EFFECTS OF VARIOUS PROTEASE INHIBITORS ON PHAGOCYTOSIS OF ANTIGEN-ANTIBODY COMPLEX BY MACROPHAGES*

ICHIRO FUKAZAWA** AND JIRO KOYAMA***

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Hokkaido, 060, Japan

(Received April 12, 1982)

In order to characterize proteases of macrophages involved in phagocytosis, the effects of various protease inhibitors were investigated in measuring the uptake and digestion of *Bacillus subtilis* α -amylase complex of guinea pig IgG2 antibody by homologous peritoneal macrophages. The inhibitory effects were observed with diisopropyl fluorophosphate (DFP), L-1-*p*-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), antipain, chymostatin, elastatinal and leupeptins, but not with pepstatins. DFP and TPCK reduced both the amounts of antigen-antibody (Ag-Ab) complex taken up by macrophages and of digested products released from the cells. TLCK, antipain, chymostatin, elastatinal and leupeptins also reduced the amount of digested products, but increased that of Ag-Ab complex associated with macrophages.

These results suggest that the inhibitory mechanism of DFP and TPCK toward the digestion of soluble Ag-Ab complex by macrophages differs from those of other inhibitors examined.

Keywords—macrophage; phagocytosis; diisopropyl fluorophosphate; L-1-*p*-tosylamido-2-phenylethyl chloromethyl ketone; 1-chloro-3-tosylamido-7-amino-L-2-heptanone; antipain; chymostatin; elastatinal; leupeptins

INTRODUCTION

The first step of phagocytosis of antigen-antibody (Ag-Ab) complex is the binding of complex to Fc receptors on the cell surface of phagocytes. The complex bound is taken up into the cells and then digested by the intracellular enzymes. With the development of the lysosomal concept, the processes governing the intracellular digestion of ingested Ag-Ab complex have been clarified.¹⁻³⁾ The mechanism which initiates the ingestive process has, however, remained to be solved. Through the use of many potent inhibitors of serine proteases, Perlman *et al.*⁴⁾ and Musson and Becker⁵⁾ presented evidence that certain serine proteases are essential for the ingestive process of

phagocytosis in neutrophils. Nagai *et al.*⁶⁾ also observed using sensitized sheep erythrocytes that a certain chymotrypsin-like protease susceptible to inhibition with diisopropyl fluorophosphate (DFP), L-1-*p*-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and chymostatin may be involved in the ingestive activity of macrophages.

In order to identify further the possible protease operative in the phagocytosis, the effects of various protease inhibitors with known specificities were determined by measuring the uptake and digestion of *Bacillus subtilis* α -amylase complex of guinea pig IgG2 antibody by homologous peritoneal macrophages. In this paper, the results are presented, suggesting that certain protease(s)

* The abbreviations used are; DFP, diisopropyl fluorophosphate; TPCK, L-1-*p*-tosylamido-2-phenylethyl chloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; B α A, *Bacillus subtilis* α -amylase; Ag-Ab complex, antigen-antibody complex.

** Present address: Medical Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan.

*** To whom correspondence should be addressed.

inactivated with DFP and TPCK is involved even in the ingestive process of soluble Ag-Ab complex. In addition, the effects of 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), antipain, chymostatin, elastatinal, leupeptins and pepstatins are reported.

MATERIALS AND METHODS

Protease Inhibitors — DFP, TPCK and TLCK were purchased from Sigma Chemical CO. (U.S.A.). Various protease inhibitors from actinomycete fermentation, *i.e.*, antipain, chymostatin, elastatinal, leupeptins and pepstatins were kindly supplied by the Institute for Microbial Chemistry, Tokyo, Japan.⁷⁾

Antibody — The Ag-Ab complex used as a stimulus was *Bacillus subtilis* α -amylase ($B\alpha A$, EC 3.2.1.1) complex of guinea pig IgG2 antibody. The IgG2 antibody was specifically purified from hyperimmune serum obtained by immunization of guinea pigs with crystalline $B\alpha A$. The IgG2 was isolated from the serum by fractionation with 40% ammonium sulfate followed by DEAE-cellulose chromatography.⁸⁾ Specific purification of anti- $B\alpha A$ antibody from the IgG2 was performed using a column of the antigen-bound Sepharose 6B which was prepared by the CNBr method.^{9,10)}

Labeling of the antibody with radioactive iodine, ^{125}I , was performed by the IC1 method.⁸⁾ Some aggregated antibody molecules formed during the purification and labeling were removed by gel filtration on a column of Sephadex G-200.

