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The use of monoclonal antibodies to distinguish several chemically modified forms of human α_1-proteinase inhibitor

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The purpose of our investigation was to obtain monoclonal antibodies that could distinguish three forms of α_1-proteinase inhibitor (α_1-PI): native α_1-PI, *N*-chlorosuccinimide-oxidized α_1-PI (Ox-α_1-PI) and proteolytically modifed α_1-PI (α_1-PI*). Three specific monoclonal antibodies were characterized as to their binding properties. By using the Bio-Dot assay, it was found that all three forms of α_1-PI were capable of binding to antibody $6D_4$-6-18, that only Ox-α_1-PI, but not native α_1-PI or α_1-PI*, could bind to antibody $6C_7$-5, and that α_1-PI* and a complex between α_1-PI and trypsin uniquely were not able to bind to antibody $5C_{12}$-8-7. Thus it was concluded that it is possible to use monoclonal antibodies with different epitopic specificities to distinguish two chemically modified forms of α_1-PI from the native protein.

INTRODUCTION

Leucocytic elastase has been implicated (Janoff *et al.*, 1979) in the pathogenesis of chronic lung diseases such as emphysema. A plasma protein, α_1-proteinase inhibitor (α_1-PI), has been proposed (Gadek *et al.*, 1980) to be the major elastase inhibitor that protects the lung from enzyme-induced damage. It has been suggested (Carp *et al.*, 1982; Burnett & Stockley, 1980) that several forms of α_1-PI may be present in fluid obtained from lungs, particularly from persons who smoke or have pulmonary infection. For instance, there may be present (Burnett & Stockley, 1980) a proteolytically modified form, of smaller molecular mass and devoid of inhibitory activity, that may be present in lung secretions from patients suffering from chest infection. Also, oxidation of α_1-PI in the lung may result from cigarette smoking (Gadek *et al.*, 1979; Carp *et al.*, 1982), yielding a defective inhibitor. Thus it is often suggested that these inactive inhibitors may play an important role in the pathogenesis of lung disease. However, it should be pointed out that other investigators (Stone & Calore, 1983; Abboud *et al.*, 1985; Lellouch *et al.*, 1985) have been unable to confirm the presence of inactive inhibitors in the lung lavage fluid of smokers. Therefore, until the presence of these inhibitors in the lung has been positively demonstrated, the roles of these inactive inhibitors in the pathogenesis of chronic lung diseases cannot be properly assessed. Once the presence of these inactive inhibitors is confirmed, suitable analytical techniques must be available for determining their relative concentrations in biological samples such as sputum or lung lavage fluid. These concentrations may then be carefully monitored in various pathological states in order to determine the causal relationship between the presence of these inactive inhibitors and the extent of chronic lung diseases.

In order to accomplish the goal, our strategy was to proceed in three stages. The first was to determine the feasibility of using monoclonal antibodies to distinguish chemically modified α_1-PI from native α_1-PI, as presented in the present paper. The second was to obtain monoclonal antibodies to distinguish leucocyte or cigarette-smoke-oxidized α_1-PI from the native protein. The third was to use these antibodies with known epitopic specifications to ascertain the presence of these inactive inhibitors in the biological fluid.

EXPERIMENTAL

Preparation of native α_1-PI and oxidized α_1-PI (Ox-α_1-PI)

Native α_1-PI was isolated and purified from outdated human plasma as previously described (Schochat *et al.*, 1978). The purified native α_1-PI was oxidized with *N*-chlorosuccinimide (Johnson & Travis, 1978) in a molar ratio of 1:25 in 0.1 M-Tris/HCl buffer, pH 8.0. The reaction was allowed to proceed at room temperature for 30 min, and Ox-α_1-PI was recovered after passing the reaction mixture through a Sephadex G-25 column (2 cm × 15 cm) that had been equilibrated in 50 mM-NH_4HCO_3. The sample of Ox-α_1-PI thus obtained had lost all its inhibitory activity against pig pancreatic elastase but maintained 85% of its former inhibitory activity against trypsin. These results are in agreement with the known properties of Ox-α_1-PI, which had been previously characterized by Johnson & Travis (1979). These investigators found two methionine sulphoxide residues in the oxidized protein, but no alteration in tryptophan or other amino acid residues was observed. Furthermore, when the association rates of α_1-PI and Ox-α_1-PI with pig elastase and trypsin were measured (Beatty *et al.*, 1980), it was found that oxidation completely abolished any interaction between the oxidized inhibitor and elastase, though the rate constant for Ox-α_1-PI and trypsin was decreased only 10-fold.

