Inflamm. res. 49 (2000) 591–599
1023-3830/00/110591-09 $ 1.50+0.20/0

Inflammation Research

Specific binding of IL-8 to rabbit α-macroglobulin modulates IL-8 function in the lung

A. Kurdowska[1], N. Fujisawa[1,*], B. Peterson[2], F. K. Carr[1], J. M. Noble[1], S. M. Alden[1,], E. J. Miller[1] and M. Teodorescu[3]**

[1] Department of Biochemistry, University of Texas Health Center, Tyler, TX 75708, USA, Fax: 903-877-5101, e-mail: anna.kurdowska@uthct.edu.
[2] Department of Cell Biology, University of Texas, Health Center, Tyler, TX 75708, USA
[3] Department of Microbiology and Immunology, College of Medicine, University of Illinois, Chicago, IL 60612, USA

Received 3 January 2000; returned for revision 8 February 2000; accepted by G. W. Carter 28 April 2000

Abstract. *Objective and Design:* The purpose of this study was to compare chemotactic activity of IL-8 alone with that of IL-8 reacted with rabbit α-macroglobulins (αM) in vivo.
Methods: Initially the binding of recombinant human IL-8 (rhIL-8) to rabbit αM was studied. ^{125}I-labeled rhIL-8 was incubated with αM, and electrophoresed on native 5% gels or SDS-polyacrylamide 4–20% gradient gels. Next, rhIL-8 or rhIL-8 bound to αM was administered via an endotracheal tube to rabbit's lungs.
Treatment: An endotracheal tube was wedged into a segment of the lobe of each lung, and a sample instilled through the tube into this segment. After 4 h the lungs were lavaged.
Results: rhIL-8 bound to αM retained its full chemotactic activity in vitro but exhibited a diminished ability to induce the influx of neutrophils into the rabbit lung.
Conclusions: The data suggest that αM may facilitate IL-8 clearance from the lung.

Key words: Neutrophils – Chemokines – Carrier proteins – α_2-Macroglobulin receptors – Alveolar macrophages

Introduction

IL-8 has been reported to be involved in the pathogenesis of several human diseases including sepsis [1], and the acute respiratory distress syndrome (ARDS) [2]. ARDS is frequently associated with the accumulation of neutrophils in the lungs [3–5]. Recent studies have shown that high concentrations of IL-8 are present in the airspaces of the lungs of patients with ARDS and are associated with increased mortality [2].

Severe damage to the pulmonary alveolar-capillary membrane occurs in ARDS, and, as a consequence, the lungs become flooded with inflammatory cells and plasma proteins [6]. Therefore, one of the hallmarks of ARDS is the leakage of high molecular weight serum proteins such as α_2-macroglobulin (α_2M) into the alveoli [6]. α_2M is considered a "universal" plasma proteinase inhibitor because it interacts with proteinases belonging to four major classes [7]. More recently it became apparent that the function of α_2M extends beyond serving as a proteinase inhibitor. α_2M was found to act as a carrier and regulator of the function of many cytokines, including transforming growth factor β [8], interleukin-1 [9], and platelet derived growth factor [10].

α_2M exists both in a native ("slow") and modified ("fast") form. α_2M undergoes conformational change after reacting with proteases or when treated with primary amines. Modified α_2M migrates faster than native α_2M during electrophoresis and is referred to as the "fast" form [11]. Most cytokines bind tightly only to "fast" form of α_2M and not to native ("slow") α_2M [12]. The "fast" form which is produced in vivo by the action of proteinases can be duplicated in vitro by treatment of α_2M with methylamine [11].

Our previous studies showed that rhIL-8 binds only to "fast" (methylamine-treated) human α_2M. The binding is specific, and the estimated K_D is approximately 30 nM [13]. The effect of any association between IL-8 and human α_2M on IL-8 activity was also thoroughly examined. rhIL-8-"fast" human α_2M complexes bound to human neutrophils with comparable affinity to rhIL-8 alone, and had a neutrophil activating ability similar to that of "free" rhIL-8 [13]. In addition, complexes between rhIL-8 and "fast" human α_2M were cleared by human alveolar macrophages via α_2M receptors [14]. Furthermore, studies from our laboratory demonstrated that a significant portion (up to 60%) of IL-8 in lung fluids from patients with ARDS is associated with α_2M [13].

The ability of IL-8 to interact with α_2M both in vitro and in vivo, suggests that α_2M may modulate IL-8 activity in vivo. We used a rabbit model because rabbit IL-8 is similar to human IL-8, and human and rabbit IL-8 cross reacts with the receptors of the other species [15]. Rabbit plasma contains two α-macroglobulins (1M and α_2M) which are chemically

Correspondence to: A. Kurdowska

* *Current address:* Department of Medicine, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501, Japan
** *Current address:* Pfizer Inc., Eastern Point Road, Groton, CT 06 340, USA

and functionally similar to the human protein [16, 17]. However, macroglobulins from different species can vary significantly in their ability to interact with the same cytokines [18, 19]. Therefore, we examined first the ability of rabbit αM to bind rhIL-8, and then the influence of rabbit αM on IL-8-induced neutrophil trafficking to the rabbit lungs.

Materials and methods

Proteins

rhIL-8 was expressed in E. coli, and purified using an antibody affinity column [20]. The concentration of IL-8 was measured with an ELISA assay developed by Ko et al. [21]. Rabbit αM (a mixture of α_1M and α_2M) was purified using a TSK-Chelate HPLC column [22], and human α_2M was purchased from Biodesign International (Kennebunk, ME). The concentration of human α_2M was determined by absorbance at 280 nm, using an $A_{1\,cm,1\%}$ of 9.0 [23]. The rabbit αM concentration was measured using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacture's instructions. Sheep anti-rabbit α_2M antibody (Cappel Laboratories, Cochranville, PA) was used in some experiments. $F(ab)_2$ fragments were prepared by pepsin digestion, using the ImmunoPure™ $F(ab)_2$ Preparation Kit (Pierce, Rockford, IL).

Conversion of αM to the "fast" form

"Fast" αM was prepared by reacting native αM (10 µg) with methylamine (100 µg) in PBS overnight at 4 °C. Excess methylamine was removed by dialysis against PBS. The conversion to "fast" αM was confirmed by the loss of the ability to bind trypsin in the trypsin binding assay, and by electrophoresis (see below).

Electrophoresis

rhIL-8 was labeled with ^{125}Iodine as previously described [24]. Complexes of ^{125}I-rhIL-8 with human α_2M and rabbit αM were detected by non-denaturing polyacrylamide gel electrophoresis (5% Tris-borate) [25] and autoradiography. Fresh rabbit plasma and methylamine-treated plasma (3 µl), or "fast" αM (10 µg) was incubated with different concentrations of labeled rhIL-8 overnight at room temperature, and electrophoresed on a native 5% gel. Similarly prepared αM samples were also electrophoresed on SDS-polyacrylamide 4–20% gradient gels. Gels were then stained with Coomassie Blue and dried for autoradiography. To estimate the extent of the covalent binding, autoradiographs of native and SDS gels were scanned using an Abaton 300/GS scanner with NIH Image software (Wayne Rasband, NIH Bethesda, MD). In some experiments "fast" αM was incubated with sheep anti-rabbit αM, sheep non-immune IgG, goat anti-human α_2M, or goat non-immune IgG ($F(ab)_2$ fragments) prior to the addition of rhIL-8.