Estimation of Protein — The absorbance at 280 nm was used for calculation of protein concentration. The values of $A_{1\text{cm}}^{1\%}$ were taken as 13.5 for IgG and 25.3 for $B\alpha A$, respectively.⁸⁾

Isolation and Culture of Macrophages — The procedures for harvesting and culturing guinea pig peritoneal macrophages were essentially the same as those described by Shinomiya and Koyama.¹¹⁾ Peritoneal cells were isolated from guinea pigs of the Hartley strain which were intraperitoneally injected with liquid paraffin four days before harvest. The cells were collected from the peritoneal cavity and washed with the

MEM medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.¹²⁾ The cells were cultured in plastic dishes (Falcon, 35 $\times$ 10 mm), using the MEM medium supplemented with 5% fetal calf serum. After incubation for 18 h at 37°C in a CO₂-air incubator, the cells adhering on the dishes were washed twice with the MEM medium containing 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid to remove the non-adherent cells. The cells thus isolated were used as macrophages since more than 95% of the cells were typical, well-spread macrophages forming an interrupted monolayer. The amount of macrophage cells was estimated by direct counting of cells or by measuring the amount of cellular proteins by the Folin reagent.

Measurement of Uptake and Digestion of Ag-Ab Complex by Macrophages — The measurement of uptake and digestion of Ag-Ab complex was performed as described by Shinomiya and Koyama.¹¹⁾ Macrophages adhering on dishes were incubated with $B\alpha A$ complex of ^{125}I -labeled IgG2 antibody (20 μ g) in the MEM medium supplemented with 0.1% bovine serum albumin, which had been added to prevent non-specific adsorption of the complex to the dishes or the cells. After incubation at 37°C in a CO₂-air incubator, the uptake and digestion of Ag-Ab complex were measured.

To measure the digestion of Ag-Ab complex, the culture supernatants were centrifuged at 4°C for the removal of some contaminating cells and then fractionated by addition of an equal volume of 10% trichloroacetic acid. The amount of digested products released into the culture medium was estimated by measuring the radioactivity of the trichloroacetic acid-soluble fraction (non-protein-bound ^{125}I).

The cell monolayer was washed twice with the MEM medium and the cellular materials were extracted with 0.1 N NaOH by gently scraping the cells from the dishes with a rubber policeman. After addition of 5 mg bovine serum albumin, the cell-associated ^{125}I -labeled materials were fractionated to the protein-bound and non-protein-bound ^{125}I with 5% trichloroacetic acid.

On the basis of specific radioactivity of antibody and number of cells contained in each dish, each amount of ^{125}I -labeled materials was expressed in terms of μg antibody processed by 1×10^7 cells. Each experiment was run in duplicate and repeated two or three times.

Measurement of Binding of Ag-Ab Complex to Macrophages — The binding reaction of Ag-Ab complex to macrophages was measured by essentially the same method as described by Leslie and Cohen.¹³⁾ Macrophages on dishes were detached by flushing with the MEM medium. Duplicate reaction mixtures containing 2×10^6 cells and various amounts of B α A complex of ^{125}I -labeled IgG2 antibody in 1.0 ml the MEM medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 0.1% bovine serum albumin and 0.02% sodium azide were incubated with constant rotation for 1 h at 4°C.

After incubation, the test tubes containing the

reaction mixtures were centrifuged and the cells precipitated were washed three times with the same medium. The radioactivity of cells and supernatants was measured and used for calculation of each amount of cell-bound and free Ag-Ab complexes. An apparent association constant and number of Fc receptors per cell were estimated by Scatchard plot, as described by others.^{13,14)}

Materials — The crystalline B α A used as an antigen was purchased from Seikagaku Kogyo Co. (Tokyo). Cytochalasin B and the MEM medium used were purchased from Sigma Chemical Co. (U.S.A.) and Nissui Seiyaku Co. (Tokyo), respectively.