Preparation of proteolytically modified α_1-PI (α_1-PI*)

Native α_1-PI (0.22 μmol) and trypsin (0.176 μmol) were incubated in 5 mM-Tris/HCl buffer, pH 8.6,

Abbreviations used: α_1-PI, α_1-proteinase inhibitor (formerly α_1-antitrypsin); Ox-α_1-PI, *N*-chlorosuccinimide-oxidized α_1-proteinase inhibitor; α_1-PI*, proteolytically modified α_1-proteinase inhibitor.

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containing 20 mM-$CaCl_2$, for 10 min at room temperature. Afterwards, 0.17 ml of 0.1 M-phenylmethanesulphonyl fluoride, a serine-proteinase inhibitor, was added. The mixture was resolved on a DEAE-Sephacel column (1 cm × 18 cm) that had been equilibrated with 5 mM-Tris/HCl buffer, pH 8.6, containing 1 mM-phenylmethanesulphonyl fluoride. After the sample was applied to the column, 50 ml of the equilibrating buffer was allowed to pass through the column, followed by a linear gradient (200 ml) of 0–0.2 M-NaCl in the same buffer. Under these conditions free trypsin emerged first, followed by the α_1-PI–trypsin complex, and free α_1-PI was eluted subsequently during the gradient. The fractions containing the complex were pooled.

To the α_1-PI–trypsin complex (45 ml) was added 0.05 ml of 0.1 M-phenylmethanesulphonyl fluoride. The pH of this solution was adjusted to 12 by dropwise addition of 20% (w/v) NaOH. The complex was allowed to dissociate at room temperature for 2 h. The pH was then lowered to 8.5 by the addition of 4 M-HCl. The solution, containing the dissociated products α_1-PI* and trypsin, was dialysed overnight against 5 mM-Tris/HCl buffer, pH 8.5, and then applied to a DEAE-Sephacel column and developed similarly to that described above. The fractions containing α_1-PI* were collected after the gradient began, and were pooled, dialysed and freeze-dried.

Samples of native α_1-PI, Ox-α_1-PI, α_1-PI* and the complex between α_1-PI and trypsin were subjected to amino acid analysis (Liao *et al.*, 1973), and, from the known amino acid compositions of the proteins, the precise concentrations of these proteins were calculated. SDS/polyacrylamide gels were prepared and run in accordance with Laemmli (1970).

Monoclonal-antibody production

A standard procedure (Goding, 1980) of hybridoma technology was followed. The antigen used for immunization was chemically oxidized α_1-PI (Ox-α_1-PI). Each of six BALB/c mice was immunized intraperitoneally with 200 μg of Ox-α_1-PI emulsified in 0.2 ml of Freund's complete adjuvant. Two additional injections were performed with 200 μg of the same antigen emulsified with incomplete adjuvant. At 6 weeks after the initial immunization mice were injected intravenously with 100 μg of the protein through the tail vein. Then 3 days later serum from each mouse was tested for its capacity to bind Ox-α_1-PI, and the spleens of the three mice whose sera showed the highest binding capacity were used for cell fusion. Fusion was carried out with an Sp 2/0–Ag 14 mouse myeloma cell line and immune spleen cells with poly(ethylene glycol) as the fusion agent. The hybrids were cultured in 96-well micro-titre plates with HAT (hypoxanthine/aminopterin/thymidine) selective medium. The supernatants from the culture medium containing the hybrid cells were tested 2 weeks after fusion for their ability to bind Ox-α_1-PI.

Positive hybrid colonies were further cloned two or three times by limiting dilution. The supernatants of the culture fluid were tested for their ability to bind with native α_1-PI, Ox-α_1-PI and α_1-PI*. The subclass of these antibodies was determined by an immunoassay procedure with a mouse immunoglobulin subtype identification kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.).

Two selected clones, $5C_{12}$-8-7 and $6D_4$-6-18, were expanded first in culture and then injected intraperitoneally into syngeneic mice that had been primed with 0.3 ml of pristane (2,6,10,14-tetramethylpentadecane) 7–9 days previously. Ascites fluid was collected, and the IgG fraction was isolated on a Protein A–Sepharose column in accordance with a published procedure (Zola & Brooks, 1982). One clone, $6C_7$-5, was grown in culture in a serum-free medium. The culture fluid, which contained the secreted IgG, was used directly as the primary antibody in the assay.

