IMMUNOINHIBITION OF INTRACELLULAR PROTEIN DIGESTION IN MACROPHAGES*

By JOHN T. DINGLE,‡ A. ROBIN POOLE, GERALD S. LAZARUS,§ AND ALAN J. BARRETT

(From the Tissue Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, England)

(Received for publication 18 December 1972)

The endocytosis of extracellular macromolecules and their subsequent digestion have been shown to occur in a variety of cell types, the focus of digestive activity being the secondary lysosome (1). This process has been studied in detail in the macrophage, by Ehrenreich and Cohn (2), for example, who demonstrated the endocytosis of [^{125}I]hemoglobin and its hydrolysis to peptides and amino acids. It is usually assumed that this degradation is due to the action of lysosomal acid proteinases, although the enzymes concerned have not been identified.

As part of an investigation of the catabolism of extracellular macromolecules of connective tissues, cathepsin D has been highly purified (3) and used to raise specific antisera with which to inhibit (4) and localize (5) cathepsin D. The present report describes the use of sheep anti-(rabbit cathepsin D) serum (As-D)[1] to inhibit specifically the intralysosomal activity of cathepsin D in alveolar macrophages and thus to examine its role in intracellular digestion.

Materials and Methods

Chemicals and Radiochemicals.—Hemoglobin was prepared from beef blood, as described by Barrett (3); the casein was the Hammersten grade from BDH Chemicals Ltd., Poole, Dorset, England. [^{3}H]Hemoglobin and [^{3}H]casein were prepared by acetylation as follows. Protein (100 mg) was dissolved in 2.0 ml phosphate-buffered saline solution (PBS-1) containing 50 mM sodium acetate and cooled to 0°C. ^{3}H-labeled acetic anhydride (500 μCi in 5 μl benzene, obtained from the Radiochemical Centre, Amersham, Bucks, England, as type A1 P2A) was introduced with thorough mixing, and the reaction mixture was allowed to stand

* This study was supported by grants from the Medical Research Council, the Nuffield Foundation, and the Arthritis and Rheumatism Council. We also acknowledge support from the Schering Corp., Bloomfield, N. J., for assistance toward publication costs.

‡ Member of the external staff of the Medical Research Council.

§ Fellow of the Arthritis Foundation and Carl Herzog Fellow of the American Dermatological Association. Present address: Section of Dermatology, Department of Medicine, Montefiore Medical Centre, Bronx, N. Y.

[1] *Abbreviations used in this paper:* As-D, sheep anti-(rabbit cathepsin D) serum; Fab', monovalent antibody prepared from IgG by digestion with pepsin (11); Ns, normal sheep serum; PBS-1, phosphate-buffered saline solution (4); PBS-2, phosphate-buffered saline solution containing 147 mM NaCl, 90 mM Na_2HPO_4 and 1 mM NaH_2PO_4.

at 0°C for 1 h. The labeled protein was then freed of [^{3}H]acetate by exhaustive dialysis against large volumes of PBS-1 containing 50 mM sodium acetate. The efficiency of incorporation of tritium into protein was of the order of 20%.

[^{14}C]Dextran (mol wt range 16,000–19,000) was purchased from New England Nuclear Corp., Boston, Mass. ^{35}S-labeled proteoglycan from bovine nasal cartilage was prepared biosynthetically as follows. The nasal septum, removed soon after death, was freed of soft tissue, surface sterilized for 30 s in 70% (vol/vol) aqueous ethanol, and aseptically cut into 1-mm-thick slices. A modication of BGJ_5 culture medium (6) containing no inorganic sulfate, 0.70 mg of streptomycin hydrochloride/ml, 500 U of penicillin/ml, and 100 U of nystatin (obtained as Mycostatin from E. R. Squibb & Sons, London)/ml, together with 5 mCi of carrier-free [^{35}S]sodium sulfate (Radiochemical Centre)/25 ml, was prepared. The cartilage slices (10 g) were incubated in 25 ml of the culture medium in a roller bottle, gassed with 5% CO_2, 75% N_2, and 20% O_2 at 37°C overnight. [^{35}S]Proteoglycan (1–2 mCi) was isolated from the labeled tissue by extraction with 4 M guanidinium hydrochloride, pH 7.6 (7), and converted into its potassium salt by treatment with 1 M potassium acetate and reprecipitation with ethanol.

Preparation of Antisera, IgG, and Fab' Fractions.—Antisera were raised against cathepsin D as described by Dingle et al. (4). Partially purified sheep immunoglobin G (IgG) was prepared from sera by precipitating three times at 33% saturation with ammonium sulfate at 20°C. Pure IgG was obtained from this fraction by the method of Aarlund et al. (8).

Rabbit anti-(sheep IgG) serum for use in cytochemical studies was raised by three separate intramuscular injections at 2-wk intervals, each of 5 mg of pure IgG in 1 ml of 0.9% NaCl, emulsified with 1 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Animals were exsanguinated by cardiac puncture 2 wk after the final injection. Rabbit anti-(sheep serum protein) serum was prepared in the same way, 1 ml of serum being administered at each injection.

The purity of rabbit and sheep IgG was determined by immunoelectrophoresis and double immunodiffusion against anti-(whole serum protein) sera raised in goat and rabbit, respectively (Wellcome Reagents, Ltd., Beckenham, Kent, England) and specific pig anti-(rabbit IgG) IgG (Mercia Diagnostics, Ltd., Watford, England) or rabbit anti-(sheep IgG) serum raised as described above.

The concentration of solutions of IgG was determined spectrophotometrically, assuming $E^{1\%}_{1\,cm} = 14.0$ at 280 nm by analogy with other species (9). Sheep IgG was also determined quantitatively (where stated) by radial immunodiffusion analysis, using a method based on that described by Mancini et al. (10). The IgG concentrations in normal sheep serum (Ns) and As-D determined by immunodiffusion were 16.6 mg/ml and 44.0 mg/ml, respectively.

A pepsin digest containing Fab' was prepared for use in immunocytochemical studies from partially purified sheep IgG by the method of Nisonoff et al. (11), and was dialyzed against phosphate-buffered saline solution containing 147 mM NaCl, 90 mM Na_2HPO_4, and 1 mM NaH_2PO_4 (PBS-2) before use.

Labeling of Proteins with Fluorochromes.—Hemoglobin, IgG, and Fab' were labeled with fluorescein isothiocyanate for 1 h at room temperature (12). Hemoglobin was also labeled with lissamine rhodamine B200 by the method of Goldman (13), free fluorochrome being removed by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The molar ratio of rabbit anti-(sheep IgG) IgG to fluorescein was 1:2.0 and that of hemoglobin to lissamine rhodamine, 1:0. 76 (13, 14).

Fluorescein-conjugated IgG and Fab' were treated for 30 min with 5 mg/ml chicken liver acetone-dried powder (15) and then centrifuged for 2 min on a microhematocrit centrifuge (Gelman-Hawksley, Lancing, Sussex, England), to remove any particulate material, before use.

Isolation of Macrophages.—Alveolar macrophages were isolated from 9–20-wk-old New Zealand White rabbits by a modification of the method of Myrvik et al. (16). Rabbits were

injected intravenously (marginal ear vein) over a period of 1 min with an emulsion of 5 mg of freeze-dried, heat-killed (60°C, 1 h) *Mycobacterium tuberculosis* var. BCG (a gift from Glaxo Laboratories, Greenford, Middlesex, England) in 0.5 ml of saline and 0.5 ml of Markol 52 (Wellcome Research Ltd., Dartford, Kent, England). Animals were killed 8 days later by exsanguination. Lungs were lavaged aseptically four times with 25 ml of sterile PBS-1 containing 5 U of heparin/ml. A total volume of approximately 80 ml of cell suspension was obtained.