RESULTS

Uptake and Digestion of Ag-Ab Complex

The uptake and digestion of B α A complex of IgG2 antibody by macrophages were measured,

FIG. 1. Effects of 5 mM DFP and 100 $\mu\text{g}/\text{ml}$ Antipain on Phagocytosis of Ag-Ab Complex by Macrophages. Macrophages were incubated with antigen complex of ^{125}I -labeled IgG2 antibody in the absence (—) or presence (---) of DFP or antipain. After varying incubation periods at 37°C, the amounts of protein-bound ^{125}I (○) and non-protein-bound ^{125}I (△) associated with cells, and non-protein-bound ^{125}I (●) released into medium were determined and expressed by μg of IgG2 antibody processed by 1×10^7 cells.

using the complex of ^{125}I -labeled IgG2 antibody prepared at a molar antigen to antibody ratio of 0.7 (an equivalence point of the precipitin reaction); the complex with this ratio was most easily incorporated and digested by macrophages among other complexes with different ratios.

As in the case of ovalbumin-antibody complexes,¹¹⁾ the release of digested ^{125}I -labeled materials into culture medium continued during the entire course of incubation (Fig. 1). The amount of protein-bound ^{125}I associated with macrophages, on the other hand, reached a constant level 2 h after exposure of the cells to Ag-Ab complex. The absence of immune precipitation of the complex was confirmed by the low level of radioactivity retained in cell-free controls. Hence the amount of protein-bound ^{125}I associated with cells was assumed to represent the combined amount of Ag-Ab complex bound to Fc receptors on the cell surface and of that ingested into the cells but still undigested. Non-protein-bound ^{125}I was also detectable in the cells, but its amount was markedly less than that of protein-bound ^{125}I .

These results indicate that the binding reaction of Ag-Ab complex to macrophages proceeds relatively fast that protein-bound radioactivity associated with cells reaches the maximum level within 2 h. In addition, the rate-limiting step of elimination of Ag-Ab complex appears to be either ingestion of the complex bound to Fc receptors or intracellular digestion of the ingested complex, but not the release of digested ^{125}I -labeled materials from the cells.

Inhibition of Digestion of Ag-Ab Complex by Various Protease Inhibitors

The effects of various protease inhibitors on intracellular digestion of Ag-Ab complex by macrophages were investigated by measuring the amount of digested ^{125}I -labeled materials released from the cells. For the purpose, DFP, TPCK, TLCK, antipain, chymostatin, elastatinal, leupeptins and pepstatins were used since the inhibitory activities of these inhibitors have been extensively studied.^{7,15)}

DFP was dissolved in 2-propanol immediately before addition to reaction mixtures. TPCK and

chymostatin were dissolved in dimethylsulfoxide and added to reaction mixtures. Other inhibitors were added by dissolving in water. The final concentrations of 2-propanol and dimethylsulfoxide in reaction mixtures were 2 and 5%, respectively. At each concentration used, neither 2-propanol nor dimethylsulfoxide affected significantly phagocytic activity of macrophages (Fig. 2).

Inhibition of the digestion of Ag-Ab complex was observed with all the inhibitors except pepstatins, when the effects were estimated after incubation for 4 h at 37°C (Fig. 2). Marked inhibition occurred with DFP, TPCK, TLCK, antipain and leupeptins. Chymostatin and elastatinal were less effective. No effect was observed with

FIG. 2. Effects of Various Inhibitors on Digestion of Ag-Ab Complex by Macrophages

Macrophages were incubated with antigen complex of ^{125}I -labeled IgG2 antibody in the presence of 5 mM DFP (I), 50 $\mu\text{g}/\text{ml}$ TPCK (II), 100 $\mu\text{g}/\text{ml}$ TLCK (III), antipain (IV), chymostatin (V), elastatinal (VI), leupeptins (VII) or pepstatins (VIII), respectively. After incubation for 4 h at 37°C, inhibitory effects were determined by measuring the amounts of non-protein-bound ^{125}I released into medium. The effects of 2% 2-propanol (IX) and 5% dimethylsulfoxide (X) used for dissolving DFP, TPCK and chymostatin, respectively, were also determined.

pepstatins.

