CHARACTERIZATION OF MACROPHAGE PROTEASES INVOLVED IN THE INGESTION OF ANTIGEN–ANTIBODY COMPLEXES BY THE USE OF PROTEASE INHIBITORS

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1. Introduction

The ingestion of antigen–antibody complexes preceding the intracellular digestion by phagocytes can be inhibited by potent inhibitors of serine esterases such as diisopropylphosphofluoridate (DFP), *p*-nitrophenyl phosphonates, cyclohexyl alkylphosphonofluoridates, and cyclohexyl phenylalkylphosphonofluoridates [1,2]. The available evidence suggests that active and/or stimulus-activated serine esterases are required for the process of ingestion.

In order to characterize the serine esterases involved, it was attempted to study effects of various protease inhibitors with known specificities on the ingestive activity of guinea pig peritoneal macrophages against sensitized sheep erythrocytes [EA]. Among the inhibitors tested, DFP, 1-L-tosylamido-2-phenylethylchloromethylketone (TPCK), and chymostatin alone were found to reduce the ingestive activity of macrophages. These results are reported in this paper, indicating that the esterases required for the ingestion are presumably chymotrypsin-like proteases.

2. Materials and methods

2.1. *Chemicals*

DFP, TPCK, and 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK) were products of Sigma Chemical Company (USA). Various protease inhibitors from actinomycete fermentation, i.e., chymostatin, antipain, pepstatin, elastatinal, and leupeptins were kindly supplied by the Institute for Microbial Chemistry, Tokyo.

Abbreviations: DFP, diisopropylphosphofluoridates; EA, sensitized sheep erythrocytes; TPCK, 1-L-tosylamido-2-phenylethylchloromethylketone; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; MEM-HEPES, the minimal essential medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; DMSO, dimethylsulfoxide

2.2. *Macrophages*

The methods for harvesting and cultivating guinea pig peritoneal macrophages were essentially the same as those described by Shinomiya and Koyama [3]. Peritoneal macrophages were obtained from guinea pigs injected intraperitoneally with 20 ml of sterilized liquid paraffin 4 days before harvest. Peritoneal cells were washed 3 times with the minimal essential medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (MEM-HEPES) [4], and 2.5×10^5 peritoneal cells in 2.5 ml of MEM containing 5% fetal calf serum were plated in plastic dishes (Falcon, 35 × 10 cm), each containing a cover glass. After incubation at 37°C for 20 h in a CO_2-air incubator, the cover glasses containing the adherent macrophages were washed 3 times with MEM-HEPES to remove the non-adherent cells.

2.3. *Sensitized sheep erythrocytes*

One ml of a 10% suspension of sheep erythrocytes in MEM-HEPES was incubated with 1 ml of rabbit IgG containing 4 AU of antibody to erythrocyte at 37°C for 15 min, and used for experiments after 10-fold dilution with MEM-HEPES. The IgG used was purified from rabbit antiserum by fractionation with ammonium sulfate and DEAE-cellulose chromatography [5]. One AU was defined as a minimum amount of antibody capable of agglutinating 0.025 ml of a 2% suspension of erythrocytes.

2.4. *Ingestion of sensitized erythrocytes by macrophages*

The method for measuring the ingestive activity of macrophages was essentially the same as that of Griffin et al. [6]. The cover glass cultures of macrophages were covered with 2 ml of MEM-HEPES in dishes and kept at 37°C for 15 min. 0.2 ml of EA was added to each dish, and the reaction mixtures were incubated at 37°C for 60 min in a CO_2–air incubator.

After incubation, the macrophage monolayers were washed 3 times with MEM-HEPES and further incubated with 2 ml of 10-fold diluted MEM-HEPES to lyse EA not ingested. The macrophage monolayers thus treated were fixed with 2.5% glutaraldehyde in 0.1 ml of sodium cacodylate buffer, pH 7.2, at 4°C for 10 min [7], and number of EA ingested per each cell of macrophage was counted by a microscope. The ingestive activity of macrophages was expressed by an average number of ingested EA with the standard error, which was calculated using 30 cells of macrophage.

Under the condition used, number of EA ingested by each cell of macrophage increased rapidly up to 60 min (fig.1).

