Monoclonal Antibody-Based Immunoradiometric Assay for Rat α_1 -Protease Inhibitor-Bound Anionic Trypsin

Toshihide KAWAI,^{1,*} Reiji MORISHITA,¹ Tadashi EGUCHI,¹ Wen-Liang CHEN,¹
Kagemasa KURIBAYASHI,² Tohru MASUDA,² and Haruto UCHINO¹

¹*The First Division, Department of Internal Medicine, and* ²*Institute for Immunology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan*

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Summary Two monoclonal antibodies that react with epitopes on the α_1 -protease inhibitor moiety of rat α_1 -protease inhibitor-bound anionic trypsin were generated in mice immunized with the complex. They showed high specificity for the complex in comparison with the free α_1 -protease inhibitor. By use of these monoclonal antibodies and another monoclonal antibody that reacts with an epitope on the anionic trypsin moiety of α_1 -protease inhibitor-bound anionic trypsin, we established immunoradiometric assays that are specific for the complex. These immunoassays did not give positive results with either free anionic trypsinogen or α_1 -protease inhibitor. The immunoradiometric assays were applicable to quantification of α_1 -protease inhibitor-bound anionic trypsin in rat sera, and we found that the mean half-life of the complex in the circulation was 140 ± 16.6 min (mean $\pm$ SD) ($n=7$). Sandwich enzyme-linked immunosorbent assays were also tried. However, they did not give enough specificities to quantify the complex in the rat sera.

Key Words: rat α_1 -protease inhibitor-bound anionic trypsin, monoclonal antibody, immunoradiometric assay

In humans and some experimental animals with pancreatitis, many digestive zymogens and their derivatives, including those of amylase, lipase, etc., are present in the bloodstream [1]. The presence of trypsinogen in the serum indicates that only inactive zymogen is released from the pancreas. However, the presence of α_1 -protease inhibitor (α_1 PI)-bound trypsin in the serum indicates that trypsinogen had been activated under certain circumstances and then inactivated by the α_1 PI. Trypsin has been speculated to be one of the triggers of intrapancreatic activation

*To whom correspondence should be addressed. Present address: Institute of Applied Biochemistry, Yagi Memorial Park, Gifu 505-01, Japan.

of digestive zymogens in the early stage of the disease [2]. So we expected to obtain more information on the pathophysiology of pancreatitis by measuring trypsinogen and α_1 PI-bound trypsin in the serum separately.

Previously we reported on the production of monoclonal antibodies against rat anionic trypsinogen, and a monoclonal antibody-based anionic trypsinogen-specific enzyme-linked immunosorbent assay (ELISA) [3]. In this paper, we describe monoclonal antibody-based immunoassays for rat α_1 PI-bound anionic trypsin: sandwich ELISA's and immunoradiometric assays (IRMA's). By the IRMA's, serum α_1 PI-bound anionic trypsin was quantitatively measured directly, and the half-life of the complex in the circulation was determined.

MATERIALS AND METHODS

Materials. The sources of materials used in this paper were described previously [3]. The sources of other materials were as follows: sodium iodine-125 from Amersham International, Buckinghamshire, England; and Enzymobead, from Bio-Rad, Richmond, CA.

The following methods described in this paper were essentially the same as those described previously [3]: the trypsin enzyme assay and its related assays including the trypsinogen assay and the α_1 PI assay, the preparation of α_1 PI-bound anionic trypsin (except for the autoactivation of trypsinogen, if necessary), the production of hybridomas, the screening ELISA using the immunogen as the antigen, the purification of monoclonal antibodies as IgG subclass fractions, and the sandwich ELISA using two different monoclonal antibodies.

Production of monoclonal antibodies against α_1 PI-bound anionic trypsin. Purified rat anionic trypsinogen (80 μ l) was incubated in 0.5 ml of 0.1 M Tris/HCl buffer (pH 8.0) containing 20 mM CaCl_2 for 4 h at 25°C. Under these conditions, anionic trypsinogen was autoactivated fully, and the trypsin activity of the auto-activated sample showed 92% of that activated with enterokinase. Purified rat α_1 PI (240 μ g) was then added to the mixture, and incubation was continued for 1 h more to produce a mixture of α_1 PI-bound anionic trypsin and free α_1 PI (complex : free α_1 PI = 2 : 1). The procedures for immunizing BALB/c mice with this mixture and producing hybridomas were as described previously [3]. Antibody-producing hybridomas were screened and characterized by ELISA's as described below. The amount or concentration of α_1 PI-bound anionic trypsin was expressed as the amount of anionic trypsinogen used to produce the complex, or as mol per liter.