The inhibitory effects of these inhibitors were dependent upon concentration of each inhibitor, as illustrated by the inhibition profiles of DFP and antipain (Fig. 3). When cell viability was measured by the lactate dehydrogenase method,¹⁶⁾ the maximum concentration of each inhibitor used did not significantly reduce the viability of macrophages; more than 85% of the cells was still viable even after incubation for 4 h at 37°C, as in the case of absence of any inhibitor. Hence the inhibition with these inhibitors does not seem to be due to their cytotoxic effect, but to their direct action on certain proteases operating in the digestive process in macrophages.

Effects of Inhibitors on Uptake of Ag-Ab Complex by Macrophages

To identify the process of digestion of Ag-Ab complex inhibited with the inhibitors, the effects

on the amount of protein-bound ¹²⁵I associated with macrophages were measured. DFP and TPCK decreased the amount of protein-bound ¹²⁵I (Fig. 4). On the contrary, TLCK, antipain, chymostatin and leupeptins increased the amount of protein-bound ¹²⁵I. With elastatinal, a slight effect was observed.

In the presence of DFP, the amount of protein-bound ¹²⁵I associated with cells stayed at a lower level, independently of duration of incubation (Fig. 1). This lowering of the level was dependent upon concentration of the inhibitor (Fig. 3). With TPCK, essentially the same inhibition profile was obtained. This seems to be explained by that DFP and TPCK inhibit more strongly the ingestion of Ag-Ab complex than the intracellular digestion of ingested complex, causing the decrease in the amount of protein-bound ¹²⁵I associated with cells.

FIG. 3. *Inhibition Profiles of DFP and Antipain*

Macrophages were incubated with antigen complex of ¹²⁵I-labeled IgG2 antibody in the absence or presence of varying amounts of DFP or antipain, respectively. After incubation for 6 h at 37°C, the amounts of protein-bound ¹²⁵I (○) and non-protein-bound ¹²⁵I (△) associated with cells, and non-protein-bound ¹²⁵I (●) released into medium were determined and expressed by μg of IgG2 antibody processed by 1×10^7 cells.

In the case of antipain, the increase in the amount of protein-bound ^{125}I was dependent upon duration of incubation as well as concentration of the inhibitor; it increased as the incubation period was prolonged (Fig. 1) and concentration was increased (Fig. 3).

The inhibition profiles of TLCK, chymostatin and leupeptins were similar to that of antipain; they increased the amount of protein-bound ^{125}I associated with cells. Antipain as well as these inhibitors seem to inhibit the digestive function of macrophages by the mechanisms differing from that by DFP and TPCK, resulting in the intracellular accumulation of undigested complex.

Effects of DFP and TPCK on Binding of Ag-Ab Complex to Macrophages

The effects of DFP and TPCK were further studied by measuring the binding reaction of Ag-

Ab complex to Fc receptors on the cell surface of macrophages. For this purpose, B α A complex of ^{125}I -labeled IgG2 antibody prepared at a molar antigen to antibody ratio of 5.0 was incubated with macrophages in the presence of 0.02% sodium azide for 1 h at 4°C, since this incubation condition had been established for the measurement of binding reaction of Ag-Ab complex to Fc receptors of phagocytes by many studies.^{13,14,17,18)}

The binding curves obtained are shown in Fig. 5, where the amounts of bound complex on macrophages are plotted against those of complex added. As a control, the binding of B α A complex of F(ab')₂ fragment prepared by pepsin digestion of IgG2 antibody¹⁹⁾ was similarly determined. The amounts of the complex of F(ab')₂ fragment bound, however, were less than several percent of those of its IgG2 antibody counterpart, indicating that the binding of IgG2 antibody complex is

FIG. 4. *Effects of Various Inhibitors on the Amounts of Protein-bound ^{125}I Associated with Macrophages*

Macrophages were incubated with antigen complex of ^{125}I -labeled IgG2 antibody in the presence of 5mM DFP (I), 50 $\mu\text{g/ml}$ TPCK (II), 100 $\mu\text{g/ml}$ TLCK (III), antipain (IV), chymostatin (V), elastatinal (VI) or leupeptins (VII), respectively. After incubation for 4 h at 37°C, the amounts of protein-bound ^{125}I associated with cells were determined.