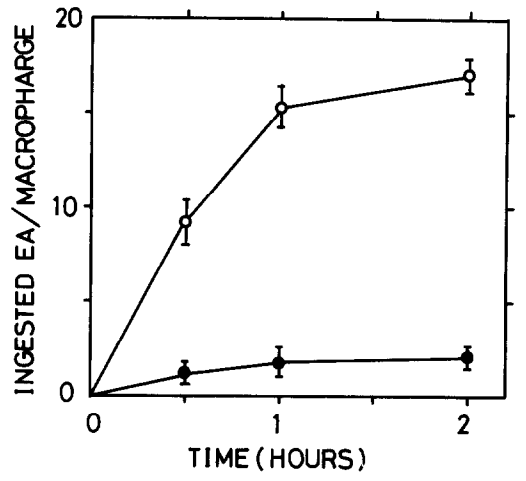

Fig.1. Ingestion of EA by macrophages. The time course of ingestion of EA was determined, as described in section 2 (o–o–o). The ingestion of erythrocytes also was measured as a control (•–•–•).

3. Results

The effects of various protease inhibitors on phagocytosis were determined by adding each inhibitor to a macrophage monolayer 15 min before the initiation of phagocytosis and comparing numbers of ingested EA in the presence or absence of the inhibitor. Among the inhibitors tested, DFP, TPCK, and chymostatin alone, each being a potent inhibitor of chymotrypsin [8–10], could inhibit the ingestive activity of macrophages (fig.2). These effects increased as the concentration of each inhibitor was increased (fig.3). On the other hand, TLCK, antipain, pepstatin, elastatinal, and leupeptins could not significantly alter the ingestive activity though these inhibitors at the same concentrations inhibited strongly trypsin, papain, pepsin, elastase, and trypsin, respectively [11–15]. The inhibition with DFP, TPCK or chymostatin may directly result from that of certain chymotrypsin-like enzyme involved in the process of ingestion since these inhibitors failed to

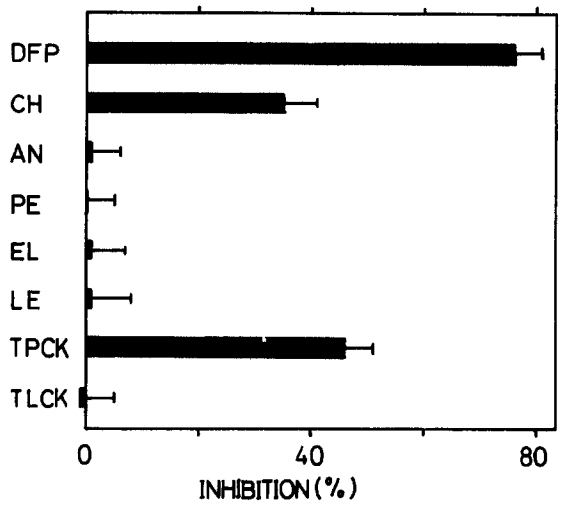

Fig.2. Inhibition of the ingestion with various protease inhibitors. Macrophages on cover glasses covered with 1.8 ml of MEM-HEPES in dishes were incubated with 0.2 ml of DFP in isopropanol, chymostatin (CH) in dimethylsulfoxide (DMSO), antipain (AN) in H_2O, pepstatin (PE) in H_2O, elastatinal (EL) in H_2O, leupeptins (LE) in H_2O, TPCK in DMSO, and TLCK in H_2O, respectively. After incubation at 37°C for 15 min, the ingestive activities of macrophages were measured, as described in section 2. The final concentration of each inhibitor was 100 μg/ml, excepting 10 mM of DFP. As controls, macrophages were similarly treated with 0.2 ml of isopropanol, H_2O, and DMSO, respectively.