Trypsin binding assay for αM

The activity of trypsin (Sigma Chemical Co., St. Louis, MO) was determined by active site titration with p-nitrophenyl-p′-guanidinobenzoate hydrochloride [26]. αM was tested for trypsin binding activity by the method of Ganrot [27] as previously described [13].

Affinity of binding of rhIL-8 to rabbit αM

Different concentrations of "fast" αM were incubated with rhIL-8 (100 ng) overnight at 25 °C. The samples were separated on a HPLC gel filtration column, TSK-250. PBS was used as elution buffer at a flow rate of 1 ml/min. The column was calibrated by using a mixture of molecular weight markers including human α_2M, albumin, and IL-8, and ranging from 8.0 to 750 kDa. The concentration of "free" and "bound" IL-8 was measured in the IL-8 ELISA assay [21]. The dissociation constant, K_D, was calculated as previously described [13].

ELISA for IL-8-α_2M complexes

IL-8-α_2M complexes were also detected by a modified IL-8 ELISA as previously described [13]. Briefly, the plates were coated with the mouse anti-human IL-8 antibody, blocked and incubated with the samples, followed by the rabbit anti-human α_2M antibody (Calbiochem, San Diego, CA) which recognizes also rabbit α_2M, and horseradish peroxidase-labeled goat anti-rabbit IgG (Dako Co., Carpinteria, CA). This later antiserum was pre-absorbed with normal mouse plasma. Nonspecific binding was measured using plates coated with an irrelevant isotypic antibody (MOPC 21, Sigma Chemical Co., St. Louis, MO).

Endotoxin removal

Endotoxin was removed from the samples using Detoxi-Gel™, and concentrations of endotoxin were measured in a Quantitative Chromogenic Lal assay; BioWhittaker, Walkersville, MD.

rhIL-8-"fast" rabbit αM complexes

Complexes between rhIL-8 and "fast" rabbit αM were prepared as previously described [13]. Briefly, to form the complexes "fast" rabbit αM was incubated with rhIL-8 overnight at 25 °C. The samples were then separated on the HPLC gel filtration column. IL-8 concentration in the purified complexes was then measured [21]. Bioactivity (chemotactic potential) of the complexes was also assessed using the leading front method of Zigmond and Hirsch as previously described [13, 28]. Finally, the complexes were aliquoted and stored at –70 °C. Our studies indicate that storage at –70 °C does not alter properties of these complexes (the activity remained the same throughout the course of the study). In addition, free IL-8 concentration (not bound to "fast" rabbit αM) was measured by subjecting the complex preparation to the HPLC gel filtration, and evaluating amount of IL-8 in ELISA [13, 21]. The concentration of free IL-8 in the complex preparation was approximately 3% of the total IL-8, and did not change in storage.

Instillation of rhIL-8 alone or rhIL-8-"fast" rabbit αM complexes to rabbit lungs

The rabbit was initially sedated with a 0.4 ml, 10 mg/ml subcutaneous dose of acepromazine. After 10–15 min, the animal was weighed, 3.0 ml of blood was withdrawn from the ear artery, and the PaO_2 (arteriolar oxygen tension) measured. Then, the rabbit was anaesthetized with 2.5 ml ketamine (100 mg/ml) + 0.3 ml xylazine (100 mg/ml) intramuscularly, and placed in a prostrate position on a metal tray. A thin guide wire was then inserted into the trachea, and the endotracheal tube (1 mm diameter) was inserted over the guide wire and through the trachea to the left or right lung. The animal received saline, rhIL-8, rhIL-8 in complex with "fast" rabbit αM, or "fast" rabbit αM alone (0.1 µg of rhIL-8/rabbit in 2–5 ml). A sample was instilled through the tube into the segment of the lung lobe into which the endotracheal tube was wedged. To identify the area which received the instillate, a sample instilled to one lung contained a 1% solution of Evans Blue (Sigma Chemical Co., St. Louis, MO), and a sample instilled to the second lung a 1% solution of carbon black (Pelikan Ink; Hanover, Germany). Following the instillation of the sample the rabbit was returned to the cage.

The animal was monitored every 30 min using both subjective (body posture etc.) and objective (capillary refill time, respiratory rate) assessment. After 2 h a blood sample (3.0 ml) was taken to determine if there were any peripheral effects of treatment. After 4 h the animal was sedated with a 0.4 ml, 10 mg/ml subcutaneous dose of acepromazine and 10–15 min later, 3.0 ml of blood was withdrawn from the ear artery and the blood gases were measured to determine changes in arterial PO_2 which might indicate the presence of lung injury.

Lung lavage

The rabbit was anaesthetized with 2.5 ml ketamine (100 mg/ml) and 0.3 ml xylazine (100 mg/ml) intramuscularly. Following anaesthesia the carotid artery was cannulated, and the animal exsanguinated. The lungs were then lavaged by the method of Peterson [29]. Briefly, following exsanguination of the animal, the chest cavity was opened, and the lungs removed. A tube (4 mm diameter) was wedged into the trachea. One lung was clamped, and 20 ml of sterile saline was rapidly instilled into the other lung. The fluid was then rapidly withdrawn into a series of four syringes. A further sample was collected from the tubing. Each of the samples was analyzed separately for cellular content. An average of 12 ml of lavage was recovered from each rabbit.

Cell analysis

Total white cell numbers were assessed using a Coulter Counter Model F_N (Coulter Electronics Inc., Hialeah, FL). Slides of each sample were stained using a Leukostat stain kit (Fisher Diagnostics, Pittsburgh, PA), and a differential white count performed on each slide.

Biochemical analyses

Total protein, IL-8 and urea were measured using commercially available kits (Pierce, Rockford, IL (protein), R&D Systems, Minneapolis, MN (IL-8), Sigma Chemical Co., St. Louis, MO (urea), respectively). The concentration of each parameter in the epithelial lining fluid (ELF) was calculated using the following equation:

$$C_A = C_M \times \frac{\text{Concentration of urea in plasma}}{\text{Concentration of urea in lung wash}}$$

where C_M was the measured concentration in the lavage fluid [29].

In addition, IL-8 bioactivity (chemotactic potential) was assessed by the leading front method of Zigmond and Hirsch as previously described [13, 28].

Statistical analysis

Differences between groups were analyzed by a simple one way analysis of variance (ANOVA) or, if the data was not normally distributed, by a Kruskal-Wallis ANOVA on ranks. The direct comparisons between any two treatment groups were performed using the Student's t-test, or the nonparametric Mann-Whitney test when the data sets were not normally distributed. Correlation between the IL-8 and neutrophil concentrations in the ELF was determined by a Spearman Rank Order Correlation method (the residuals or distances of the data points from the regression line were not normally distributed). All statistical analyzes were performed using SIGMASTAT (SPSS Science Inc., Chicago, IL).