Cells were centrifuged at 750 g_{av} for 10 min and washed twice at room temperature with 50 ml of BGJ_W at 750 g_{av} for 10 min. (BGJ_W has the same composition as BGJ_5 [6]—except that it contains 6 mM sodium bicarbonate and 10-fold greater antibiotic concentration.) Cells were counted with a hemacytometer slide (improved Neubauer pattern). Cell viability was estimated by dye exclusion using eosin Y at a concentration of 1.5 mg/ml in PBS-2. The total yield of cells per animal was 5.4×10^8 and the viability was 89% (means from seven animals). Over 95% of the cells were macrophages, the remainder being small round cells.

Incubation of Macrophages with Sera and Radiochemicals.—Cells (2×10^7) were resuspended in 2 ml of medium BGJ_5 and incubated at 37°C after gassing with a mixture of 5% CO_2, 20% O_2, and 75% N_2. The media contained either 14.3% (vol/vol) Ns or 14.3% As-D, unless otherwise stated. Sera were heated at 56°C for 30 min to inactivate complement before use. The following amounts of radioactive macromolecules were included in 1 ml of medium, for use with 10^7 cells: [^{3}H]hemoglobin (0.5×10^6 cpm/mg), 150 μg; [^{14}C]dextran (2.47×10^6 cpm/mg), 81 μg; [^{35}S]cartilage proteoglycan (6.7×10^6 cpm/mg), 21 μg; and [^{3}H]casein (0.62×10^6 cpm/mg), 17 μg.

Cells were isolated from incubation media by centrifugation at 750 g_{av} for 10 min and, where indicated, washed with 2 equal vol of BGJ_W. Cells were then resuspended in medium BGJ_5. Experiments were carried out in triplicate.

Radiochemical Analyses.—In experiments with [^{3}H]hemoglobin and [^{3}H]casein, protein in cells and supernatant media was precipitated with 2.5% (wt/vol) TCA. [^{35}S]Proteoglycan was precipitated with 3 vol of acid ethanol (0.27% concentrated sulfuric acid in ethanol). Precipitates were dissolved at 90°C for 15 min in 98% formic acid for scintillation counting. In studies with [^{14}C]dextran, formic acid digests were prepared directly from washed cells. All digests were centrifuged at 1,300 *g* for 10 min. Samples (100 μl) of digests and supernatants were counted in 10 ml of scintillant solution (17). On six separate samples of cells showing a mean uptake of precipitable [^{3}H]hemoglobin of 9,900 cpm/10^7 cells, the standard error of the mean was 180; in the same experiments, TCA-soluble ^{3}H counts were 1,920, with a standard error of 35.

Cytochemical Studies.—Smears were prepared on cover slips with 5×10^5 cells; they were allowed to air-dry at room temperature for 15 min and were stored at −20°C for up to 1 wk. The smears were fixed for 10 min in formaldehyde freshly prepared from 4% (wt/vol) paraformaldehyde in PBS-2 (18). They were washed in PBS-2 for three 10-min periods and then were mounted for microscopic examination (5).

Intracellular cathespin D was localized by staining cells for 60 min with fluorescein-labeled As-D Fab′ (initial protein concentration before adsorption, 0.42 mg/ml; initial E_{495}, 0.592) in the presence of 1 mM cysteine to maintain Fab′ in its monomeric state. Controls were prepared by pretreating cells for 60 min with 12 times this concentration of unlabeled Fab′ from either As-D or Ns.

Sheep IgG was detected by staining cells for 30 min with fluorescein-labeled rabbit anti-(sheep IgG) IgG at an initial concentration of 0.18 mg protein/ml (molar ratio of IgG to fluorescein, 2:1). Controls were prepared by pretreating cells for 30 min with either nonimmune rabbit serum or rabbit anti-(sheep IgG) serum, diluted 1:10 with PBS-2. Cells were washed with PBS-2 for three 10-min periods after each immunoglobulin treatment. Cells were finally mounted in 0.2 M Tris buffer (pH 8.6)/glycerol (1:9 vol/vol), and were examined by dark ground fluorescence microscopy. The green fluorescence of fluorescein was observed and photographed as previously described (5), except that BG 38 filtration with a total filter

thickness of 8 mm was used to remove all red contrast light, normally passed by the excitation filter. Lissamine rhodamine B200 fluorescence was observed, the appropriate matched Olsen exciter and barrier filters, supplied by Polaron (London, England), being used.

Electron Microscopy.—Cells were examined immediately after isolation from the animal and after incubation, as indicated. Cells (2×10^7) were fixed for 30 min with 1 ml of cold 2.5% glutaraldehyde in 0.09 M cacodylate buffer, pH 7.2, containing 3 mM calcium chloride. They were then centrifuged at 1,000 g_{av} for 10 min, after which the fixative was replaced by 2.0 ml. of cold 0.1 M cacodylate buffer, pH 7.2. Cells were postfixed in Zetterqvist's buffered 1% osmium tetroxide (19) for 1 h, washed, and stained with 0.5% uranyl acetate in Zetterqvist's buffer for 1 h; they were dehydrated in ethanol and embedded in Araldite. Sections 0.05–0.10 μm thick were cut on an LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.) fitted with a glass knife. The sections were mounted on copper grids coated with collodion-carbon films and were stained with 1% aqueous uranyl acetate for 10 min. They were then stained with lead citrate (20) for 5 min and were examined in an AEI EM 6B electron microscope (AEI Scientific Apparatus, Inc., Elmsford, N. Y.). Five grids and at least 200 cells were examined from each preparation.

RESULTS

Immunoinhibition of the Proteolytic Activity of Macrophage Extracts.—Macrophage extracts digested hemoglobin over a wide range of pH values (Fig. 1). Removal of cathepsin D from the extracts by immunoprecipitation revealed

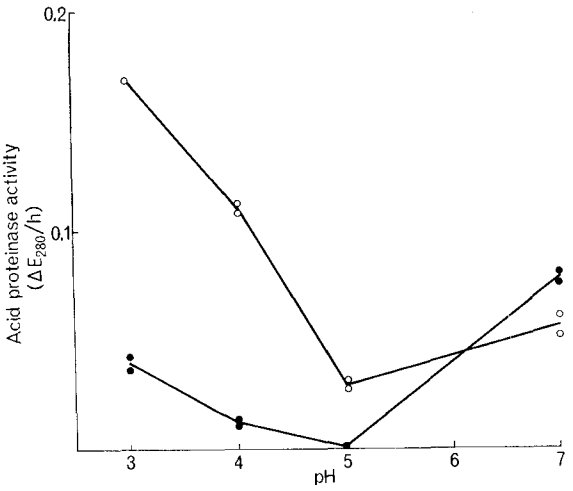


FIG. 1. Digestion of hemoglobin by a cell extract. A homogenate of rabbit alveolar macrophages (7×10^8 cells) was prepared with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, W. Germany). Cell debris was removed by centrifugation at 11,000 g_{av} for 20 min, and the supernatant was diluted to 1 U cathepsin D/ml. To 5 ml of this extract were added 250 μl of As-D or Ns; the mixtures were incubated at 37°C for 15 min, and then at 4°C for 18 h. After centrifugation at 42,000 g_{av} for 30 min to remove any precipitates, supernatants were assayed for acid proteinase activity in the presence of 4 mM cysteine and 4 mM EDTA, at pH 3.0 (formic acid-sodium formate buffer), pH 4.0, pH 5.0 (acetic acid-sodium acetate buffer), and pH 7.3 (phosphate buffer). The method was otherwise as described for cathepsin D (3). The results are means of determinations of enzyme activity in extracts after treatment with Ns (○) or As-D (●), in three experiments.

that this enzyme was responsible for most of the proteolytic activity recorded at pH 3.0–5.0. However, hemoglobin-degrading activity at pH 7.0 was unaffected by immunoprecipitation and hence was not due to cathepsin D.

Immunoinhibition of the Proteolytic Activity of Living Macrophages.—The total cellular proteolytic activity of macrophage extracts at pH 5.0 remained unchanged during a period of 4 h exposure to Ns (Fig. 2). Cells incubated with As-D for the same period contained sufficient endocytosed antibody to give 42% inhibition of the total proteolytic activity measured after subsequent cellular disruption.

