Biochem. J. (1979) **180**, 339–345
Printed in Great Britain

Macrophage Protein Turnover

EVIDENCE FOR LYSOSOMAL PARTICIPATION IN BASAL PROTEOLYSIS

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(*Received* 18 *October* 1978)

1. Turnover of intracellular proteins in cultured mouse macrophages was found to be slightly accelerated by the omission of serum from the culture medium. Media containing 10% (v/v) or more of serum established basal degradation rates in the cultures. 2. Basal degradation rates varied considerably between experiments, probably as a result of variable activation *in vivo* of the macrophages. 3. The selective carboxyl proteinase inhibitor pepstatin, which appeared to enter the lysosomes of the cells by pinocytosis, gave a progressive inhibition of basal proteolysis up to a maximum of about 40%. Cellular cathepsin D was largely inhibited after 48h of cultivation with pepstatin (100μg/ml). 4. Leupeptin and 7-amino-1-chloro-3-tosylamidoheptan-2-one are less selective proteinase inhibitors. They also induced 25–35% inhibition of degradation, but their actions may not have been restricted to lysosomes. 5. Several solutes and particles that are endocytosed by macrophages and stored in lysosomes induce some inhibition of basal proteolysis, whether or not they themselves are substrates for proteolysis. 6. Colchicine was without effect on protein degradation, but cytochalasin B and the local anaesthetics lidocaine and procaine, all of which have effects on microfilaments, were significantly inhibitory. This inhibition may result from a decrease in the rate of autophagy, and thus of lysosomal proteolysis, due to prevention of microfilament action.

It is now generally accepted that lysosomes carry out the degradation of much intracellular protein of cells and organs under conditions of nutritional deprivation (Dean, 1975; Dean & Barrett, 1976; Goldberg & St. John, 1976; Amenta *et al.*, 1977, 1978; Ballard, 1978). Nutritionally deprived (or 'step-down') conditions occur when organs are perfused in the absence of insulin or amino acids, or cells are grown without serum, hormones or amino acids. Under these circumstances of 'accelerated turnover', autophagic vacuoles become a more pronounced feature in electron micrographs of cells (Amenta *et al.*, 1978), although it is not yet known what alterations in the rate of autophagic sequestration and degradation occur (Dean & Barrett, 1976; Dean, 1977). In contrast, there is only limited evidence (Dean, 1975) for involvement of lysosomes in 'basal degradation' occurring in nutritionally complete conditions (Ballard, 1978).

The present paper describes the general characteristics of protein degradation in cultured mouse peritoneal macrophages and provides evidence that basal turnover is affected by agents which modify the function of the lysosomal system. Preliminary reports of some of this work have appeared (Dean, 1977, 1978).

Materials and Methods

Materials for cell culture

Plastic Petri dishes were from Sterilin, Teddington, Middx., U.K. Medium 199 and sera were from Bio-Cult Laboratories, Glasgow, Scotland, U.K.

Biochemical reagents

7-Amino-1-chloro-3-tosylamidoheptan-2-one ('TLCK'), penicillin, phorbol myristate acetate, streptomycin, and zymosan from the yeast *Saccharomyces cerevisiae*, were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K., dextran sulphate (DS500) was from Serva, Heidelberg, Germany; polystyrene latex particles (0.81μm diameter; 5%, v/v) were from Difco, Detroit, MI, U.S.A.; and heparin, preservative-free, was from Paines and Byrne, Greenford, Middx., U.K. L-[U-^{14}C]Leucine was from The Radiochemical Centre, Amersham, Bucks., U.K. Sepharose 4B was from Pharmacia (G.B.), London W.5. U.K., and was weighed as a centrifuged wet slurry. Pepstatin and leupeptin were obtained from the Protein Research Foundation, 476, Ina, Minoh-Shi, Osaka 562, Japan. Pepstatin was dissolved in methanol at 10mg/ml, and was diluted from this stock into media at the specified

concentration. Cytochalasin B was dissolved in dimethyl sulphoxide at 10mg/ml, and similarly diluted into medium. The ionophore A23187, kindly given by Eli Lilly, Basingstoke, Hants., U.K., was dissolved in dimethyl sulphoxide (at 10mg/ml) before dilution into medium. Control experiments with solvent alone were always conducted in parallel with those with the inhibitors present. The solvents had no observable effects on the parameters under study, and only methanol caused a slightly increased rate of cell death. Methanol was at a final concentration of 1% (v/v), and dimethyl sulphoxide at 0.1% (v/v).

