The specific activity in medium A (1.55 Ci/mol) was thus low enough to avoid dilution of intracellular pools of phenylalanine due to protein degradation [3,6,7]. Samples of incubation medium was prepared and counted as described above for determination of [^{14}C]phenylalanine specific activity. Cellular protein on the tissue culture dishes was treated with 5% trichloroacetic acid dissolved in 1 ml 1 M NaOH, and counted as described above. From 90—150 μg protein were measured per dish [8]. Incorporated counts were corrected for zero time blank, quenching, and volumes of sampling, divided by the specific activity of [^{14}C]phenylalanine in the medium and expressed as pmol phenylalanine incorporated per μg protein/h.

Isotopes and chemicals. L-[U-^{14}C]phenylalanine and L-[2,3-^{3}H]phenylalanine were obtained from Schwarz-Mann, Orangeburg, NY, ^{3}H and ^{14}C toluene standards, Aquasol and 2,5 diphenyloxazole from New England Nuclear, heparin from Organon, West Orange, NJ, streptomycin sulfate and potassium penicillin G from Pfizer, NY, and Ficoll-Paque from Pharmacia Fine Chemicals, Piscataway, NJ. Other reagents were the highest commercial grade obtainable. grade obtainable.

Results

Greatly accelerated rates of protein degradation resulted when prelabeled alveolar macrophages were treated with lyophilized sporulating *B. licheniformis.* For macrophages prelabeled for 1 h, the rate of proteolysis increased by 56% ($P < 0.05$), from 8.2 to 12.8% per h (5 experiments). For the slow turning over proteins labeled for 20 h the relative increase in the rate of proteolysis was even greater: 82% ($P < 0.05$), from 2.7 to 4.9% per h (3 experiments). During exposure to the bacterial preparation the macrophages appeared to be in good condition by phase contrast microscopy. At the end of 90 min exposure, the viability by Trypan Blue exclusion was 89 ± 3% (10 experiments) for treated macrophages compared to 93 ± 2% (10 experiments) for controls (not significant). Incorporation of phenylalanine into cellular protein for 2 h was also measured to indicate the health of the macrophages. No change in the rate of protein synthesis was observed (7.8 ± 1.1 pmol/μg protein/h for cells treated with bacteria, 7.9 ± 0.4 for controls (4 experiments not significant)).

We wished to ascertain whether the stimulation of proteolysis could be due to a general activation of the macrophages and induction of new protein synthesis. However, we found that the effect was retained in the presence of 20 μM cycloheximide, a concentration which inhibited protein synthesis by more than 90% in rabbit alveolar macrophages (results not shown). Thus the stimulation of proteolysis by lyophilized sporulating bacteria occurs even in the absence of new protein synthesis.

The response of proteolysis to increasing amounts of lyophilized sporulating bacilli was assessed in 20-h prelabeled macrophages. See Fig. 1. When the particle to cell ratio was increased from 150 : 1 to 600 : 1, a linear response of the rate of protein degradation was apparent. The highest rate, produced

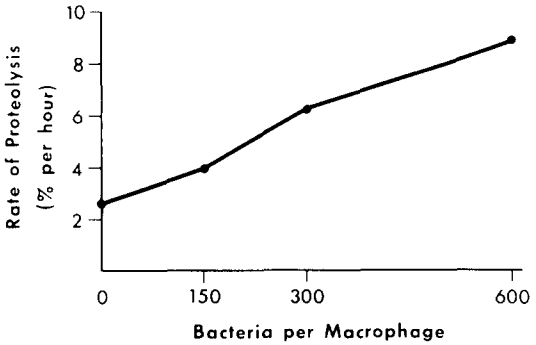

Fig. 1. Dose-response effect of sporulating *B. licheniformis* on proteolysis in macrophages. Prelabeled (20 h) and washed macrophages were exposed to the amounts of bacetrial particles as shown on the abscissa. Protein degradation was then determined, and each point represents the mean of two experiments.

by the 600 : 1 particle to cell ratio, was about 3 times the control rate of proteolysis.

With the above results in hand, we asked whether the lyophilized spores themselves, or normal vegetative *B. licheniformis* organisms might stimulate the rates of protein degradation. Accordingly, 1-h prelabeled macrophages were exposed to lyophilized *B. licheniformis* spores or vegetative cells in the same 300 : 1 particle to cell ratio as for the effective preparations of sporulating bacteria and the results are shown in Table I. The rate of breakdown of rapidly turning over cell proteins was unaffected by these preparations. A portion of the lyophilized sporulating *B. licheniformis* was autoclaved 20 min at 121°C, cooled, washed and resuspended as usual and was also found to be without effect on proteolysis. Only the lyophilized sporulating preparation was effective, also shown for comparison in Table I.

The possibility was considered that a soluble mediator might be released from the macrophages when in contact with the lyophilized bacteria. There-

TABLE I

EFFECT OF SPORES, VEGETATIVE CELLS, AND AUTOCLAVED SPORULATING BACTERIA ON PROTEOLYSIS

Preparations of bacteria were suspended in medium and presented to prelabeled (1 h) washed macrophages at a ratio of 300 bacteria/macrophage. Rates of proteolysis of rapidly turning over proteins were determined at 0.5—1.5 incubation. Control dishes were incubated in parallel, without addition of bacterial preparations. Results are followed by S.E. and the number of experiments in parentheses. Significance was determined by Student's *t*-test. n.s.: not significant.