Screening and characterization of monoclonal antibodies. Screening ELISA of specific-antibody-producing hybridomas was carried out by use of the immunogen mixture as the antigen. The wells that were positive in the screening ELISA were applied to ELISA's for characterization. Anti- α_1 PI and anti-anionic trypsinogen reactivities in the culture fluid of the hybridomas were determined by ELISA's using α_1 PI and anionic trypsinogen, respectively, as the antigen.

One additional characterization was performed by indirect sandwich ELISA

for α_1 PI-bound anionic trypsin: for this we used a monoclonal antibody (5C5) reacting with the epitope on the anionic trypsin moiety of the complex [3] and the culture fluid of the hybridomas [4]. One hundred microliters of 5C5 (3 μ g/ml) in 50 mM Tris/HCl buffer (pH 8.6) (coating buffer) was added to each well of a microtiter plate, and incubation was carried out overnight at 4°C. After blockage with BSA, 100 μ l of α_1 PI-bound anionic trypsin (3 μ g/ml) in 50 mM Tris/HCl buffer (pH 7.2) containing 0.3 M NaCl, 5 mg/ml bovine serum albumin (BSA), and 0.05% Tween 20 (dilution buffer) was added and incubation continued for 2 h at the same temperature. Culture fluid of the hybridoma (100 μ l) was incubated with 100 μ l of rabbit anti-mouse IgG that had been previously conjugated with peroxidase and diluted appropriately, in a microtube for 2 h at 37°C. Pooled normal BALB/c mouse sera (40 μ l) was added to each microtube and incubation was continued for 1 h more. By these procedures, a complex of monoclonal antibody and rabbit anti-mouse IgG was produced, and the residual binding capacity of rabbit anti-mouse IgG was blocked by mouse IgG in the normal BALB/c sera. The preparation containing the IgG complex (100 μ l) was added to each well of a microtiter plate, and the plate was incubated for 2 h at 25°C. Then the peroxidase activity of each well was determined. Two wells containing positive hybridomas were cloned, and their monoclonal antibodies were purified to the IgG subclass level.

Sandwich ELISA for α_1 PI-bound anionic trypsin. α_1 PI-bound anionic trypsin, α_1 PI, or anionic trypsinogen was applied to each well of a microtiter plate that had been coated with one of the monoclonal antibodies reacting with the α_1 PI moiety of α_1 PI-bound anionic trypsin. Then 5C5 conjugated with horseradish peroxidase was applied to each well, and the peroxidase activity was determined. The inhibitory effect of free α_1 PI on the binding of α_1 PI-bound anionic trypsin to the monoclonal antibody bound to the microtiter plate was measured by addition of different amounts of α_1 PI to samples containing the complex and subjection of them to the ELISA.

Immunoradiometric assay. Thirty-six milligrams of 5C5 was bound to 36 ml of activated Sepharose 4B. The resulting 5C5-Sepharose was resuspended in 0.1 M Tris/HCl buffer (pH 8.0) containing 20 mM CaCl_2 and 0.05% NaN_3 in a total volume of 100 ml, divided into small aliquots, and stored at 4°C. Monoclonal antibodies reacting with the α_1 PI moiety of α_1 PI-bound anionic trypsin were labeled with iodine-125 by use of Enzymobead [5]. Antiserum obtained from mice immunized with the α_1 PI-bound anionic trypsin was purified to the IgG fraction level, and these antibodies were also labeled with iodine-125.