Determination of binding of the monoclonal antibodies

The Dot immunoassay (Hawkes *et al.*, 1982) was used for this purpose. In this procedure and in the procedure described in the next paragraph, several common solutions were used, and the compositions of these solutions are as follows: (1) phosphate-buffered saline was 10 mM-sodium phosphate, pH 7.2, containing 0.15 M-NaCl; (2) Tween phosphate-buffered saline was phosphate-buffered saline containing 0.05% Tween 20; (3) blocking solution was phosphate-buffered saline containing 5% (w/v) dried milk powder; (4) secondary antibody solution contained peroxidase-linked affinity-purified goat anti-mouse IgG antibodies (catalogue no. 14-18-06; Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) that had been diluted 1:2000 with the blocking solution; (5) stain solution was 2 ml of 4-chloro-1-naphthol (3 mg/ml in methanol), 8 ml of phosphate-buffered saline and 10 μl of H_2O_2.

The procedure for screening positive hybridomas is described below. Nitrocellulose paper was cut into 3 mm × 12 mm strips. Four grids were printed on to each strip with a Millipore 3 mm × 3 mm grid printer. Approx. 0.1 μl of antigen at a concentration in the range 2–20 nmol/ml was dotted on to the paper. Since there were four grids on each strip of nitrocellulose paper, each strip could accommodate four concentrations of an antigen or four different antigens. After 2 h of drying at room temperature, the strips were rinsed first in distilled water and then in blocking solution. Each strip was then inserted into a well of a 96-well Linbro micro-titration plate, 200 μl of culture fluid to be tested for binding capacity with the antigen was introduced into each well, and the plate was left to sit overnight at room temperature. The culture fluid was removed by aspiration, and the strips in the wells were washed once with 200 μl of Tween/phosphate-buffered saline and twice with phosphate-buffered saline and incubated with the blocking solution for 15 min. Into each well was introduced 100 μl of the secondary antibody solution, and the plate was left to incubate for 2 h at room temperature. Afterwards the antibody solution was removed by aspiration, and the strips in the wells were washed sequentially once with Tween/phosphate-buffered saline and five times with phosphate-buffered saline. Stain solution in a volume of 100 μl was introduced to each well, and colour development took place in 2–15 min. The appearance of blue colour within a grid indicated positive binding between the antigen applied within the grid and the antibodies present in the culture fluid.

Relative binding affinity of three selected monoclonal antibodies

The relative binding of three monoclonal antibodies to native α_1-PI, Ox-α_1-PI and α_1-PI* was determined by the

Bio-Dot immunoassay (Hawkes *et al.*, 1982). A piece of nitrocellulose paper was washed in distilled water and, while still wet, the paper was assembled in a Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Excess water in the paper was removed by gentle suction in the apparatus. Native α_1-PI, Ox-α_1-PI or α_1-PI* of a known concentration in a volume of 50 μl was then applied to each well in the apparatus and allowed to absorb into the paper. After drying overnight in the apparatus, the paper was removed, washed with phosphate-buffered saline and incubated with the blocking solution for 30 min. The paper was then immersed in a solution containing the monoclonal antibody to be tested as specified in each experiment. After being washed once with Tween/phosphate-buffered saline and then twice with phosphate-buffered saline, for 10 min each, the paper was incubated in blocking buffer for 15 min followed by the secondary antibody solution for 2 h. After being washed once with Tween/phosphate-buffered saline and then four times with phosphate-buffered saline, each for 5 min, the paper was immersed in the stain solution for colour development, which was complete within 10 min. The intensity of the colour spot (blue) was monitored by a reflective densitometer (Bio-Rad Laboratories). In the procedure described above, washing was carried out by placing the nitrocellulose paper in a washing solution with continuous gentle rocking.

RESULTS

Samples of native α_1-PI, Ox-α_1-PI and α_1-PI* were subjected to SDS/polyacrylamide-gel electrophoresis. As is shown in Fig. 1, a single band was revealed for each of these proteins, native α_1-PI and Ox-α_1-PI having similar molecular masses and α_1-PI* having a slightly smaller molecular mass.