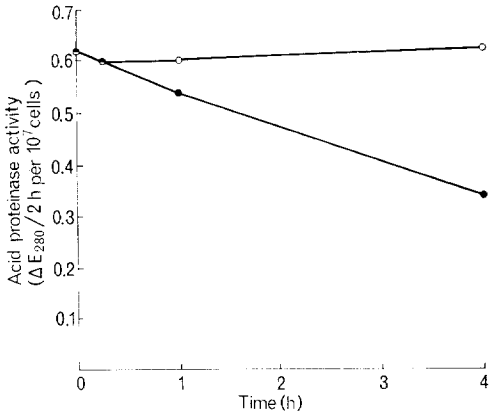


FIG. 2. Effect of endocytosed As-D on total cellular acid proteinase activity (3) at pH 5.0. Macrophages were incubated with either Ns (○) or As-D (●). At various time intervals, cells were centrifuged, washed, and resuspended in 2 ml of 0.2% Triton X-100 in distilled water. They were assayed at pH 5.0 in order to detect any inhibition of enzyme activity. The results are means of two experiments. The E_{280} of the cell lysates was also measured and found to be constant with time.

Incubation of macrophages with [^{3}H]hemoglobin resulted in the progressive accumulation of precipitable radioactivity within these cells up to 4 h, after which time no further increase was detected in the presence of Ns (Fig. 3 *a*). When the medium contained As-D, hemoglobin accumulation was greater at all the time intervals studied.

Extracellular TCA-soluble radioactivity also increased with time (Fig. 3 *b*). Cells incubated in the presence of As-D released less TCA-soluble radioactivity than the controls with Ns; the difference was most marked at 4 h. The degree of inhibition of release of TCA-soluble radioactivity was found to be directly proportional to the concentration of As-D in the medium, up to 15% (vol/vol).

Effect of As-D on the Endocytosis of Macromolecules.—In order to determine whether the antiserum increased cellular accumulation of macromolecules through a general stimulation of endocytosis, rather than by inhibiting pro-

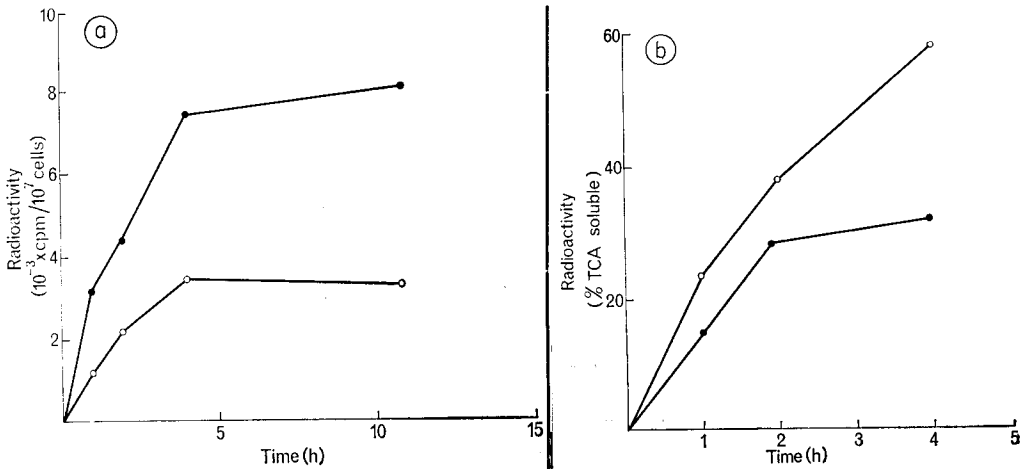


FIG. 3. The effect of As-D on (*a*) uptake of [^{3}H]hemoglobin and (*b*) production of extracellular TCA-soluble [^{3}H]hemoglobin digestion products. Macrophages were incubated with hemoglobin and Ns (○) or As-D (●). Roller tubes were removed at intervals, cells were washed, and determinations were made of (*a*) total cellular ^{3}H and (*b*) extracellular TCA-soluble ^{3}H expressed as a percentage of the total counts per minute per roller tube. Results are expressed as means of three determinations.

teolytic digestion, the uptake of [^{14}C]dextran (which cannot be digested by these cells) was measured. No stimulation of endocytosis was found (Fig. 4).

The Accumulation and Digestion of Casein and Proteoglycan.—Like hemoglobin, both [^{3}H]casein (1,100 cpm/10^7 cells, 82% radioactivity as TCA-soluble digestion products) and [^{35}S]cartilage proteoglycan (2,900 cpm/10^7 cells, 60% radioactivity as ethanol-soluble digestion products) were accumulated and digested by macrophages during incubation for 5 h in normal serum. Similar incubation of the cells in As-D increased the accumulation of proteoglycan (4,540 cpm/10^6 cells, 46% radioactivity is ethanol soluble), but did not appreciably affect the cell content of casein (1,250 cpm/10^6 cells, 74% radioactivity is TCA soluble).

Loss of [^{3}H]Hemoglobin from Macrophages after Endocytosis in the Presence of As-D or Ns.—A study of the rate of digestion of accumulated [^{3}H]hemoglobin in macrophages containing As-D or Ns gave the results shown in Fig. 5. Hemoglobin was lost much less rapidly during the first 5 h from cells continuously exposed to a partially purified IgG fraction from As-D. Subsequently, the rate of loss remained almost unchanged and paralleled that recorded for cells exposed to IgG from Ns.

Intracellular Localization of Cathepsin D.—The localization of this enzyme was investigated immunocytochemically (Fig. 6). Cells treated with As-D Fab′ labeled with fluorescein exhibited an intense green staining associated

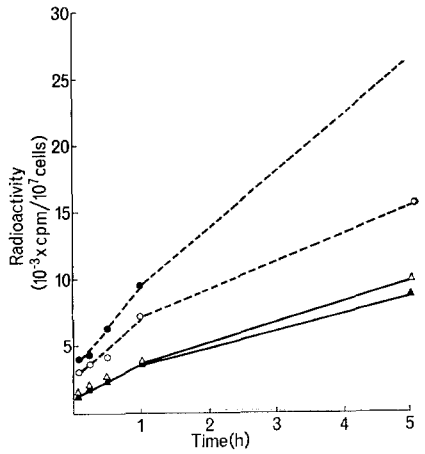


FIG. 4. The effect of As-D on the uptake of dextran compared with that of hemoglobin. Macrophages were incubated with [^{3}H]hemoglobin and Ns (○) or As-D (●), or [^{14}C]dextran and Ns (△) or As-D (▲). Cells were removed and washed at intervals. Total cellular ^{3}H and ^{14}C were determined. The results are expressed as means of four determinations.

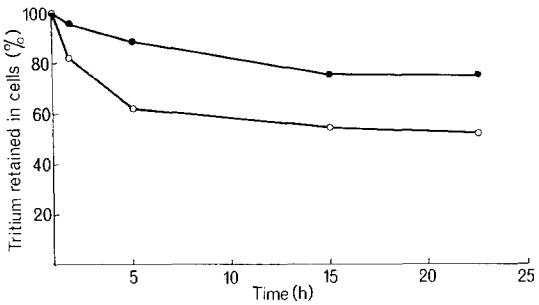


FIG. 5. Intracellular digestion of [^{3}H]hemoglobin. Macrophages were incubated for 3 h in BGJ_b containing 1% Ns and 5.5 mg partially purified IgG from Ns (○) or As-D (●). [^{3}H]-Hemoglobin was then introduced, and the incubation was continued for a further 3 h. Cells were washed and resuspended in fresh media of the original compositions, but without hemoglobin. Cells were incubated for varying periods, washed, and examined by scintillation counting. The total cellular radioactivity (means of three determinations) is expressed as a percentage of the zero-time value.

with cytoplasmic organelles. Some weak diffuse cytoplasmic staining was also observed. Nuclei were unstained.