Results

Analysis of the interaction of rhIL-8 with rabbit αM

The interaction between rhIL-8 and rabbit αM was revealed by gel electrophoresis (Fig. 1–5). Fresh rabbit plasma was mixed with labeled rhIL-8 in the absence and presence of methylamine, and subjected to electrophoresis on a non-denaturating polyacrylamide gel and autoradiography (Fig. 1). rhIL-8 has an isoelectric point of 8–8.5 [30], and therefore, it does not migrate well in native polyacrylamide gels of pH 8.6 (the top band in lanes 1 and 2 corresponds to uncomplexed ^{125}I-rhIL-8). In the presence of methylamine ^{125}I-rhIL-8 formed complexes with high molecular weight proteins migrating in positions identical to "fast" rabbit α_1M and α_2M (lane 1) while in the absence of methylamine the bands corresponding to ^{125}I-rhIL-8 complexes with rabbit α_1M and α_2M (lane 2) were not present (Fig. 1A). Therefore, it can be concluded that ^{125}I-rhIL-8 does not bind to "slow" α_1M and α_2M. In addition, ^{125}I-rhIL-8 bound mainly to "fast" rabbit α_2M, although both rabbit macroglobulins are similarly represented in plasma [16] as seen on the Coomassie-stained gel of "fast" and native rabbit plasma α_1M and α_2M (Fig. 1B, lane 3 and 4, respectively). Preferential binding to α_2M was confirmed by scanning the gels, and dividing the amount of rhIL-8 bound to α_1M and α_2M (measured intensity was 11,760 and 47,040 for α_1M and α_2M bands, respectively) by the amount of protein in the corresponding lanes (measured intensity was 7,728 and 6,376 for α_1M and α_2M bands, respectively). Rabbit α_2M bound approximately 5-times as much of rhIL-8 than α_1M.

In the next series of experiments the binding of rhIL-8 to purified rabbit αM was studied. The preparations of rabbit αM obtained by us contained mixture of α_1M and α_2M. Since ^{125}I-rhIL-8 preferentially bound to "fast" forms of rabbit α_1M and α_2M only methylamine-treated αM was used in the following experiments. The binding of rhIL-8 to rabbit αM was concentration-dependent (Fig. 2A) and specific, i.e., the binding of labeled rhIL-8 to αM was inhibited by the excess of unlabeled rhIL-8 (Fig. 2A, lane 5).

The nature of the interaction of ^{125}I-rhIL-8 with "fast" rabbit αM was further investigated using SDS gel electrophoresis (Fig. 3A). Rabbit α_1M and α_2M migrate in SDS gels as one band [31]. In addition, the upper band (first from the top) in Fig. 3B (stained gel) represents the portion of

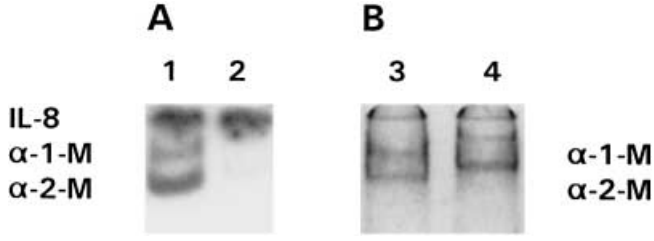

Fig. 1. Native gel analysis of binding of ^{125}I-rhIL-8 to rabbit plasma proteins in the absence and presence of methylamine. Fig. 1A; Lane 1: 3 µl of rabbit plasma with methylamine and ^{125}I-rhIL-8 (5 ng); Lane 2: 3 µl of untreated rabbit plasma and ^{125}I-rhIL-8 (5 ng). Fig. 1B represents Coomassie blue staining of untreated plasma (lane 3) and methylamine-treated plasma (lane 4).

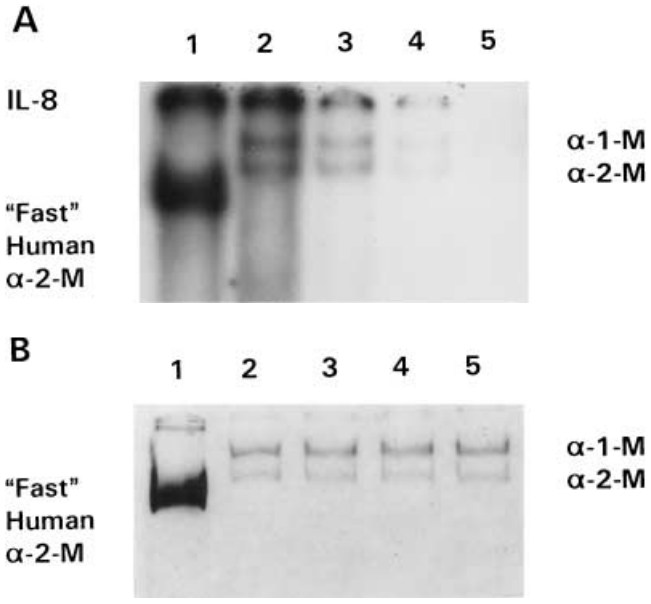

Fig. 2. Specific and concentration-dependent binding of ^{125}I-rhIL-8 to "fast" form of rabbit αM. Fig. 2A represents autoradiogram, and Fig. 2B Coomassie blue staining of the native gel. Lane 1: "fast" form of human α_2M (10 µg) complexed with ^{125}I-rhIL-8 (5 ng); Lane 2,3,4: "fast" form of rabbit αM (10 µg) with different concentrations of ^{125}I-rhIL-8 (5 ng, 2.5 ng, and 1.25 ng); Lane 5: same as 3 but reacted with excess unlabeled rhIL-8 (250 ng).

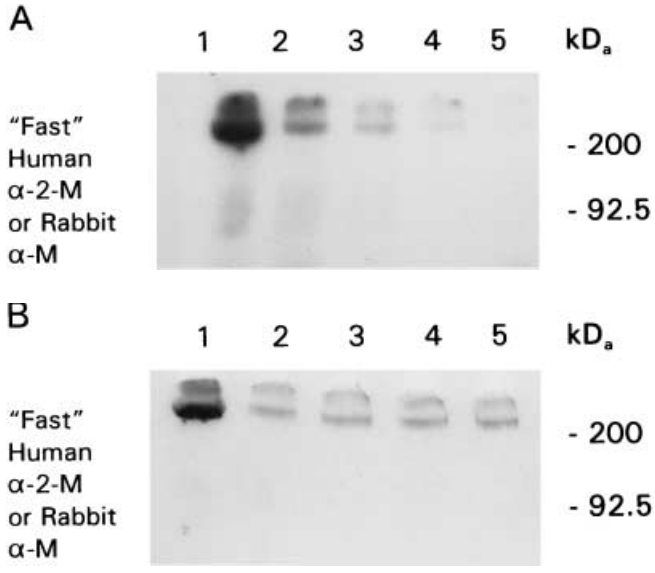

Fig. 3. SDS-PAGE analysis of the binding of ^{125}I-rhIL-8 to "fast" form of rabbit αM. Fig. 3A represents autoradiogram, and Fig. 3B Coomassie blue staining of the gel. Lane 1: "fast" form of human α_2M (10 µg) complexed with ^{125}I-rhIL-8 (5 ng); Lane 2,3,4: "fast" form of rabbit αM (10 µg) with different concentrations of ^{125}I-rhIL-8 (5 ng, 2.5 ng, and 1.25 ng); Lane 5: same as 3 but reacted with excess unlabeled rhIL-8 (250 ng). The upper band (first from the top) in Fig. 3A represents ^{125}I-rhIL-8-"fast" human α_2M/"fast" rabbit αM complexes that do not penetrate the gel, and in Fig. 3B the portion of human α_2M or rabbit αM that does not penetrate the gel.