Macrophage collection and culture

Macrophages were obtained by peritoneal lavage of normal Swiss mice (T.O. strain) with 5ml of cold Medium 199 containing 100 units of penicillin/ml, 100 units of streptomycin/ml and 10i.u. of heparin/ml. Samples (5ml) of the peritoneal exudate cell suspension (containing 0.75×10^6–1.3×10^6 cells/ml) were placed in 50mm diameter Petri dishes and incubated at 37°C in a humidified atmosphere of CO_2/air (1:19) for 1–2h. Non-adherent cells were then removed by washing four times with phosphate-buffered saline, comprising 136mM-NaCl, 2.7mM-KCl, 8.1mM-NaH_2PO_4 and 1.5mM-KH_2PO_4, without calcium and magnesium. After washing, the cells were cultured in 5ml of Medium 199 containing 10% (v/v) heat-inactivated pig serum. The heat-inactivation was performed at 56°C for 30min, and the serum was also acid-treated (pH3, 30min) to destroy some proteinase inhibitors (Werb & Gordon, 1975). Cultures gave a sheet of spread cells within a few hours, and experimental treatments began after incubation overnight (at which point the cell sheets were considered to be established). At this point the medium was changed, and 1μCi of [^{14}C]leucine/ml included in the new medium. The cells were normally allowed to incorporate the label into protein for 24h, after which period the cells were washed four times with phosphate-buffered saline containing 10mM-leucine. This washing procedure was found to deplete the cells of radioactive low-molecular-weight substances; less than 4% of the radioactivity associated with the cells was found to be soluble in 5% (w/v) trichloroacetic acid. Cells then received fresh Medium 199 normally supplemented with 10mM-leucine, and sometimes containing various inhibitors, particles or other agents (see the Results section). Degradation was measured for up to 48h, with triplicate cultures at every time point. To measure degradation at each chosen time, the culture medium was removed, and the cells lysed with phosphate-buffered saline containing 0.1% (v/v) Triton X-100 (2.5ml per culture). The cells were then scraped off with sterile silicone-rubber bungs and 0.5ml samples of the medium and cell suspensions were then precipitated with 0.5ml of 10% (w/v) trichloroacetic acid containing 10mM-leucine. To the cell suspensions, 50μl of 10% bovine serum albumin was added before the trichloroacetic acid, to act as carrier. The precipitates were then sedimented in a bench centrifuge, and portions of the supernatant counted in a Triton X-100/toluene (3:7, v/v) scintillant. Quench correction was by the automatic external-standards-ratio method. Pellets from precipitation with trichloroacetic acid were resuspended and washed twice with 5% trichloroacetic acid/10mM-leucine, and the final pellet redissolved in 1ml of formic acid (98–100%) for counting radioactivity. Trichloroacetic acid-soluble radioactivity was taken to represent degradation products, and the trichloroacetic acid-precipitable radioactivity to represent intact macromolecules. Degradation is expressed as trichloroacetic acid-soluble radioactivity as a percentage of the total radioactivity in the system. Trichloroacetic acid-precipitable material in the medium gave an upper estimate to the amount of cell death occurring in the system, since separate experiments showed that little degradation of macrophage protein occurs in a medium in which macrophages have previously been grown for up to 48h, in the absence of intact cells.

In experiments in which cathepsin D activity or protein in the cells was measured, the cell layer was washed twice with phosphate-buffered saline after removal of the medium, to decrease contamination of the cells by serum protein from the medium. In addition, for cathepsin D assays, the cells were harvested in 50mM-sodium acetate/acetic acid buffer, pH5.0, containing 0.1% Triton X-100.

Assays

Protein was measured by the Lowry *et al.* (1951) procedure, with bovine serum albumin as standard. Cathepsin D was measured by Method 2 described by Barrett & Heath (1977).