The following controls were made to establish the specificity of staining. Cells were pretreated with unlabeled Ns Fab′ before incubation with labeled As-D Fab′; they exhibited unchanged staining patterns. Cells were also pretreated with unlabeled As-D Fab′ before incubation with labeled Fab′. They exhibited virtually no staining (too weak to photograph), indicating the specific attachment of labeled antibody Fab′ molecules to cell-bound cathepsin D.

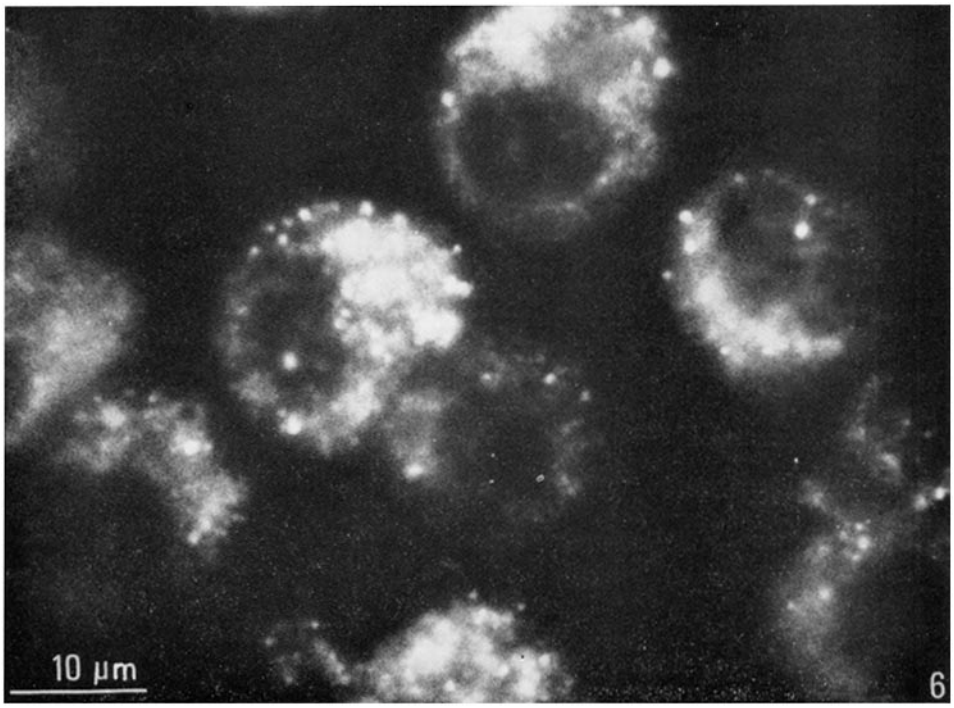


FIG. 6. Intracellular localization of cathepsin D in rabbit alveolar macrophages incubated with Ns for 5 h. Smears were treated with Fab′ from IgG of As-D or Ns, followed by fluorescein-conjugated Fab′ from IgG of As-D. Virtually no fluorescence was exhibited by cells pretreated with As-D Fab′ (too weak to photograph); all cells pretreated with Ns Fab′ showed fluorescence in the cytoplasm, both in diffuse and particulate localizations.

Relative Intracellular Localizations of IgG and Fluorescein-Labeled Hemoglobin.—Macrophages were exposed to lissamine rhodamine-labeled hemoglobin and partially purified IgG from As-D or Ns. The results (Fig. 7 *a*, *b*) indicate that both proteins were endocytosed and accumulated in cytoplasmic vacuoles in 88–97% of the cells. Often, the fluorescent emissions of rhodamine and fluorescein were observed within the same vacuoles (Fig. 8 *a*, *b*), but vacuoles in which only rhodamine was detected were also present.

Relative Intracellular Localizations of Cathepsin D and Fluorescein-Labeled Hemoglobin.—It can be seen from Fig. 9 that many of the vacuoles containing fluorescein also were stained for cathepsin D, and thus resembled secondary lysosomes.

Loss of IgG from Cells.—Cells were allowed to accumulate IgG for 5 h from media containing 1 mg/ml of IgG (determined by radial immunodiffusion) partially purified from either As-D or Ns. They were then washed, maintained in the absence of sheep IgG, and examined at varying time intervals. Initially,

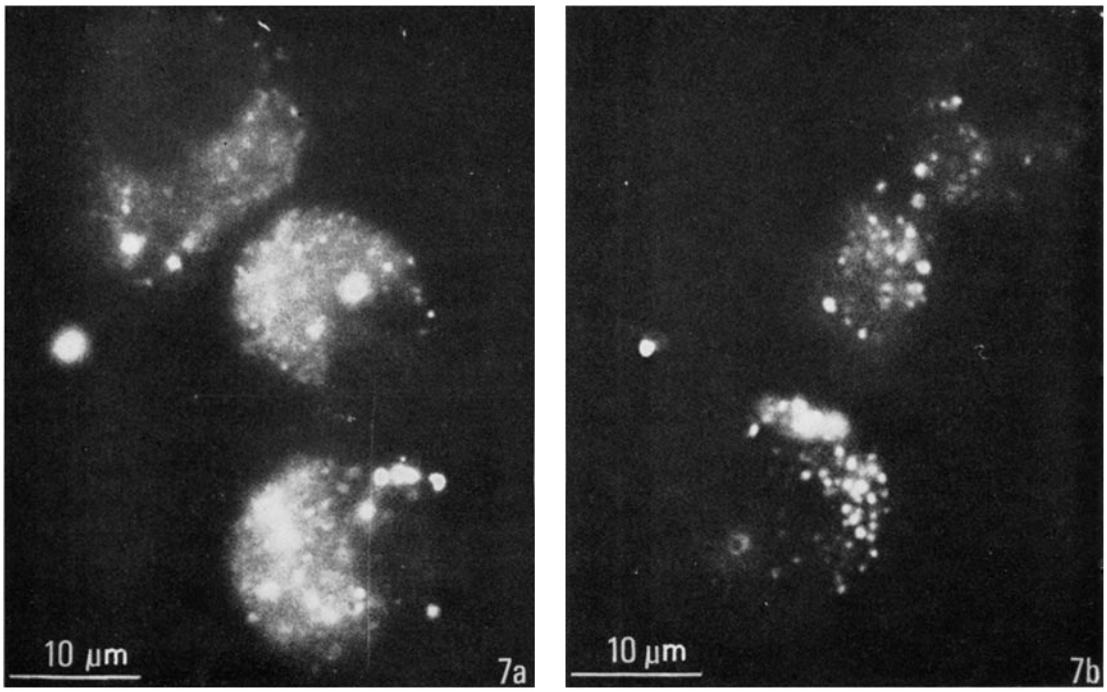
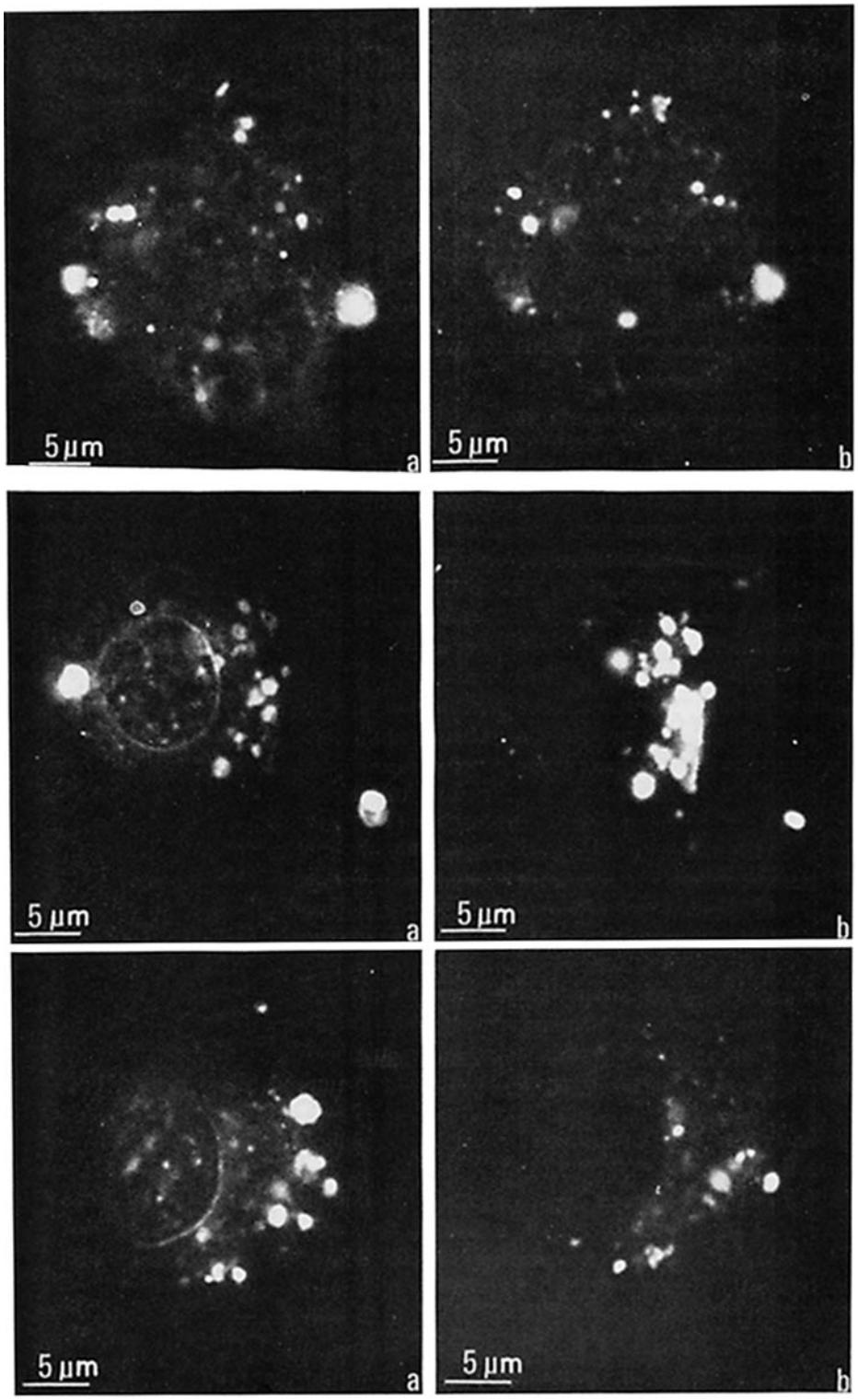