human α_2M or rabbit αM (rabbit α_1M and α_2M migrate in SDS gels as one band) that does not penetrate the gel. Rabbit αM or human α_2M is dissociated into halves by treatment with SDS under non-reducing conditions [31, 32]. Incomplete dissociation and/or aggregation will result in generating a moiety which precedes a dimer (half molecule) in the electrophoretic run. Our previous studies also indicate that only a very small portion of ^{125}I-rhIL-8 does not enter non-reducing SDS gels [13]. Therefore, the upper band (first from the top) in Fig. 3A (autoradiograph) represents mainly ^{125}I-rhIL-8-human α_2M/rabbit αM complexes that do not penetrate the gel. The remaining band (Fig. 3A) depicts ^{125}I-rhIL-8-αM complexes which could be still detected after the SDS treatment in the absence of mercaptoethanol. Furthermore, scanning of the autoradiographs of native and SDS gels revealed that 60% of rhIL-8 complexed to αM was dissociated by SDS, suggesting that the binding of ^{125}I-rhIL-8 to αM was mostly non-covalent in nature. It

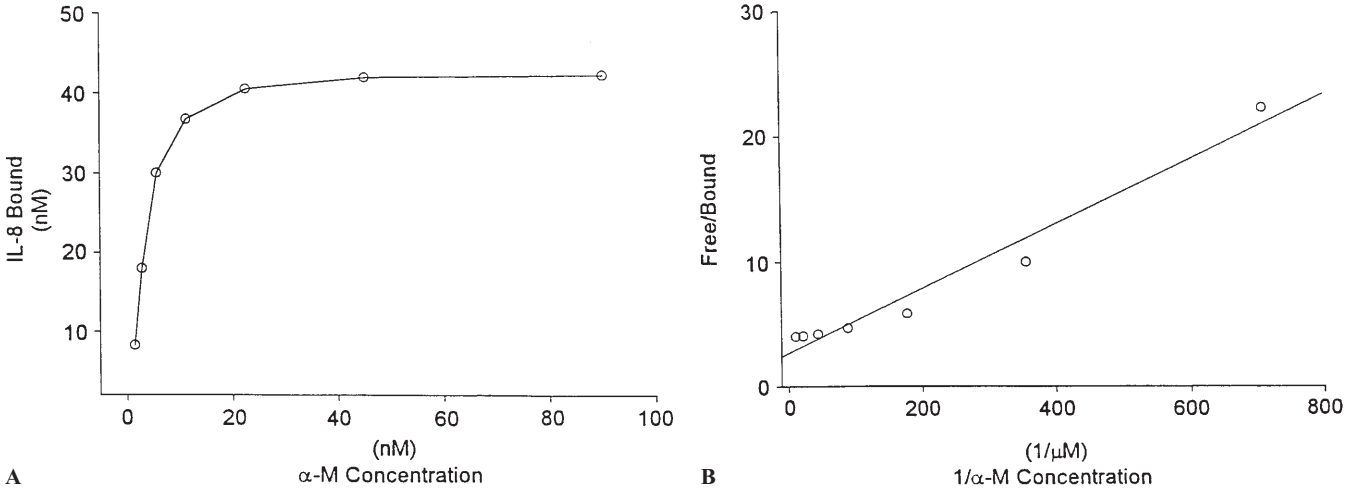

Fig. 4. Affinity of the binding of rhIL-8 to "fast" rabbit αM. Fig. 4A. Different concentrations of "fast" rabbit αM were incubated with rhIL-8 (100 ng) overnight at 25 °C. The concentration of "free" and "bound" IL-8 was measured in the IL-8 ELISA assay. Figure 4B. Calculation of the KD for the interaction of rhIL-8 with "fast" rabbit αM. The results represent the average of three experiments.

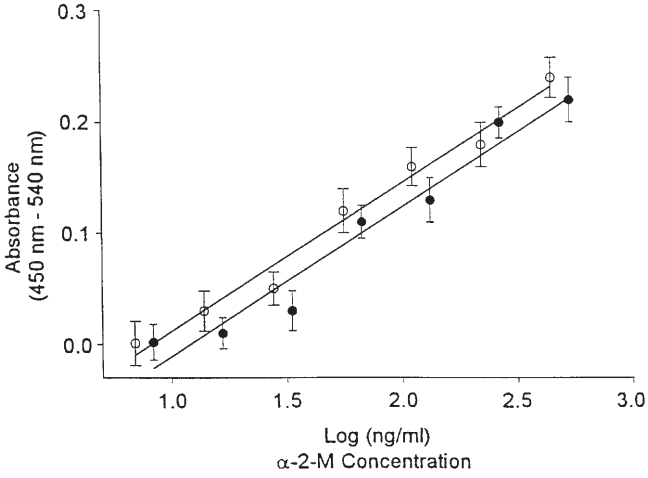

Fig. 5. Detection of rhIL-8 bound to "fast" rabbit or human α_2M using the ELISA assay specific for rhIL-8-human α_2M complexes. "Fast" rabbit αM or human α_2M was incubated with rhIL-8 overnight at 25 °C. The rhIL-8-"fast" human α_2M complexes(●)/rabbit α_2M complexes (○) were detected in the ELISA. The graph represents average of four experiments.

Table 1. Components of the ELF[a]

Sample (n)	Total Protein (mg/ml)	IL-8 (ng/ml)	PMN ($\times 10^6$/ml)
Control (8)	7.0 ± 10.6	2.4 ± 2.5	4.7 ± 6.4
Saline control (8)	7.8 ± 4.3	8.8 ± 3.2	3.2 ± 1.8
rhIL-8 (5)	7.4 ± 2.6	26.1 ± 12.7[b,c]	14.6 ± 8.9[b]
rhIL-8-"fast" rabbit αM complexes (5)	10.3 ± 5.9	11.9 ± 5.0[c]	4.2 ± 3.7
"Fast" Rabbit αM (4)	6.2 ± 4.2	2.4 ± 2.0	4.9 ± 6.3

[a] ELF was evaluated 4 h after the instillation of the samples as described above. Control represents the lungs to which no samples were instilled. There was no significant difference between any groups for total protein ($p > 0.05$).

[b] When rhIL-8 was instilled to the rabbit lungs a significant increase both in the IL-8 concentration and PMN number ($p < 0.05$) was observed.

[c] The concentrations of bioactive IL-8 (as established in the chemotactic assay) were 29.1 ± 15.0 ng/ml and 5.4 ± 4.7 ng/ml for the instillation of rhIL-8 alone and rhIL-8-"fast" rabbit αM complexes, respectively.

should also be mentioned that we used in this study preformed "fast" αM. Namely, αM was converted to the "fast" form (methylamine treatment), dialyzed and then reacted with IL-8. Therefore, the only possible covalent interaction would involve S−H groups [31]. Indeed, we have recently shown that thiol groups are involved in covalent interaction between IL-8 and human α_2M (Kurdowska A, Noble JM, Alden SM, Carr FK. Cysteine-949 is involved in the binding of interleukin-8 to α-2-macroglobulin. Proc Soc Exp Biol Med, second review). Furthermore, binding of ^{125}I-rhIL-8 to "fast" rabbit αM was inhibited by an antibody to rabbit αM (not shown).