Results and Discussion

In initial experiments the effects of addition of various concentrations of unlabelled leucine on the apparent degradation were assessed. Measured degradation increased slightly as added leucine was increased from 1 to 5mM; values obtained with 5mM- and 10mM-leucine were not distinguishable statistically. It was concluded that the addition of 10mM-leucine (giving a final concentration of 10.9mM-leucine, because of the presence of leucine in Medium 199) virtually suppressed isotope reutilization, and thus all further experiments were conducted under these conditions.

Fig. 1 shows the kinetics of degradation in a representative experiment. Trichloroacetic acid-soluble radioactivity in the cells remains at a low value throughout the experiments, while trichloro-

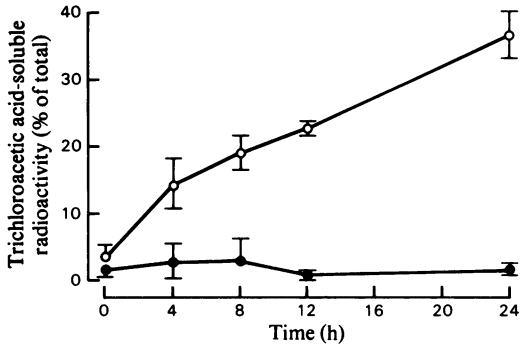

Fig. 1. *Kinetics of degradation of intracellular proteins in macrophages*
The accumulation of trichloracetic acid-soluble degradation products within the cells (●) and in the medium (○) is shown. Results are means ± S.D. for three cultures.

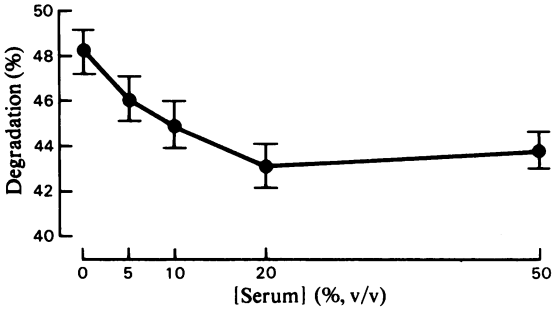

Fig. 2. *Dependence of macrophage protein turnover on the concentration of serum in the medium*
Cells were pre-labelled under normal standard conditions, and, after washing, medium containing the chosen concentrations of acid-treated foetal-calf serum was added. Degradation was measured after 24h. Results are means ± S.D. for three cultures.

acetic acid-soluble degradation products continue to accumulate in the medium. Degradation is approximately exponential, with some indication of an early fast phase completed within 4h. This has not been studied in detail. The rate of degradation under these standard conditions varies quite considerably from experiment to experiment, so that the extent of degradation at 24h ranged from 32 to 61%. High rates were observed mainly in summer, and low rates in winter (when the incidence of infection among the mice may be higher than in summer). Evidence presented below shows that various agents that activate macrophages in certain respects (see Davies & Allison, 1976) also modify the rate of degradation, so it is quite likely that these variations reflect different extents of activation *in vivo* of the macrophages due to infection and immunological stimuli.

Fig. 2 shows the dependence of degradation rate on the concentration of serum in the medium. There is a slight acceleration in 'step-down' conditions (no serum), but this is very much less marked than in other systems (Knowles & Ballard, 1976; Amenta *et al.*, 1978; Goldberg & St. John, 1976). It seems that concentrations of serum of 10% or more provide conditions for basal degradation; this is confirmed by experiments that show that insulin (1 pM–1 μM) has only slight effects on degradation rate in 10% serum (results not shown). Glucagon (10 μM) also has negligible effects. It is noteworthy that when mouse macrophages are cultured in the presence of high concentrations of serum (20–50%, v/v), they progressively accumulate both lysosomal enzymes and secondary lysosomes (see Cohn & Fedorko, 1969). This increase in lysosomal enzyme activity is not accompanied by increased endogenous protein turnover (Fig. 2). The normal enzyme activity therefore seems quite sufficient to degrade the required flux of substrate.