FIG. 7. Intracellular localizations of (*a*) hemoglobin and (*b*) sheep IgG. Macrophages were incubated for 5 h in BGJ_5 medium with 1 mg partially purified IgG from Ns/ml and with hemoglobin labeled with lissamine rhodamine at a concentration of 0.5 mg/ml. Cells were washed, smeared, fixed, and stained with fluorescein-labeled rabbit anti-(sheep IgG) IgG. Preparations were examined for both fluorochromes. The fluorochromes showed particulate cytoplasmic localizations.

90% of cells (436 examined) exhibited particulate cytoplasmic staining for IgG or antigenically active fragments. After 3 h, 23% of the cells previously exposed to IgG from Ns stained for IgG (172 cells examined), whereas 45% of those previously exposed to IgG from As-D (183 examined) still exhibited particulate cytoplasmic staining for IgG. Similar differences were observed in other experiments.

FIG. 8. Localizations of (*a*) sheep IgG and (*b*) hemoglobin in three individual cells prepared as for Fig. 7 and examined for both fluorochromes. Each field was first examined for (*a*) fluorescein fluorescence, because this was more rapidly quenched, and then for (*b*) lissamine rhodamine fluorescence. Fluorescein fluorescence was not detected with lissamine rhodamine filtration, and vice versa. The fluorochromes are often observed in the same organelles. Neither red nor green fluorescence was detected in control cell preparations that contained neither hemoglobin nor IgG, respectively. Autofluorescent emission was too weak to photograph.


5 µm
a
5 µm
b
5 µm
a
5 µm
b
5 µm
a
5 µm
b

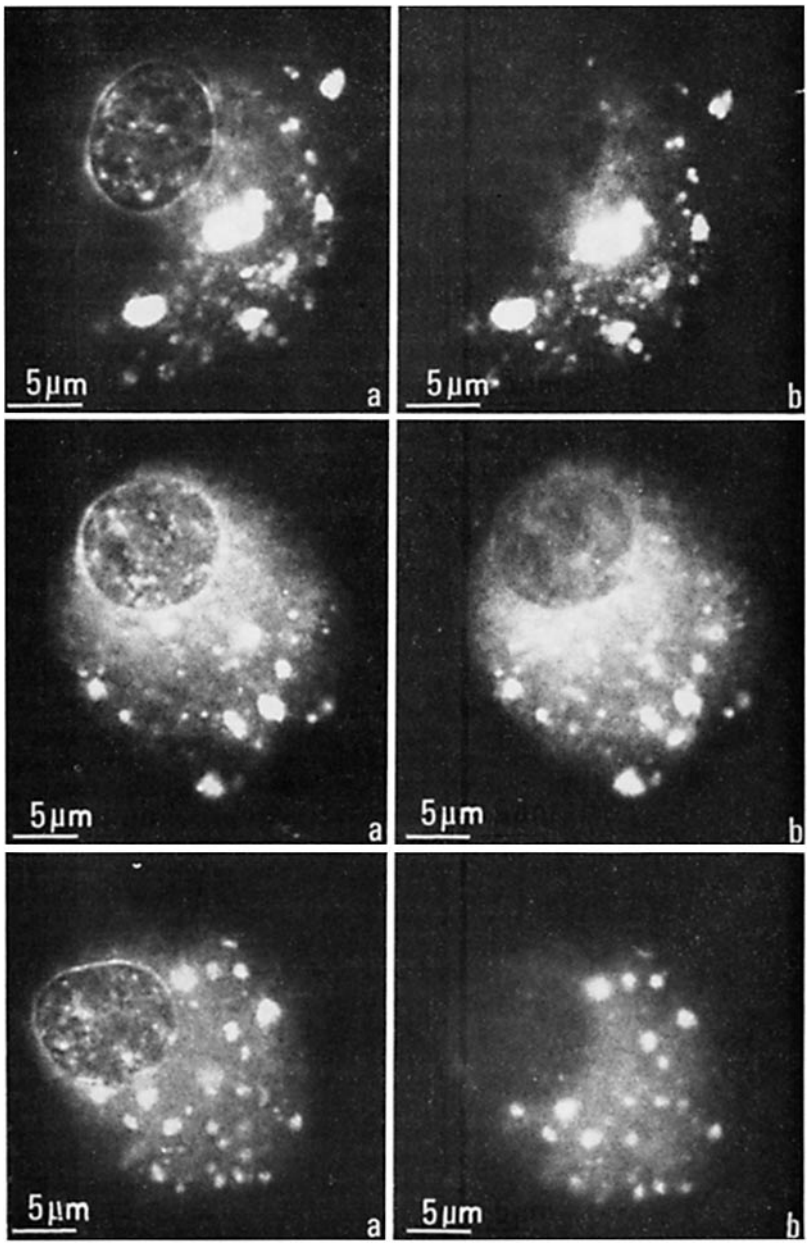


FIG. 9. Localizations of (*a*) cathepsin D and (*b*) hemoglobin in three individual cells. Macrophages were incubated, prepared for staining, and examined as described in Figs. 7 and 8, except that they were stained with fluorescein-labeled Fab′ from As-D, instead of anti-(sheep IgG). Controls similar to those described in Fig. 8 were run. It can be seen that most organelles display both types of fluorescence.