Evidence for the binding of rhIL-8 (unlabeled) to "fast" rabbit αM was also obtained by IL-8 ELISA analysis of samples generated using the HPLC TSK-250 column. The binding was saturable (Fig. 4A), and the calculated K_D was approximately 26 nM (Fig. 4B). In addition, the binding of unlabeled rhIL-8 with "fast" α_2M was detected in the ELISA specific for IL-8-α_2M complexes (Fig. 5). The values obtained for rabbit α_2M did not differ significantly from those for human α_2M ($p > 0.05$).

In vivo studies. The effect of "fast" rabbit αM on IL-8 ability to attract neutrophils to the rabbit lung

The purpose of the in vivo experiments was to determine whether αM plays a role in modulating the IL-8 induced neutrophil trafficking to the lung. rhIL-8 or saline (control) was instilled into one lung, and rhIL-8 in complex with "fast" rabbit αM or "fast" rabbit αM alone into the other. The sample instilled into one lung contained a 1% solution of Evans Blue, and the sample instilled into the second lung contained a 1% solution of carbon black. Before the lavage, the lungs were assessed visually. The presence of the dye indicated that the instillation was successful. The results described below represent the analysis of 20 separate experiments. It should also be noted that the instillation of either dye did not cause a significant change in measured parameters as compared to no instillation of any kind (see Table 1).

The concentration of IL-8 and the number of neutrophils in the lung lavage were determined following instillation. The concentration of IL-8 was significantly increased ($p < 0.05$) when rhIL-8 alone was instilled to the lung (Table 1). Significantly less IL-8 was detected following the instillation of rhIL-8-"fast" rabbit αM complexes, "fast" rabbit αM alone or saline ($p < 0.05$). In addition, to estimate the amount of bioactive IL-8, lung fluids were analyzed in the chemotactic assay. The activity of IL-8 present in these fluids was compared to that of the same concentration of control IL-8. Samples obtained by instillation of rhIL-8 alone displayed about 112% activity suggesting that the concentration of IL-8 was actually higher from the one measured using the ELISA assay. On contrary, lung fluids collected after administering rhIL-8-"fast" rabbit αM complexes exhibited only 45% activity. Furthermore, the concentrations of bioactive IL-8 for the instillation of rhIL-8 alone and rhIL-8-"fast" rabbit αM complexes were significantly different ($p < 0.05$) (included in the legend to Table 1). In addition, the amounts of IL-8 in ELF collected from the rabbits which received rhIL-8-"fast" rabbit αM complexes or "fast" rabbit αM alone did not differ.

In addition, there were fewer neutrophils ($p < 0.05$) after instillation of rhIL-8-"fast" rabbit αM complexes than after the instillation of rhIL-8 alone (Table 1). Similarly, the percentage of neutrophils was significantly decreased ($p < 0.001$) when rhIL-8-"fast" rabbit αM complexes, "fast" rabbit αM alone or saline was instilled into rabbit lungs (Fig. 6). Furthermore, the direct comparison between the instillation of rhIL-8 alone and rhIL-8-"fast" rabbit αM complexes revealed that the amount of neutrophils was significantly higher ($p < 0.05$) in case of rhIL-8 instillation (Table 1, Fig. 6). In addition, there was a significant correlation between the IL-8 and neutrophil concentrations in the ELF ($p < 0.05$) (Fig. 7). The correlation coefficient is 0.43 when the concen-

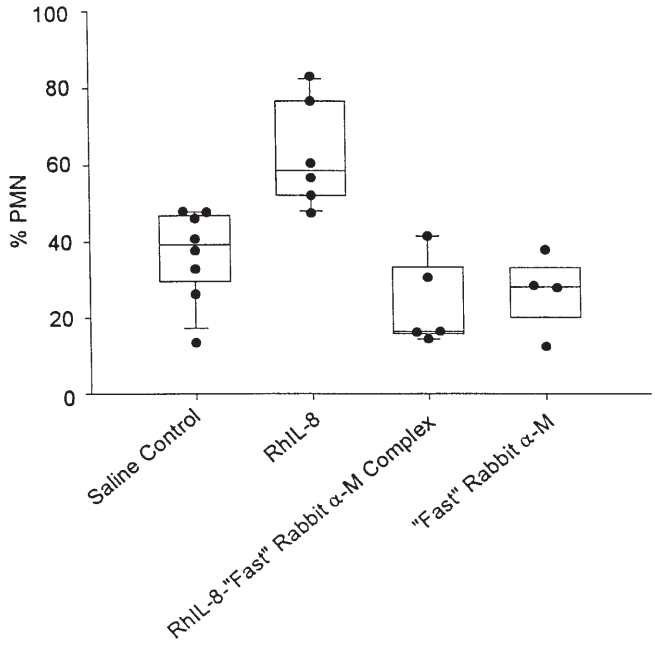

Fig. 6. The percentage of neutrophils in lung fluid samples after instilling saline (control), rhIL-8, rhIL-8 in complex with "fast" rabbit αM or "fast" rabbit αM alone. The percentage of neutrophils was assessed in lung fluid samples after instilling of the following samples: saline control, rhIL-8, rhIL-8 in complex with "fast" rabbit αM, and "fast" rabbit αM. The figure represents box and whisker plot with the $10^{th}/90^{th}$ percentiles as lines and the $5^{th}/95^{th}$ percentiles as error bars.

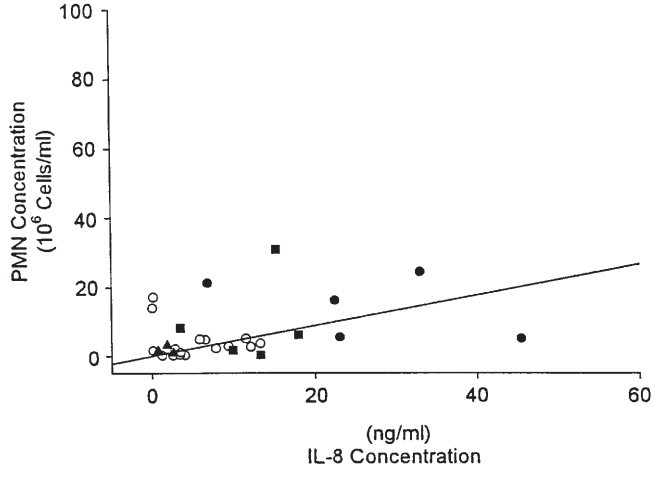

Fig. 7. The correlation between IL-8 and neutrophil concentrations in the ELF of rabbits after instillation of the samples. The concentrations of IL-8 and PMN were significantly correlated ($p < 0.05$, $r = 0.43$) after instilling of the following samples: no sample, or saline control (○), rhIL-8 (●), rhIL-8 in complex with "fast" rabbit αM (■), and "fast" rabbit αM (▲).

trations of IL-8 and PMN in the ELF samples from all the treatment groups are evaluated. Nonetheless, it raises to 0.60 for the ELF obtained after the instillation of rhIL-8, rabbit "fast" αM, and rhIL-8-"fast" rabbit αM complexes. The latter value parallels the values reported previously for IL-8 and PMN [33, 34].