Inhibition of degradation by selective proteinase inhibitors

Pepstatin, the specific carboxyl-proteinase inhibitor (Umezawa & Aoyagi, 1977), has previously been used to demonstrate lysosomal involvement in accelerated turnover in perfused liver (Dean, 1975). Because macrophages grown in 10% serum seem to represent

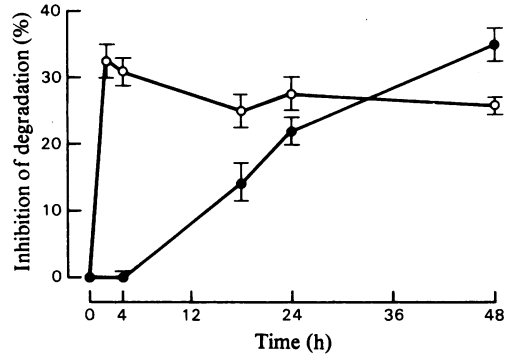

Fig. 3. *Inhibition of macrophage protein degradation by pepstatin and leupeptin*
The degree of inhibition (% of control) observed at various times after exposure to pepstatin (100 μg/ml, ●) and leupeptin (50 μg/ml, ○) is shown. Results are means ± S.D. for three cultures. Pepstatin inhibition is expressed as a percentage of the degradation observed in control cultures with 1% methanol, and leupeptin inhibition as a percentage of the degradation in controls free of organic solvents. Degradation in the two control groups did not significantly differ.

a system undergoing basal turnover, similar experiments with pepstatin were performed on these cells.

Fig. 3 shows the effect of pepstatin on macrophage degradation. Inhibition is progressive, reaching a maximum at about 48h. This progressive inhibition presumably reflects the time taken by the macrophages to pinocytose the inhibitor and transfer it to the lysosomes. The activity of cathepsin D recoverable from the cell suspensions declines as the degree of inhibition of degradation increases; in one such experiment, with pepstatin at 100μg/ml, the activities per culture at 4, 12, 24 and 48h were respectively 100±5.5%, 74±3.8%, 45.1±7.7% and 3.4±4.4% of the corresponding control values in cells cultivated in parallel without inhibitor. Although the inhibitor causes an acceleration of cell death (see Table 1), this can only explain a small part of the decrease of activity in the treated cultures, and this was confirmed by assessing the cathepsin D activity per mg of cell protein.

Further confirmation that the uptake is by pinocytosis and not by permeation was obtained by treating a set of cultures for 30min only with 100μg of pepstatin/ml, then removing the medium, replacing it with fresh medium, and following subsequent degradation in these cultures and control cultures treated with methanol (1%, v/v) and comparably washed. No differences were detectable, so that no significant entry of pepstatin into the cells had occurred by 30min; similarly there was no apparent inhibition of cellular cathepsin D.

Table 1 shows that inhibition of degradation observed after 24h increases with the concentration of pepstatin used (up to 100μg/ml). This may be a near-maximal effect, but it is difficult to use higher concentrations of the inhibitor because of problems of solubility and toxicity. Both the kinetics and the extent of inhibition vary from experiment to experiment, and this is probably due again to variation in the degree of activation of the cells, with concomitant variation in degradation rate and pinocytic rate (Edelson *et al.*, 1975), both of which might affect the characteristics of inhibition by pepstatin. This evidence demonstrates lysosomal involvement in basal proteolysis in macrophages.

Leupeptin, in contrast, is a less selective inhibitor of thiol proteinases (Umezawa & Aoyagi, 1977) and to some extent other proteinases, and seems to penetrate quite rapidly into cells (Dunn & Aronson, 1977; Seglen, 1978). It causes a substantial inhibition of macrophage degradation (approx. 25–35%) (Fig. 3), and there seems to be no lag in the onset of degradation, consistent with entry by permeation. Similarly, 7-amino-1-chloro-3-tosylamidoheptan-2-one, a serine proteinase inhibitor that also affects some thiol enzymes, has a rapid inhibitory action, giving approx. 35% inhibition at 24h. However, this reagent was quite lytic to the cells (65.7±1.1% release of trichloroacetic acid-precipitable material in 24h). Nevertheless, the results still indicate significant inhibition of degradation when degradation is taken as a percentage of total radioactivity excluding trichloroacetic acid-precipitable radioactivity in the medium. Since both these inhibitors seem to penetrate through cell membranes, they might be acting on proteinases in any part of the cell. In recent work (E. N. Shaw & R. T. Dean, unpublished work), we have shown that a selective thiol proteinase inhibitor benzyloxycarbonylphenylalanylalanine diazomethyl ketone (Leary & Shaw, 1977) inhibits macrophage basal protein turnover concomitant with inhibition of cellular cathepsin B. Entry seems to be by pinocytosis, and the effects of the inhibitor are thus on lysosomal activity.