Electron Microscopy of Cells Exposed to Hemoglobin, As-D, and Ns.—Freshly isolated macrophages were characterized by villus-like projections from the cell surface, prominent Golgi regions, large numbers of mitochondria, and some granules, with homogeneous contents and dense bodies; occasional vacuoles were seen (Fig. 10 *a*). After incubation for 5 h with As-D and hemoglobin, approximately 75% of the macrophages exhibited massive vacuolation (Fig. 10 *b*). Inside some vacuoles, membranous whorls were seen; others were relatively free of electron-opaque material. Some vacuoles containing peripherally arranged electron-opaque material were also observed.

By contrast, cells that had been incubated in Ns and hemoglobin had some electron-opaque granules and numerous vacuoles that contained peripherally arranged electron-opaque material (Fig. 10 *c*). Vacuoles were smaller and far fewer in number than in cells incubated with As-D. Subsequent incubation of As-D-treated cells in Ns medium resulted, within 2 h, in a significant decrease in vacuolation and the appearance of numerous homogeneous granules, small vacuoles containing electron-opaque material peripherally arranged, and dense bodies.

DISCUSSION

This work was done in order to test the hypothesis that cathepsin D plays a role in the digestion of proteins endocytosed by macrophages, and that this digestion occurs within secondary lysosomes. Previous work, which has made use of cell fractionation techniques, has provided support for this idea; but the availability of specific antisera to cathepsin D has now made possible the direct examination of protein digestion in the living cells.

Rabbit alveolar macrophages were allowed to take up radiochemically labeled proteins, and the conversion of these by macrophages to TCA-soluble fragments was followed during incubation of the cells with normal serum or antiserum to rabbit cathepsin D. Marked inhibition of digestion of both hemoglobin and cartilage proteoglycan was obtained in the presence of the antiserum, and similar results were obtained with IgG prepared from the sera. Since it was shown that the antiserum had no effect on the rate of endocytosis by the cells, the results were interpreted as showing that cathepsin D contributes to the digestion of these macromolecules in the living cell.

The locus of the intracellular digestion susceptible to immunoinhibition was sought by immunocytochemical methods. Cathepsin D was detected within cytoplasmic organelles with the size, shape, and distribution characteristic of secondary lysosomes, as has been found previously (5). The majority of these organelles also contained endocytosed hemoglobin, and some contained sheep IgG. These findings are fully consistent with the hypothesis that digestion of endocytosed proteins by cathepsin D occurs within secondary lysosomes.

The inhibition of intracellular digestion by antiserum to cathepsin D was incomplete. This is partly accounted for by the observed absence of sheep antibody

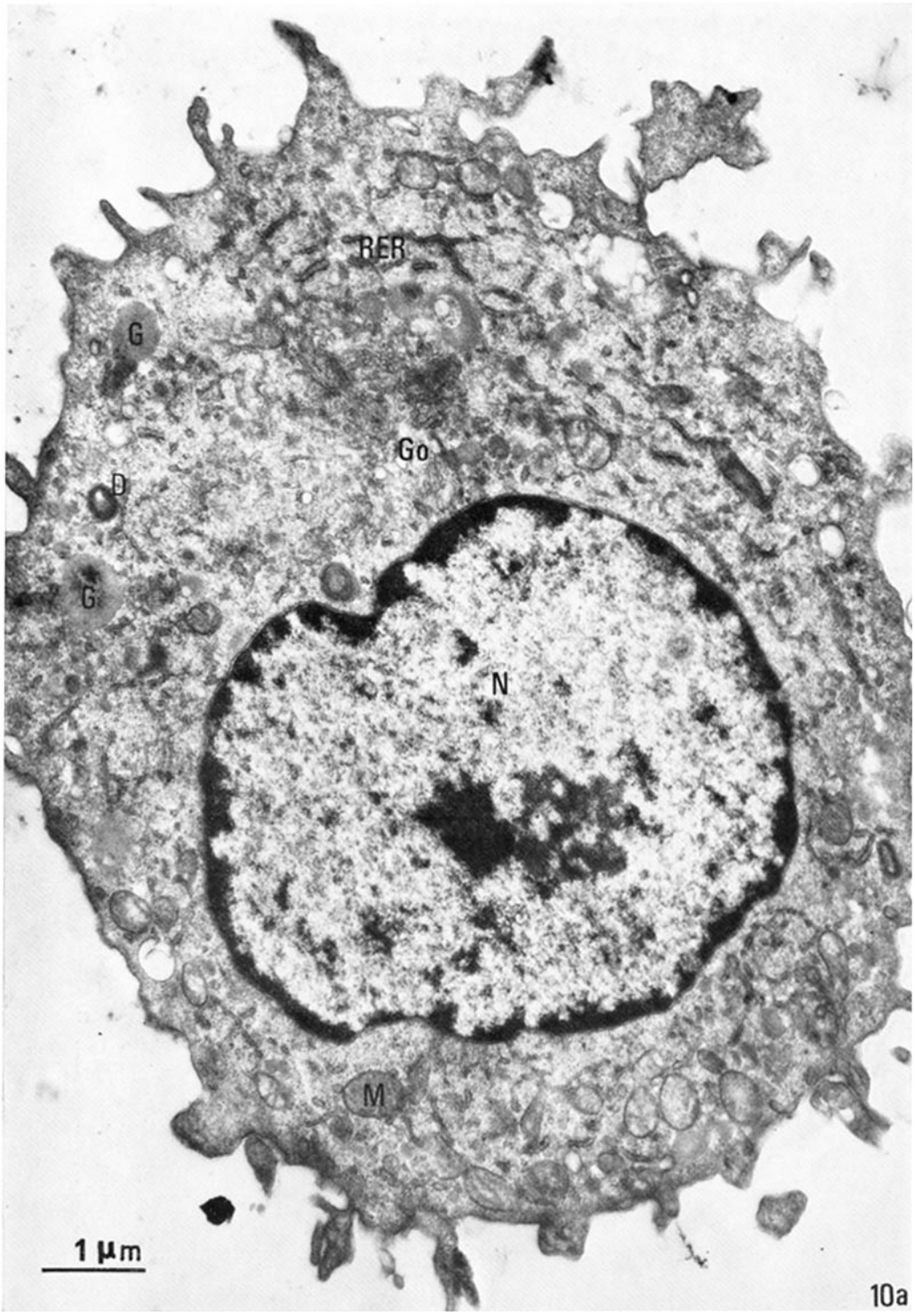


FIG. 10. Macrophage ultrastructure; ultrathin sections stained with uranyl acetate and lead citrate (see Methods). *N*, nucleus; *M*, mitochondrion; *RER*, rough endoplasmic reticulum; *Go*, Golgi apparatus.

FIG. 10 *a*. Alveolar macrophage immediately after isolation from rabbit lung containing granules (*G*) and dense bodies (*D*).