It should also be noted that the concentrations of neutrophils and IL-8 in the lung fluids from control rabbits as well as animals which had only saline instilled are consistent with the previous findings [35–37]. For example, approximately 0.4×10^6 neutrophils were present in lung washes obtained from normal rabbits. The value reported in our study (about 5×10^6) was obtained by multiplying the actual measurement by the ratio of urea concentrations between plasma and lavage fluid (these ratios ranged from 10 to 30). Analogous explanations apply to concentrations of other measured parameters. We have used urea to correct for dilution in the lung so that we could express all results per unit volume of epithelial lining fluid. In addition, we recovered very similar volumes of lavage fluids, therefore, the data presented either as the total number of neutrophils (Fig. 6) or the concentrations of neutrophils does not differ (not shown).

Furthermore, the instillation of the samples had no significant effect ($p = 0.68$) on the total protein concentration in the lung fluids (Table 1). In addition, there was a 40% decrease in the number of circulating white blood cells two hours after the instillation. By four hours the neutrophil counts reached about 90% of the initial values. Similar decreases occurred for all types of samples instilled. This transient decrease in number of circulating neutrophils at two hours reflects a well-recognized response to minor procedures performed on animals [38, 39].

Discussion

The precise role of α_2M is still not known although several studies suggested its involvement in various aspects of the immune response [40, 41]. In addition, it should be noted that in humans a complete α_2M deficiency has never been described. The physiologic significance of cytokine – α_2M interactions also remains unclear despite extensive studies during the last two decades [41]. In patients with ARDS, the concentration of α_2M is elevated in lung fluids due primarily to increased lung permeability [6]. In addition, the majority of α_2M present in these fluids is complexed to human neutrophil elastase ("fast" form of α_2M) [42]. Furthermore, ARDS is frequently associated with an accumulation of neutrophils [4], and IL-8 is a potent neutrophil attractant and activator in lung fluids from patients with ARDS [2].

We have shown previously that rhIL-8 binds to "fast" (methylamine-treated) human α_2M [13]. Native or "slow" α_2M can be easily converted to the "fast" form by treatment with methylamine [43]. In vivo proteinases reacting with α_2M transform it to the "fast" form [11, 43]. However, cytokines bind equally well to methylamine and proteinase-treated α_2M [44, 45].

In this study we report that rhIL-8 binds to "fast" forms of rabbit α_1M and α_2M. However, the autoradiographs revealed that α_2M binds approximately 5-times more of rhIL-8 than α_1M (see Fig. 1). In addition, most of rhIL-8 complexed to rabbit αM was dissociated by SDS, suggesting that the binding was mostly non-covalent in nature. This is in agreement with our previous findings with rhIL-8 and "fast" human α_2M [13]. Similarly, the binding was also specific and unlabeled rhIL-8 competed with ^{125}I-rhIL-8 for the binding to "fast" rabbit αM.

Our data indicate that the binding of rhIL-8 to "fast" forms of rabbit αM was saturable, and the calculated K_D was approximately 26 nM. Similar results were obtained for the binding of rhIL-8 to "fast" form of human α_2M. Even though "fast" rabbit αM contains two IL-8-binding moieties (α_1M and α_2M), the curve fitted only a one-site model when it was analyzed using the Lundon computer program (Lundon Software Inc., Chagrin Falls, OH). However, taking into consideration that approximately 5-times less of rhIL-8 binds to "fast" rabbit α_1M (scanning the autoradiographs), we calculated K_D for "fast" rabbit α_1M and α_2M alone which was about 80 and 15 nM, respectively. Our findings agree with the observations made by De Lean et al. [46]. The authors found that to achieve a resolution of the two sites parameters, the ratio of the K_{Ds} must be at least 6 providing that one site binds approximately 5-times less of the ligand [46].

In addition, the amount rhIL-8 bound to "fast" rabbit α_2M was similar to that for "fast" human α_2M as measured using the ELISA assay specific for IL-8-α_2M complexes ($p > 0.05$).

In the previous study, we showed that rhIL-8-"fast" human α_2M complexes bound to IL-8 receptors on human neutrophils, and did not differ from "free" IL-8 in their ability to activate and attract neutrophils [13]. This would suggest that interaction with α_2M did not affect the regions of the IL-8 molecule responsible for the binding to the specific IL-8 receptors [13]. Since the affinity of the binding of rhIL-8 to rabbit αM is similar for that for human α_2M, it is very likely that the former also would not change the activity of rhIL-8. Indeed, rhIL-8 in complex with "fast" rabbit αM retained its chemotactic activity.

IL-8 plays an important role in the pathogenesis of ARDS as a neutrophil attractant and activator. In addition, the results of our earlier studies showed that IL-8-α_2M complexes are present in bronchoalveolar (BAL) fluids from ARDS patients [13], and that IL-8 can induce neutrophil trafficking to the lungs [33]. Presently we report that the ability of rhIL-8 to attract neutrophils to the rabbit lungs is reduced when it is in complex with "fast" rabbit αM. Most of rhIL-8 binds noncovalently to "fast" rabbit αM and could be released from the complex over time. However, instillation of rhIL-8-"fast" rabbit αM complexes did not increase the amount of IL-8 in lung fluid samples when compared with instillation of saline or "fast" rabbit αM alone. It should also be noted that the same amount of rhIL-8 was instilled as "free" or complexed rhIL-8, and the amount of "free" IL-8 in the complex preparation was only approximately 3% of the total IL-8. Finally, rhIL-8-"fast" rabbit αM complexes exhibited unchanged IL-8 bioactivity (before the instillation) when assessed in the chemotactic assay.

We also calculated the concentrations of "bioactive" IL-8 based on the measurement of the chemotactic activity of the ELF obtained after the instillation of rhIL-8 alone and rhIL-8-"fast" rabbit αM complexes. These concentrations were 29.1 ± 15.0 ng/ml and 5.4 ± 4.7 ng/ml for the instillation of rhIL-8 alone and rhIL-8-"fast" rabbit αM complexes, respectively. In comparison IL-8 concentrations calculated based on the ELISA assay were 26.1 ± 12.7 ng/ml and 11.9 ± 5.0 ng/ml. This discrepancy may be due to the fact that many ELISA systems vary in the detection of monomeric and polymeric forms of cytokines [47]. However, all forms of IL-8 are equally active [48]. In addition, αM was not detected in lavage fluids. Therefore, it could not interfere with IL-8 detection in the ELISA assay [13]. Nevertheless, significantly less IL-8 was recovered during the lavage when the complex was used instead of "free" IL-8, suggesting increased IL-8 clearance from the lung when complexed to αM. The increased IL-8 concentration in the lung fluid after instillation of IL-8 alone may represent residual protein. We administered 100 ng of IL-8 per rabbit, and recovered approximately 26 ng. Similarly, Frevert et al. reported that 4 h after instillation of iodinated IL-8 to the rabbit lung about 50% of radioactivity remained in the lung [49]. Moreover, approximately 31% labeled IL-8 was detected in the bronchoalveolar lavage fluid and 28% in lung tissue when the mixture of iodinated IL-8 with 495 ng of unlabeled IL-8 was administered [50]. In addition, in our experiments there was no detectable IL-8 in plasma. Furthermore, the fact that no αM was detected in the lavage fluids father supports the interpretation of a clearance mechanism. This increased clearance may be due to uptake by alveolar macrophages. Complexes between α_2M and cytokines are believed to be cleared through α_2M receptors [41], and alveolar macrophages (human and rabbit) have receptors for α_2M [51, 52]. In addition, we showed previously that complexes between rhIL-8 and "fast" human α_2M are cleared by human alveolar macrophages via α_2M receptors [14]. IL-8, on the other hand, does not bind to macrophages [14]. Furthermore, histological analysis of slides using a monoclonal antibody to rabbit αM revealed that macrophages were the only cells stained positive for rabbit αM (data not shown). Thus while complexed rhIL-8 retains its biological activity [13] increased clearance of IL-8-αM complexes by macrophages, would decrease IL-8 effectiveness. αM could be an important mediator of IL-8 clearance.