The binding capacity of 5C5-Sepharose for α_1 PI-bound anionic trypsin or anionic trypsinogen was assayed. 5C5-Sepharose suspension (100 μ l) was added to 900 μ l of dilution buffer in an assay tube. Increasing amounts of α_1 PI-bound anionic trypsin or anionic trypsinogen were added to separate tubes, and the suspension was then stirred for 2 h at 25°C. The supernatant was obtained after centrifugation, and assayed by the ELISA for α_1 PI-bound anionic trypsin and by

that for anionic trypsinogen (9G6&5C5:PO ELISA [3]) to measure unbound α_1 PI-bound anionic trypsin and anionic trypsinogen, respectively, in the supernatant. The binding capacity was determined from the difference between the amount applied and the unbound fraction.

In the IRMA, the dilution buffer containing 1 mM diisopropylfluorophosphate (DFP), 50 μ l of 5C5-Sepharose suspension, and a sample (<100 μ l) were added to an assay tube for a final volume of 1 ml and stirred for 2 h at 25°C. After the 5C5-Sepharose in the assay tube had been thoroughly washed with the washing buffer, a measured amount of monoclonal antibody or polyclonal antibodies labeled with iodine-125 was added to each tube (total volume, 1 ml). Then each tube was stirred for 2 h at 25°C. After 5C5-Sepharose in the tube had been washed, the radioactivity of each tube was counted with a gamma scintillation-counter. An α_1 PI-bound anionic trypsin standard was prepared by the mixing together of anionic trypsin (activated with enterokinase) and an excessive amount of α_1 PI. Standard and sample concentrations were determined in duplicate.

Measurement of α_1 PI-bound anionic trypsin produced by the reaction of anionic trypsin with rat serum. α_1 PI-bound anionic trypsin was produced by the reaction of anionic trypsin with rat serum obtained from Slc:Wistar rats or non-Slc:Wistar rats by two different methods: by the reaction of a quantified amount of anionic trypsin with rat serum containing excessive total protease-inhibitor activity, and by the reaction of an excessive amount of anionic trypsin with a quantified amount of rat serum. In the former experiment, 3.6 μ g of anionic trypsinogen was activated with enterokinase, and incubated with 10 μ l of rat serum or 20 μ l of purified α_1 PI (0.95 mg/ml) in the enzyme assay buffer in a total volume of 0.5 ml for 1 h at 25°C. In the latter experiment, purified α_1 PI and rat sera were diluted ten-fold. Anionic trypsinogen (4.5 μ g) was activated with enterokinase, and incubated with 10 μ l of the diluted α_1 PI or rat serum as described above. Residual anionic trypsin was inactivated by the addition of DFP (5 mM) and by the incubation for 1 h more. In both experiments, each sample was assayed by the IRMA to measure the amount of α_1 PI-bound anionic trypsin produced.

Measurement of the normal level and the half-life of α_1 PI-bound anionic trypsin in the circulation. α_1 PI-bound anionic trypsin produced by the reaction between autoactivated anionic trypsin and α_1 PI was doubly diluted with 1% BSA in saline, applied to Sephadex G-25 to change the buffer to saline, and stored at -80°C in small aliquots. Male Slc:Wistar rats weighing 250-350 g were anesthetized by intraperitoneal injection of pentobarbital sodium, and 200 μ l of α_1 -PI-bound anionic trypsin (3.8 μ g/ml) was injected into the left jugular vein. At specified times 200 μ l blood samples were collected from the right jugular vein, and allowed to clot. Serum was collected and stored at -80°C until assayed. The serum α_1 PI-bound anionic trypsin level of the fasting normal Slc:Wistar rat was also measured.

RESULTS AND DISCUSSION

Production and characterization of monoclonal antibodies

Spleen cells (1.6×10^8) from a mouse immunized with a mixture of α_1 PI and α_1 PI-bound anionic trypsin were fused with myeloma cells (0.4×10^8). Hybridomas were placed into 96-well plates. Twenty wells showing the highest (scale out) response to the screening ELISA were applied to the characterization ELISA's (Fig. 1). Hybridomas reacting with neither α_1 PI nor anionic trypsin, and showing a high response in the indirect sandwich ELISA between 5C5 and the culture fluid (e.g., 3D3), were selected and cloned. Finally two monoclonal antibodies (3D3 and 3H7) were cloned and purified to their IgG subclass level. The two hybridomas were characterized further following cloning. Both monoclonal antibodies crossreacted weakly with α_1 PI, but neither reacted with anionic trypsinogen. The cross-reactivity of 3D3 with α_1 PI was weaker than that of 3H7. These results show that the epitopes recognized by 3D3 and 3H7 were on the α_1 PI moiety of α_1 PI-bound anionic trypsin.