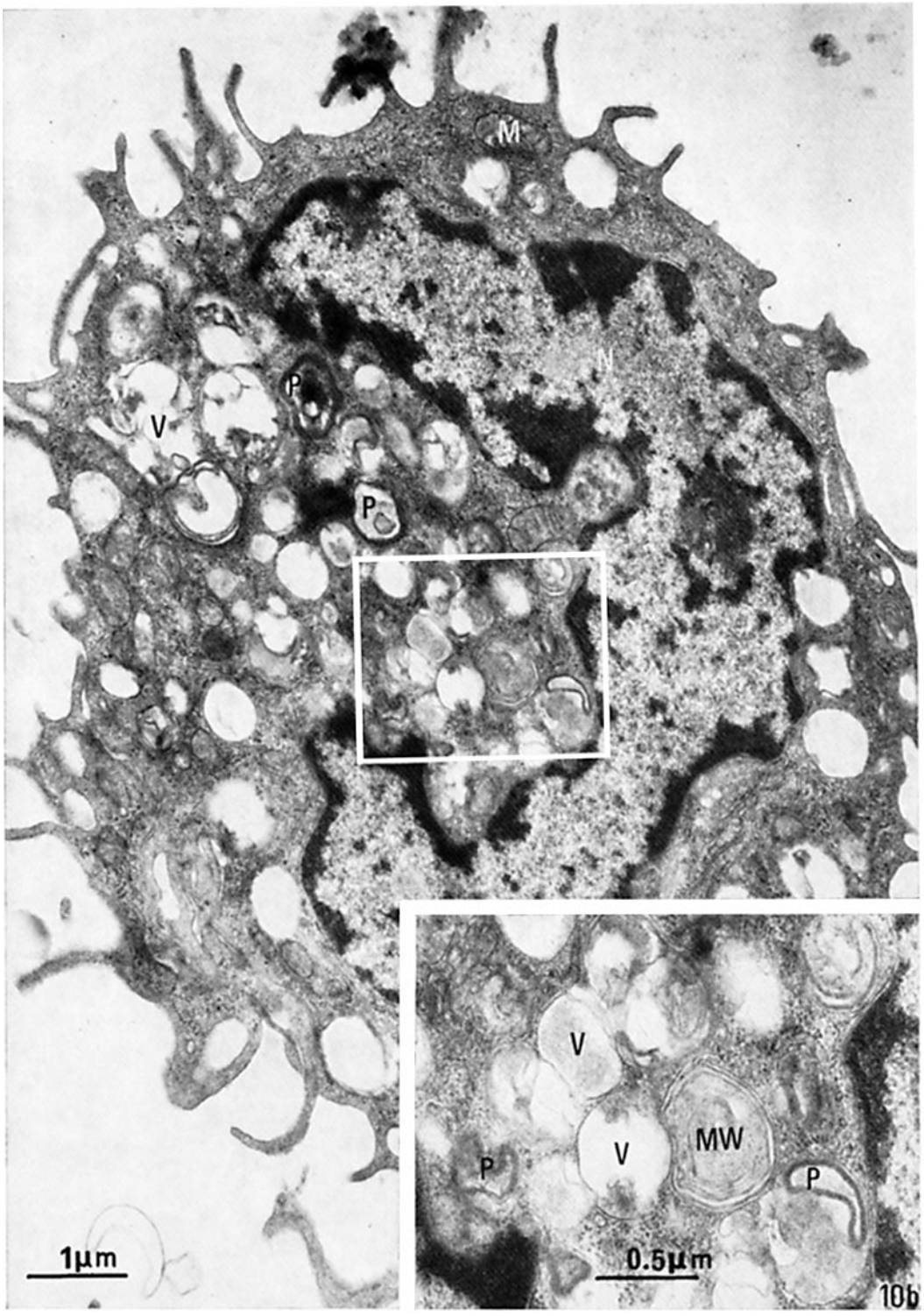


FIG. 10 *b*. Alveolar macrophage after 5 h of incubation with As-D and hemoglobin. Many large vacuoles (*V*) can be seen, some of which contain membranous whorls (*MW*). Occasional vacuoles (*P*) containing peripheral electron-opaque material were also present.

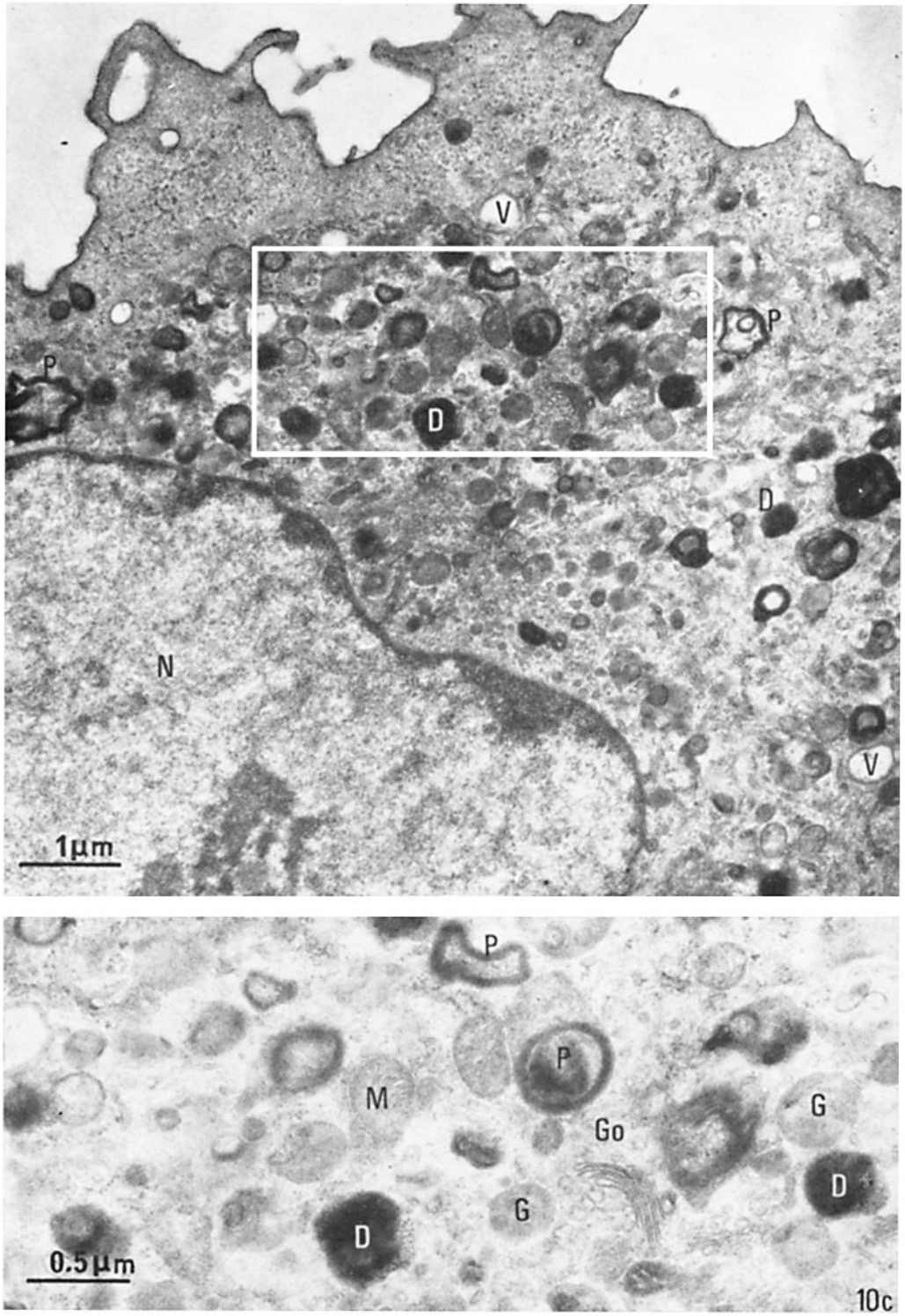


FIG. 10 *c*. Alveolar macrophage after 5 h of incubation with Ns and hemoglobin. The cells contain cytoplasmic granules (*G*), dense bodies (*D*), and numerous vacuoles with peripheral electron-opaque inclusions (*P*). Relatively few empty vacuoles (*V*) were seen.

from some vacuoles containing substrate,[2] so that digestion is likely to have continued normally in a proportion of the organelles. Since, in the living cell, the flow of cathepsin D from primary to secondary lysosomes presumably continued during the experiments, the large excess of antibody necessary for complete inhibition (4) may not have been maintained. It should be remembered that cells accumulated only sufficient antibody to produce, at most, 50% inhibition of the total cellular cathepsin D activity.

The results do not exclude the possibility that proteolytic enzymes other than cathepsin D are also involved, acting either separately or synergistically with cathepsin D. Indeed, the poor inhibition of digestion of casein by antiserum to cathepsin D suggests that some other enzyme can bring about the catabolism of this protein.