The data suggests that αM increases the rate of uptake of IL-8 and this way decreases the amount of IL-8 available to activate PMN. These findings are particularly relevant to inflammatory conditions involving IL-8, as for example, ARDS, and may help in understanding the mechanisms involved in regulating IL-8 activity in the lung. Our earlier studies indicate that a significant amount of IL-8 is bound to "fast" α_2M in lung fluids from patients with ARDS. The concentration of these complexes declines notably over time (between days 1 and 7 of ARDS) but only in ARDS survivors. This phenomenon is independent of the drop in total protein concentration. Then, on days 14 and 21 the concentration of IL-8 associated with α_2M significantly increases whereas the concentration of IL-8 exhibits declining tendency [13, 53]. One possibility is that formation of IL-8-α_2M complexes facilitates clearance of IL-8 by alveolar macrophages. It is also worth mentioning that Ramdin et al. detected between 1 and 10 ng/ml of α_2M in lung fluids collected from normal subjects [54]. Nevertheless, mechanisms that regulate IL-8 transport from the lungs need to be studied further. α_2M may affect activity of other chemokines as well. Our studies, for example, indicate that IL-8 competes with growth related oncogene α (GRO α) for binding to α_2M [13]. In addition, Perks et al. reported the presence of complexes with GRO α, neutrophil activating peptide-2, RANTES (regulated on activation, normal T expressed and secreted), and epithelial neutrophil activating peptide-78 in sputum from patients with asthma and cystic fibrosis [55].

Acknowledgements. This work was supported by a grant from the American Heart Association, Texas Affiliate Inc., and grants R29-HL56768 and R29-HL55622 from the National Heart, Lung and Blood Institute.

References

[1] Endo S, Inada K, Ceska M, Takakuwa T, Yamada Y, Nakae H, et al. Plasma interleukin 8 and polymorphonuclear leukocyte elastase concentrations in patients with septic shock. J Inflammation 1995; 45: 136–42.

[2] Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, et al. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. Am Rev Respir Dis 1992; 146: 427–32.

[3] Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, Gadek JE. Lung neutrophils in the adult respiratory distress syndrome. Clinical and pathological significance. Am Rev Respir Dis 1986; 133: 218–25.

[4] Martin TR, Pistorese BP, Hudson LD, Maunder RJ. Function of lung and blood neutrophils in patients with the adult respiratory distress syndrome. Implications for the pathogenesis of lung infections. Am Rev Respir Dis 1991; 144: 254–62.

[5] Gadek JE. Adverse effects of neutrophils on the lung. Am J Med 1992; 92: S27–31.

[6] Holter JF, Weiland JE, Pacht ER, Gadek JE, Davis WB. Protein permeability in the adult respiratory distress syndrome. Lose of size selectivity of the alveolar epithelium. J Clin Invest 1986; 78: 1513–22.

[7] Travis J, Salvesen GS. Proteinase inhibitors. Ann Rev Biochem 1983; 52: 684–95.

[8] O'Connor-McCourt MD, Wakefield LM. Latent transforming growth factor-β in serum, a specific complex with α_2-macroglobulin. J Biol Chem 1987; 262: 14090–99.

[9] Borth W, Luger TA. Identification of α_2-macroglobulin as a cytokine binding plasma protein. Binding of interleukin-1β to "F" α_2-macroglobulin. J Biol Chem 1989; 264: 5818–25.

[10] Bonner JC, Goodell AL, Lasky JA, Hoffman MR. Reversible binding of platelet-derived growth factor-AA, -AB, and -BB isoforms to a similar site on the "slow" and "fast" conformations of α_2-macroglobulin. J Biol Chem 1992; 267: 12837–44.

[11] Gonias SL, Reynolds JA, Pizzo SV. Physical properties of human 2-macroglobulin following reaction with methylamine and trypsin. Biochim Biophys Acta 1982; 705: 306–14.

[12] Crookston KP, Webb DJ, Wolf BB, Gonias SL. Classification of α_2-macroglobulin-cytokine interactions based on affinity of noncovalent association in solution under apparent equilibrium conditions. J Biol Chem 1994; 269: 1533–40.

[13] Kurdowska A, Carr FK, Stevens MD, Baughman RP, Martin TR. Studies on the interaction of interleukin-8 (IL-8) with human plasma α_2-macroglobulin (α_2M). Evidence for the presence of IL-8 complexed to α_2M in lung fluids of patients with the adult respiratory distress syndrome. J Immunol 1997; 158: 1930–40.

[14] Kurdowska A, Alden SM, Noble JM, Stevens MD, Carr FK. Involvement of α_2-macroglobulin receptor in clearance of interleukin-8-α_2-macroglobulin complexes by human alveolar macrophages. Cytokine 2000; 12: 1046–53.

[15] Thomas KM, Taylor L, Prado G, Romero J, Moser B, Car B, et al. Functional and ligand binding specificity of the rabbit neutrophil IL-8 receptor. J Immunol 1994; 152: 2496–500.

[16] Berne BH, Dray S, Knight KL. Immunological relationships of serum α-macroglobulins in the human, rat, rabbit. Proc Soc Exp Biol Med 1971; 138: 531–35.

[17] Hinata A, Ijima M, Nakano Y, Sakamoto T, Tomita M. Chemical characterization of rabbit α_2-macroglobulin. Chem Pharm Bull 1987; 35: 271–6.

[18] Webb DJ, Crookston KP, Figler NL, Lamarre J, Gonias SL. Differences in the binding of transforming growth factor β1 to the acute-phase reactant and constitutively synthesized α-macroglobulins of rat. Biochem J 1995; 312: 579–86.

[19] Webb DJ, Wen J, Lysiak JJ, Umans L, Van Leuven F, Gonias SL. Murine α-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor-β and as inducers of nitric oxide synthesis. J Biol Chem 1996; 271: 24982–88.

[20] Miller EJ, Cohen AB, Carr FK, Hayashi S, Chiu CY, Lee-Ng CT, et al. High yields of interleukin-8 produced by a synthetic gene expressed in Escherichia coli and purified with a single antibody affinity column. Protein Expression and Purification. 1995; 6: 357–62.