Fig. 1. Characterization of monoclonal antibody-producing hybridomas. Hybridomas showing the highest response to the screening ELISA using the immunogen (a mixture of α_1 PI-bound anionic trypsin and α_1 PI) as the antigen, were assayed for anti-anionic trypsinogen (Δ) and anti- α_1 PI ($\circ$) reactivity. Further, indirect sandwich ELISA for α_1 PI-bound anionic trypsin ($\bullet$) was carried out, by use of a monoclonal antibody (5C5) reacting with the anionic trypsin moiety of the complex and culture fluid of the hybridoma. Serum (diluted $\times 10^4$) of a BALB/c mouse immunized with the immunogen (poly Ab) and the supernatant from the myeloma cell line (NS-1) were included as positive and negative controls, respectively. Points above break marks indicate values at scale out.

Sandwich ELISA for α_1 PI-bound anionic trypsin

Samples containing α_1 PI-bound anionic trypsin, α_1 PI or anionic trypsin were applied to the wells of microtiter plates that had been coated with 3D3. Then, 5C5 conjugated with horseradish peroxidase (5C5:PO) was applied (3D3&5C5:PO ELISA). Neither α_1 PI nor anionic trypsinogen was detected by this ELISA (Fig. 2). However, the addition of various amounts of α_1 PI to samples containing α_1 PI-bound anionic trypsin inhibited the ELISA in a dose-dependent manner (Fig. 3). These inhibitory effects may be due to a lack of specificity of 3D3. Therefore, the 3D3&5C5:PO ELISA may not be suitable for measurement of α_1 PI-bound anionic trypsin in samples such as sera containing excessive amounts of free α_1 PI.

IRMA for α_1 PI-bound anionic trypsin

The binding capacity of 5C5-Sepharose for α_1 PI-bound anionic trypsin or anionic trypsinogen was more than $4\ \mu\text{g}$ per $100\ \mu\text{l}$ of the suspension (Fig. 4). Three different IRMA's were performed with combinations of 5C5-Sepharose and labeled 3D3, 3H7, or polyclonal antibodies. Figure 5 shows the results of one IRMA consisting of 5C5-Sepharose and labeled 3D3 (5C5-Sepharose&3D3 IRMA). Basal evaluations for this 5C5-Sepharose&3D3 IRMA were performed. The intra- and inter-assay coefficients of variation were calculated, based on pooled Slc:Wistar rat sera treated with exogenous α_1 PI-bound anionic trypsin, to be 1.6% ($n=10$) and 3.2% ($n=8$), respectively (the concentration of α_1 PI-bound

Fig. 2. Sandwich ELISA for α_1 PI-bound anionic trypsin. Sandwich ELISA was established by use of a monoclonal antibody (3D3) (reacting with the α_1 PI moiety of α_1 PI-bound anionic trypsin) bound to microtiter plate and another monoclonal antibody (5C5) (reacting with the anionic trypsin moiety of the complex) conjugated with horseradish peroxidase (3D3&5C5:PO ELISA). α_1 PI-bound anionic trypsin (●), anionic trypsinogen (▲), or α_1 PI (△) was applied to the ELISA.

Fig. 3. Inhibitory effect of free α_1 PI on the 3D3&5C5:PO ELISA. α_1 PI was added to samples containing α_1 PI-bound anionic trypsin in different concentrations and the samples were applied to the 3D3&5C5:PO ELISA. The ratio of α_1 PI-bound anionic trypsin : α_1 PI (mol : mol) was 1 : 1 (●), 1 : 400 (Δ), 1 : 4,000 (▲), or 1 : 40,000 (□).