FIG. 5. *Effects of Inhibitors on the Binding of Ag-Ab Complex to Macrophages*

Macrophages were incubated with varying amounts of antigen complex of ^{125}I -labeled IgG2 antibody for 1 h at 4°C in the presence of 0.02% sodium azide. After incubation, the amounts of cell-bound complex were determined ($\circ$). The binding reaction was also determined in the presence of 10 mM DFP ($\bullet$), 100 $\mu\text{g/ml}$ TPCK ($\blacktriangle$) or 10 $\mu\text{g/ml}$ cytochalasin B ($\triangle$), respectively.

mediated by the Fc part of the antibody molecule.

Based on the binding curves, an apparent association constant and number of Fc receptors per cell were found to be $3.5 \times 10^7 \text{ M}^{-1}$ and 7.8×10^5 sites/cell, respectively. These values are of the same order of magnitude as those by others.^{13,14)}

Neither DFP nor TPCK affect the binding reaction since identical binding curves were obtained, independently of the presence or absence of the inhibitors. Thus, the inhibitory activities of these inhibitors may not direct to the binding of Ag-Ab complex to Fc receptors, but to other process involved in phagocytosis of the complex.

Effect of Cytochalasin B

Since cytochalasin B is known to inhibit phagocytic activity of phagocytes toward immune complexes,^{18,20)} it seems likely that cytochalasin B gives an inhibition profile similar to those of DFP and TPCK. In fact, it was found that cytochalasin B inhibited the digestion of Ag-Ab complex and concomitantly decreased the amount of protein-bound ^{125}I associated with cells (Fig. 6). Furthermore, it did not inhibit the binding reaction of Ag-Ab complex to Fc receptors (Fig. 5). These results suggest that DFP and TPCK inhibit more strongly the ingestion of Ag-Ab complex than the intracellular digestion of ingested complex.

DISCUSSION

The ingestion of Ag-Ab complex preceding the intracellular digestion by neutrophils is inhibited by many potent inhibitors of serine proteases.^{4,5)} With guinea pig macrophages, Nagai *et al.*⁶⁾ reported the involvement of a certain chymotrypsin-like protease in the ingestive process; the uptake of sensitized sheep erythrocytes by the cells is inhibited with DFP, TPCK and chymostatin, but not with TLCK, antipain, pepstatins, elastatinal and leupeptins.

The results presented in this paper show that DFP and TPCK decrease the amount of protein-bound ^{125}I associated with macrophages, as does cytochalasin B, and confirm that a certain

chymotrypsin-like protease may operate even in the ingestive process of soluble Ag-Ab complexes. Furthermore, the inhibitory effects of these two inhibitors was found to direct to any other process than the binding of immune complexes to Fc receptors on the cell surface.

The results with chymostatin, on the other hand, did not reveal the involvement of a chymotrypsin-like protease since it did not decrease the amount of protein-bound ^{125}I associated with macrophages, but rather increased it. This inhibition profile might be in conflict with that by Nagai *et al.*⁶⁾ Chymostatin also may,

FIG. 6. An Inhibition Profile of Cytochalasin B
Macrophages were incubated with antigen complex of ^{125}I -labeled IgG2 antibody in the presence of varying concentrations of cytochalasin B. After incubation for 4 h at 37°C , the amounts of protein-bound ^{125}I (○) and non-protein-bound ^{125}I (△) associated with cells, and non-protein-bound ^{125}I (●) released into medium were determined and expressed by μg of IgG2 antibody processed by 1×10^7 cells.

however, inhibit the ingestion of Ag-Ab complex, but do more strongly the intracellular digestion of the complex, resulting in the increase in the amount of protein-bound ^{125}I associated with macrophages; chymostatin is known to inhibit strongly many lysosomal proteases [cathepsin A (EC 3.4.12A.1), B (EC 3.4.22.1), D (EC 3.4.23.5) and L (EC 3.4.22.15).^{21,22}]

TLCK, antipain, elastatinal and leupeptins also were found to inhibit the digestion of Ag-Ab complex. These inhibitors are known to inhibit many lysosomal proteases^{7,15,23}; for instance, cathepsin A is inhibited with antipain,²⁴ cathepsin B with TLCK,²⁵ antipain²⁴ and leupeptins,⁷ cathepsin L with TLCK, antipain and leupeptins,^{22,26} and elastase (EC 3.4.21.11) with elastatinal,²⁷ respectively. Since these inhibitors do not inhibit the uptake of sensitized sheep erythrocytes,⁶ the intracellular accumulation of protein-bound ^{125}I seems to be explained by the inhibitory activities toward lysosomal proteases operating in the digestion of soluble Ag-Ab complex; previous experiments with sensitized sheep erythrocytes were not designed for the determination of these activities.⁶