[21] Ko Y, Mukaida N, Panyutich A, Voitenok NN, Matsushima K, Kawai T, et al. A sensitive enzyme-linked immunosorbent assay for human interleukin-8. J Immunol Methods 1992; 149: 227–35.

[22] Kato Y, Nakamura K, Hashimoto T. High-performance metal chelate chromatography of proteins. J Chromatogr 1986; 354: 511–7.

[23] Salvesen G, Enghild JJ. α-Macroglobulins: detection and characterization. In: Lorand L, Mann KG, editors. Methods in Enzymology: Proteolytic Enzymes in Coagulation, Fibrinolysis, and Complement Activation. vol. 223. Academic Press, New York, 1993: 121–41.

[24] Hunter WM, Greenwood FC. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 1962; 194: 495–6.

[25] Nelles LP, Hall PK, Roberts RC. Human alpha$_2$-macroglobulin: Studies on the electrophoretic heterogeneity. Biochym Biophys Acta 1980; 623: 46–56.

[26] Chase T, Shaw EP. P-nitrophenyl-p′-guanidinobenzoate HCl: A new active site titrant for trypsin. Biochem Biophys Res Commun 1967; 29: 508–14.

[27] Ganrot PO. Determination of α_2-macroglobulin as trypsin protein esterase. Clin Chim Acta 1966; 14: 493–501.

[28] Zigmond S, Hirsch J. Leukocyte locomotion and chemotaxis. J Exp Med 1963; 137: 387-410.

[29] Peterson BT, Griffith DE, Tate RW, Clancy SJ. Single-cycle bronchoalveolar lavage to determine solute concentrations in epithelial lining fluid. Am Rev Resp Dis 1993; 147: 1216–22.

[30] Matsushima K, Oppenheim JJ. Interleukin-8 and MCAF: Novel inflammatory cytokines inducible by IL-1 and TNF. Cytokine 1989; 1: 2–13.

[31] Schlesinger C, McEntire J, Wallman J, Skosey JL, Hanly WC, Teodorescu M. Covalent binding to α-macroglobulins of a protein with free SH groups produced by activated B cells: Blocking by D-penicillamine and gold compounds. Mol Immunol 1989; 26: 255–67.

[32] Harpel PC. Studies on human plasma α_2-macroglobulin-enzyme interactions. J Exp Med 1973; 138: 508–21.

[33] Miller EJ, Cohen AB, Peterson BP. Peptide inhibitor of interleukin-8 (IL-8) reduces staphylococcal enterotoxin-A (SEA) induced neutrophil trafficking to the lung. Inflammation Research 1996; 45: 393–7.

[34] Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, et al. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. Am J Respir Crit Care Med 1996; 154: 602–11.

[35] Cohen AB, Batra G, Petersen R, Podany J, Nguyen D. Size of the pool of alveolar neutrophils in normal rabbit lungs. J Appl Physiol 1979; 47: 440–4.

[36] Folkesson HG, Matthay MA, Hébert CA, Broaddus VC. Acid aspiration-induced lung injury in rabbits is mediated by interleukin-8-dependent mechanism. J Clin Invest 1995; 96: 107–16.

[37] Fox-Dewhurst R, Alberts MK, Kajikawa O, Caldwell E, Johnson MC, Skerrett SJ, et al. Pulmonary and systemic inflammatory responses in rabbits with gram-negative pneumonia. Am J Respir Crit Care Med 1997; 155: 2030–40.

[38] Fox-Dewhurst R, Alberts MK, Kajikawa O, Caldwell E, Johnson II MC, Skerrett SJ, et al. Pulmonary and systemic inflammatory responses in rabbits with gram-negative pneumonia. Am J Respir Crit Care Med 1997; 155: 2030–2040.

[39] Doerschuk CM, Mizgerd JP, Kubo H, Qin L, Kumasaka T. Adhesion molecules and cellular biomechanical changes in acute lung injury. Chest 1999; 116: 37–43.

[40] James K. Interactions between cytokines and alpha$_2$-macroglobulin. Immunol Today 1990; 11:163–6.
[41] LaMarre J, Wollenberg GK, Gonias SL, Hayes MA. Biology of disease. Cytokine binding and clearance properties of proteinase-activated 2-macroglobulin. Lab Invest 1991; 65: 3–14.
[42] Wewers MD, Herzyk DJ, Gadek JE. Alveolar fluid neutrophil elastase activity in the adult respiratory distress syndrome is complexed to alpha$_2$-macroglobulin. J Clin Invest 1988; 82: 1260–7.
[43] Björk I, Fish WW. Evidence for the similar conformational changes in alpha$_2$-macroglobulin on reaction with primary amines or proteolytic enzymes. Biochem J 1982; 207: 347–56.
[44] Hall SW, LaMarre JU, Marshall LB, Hayes MA, Gonias SL. Binding of transforming growth factor-β1 to methylamine-modified α_2-macroglobulin and to binary and ternary α_2-macroglobulin-proteinase complexes. Biochem J 1992; 281: 569–75.
[45] Crookston KP, Webb DJ, LaMarre J, Gonias SL. Binding of platelet-derived growth factor-BB and transforming growth factor-β1 to α_2-macroglobulin in vitro and in vivo: Comparison of receptor-recognized and non-recognized α_2-macroglobulin conformations. Biochem J 1993; 293: 443–50.
[46] De Lean A, Hancock AA, Lefkowitz RJ. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. Mol Pharmacol 1982; 21: 5–16.
[47] Petyovka N, Lyach L, Voitenok NN. Homologous ELISA for detection of oligomeric human TNF: properties of the assay. J Immunol Methods 1995; 186: 161–70.
[48] Schnitzel W, Monschein U, Besemer J. Monomer-dimer equilibria of interleukin-8 and neutrophil activating peptide 2. Evidence for IL-8 binding as a dimer and oligomer to IL-8 receptor B. J Leukocyte Biol 1994; 55: 763–70.
[49] Frevert CW, Goodman RB, Wynant K, Kajikawa O, Hagen T, Martin TR. The effect of neutrophils on ^{125}I-IL-8 clearance in lungs and skin. Am J Respir Crit Care Med 1999; 159: A108.
[50] Frevert CW, Goodman RB, Kinsella M, Stelzner M, Ballman K, Martin TR. Simple diffusion can not account for the movement of IL-8 from the alveolar space into blood. FASEB J 2000; 14: A179.
[51] Moestrup SK, Gliemann J, Pallesen G. Distribution of the alpha$_2$-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. Cell Tissue Res 1992; 269: 375–82.
[52] Kaplan J, Nielsen ML. Analysis of macrophage surface receptors. J Biol Chem 1979; 254: 7323–28.
[53] Kurdowska A, Noble JM, Steinberg KP, Ruzinski J, Martin TR. IL-8-binding proteins in alveolar fluid from patients with the acute respiratory distress syndrome (ARDS) FASEB J 1998; 12: A879.
[54] Ramdin L, Perks B, Sheron N, Shute JK. Regulation of interleukin-8 binding and function by heparin and α_2-macroglobulin. Clin Exp Allergy 1998; 28: 616–24.
[55] Perks B, Hillaby CH, Warner JO, Shute JK. Macromolecular forms of chemokines in the airways in asthma and cystic fibrosis. Resp Med 1997; 91: A43.