There has been much discussion concerning the pH within secondary lysosomes. Since, in vitro, immunoinhibition of cathepsin D is reduced at pH 4 and absent at pH 3 (4), our results indicate a lower limit for this pH. Because of the acidic pH optimum of almost all lysosomal enzymes (23), and especially the negligible activity of cathepsin D against hemoglobin near neutral pH, pH 6.5 would probably represent an upper limit for the pH within these organelles.

The immunoinhibition of digestion of hemoglobin within secondary lysosomes was correlated with characteristic ultrastructural changes in the cells. The cytoplasmic vacuoles of cells incubated with hemoglobin and Ns, which contained electron-opaque peripheral aggregates, resembled those of macrophages engaged in digestion of erythrocytes in splenic pulp (24). Daems (24) thought that the aggregates were a product of the digestion of hemoglobin, possibly hemosiderin. By contrast, cells exposed to As-D contained fewer such organelles and hardly any dense bodies, presumably as a result of an inhibition of protein digestion. The appearance within these cells of large vacuoles with few recognizable contents may also be directly related to an inhibition of protein digestion; the vacuoles resembled those reported for rat fibroblasts cultured in the presence of an anti-rat lysosome serum (25).

The other class of large vacuoles seen in As-D-cultured cells, which contained membranous whorls, may represent an autophagic response of the cells to an impairment of protein digestion. The large vacuoles resembled in ultrastructure those that appeared in mouse peritoneal macrophages within 15 min of exposure to chloroquine (26); they also resembled autophagic vacuoles of cells of animals either starved or given inhibitors of protein synthesis (27).

The disappearance of both types of vacuoles when the antiserum was removed was correlated with the increased protein digestion measured biochemically. Some of the characteristics of the macrophages subjected to intravacuolar

---

[2] The difference in uptake of IgG and hemoglobin may be accounted for by the presence of an Fc receptor site at the surface of these cells, as has been demonstrated for other macrophages (21, 22).

immunoinhibition of cathepsin D were reminiscent of cells from persons suffering from genetic deletion of a lysosomal enzyme. This suggests that immunoinhibition of lysosomal enzymes in other cell types may provide model systems for the study of storage diseases.

This immunoenzymic investigation of cathepsin D activity has demonstrated that the enzyme plays a role in the digestion of proteins within lysosomes in living cells that probably varies in importance depending on the substrate. The use of antisera to the other major tissue proteinases should lead to a more complete understanding of intralysosomal proteolytic activity.

SUMMARY

Specific anti-(rabbit cathepsin D) serum, previously shown to inhibit cathepsin D, arrested the intracellular digestion of sheep IgG and radiochemically labeled hemoglobin and proteoglycan in rabbit alveolar macrophages. In the presence of antiserum, cells remained viable, but became very vacuolated. Both sheep IgG and hemoglobin were demonstrated immunocytochemically in vacuoles most of which could also be shown to contain cathepsin D. When the antiserum was removed, cells regained their normal morphology, and digestion of endocytosed proteins returned to normal. These results indicate that cathepsin D can be inhibited within lysosomes of viable cells, in which it plays a major role in the intracellular digestion of certain proteins.

We wish to thank Mr. P. E. N. Martin, Mrs. B. Wright, Miss C. Camus, and Mr. R. Parker for their technical assistance. Mr. M. Applin assisted in the preparation of the figures. We thank Dr. Audrey M. Glauert for her advice in preparing the electron micrographs.

REFERENCES

1. Jacques, P. J. 1969. Endocytosis. *In* Lysosomes in Biology and Pathology. J. T. Dingle and H. B. Fell, editors. North-Holland Publishing Co., Amsterdam. **2**:395.
2. Ehrenreich, B. A., and Z. A. Cohn. 1968. Fate of hemoglobin pinocytosed by macrophages *in vitro*. *J. Cell Biol.* **38**:244.
3. Barrett, A. J. 1970. Cathepsin D: purification of isoenzymes from human and chicken liver. *Biochem. J.* **117**:601.
4. Dingle, J. T., A. J. Barrett, and P. D. Weston. 1971. Cathepsin D: characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. *Biochem. J.* **123**:1.
5. Poole, A. R., J. T. Dingle, and A. J. Barrett. 1972. The immunocytochemical demonstration of cathepsin D. *J. Histochem. Cytochem.* **20**:261.
6. Dingle, J. T., H. B. Fell, and R. R. A. Coombs. 1967. The breakdown of embryonic cartilage and bone cultivated in the presence of complement-sufficient antiserum. *Int. Arch. Allergy Appl. Immunol.* **31**:283.
7. Sajdera, S. W., and V. C. Hascall. 1969. Proteinpolysaccharide complex from bovine nasal cartilage. A comparison of low and high shear extraction procedures. *J. Biol. Chem.* **244**:77.
8. Aarlund, O., J. W. Osebuld, and F. A. Murphy. 1965. Isolation and characterisation of ovine gamma globulins. *Arch. Biochem. Biophys.* **109**:142.

9. Little, J. R., and H. Donahue. 1968. Spectral properties of proteins and small molecules of immunological interest. *Methods Immunol. Immunochem.* **2**:343.
10. Mancini, G., A. O. Carbonera, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2**:235.
11. Nisonoff, A., F. C. Wissler, L. N. Lipman, and D. C. Woernly. 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem. Biophys.* **89**:230.
12. The, T. H., and T. E. W. Feltkamp. 1970. Conjugation of fluorescein isothiocyanate to antibodies. II. A reproducible method. *Immunology.* **18**:875.
13. Goldman, M. 1968. Fluorescent Antibody Methods. Academic Press, Inc., New York. 110.
14. Nairn, R. C. 1969. Fluorescent Protein Tracing. E. & S. Livingstone Ltd., Edinburgh. 3rd edition.
15. Tabor, H. 1955. Acetylation of amines with pigeon liver enzyme. *Methods Enzymol.* **1**:608.
16. Myrvik, Q. N., E. S. Leake, and S. Oshima. 1970. A study of macrophages and epithilioid-like cells from granulomatous (BCG-induced) lungs of rabbits. *J. Immunol.* **89**:745.
17. Hall, T. C., and E. C. Cocking. 1965. High-efficiency liquid scintillation counting of ^{14}C-labelled material in aqueous solution and determination of specific activity of labelled proteins. *Biochem. J.* **96**:626.
18. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**:137A.
19. Glauert, A. M. 1965. The fixation and embedding of biological specimens. *In* Techniques for Electron Microscopy. D. Kay, editor. Blackwell Scientific Publications Ltd., Oxford. 2nd edition. 166.
20. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
21. Huber, H., M. J. Polley, W. D. Linscott, H. H. Fudenberg, and H. J. Müller-Eberhard. 1968. Human monocytes. Distinct receptor sites for the third component of complement and for immunoglobulin G. *Science (Wash. D.C.).* **162**:1281.
22. Lo Buglio, A. F., R. S. Cotron, and J. H. Jandl. 1967. Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. *Science (Wash. D.C.).* **158**:1582.
23. Barrett, A. J. 1972. Lysosomal enzymes. *In* Lysosomes: a Laboratory Handbook. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 46.
24. Daems, W. Th. 1970. Discussion following "A biochemical study of lysosomes in lymphoid tissues," by W. E. Bowers. *In* Mononulear Phagocytes. R. van Furth, editor. F. A. Davis Company, Philadelphia. 96.
25. Tulkens, P., A. Trouet, and F. van Hoof. 1970. Immunological inhibition of lysosome function. *Nature (Lond.).* **228**:1282.
26. Fedorko, M. E., J. G. Hirsch, and Z. A. Cohn. 1968. Autophagic vacuoles produced *in vitro*. I. Studies on cultured macrophages exposed to chloroquine. *J. Cell Biol.* **38**:377.
27. Swift, H., and Z. Hruban. 1964. Focal degradation as a biological process. *Fed. Proc.* **23**:1026.