With pepstatins, we could not observe any significant inhibitory activity, whereas pepstatins are potent inhibitors of cathepsin D (EC 3.4.23.5).²⁸ The cathepsin D, which was highly purified from guinea pig liver by the method of Smith and Turk,²⁹ was completely inhibited at the concentration of pepstatins used. Hence the ineffectiveness of pepstatins on phagocytosis may be caused by its incapability to enter into macrophage cells or the phagolysosomes in the cells.

On the other hand, there seems to be another possibility that the majority of the digestion of Ag-Ab complex observed does not occur through the phagocytic function of macrophages, but is taken place extracellularly by the cathepsins released by degranulation of the cells stimulated with Ag-Ab complex; these cathepsins and perhaps other neutral proteases might affect the hydrolysis of the complex in the reaction medium. When this possibility was tested by

measuring the supernatants from Ag-Ab complex-treated macrophages for the hydrolytic activity toward the complex of ^{125}I -labeled antibody, no significant amount of trichloroacetic acid-soluble, digested products was formed. The digestion of Ag-Ab complex measured in this study, therefore, was confirmed to occur intracellularly, but not extracellularly.

The results presented in this paper indicate that the protease inhibitors used give useful tools for analyzing the phagocytic mechanism consisting of many complicated processes. No available information has been obtained on intracellular localization of the chymotrypsin-like protease possibly essential for the ingestive activity of macrophages and its activity whereby the ingestive process may be initiated. Further studies will be required for elucidation of these problems.

Acknowledgements

We thank Dr. T. Aoyagi, the institute for Microbial Chemistry, Tokyo, for the generous gift of protease inhibitors from actinomycete fermentation. This work was supported in part by a grant from the Ministry of Welfare of Japan.

REFERENCES

- 1) C.De Duve and R.Wattiaux: Function of lysosomes, *Ann. Rev. Physiol.*, **28**, 435–492 (1966).
- 2) I.M.Goldstein: Polymorphonuclear leukocytes and immune tissue injury, *Prog. Allergy*, **20**, 301–340 (1976).
- 3) S.C.Silverstein, R.M.Steinman and Z.A.Cohn: Endocytosis, *Ann. Rev. Biochem.*, **46**, 669–722 (1977).
- 4) D.S.Perlman, P.A.Ward and E.L.Becker: The requirement of serine esterase function in complement-dependent erythrophagocytosis, *J. Exp. Med.*, **130**, 745–764 (1969).
- 5) R.A.Musson and E.L.Becker: The role of an activatable esterase in immune-dependent phagocytosis by human neutrophils, *J. Immunol.*, **118**, 1354–1365 (1977).
- 6) K.Nagai, T.Nakamura and J.Koyama: Characterization of macrophage protease involved in the ingestion of antigen-antibody complexes by the use of protease inhibitors, *FEBS Lett.*, **92**, 299–309 (1978).
- 7) H.Umezawa and T.Aoyagi: Activities of protease inhibitors of microbial origin, "Proteinases in mammalian cells and tissues," ed. by Barrett, North-Holland, Amsterdam, 1977, pp. 637–662.