Effects of agents accumulating in lysosomes

Many weak bases (such as chloroquine and ammonia) accumulate in lysosomes, probably by virtue of protonation within the acid internal milieu (see Dean & Barrett, 1976) and decrease the acidity. This

Table 1. *Inhibition of protein degradation in macrophages by pepstatin*

Technical details are given in the Materials and Methods section. Results are means±S.D. for groups of three cultures from a single experiment. Qualitatively similar results were obtained in three other comparable experiments. Degradation in controls containing methanol was not significantly different from that in solvent-free controls.

	Concn. (μg/ml)	Degradation in 24h (%)	Inhibition of degradation (%)	Medium trichloracetic acid-precipitable radioactivity as percentage total radioactivity
Controls (containing methanol, 1%, v/v)	—	47.4±2.5	—	19.8±2.5
Pepstatin	1	48.0±2.6	0	17.5±1.8
	10	40.8±2.4	13.9	19.0±2.0
	20	36.2±2.4	23.6	19.9±1.9
	50	32.5±1.8	31.4	24.2±2.1
	100	29.0±1.0	38.8	28.1±1.4

Table 2. *Effects of endocytosable agents and related materials on protein degradation in macrophages*
Degradation was measured during 24h of continuous exposure to the agents listed, by using methods given earlier. The materials had no significant effects on viability. 0 indicates no significant effect on degradation.

	Concn.	Mode of entry into cells	Inhibition of degradation (%)
Endocytosable agents			
Sucrose	20mM	Pinocytosis	8.0±1.2
	40mM		10.8±1.1
	80mM		29.0±2.0
Dextran sulphate	50μg/ml		6.5±0.5
Latex	1μl/ml	Phagocytosis	9.6±2.4
Zymosan	50μg/ml		9.1±2.1
Other agents			
Glucose	80mM	Permeation and transport	3.2±1.0
Sepharose 4B	10mg/ml	Does not enter	0

results in substantial (up to 90%) inhibition of protein degradation in isolated hepatocytes. Such cells show the accelerated turnover characteristic of nutritionally deprived cells (Seglen, 1977), probably because of lack of hormonal control *in vitro*. Weak bases also cause inhibition in cultured fibroblasts grown in 'step-down' conditions (Knowles & Ballard, 1976; Amenta *et al.*, 1977), although the effect is much smaller (about 30% inhibition). In the present work the effects of chloroquine and ammonia in macrophages were found to be slight. At 10μM, chloroquine gave a 6.6±0.4% inhibition of degradation in 24h, but at 100μM it produced extensive lysis of cells; 10mM-NH_4Cl gave only 10% inhibition at 24h. Similar results with ammonia, chloroquine, and another weak base, Neutral Red, have been obtained by Poole *et al.* (1977). This evidence is at least consistent with the thesis that lysosomal proteolysis contributes to basal turnover. It is not clear why the effects of these agents on turnover of endogenous macrophage proteins are so slight. Their effects on degradation of endocytosed proteins by macrophages are much greater (Poole *et al.*, 1977). These compounds have quite diverse effects (see Goldberg & St. John, 1976; Seglen & Reith, 1977) and in macrophages chloroquine causes a marked accumulation of autophagic vacuoles (Fedorko *et al.*, 1968). This may tend to accelerate endogenous turnover, and thus mask any intralysosomal inhibitory effect. Because of the diversity of activities of these compounds, interpretation of their effects is in any case difficult.

Table 2 shows the inhibitory effect of various materials that accumulate after endocytosis in macrophage lysosomes. Only zymosan contains digestible elements, so the inhibition cannot be simply attributed to competition between endocytosed and endogenous substrates. Similar inhibitory effects of sucrose on protein degradation in mouse heart organ cultures have been reported (Wildenthal *et al.*, 1978). Some of the agents (zymosan, dextran sulphate) can induce activation of normal macrophages (Davies & Allison, 1976) whereas the others do not. The only common features of the inhibitory agents are that they enter the cell by endocytosis and accumulate in lysosomes. The non-inhibitory materials are distinct in both respects, since glucose permeates cell membranes, Sepharose 4B does not enter the cells, and neither accumulates in lysosomes. Thus accumulation of various materials in lysosomes leads to suppression of protein turnover.