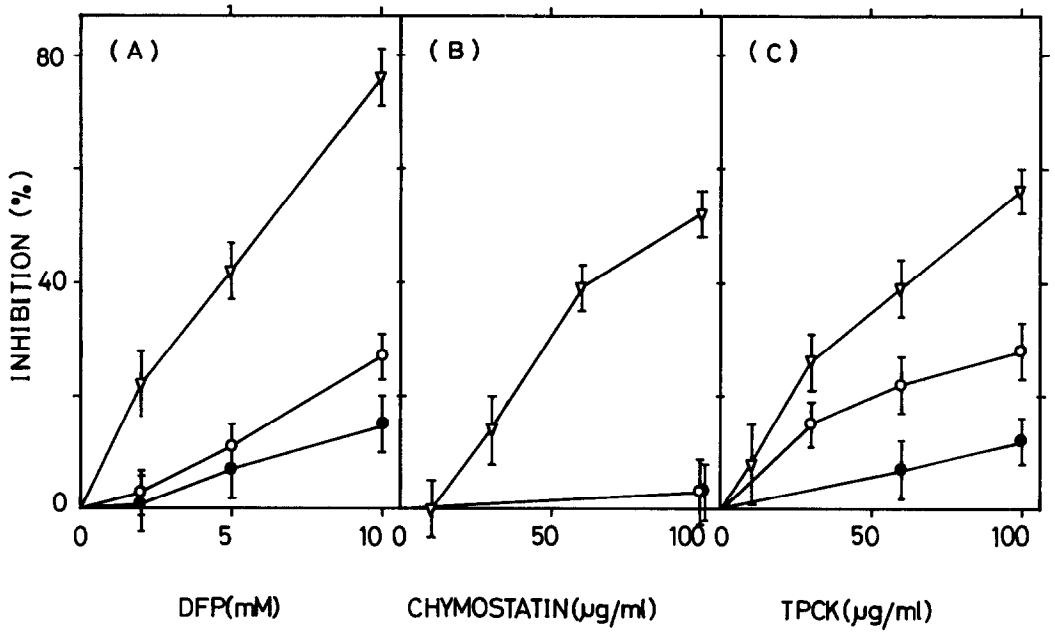

Fig.3. Inhibition of the ingestion with DFP, chymostatin, and TPCK. Inhibition was measured by two types of procedure: (1) macrophages on cover glasses covered with 1.8 ml of MEM–HEPES in dishes were incubated with 0.2 ml of DFP in isopropanol (A), chymostatin in DMSO (B), and TPCK in H_2O (C), respectively. After incubation at 37°C for 15 (●–●–●) or 60 min (○–○–○), the macrophages were washed five times with MEM-HEPES, and tested for ingestion of EA, as described in section 2; and (2) Inhibition with each inhibitor was measured by the same procedure as described in fig.1 (▽–▽–▽). In all cases, macrophages were similarly treated with 0.2 ml of isopropanol, H_2O, and DMSO, respectively, as controls. The final concentration of each inhibitor was shown on the absissa.

alter macrophage viability, as assessed by trypan blue uptake [16].

In order to discern whether pretreatment with TPCK or chymostatin affects the ingestive activity of macrophages, the effects were determined by measuring the ingestion by cells pretreated with these inhibitors and washed before the initiation of phagocytosis. As in the case of DFP [2], the pretreatment with TPCK reduced the ingestion though the inhibition was always less than that due to the presence of TPCK at the same concentration during phagocytosis (fig.2). To the contrary, the effect of pretreatment with chymostatin was essentially nonexistent. This may be because chymostatin inhibits reversibly chymotrypsin-like proteases though both DFP and TPCK do irreversibly by phosphorylating or alkylating the enzymes, respectively. presented some evidence that the serine esterase

4. Discussion

With the development of the lysosomal concept, the processes governing the intracellular digestion of ingested antigen–antibody complexes have been clarified [17,18]. In contrast, the mechanisms which regulate the ingestive process of phagocytosis have remained to be solved. Through the use of many potent serine esterase inhibitors, Pearlman et al. [1] and Musson and Becker [2] have presented evidence that serine esterases were required for the ingestive process of phagocytosis by rabbit and human neutrophils.

The results presented in this paper extended to guinea pig peritoneal macrophages the previous finding by Musson and Becker [2] that a DFP-sensitive serine esterase was involved in the ingestive activity of phagocytes. In addition, this paper

involved was a chymotrypsin-like protease, since DFP as well as two well-known inhibitors of chymotrypsin, TPCK and chymostatin, inhibited strongly the ingestive activity of macrophages. This characterization was further supported by the failure of TLCK, antipain, pepstatin, elastatinal, and leupeptins to inhibit the ingestive process of phagocytosis.

Both DFP and TPCK were found to reduce the ingestive activity even when inhibition was determined by measuring the ingestion by macrophages pretreated with these inhibitors, though the inhibition by pretreatment was less than that due to the presence of inhibitors during phagocytosis. As Musson and Becker [2] proposed, this difference in the inhibition profiles by two types of inhibition procedure seems to be accounted for by the mechanism that the ingestive process may require two types of serine esterase: an active esterase and another esterase which is normally in a proesterase form and activated by the interaction of phagocytes with antigen–antibody complexes through Fc receptors on the cell surface. If such two types of esterase participated in the ingestive process, both of them should be chymotrypsin-like enzymes. However, the results obtained so far are not sufficient to conclude that more than one type of esterase are involved in the ingestive process, since the evidence, by its nature, is indirect.

Recently, a neutral protease susceptible to inhibitory activities of DFP and chymostatin was partially purified by Suzuki and Murachi [19] from the lysate of rat peritoneal macrophages. Whether this chymotrypsin-like protease functions directly in the ingestive process has also been uncertain. Further biochemical characterization of the active and stimulus-activated esterases is required for the elucidation of molecular events involved in phagocytic processes.

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