- 8) N.Mori, T.Nakamura and J.Koyama: Immunochemical studies on non-precipitating guinea pig antibody produced by administration of an excessive dose of *Bacillus subtilis* α -amylase, *J. Biochem.*, **82**, 1–8 (1977).
- 9) J.Porath, R.Axen and S.Ernback: Chemical coupling of proteins to agarose, *Nature* (London), **215**, 1491–1492 (1967).
- 10) W.E.Dandliker and V.A.De Saussure: Antibody purification at neutral pH utilizing immunospecific adsorbents, *Immunochemistry*, **5**, 357–365 (1968).
- 11) T.Shinomiya and J.Koyama: In vitro uptake and digestion of immune complexes containing guinea-pig IgG1 and IgG2 antibodies by macrophages, *Immunology*, **30**, 267–275 (1976).
- 12) J.Calderon and E.R.Unanue: The release of antigen molecules from macrophages: Characterization of the phenomena, *J. Immunol.*, **112**, 1804–1814 (1974).
- 13) R.G.O.Leslie and S.Cohen: Comparison of the cytophilic activities of guinea pig IgG₁ and IgG₂ antibodies, *Eur. J. Immunol.*, **6**, 848–855 (1976).
- 14) K.Yagawa, K.Onoue and Y.Aida: Structural studies of Fc receptors I. Binding properties, solubilization, and partial characterization of Fc receptors of macrophages, *J. Immunol.*, **122**, 366–373 (1979).
- 15) A.J.Barrett: Introduction to the history and classification of tissue proteases, "Proteinases in mammalian cells and tissues," ed. by Barrett, North-Holland, Amsterdam, 1977, pp. 1–56.
- 16) F.Wróblewski and J.S.LaDue: Lactic dehydrogenase activity in blood, *Proc. Soc. exp. Biol. Med.*, **90**, 210–213 (1955).
- 17) R.G.Q.Leslie: Macrophage handling of soluble immune complexes. Ingestion and digestion of surface-bound complexes at 4, 20 and 37°C, *Eur. J. Immunol.*, **10**, 323–333 (1980).
- 18) R.G.Q.Leslie: Macrophage handling of soluble immune complexes. Use of specific inhibitors to study the biochemical events involved in complex catabolism, *Eur. J. Immunol.*, **10**, 799–802 (1980).
- 19) A.Nisonoff, F.C.Wissler, L.N.Lipman and D.L.Woernley: Separation of univalent fragments from the divalent rabbit antibody molecule by reduction of disulfide bonds, *Arch. Biochem. Biophys.*, **89**, 230–244 (1960).
- 20) P.Davies, R.I.Fox, M.Polyzonis, A.C.Allison, D.Phil and A.D.Haswell: The inhibition of phagocytosis and facilitation of exocytosis in rabbit polymorphonuclear leukocytes by cytochalasin B, *Lab. Invest.*, **28**, 16–22 (1973).
- 21) H.Ikezawa, T.Aoyagi, T.Takeuchi and H.Umezawa: Effect of protease inhibitors of actinomycetes on lysosomal peptide-hydrolases from swine liver, *J. Antibiot.*, **24**, 488–489 (1971).
- 22) T.Towatari, K.Tanaka, D.Yoshikawa and N. Katsunuma: Purification and properties of a new cathepsin from rat liver, *J. Biochem.*, **84**, 659–671 (1978).
- 23) T.Kato, K.Kojima and T.Murachi: Protease of macrophages in rat peritoneal exudate, with special reference to the effects of actinomycete protease inhibitors, *Biochim. Biophys. Acta*, **289**, 187–193 (1972).
- 24) H.Ikezawa, K.Yamada, T.Aoyagi, T.Takeuchi and H.Umezawa: Effect of antipain on lysosomal peptide-hydrolases from swine liver, *J. Antibiot.*, **25**, 738–740 (1972).
- 25) A.J.Barrett: Human cathepsin B1, purification and some properties of the enzyme, *Biochem. J.*, **131**, 809–822 (1973).
- 26) H.Kirschke, J.Langner, B.Wiederanders, S.Ansorge and P.Bohley: Cathepsin L, A new protease from rat-liver lysosomes, *Eur. J. Biochem.*, **74**, 293–301 (1977).
- 27) G.Feinstein, C.J.Malemud and A.Janoff: the inhibition of human leucocyte elastase and chymotrypsin-like protease by elastatinal and chymostatin, *Biochim. Biophys. Acta*, **429**, 925–932 (1976).
- 28) H.Umezawa, T.Aoyagi, H.Morishima, M.Matsuzaki, M.Hamada and T.Takeuchi: Pepstatin, a new pepsin inhibitor produced by actinomycetes, *J. Antibiot.*, **23**, 259–262 (1970).
- 29) R.Smith and V.Turk: Cathepsin D: rapid isolation by affinity chromatography on haemoglobin-agarose resin, *Eur. J. Biochem.*, **48**, 245–254 (1974).