		Rates of proteolysis (% per h)		
		Control	Experimental	%Change
Free spores	300 : 1	8.98 ± 0.53 (3)	9.24 ± 0.67 (3)	+ 3% n.s.
Vegetative cells	300 : 1	9.59 ± 0.96 (3)	8.40 ± 0.60 (3)	—12% n.s.
Autoclaved, sporulating cells	300 : 1	9.09 ± 1.42 (3)	9.28 ± 0.91 (3)	+ 2% n.s.
Sporulating cells	300 : 1	8.21 ± 1.0 (5)	12.79 ± 1.37 (5)	+57% $P < 0.05$

TABLE II

EFFECT OF CONDITIONED MEDIA ON RATES OF PROTEOLYSIS

Macrophages were attached to dishes and prelabeled for 1 h and 20 h and rates of proteolysis determined. Each result is the mean of two experiments. Control dishes contained prelabeled macrophages exposed to medium A containing 4 mM L-phenylalanine. A second batch of sporulating *B. Licheniformis* was employed in the experiments on line 2. Prior to incubation for proteolysis, another preparation of attached macrophages was incubated in dishes in medium A containing 4 mM L-phenylalanine and lyophilized sporulating *B. licheniformis*, 300 : 1. Another set of dishes contained the above suspension of lyophilized bacteria in the same medium, but without macrophages. Both these preparations were incubated 1 h at 37°C. The media were filtered through 0.45 μm Millipore filters and applied to the prelabeled macrophages at zero time.

	Rate of proteolysis	
	1 h prelabeled	20 h prelabeled
Control	8.63	3.18
B. licheniformis sporulating, 300 : 1	15.03	11.90
Medium from macrophages and bacteria (1 h)	8.76	2.41
Medium from bacteria alone (1 h)	8.83	3.01

fore, we prepared a second batch of lyophilized sporulating bacteria as above, and saved the medium from bacteria and unlabeled macrophages incubated together, and also from bacteria incubated alone. Care was taken that the medium included the 4 mM L-phenylalanine necessary for proteolysis experiments. These media were filtered through 0.45 μm filters and used without dilution in a subsequent proteolysis experiment using prelabeled macrophages. The results are shown in Table II. It can be seen that neither conditioned medium affected proteolysis, but this preparation of lyophilized sporulating bacteria greatly stimulated degradation of both rapidly and slowly turning over proteins in these cells. The rate of degradation of slowly turning over proteins was increased to 11.9% per h by this second batch of lyophilized bacteria.

Discussion

These results show that endogenous protein degradation of pulmonary alveolar macrophages is accelerated during engulfment of lyophilized *Bacillus licheniformis*. Only in the special case of sporulating bacteria was the effect seen; free spores and normal vegetative cells were ineffective. The effect was lost if the lyophilized bacilli were autoclaved, suggesting that engulfed bacterial enzymes sensitive to heat denaturation might be responsible (Table I). Studies with conditioned media (Table II) showed that soluble factors released into the medium are not involved. Similar results were also obtained in our laboratory with a sporulating culture of *B. cereus* (results not shown).

The process of spore formation is associated with a succession of striking morphologic and enzymatic changes in bacteria. Proteases in particular increase during forespore formation [9]. For example, bacterial protein turnover increases to 21—24% per h in *B. licheniformis* [10], and proteolytic enzymes are rapidly synthesized during early stages of sporulation [11]. In the present experiments, these enzymes, included during engulfment of the lyophilized

bacteria by the macrophages, may be included in the phagosome-lysosome system. Since we observe a striking increase in proteolysis, the present results suggest that the engulfed bacterial proteases participate actively in the degradation of the macrophage cell proteins. The high rate of protein turnover produced by one batch of bacteria suggests the presence of higher levels of proteases (Table II).

Lysosomal function has been altered experimentally by cellular engulfment of a variety of agents [12—14]. Liposomes or other aggregates and particles may be used to introduce drugs, inhibitors, and enzymes into the lysosomes of phagocytic cells. Some of these approaches show promise both for experimental and therapeutic purposes. Thus the introduction of proteases in phagocytic vesicles in the present experiments may be analogous to those systems. On the other hand, the substrate for these bacterial proteases was prelabeled cellular proteins. How do these proteins come in contact with engulfed particles? There is some evidence that autophagy involves a sequestration of cellular proteins in lysosomes [15,16]. There may be fusion and subsequent fission and thus communication between these two classes of digestive organelles, as suggested by DeDuve [16]. The results of such fusion would be an exchange of lysosomal contents, and increasing homogeneity of the lysosomal space.

Recent results with peritoneal macrophages suggest that separate classes of lysosomes digest exogenous and endogenous proteins. In those experiments, ammonium chloride and chloroquine were found to inhibit strongly the degradation of engulfed proteins, while having no effect on the breakdown of endogenous cellular proteins [17]. This led to the conclusion that the sites of degradation of the two types of protein could not be the same. We have confirmed that result in sheep alveolar macrophages (unpublished data). The present results, however, suggest a communication between mechanisms of degradation of endogenous proteins and engulfed particles, possibly involving fusion of various lysosomes. The difference in these conclusions may be explained by the response of the lysosomal system to engulfed lyophilized bacteria, which may somehow promote a greater degree of fusion and intercommunication than occurs during treatment with basic inhibitors [17].

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