Fig. 4. The binding capacity of 5C5-Sepharose. 5C5-Sepharose suspension (100 μ l), along with increasing amounts of α_1 PI-bound anionic trypsin (●) or anionic trypsinogen (□), was added to 900 μ l of dilution buffer in assay tubes. The mixture were stirred for 2 h at 25°C, and the supernatant of each tube was collected after centrifugation. Unbound α_1 PI-bound anionic trypsin or anionic trypsinogen in the supernatant was measured by the α_1 PI-bound anionic trypsin-specific 3D3&5C5:PO ELISA and the anionic trypsinogen-specific 9G6&5C5:PO ELISA, respectively. Bound α_1 PI-bound anionic trypsin or anionic trypsinogen was determined from the difference between the amount applied and the unbound fraction. The amount of α_1 PI-bound anionic trypsin was taken as that amount of anionic trypsinogen used to produce the complex.

Fig. 5. IRMA for α_1 PI-bound anionic trypsin. 5C5-Sepharose suspension (50 μ l) was added to samples (<100 μ l) containing α_1 PI-bound anionic trypsin and the dilution buffer in the assay tube (total volume, 1 ml), and stirred for 2 h at 25°C. After the 5C5-Sepharose in the tubes had been washed sufficiently, a definite amount of the other monoclonal antibody (3D3) labeled with radioisotope was added to each tube, and the mixture was stirred for another 2 h. After the 5C5-Sepharose in the tube had been washed, the radioactivity of each tube was counted by a gamma scintillation-counter. The background counts are shown by the datum points in parentheses.

Table 1. Recovery of α_1 PI-bound anionic trypsin added to normal rat serum.
 α_1 PI-bound anionic trypsin was added to three normal rat sera. The sera were applied to 5C5-Sepharose & 3D3 IRMA, and recoveries of the added α_1 PI-bound anionic trypsin were measured.

Added α_1 PI-bound anionic trypsin (ng)	α_1 PI-bound anionic trypsin concentration (ng/ml)	Increase in α_1 PI-bound anionic trypsin (ng)	Recovery of added α_1 PI-bound anionic trypsin (%)
Serum 1 0	1.1		
8.4	7.7	6.6	78.6
23.0	23.0	21.9	95.2
74.0	75.0	73.9	99.9
Serum 2 0	1.2		
8.4	9.7	8.5	101.2
23.0	23.6	22.4	97.4
74.0	74.0	72.8	98.4
Serum 3 0	1.1		
8.4	9.2	8.1	96.4
23.0	23.0	21.9	95.2
74.0	74.0	72.9	98.5

Mean values of duplicate experiments.

anionic trypsin was 41 ng/ml). The recoveries of exogenous α_1 PI-bound anionic trypsin added to normal Slc:Wistar rat serum ranged from 78.6 to 101.2% (Table 1). These results show that the 5C5-Sepharose&3D3 IRMA is highly quantitative

Fig. 6. Serum α_1 PI concentration measured in the form of α_1 PI-bound anionic trypsin by 5C5-Sepharose&3D3 IRMA. Slc:Wistar (Slc) or non-Slc:Wistar (non-Slc) rat sera were diluted ten-fold, and 10 μ l of each diluted serum was incubated with 4.5 μ g of anionic trypsin for 1 h at 25°C. The residual anionic trypsin was then inactivated by DFP (5 mM). These samples were subjected to the 5C5-Sepharose&3D3 IRMA to measure the α_1 PI-bound anionic trypsin produced, by which procedure the α_1 PI concentrations in the sera were determined. Error bars represent the mean $\pm$ SD.

and highly specific for measuring serum α_1 PI-bound anionic trypsin.

Sandwich immunoassays for serum α_1 PI-bound trypsin utilizing polyclonal anti- α_1 PI antibody and anti-trypsin antibody have been reported [6,7]. Regardless of which polyclonal antibodies are bound to the solid phase (e.g., microtiter plate), these assays necessarily show some inhibitory effects by serum free α_1 PI or trypsinogen [6]. Methods to overcome the inhibitory effects involve by the use of antibodies highly specific for α_1 PI-bound trypsin in comparison with free α_1 PI or trypsinogen, or the increasing of the binding capacity of the antibodies bound to the solid phase. Though we first tried the former method (3D3&5C5:PO ELISA), the specificity of 3D3 for α_1 PI-bound anionic trypsin was insufficient. So we performed IRMA for α_1 PI-bound anionic trypsin *via* the latter method (5C5-Sepharose&3D3 IRMA).