This idea is also consistent with the observation of

Table 3. *Effects of agents acting on the cytoskeletal system on macrophage protein degradation*
Cells were exposed to the drugs during the whole period of degradation. 0 indicates no detectable effect on degradation. Cytochalasin inhibition is expressed as a percentage of the degradation in control cultures containing 0.1% (v/v) dimethyl sulphoxide. Inhibition with the other agents is given as percentage of the degradation in controls free of organic solvent. The two control groups did not differ significantly from each other.

Drug	Concn.	Inhibition of degradation (%)	Duration of degradation (h)
Colchicine	10^{-4}–10^{-8}M	0	Up to 48
Cytochalasin	2μg/ml	14.4±2.0	48
	5μg/ml	24.6±2.1	48
	10μg/ml	26.4±2.1	48
Lidocaine	1mM	4.0±1.0	24
	5mM	15.5±4.2	24
Procaine	0.1mM	6.0±0.8	24
	1mM	20.2±5.0	24

inhibition of degradation by concanavalin A (20 μg/ml gave 20.4±1.1% inhibition at 24h) and phorbol myristate acetate (100ng/ml gave 16.6±1.4% inhibition at 24h). These agents stimulate pinocytosis (Edelson *et al.*, 1975) and other macrophage functions and result in an enlarged population of lysosomes. Increased intracellular storage could contribute to the inhibition of turnover by serum, which also stimulates pinocytosis (Davies *et al.*, 1973). Nevertheless, increased pinocytosis is in some cases associated with increased degradation of plasma-membrane components (Edelson & Cohn, 1976), perhaps as a direct consequence of their internalization.

Effects of agents capable of acting on cytoskeletal structures

Table 3 outlines the effects of agents that, among other actions, inhibit the functioning of cytoskeletal structures. Colchicine, which causes microtubule disaggregation, has no significant effects on macrophage protein degradation. This is consistent with the observations of Amenta *et al.* (1977) on fibroblasts in which colchicine only has an effect under conditions of accelerated turnover. However, since macrophage basal turnover involves lysosomes (judging by the evidence presented already), it follows that lysosomal-degradative mechanisms do not necessarily require maximal microtubule integrity. The inhibition of accelerated turnover by colchicine may involve inhibition of lysosomal activities distinct from those in basal turnover (perhaps autophagy rather than microautophagy; see Dean & Barrett, 1976).

The effects of the microfilament reagent cytochalasin B on macrophage turnover are also consonant with its effects on fibroblast turnover (Amenta *et al.*, 1977). In the latter system, both elevated and basal turnover can be inhibited by the drug. In macrophages (Table 3), basal turnover can be inhibited by about 30%. The inhibition can be reversed after 24h by washing the macrophages three times with 5min intervals between washes. A possible interpretation is that lysosomal activities in both basal and elevated turnover require microfilaments. This requirement may be for microfilament contraction in initiating the membrane fusions required in both autophagy and microautophagy; in a similar way, cytochalasin can inhibit endocytic processes, which involve membrane fusions (see Tanenbaum, 1978). In the light of this hypothesis, the effects of the calcium ionophore A23187, which might have induced microfilament paralysis by virtue of continuously supplying calcium necessary for contraction, were assessed. Unfortunately, the compound was too lytic to macrophages for useful results to be obtained.

The slightly inhibitory effects of the local anaesthetics lidocaine and procaine (Table 3) may be due to actions on microfilaments (see Poste *et al.*, 1975). Equally, as these drugs are weakly basic tertiary amines, they may accumulate in lysosomes and inhibit by altering the intralysosomal pH. It has been shown (Carpenter & Cohen, 1976) that the degradation of previously endocytosed epidermal growth factor can be inhibited by these local anaesthetics.

Although the drugs discussed in this section have diverse effects, and thus several interpretations of their inhibitory actions on protein degradation have been proposed, all the alternatives suggested already involve the participation of the lysosomal system. It is less plausible, though not impossible, to explain their inhibitory actions by involving effects on other cell organelles without alteration of lysosomal functions.

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