Measurement of α_1 PI-bound anionic trypsin produced by the reaction of anionic trypsinogen with serum

The α_1 PI-bound anionic trypsin produced was measured by three different IRMA's consisting of 5C5-Sepharose and labeled 3D3, 3H7, or polyclonal antibodies. When a quantified amount of anionic trypsin was incubated with rat sera or purified α_1 PI containing excessive total protease-inhibitor activity, the amount

Fig. 7. Clearance of α_1 PI-bound anionic trypsin from the circulation. Two hundred microliters of α_1 PI-bound anionic trypsin ($3.8 \mu\text{g/ml}$) was injected into the left jugular vein of male Slc:Wistar rats. Blood samples were taken from the right jugular vein after specified intervals, and the serum concentrations of α_1 PI-bound anionic trypsin were measured. To measure the mean half-life of α_1 PI-bound anionic trypsin from different experiments, we normalized the concentrations calculated for the 5 min point to 100%. Seven animals were studied. Error bars represent the SD.

of α_1 PI-bound anionic trypsin produced by the reaction of anionic trypsin with serum was only less than 5% of that obtained with purified α_1 PI. All three IRMA's yielded essentially the same results. These experiments showed that only a small part of the anionic trypsin added to the rat serum reacted with the α_1 PI in the serum under these experimental conditions. These results are consistent with those reported by other investigators [8].

When a quantified amount of rat sera or purified α_1 PI was incubated with excessive anionic trypsin, all α_1 PI in the samples was expected to react with the anionic trypsin, and the concentration of α_1 PI was expected to be measured in the form of α_1 PI-bound anionic trypsin by the IRMA for the complex. The concentration of purified α_1 PI measured by the 5C5-Sepharose&3D3 IRMA was 0.63 mg/ml (66% of that of applied α_1 PI). The α_1 PI concentrations in sera from non-Slc:Wistar rats measured by this assay were more varied than those from Slc:Wistar rats (Fig. 6). The reason for this variation in α_1 PI concentration in non-Slc:Wistar rats seems to be due to differences in the α_1 PI allotypes [9], because the range of α_1 PI concentrations in sera from non-Slc:Wistar rats measured by the enzyme assay was almost the same as that in sera from Slc:Wistar rats. These results show that we do not have to consider the effects of variation in α_1 PI allotypes, as long as Slc:Wistar

rats are used.

The normal level and the half-life of α_1 PI-bound anionic trypsin in the circulation

The mean α_1 PI-bound anionic trypsin level of normal Slc:Wistar rat sera was 0.925 ± 0.182 ng/ml (bound anionic trypsinogen) or 31.7 ± 6.07 pM (mean $\pm$ SD) ($n=20$). The clearance of α_1 PI-bound anionic trypsin from the circulation was rather slow (Fig. 7). The mean half-life of α_1 PI-bound anionic trypsin in the circulation was calculated to be 140 ± 16.6 min (mean $\pm$ SD) ($n=7$). This value is similar to the serum half-life of human α_1 PI-bound cationic trypsin reported previously [10].

We have described a monoclonal antibody-based ELISA for rat anionic trypsinogen (9G6&5C5:PO ELISA) and an IRMA for α_1 PI-bound anionic trypsin (5C5-Sepharose&3D3 IRMA), in this and a companion paper. The methods used to establish the immunoassays described in the two papers can be applied to human samples. This 5C5-Sepharose&3D3 IRMA has the same advantages as the 9G6&5C5:PO ELISA: (1) It is highly quantitative and not time-consuming. (2) It is an extremely accurate, true 'two site' sandwich immunoassay, using two different monoclonal antibodies, each of which reacts with the epitope on the α_1 PI or the anionic trypsin moiety of the complex. (3) It uses α_1 PI-bound anionic trypsin itself as the standard.

Using both immunoassays, we can separately measure anionic trypsinogen and α_1 PI-bound anionic trypsin, which have heretofore been measured together as trypsin-like immunoreactivity. These immunoassays may be useful for clarification of the mechanism involved in the development of pancreatitis. *In vivo* experiments using these two immunoassays are now in progress.

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