Hybridoma cells that secreted antibodies that bound to Ox-α_1-PI were subcloned two or three times by the procedure of limiting dilution. A panel of monoclonal antibodies secreted from the cloned hybridoma cells was tested for capacity of binding to three forms of α_1-PI, i.e. native α_1-PI, Ox-α_1-PI and α_1-PI*. In addition, the subclasses of heavy chains and light chains present in these monoclonal antibodies were determined. The results are shown in Table 1. Among the 20 selected monoclonal antibodies examined, all bound Ox-α_1-PI, the immunizing antigen; two of them, $6C_7$-5 and $3D_6$-G_4, bound only Ox-α_1-PI, but not native α_1-PI or α_1-PI*. Two monoclonal antibodies, $5C_{12}$-23 and $5C_{12}$-8-7, bound both native α_1-PI and Ox-α_1-PI, but not α_1-PI*.

Three hybridoma clones were selected for further studies. The IgG fractions from two clones, $6D_4$-6-18 and $5C_{12}$-8-7, were purified on a Protein A–Sepharose column. The IgG fraction present in the culture fluid of the third clone, $6C_7$-5, was used directly without purification. The binding capacity of each of these three monoclonal antibodies was tested against several concentrations of three forms of α_1-PI: native α_1-PI, Ox-α_1-PI and α_1-PI*. The results are illustrated in Fig. 2. In Fig. 2(*a*), with monoclonal antibody $6D_4$-6-18 as the primary antibody, it is observed that this antibody bound to all three forms of α_1-PI. In all cases linear proportionality could be established between the concentration of the respective antigen in the range 20–80 pmol/ml and the intensity of the blue colour developed in the assay. This result indicates that the Bio-Dot immunoassay method could appropriately be used for the measurement of a range of concentrations of a given antigen subject to the limitation of the binding capacity of the nitrocellulose paper to proteins (both α_1-PI and other proteins in the

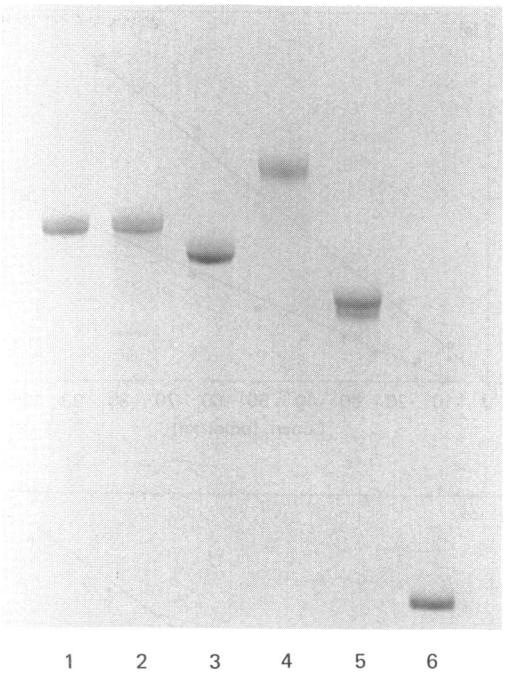

Fig. 1. SDS/10%-polyacrylamide-gel electrophoresis of native α_1-PI, Ox-α_1-PI and α_1-PI*

Lane 1, 6 μg of native α_1-PI; lane 2, 6 μg of Ox-α_1-PI; lane 3, 6 μg of α_1-PI*; lane 4, 5 μg of bovine serum albumin (68000 Da); lane 5, 5 μg of alcohol dehydrogenase (41000 Da); lane 6, 5 μg of myoglobin (17000 Da).

Table 1. Properties of a panel of monoclonal antibodies against human α_1-PI

Clone	Subclass	Binding to		
		α_1-PI	Ox-α_1-PI	α_1-PI*
$6H_4$-I	γ_1, K	+	+	+
$3F_2$-C_2	γ_3, K	+	+	+
$6C_3$-F_7	γ_3, K	+	+	+
$4E_5$-22	γ_1, K	+	+	+
$5C_{12}$-23	γ_1, K	+	+	−
$5C_{12}$-8-7	γ_1, K	+	+	−
$6D_4$-18-10	γ_1, K	+	+	+
$6D_4$-18-22	γ_1, K	+	+	+
$6D_4$-6-8	γ_{2a}, K	+	+	+
$6D_4$-3-5	γ_{2a}, K	+	+	+
$6D_4$-6-18	γ_{2a}, K	+	+	+
$6D_4$-7-5	γ_1, K	+	+	+
$6D_4$-6-22	γ_{2a}, K	+	+	+
$5C_3$-19	γ_1, K	+	+	+
$6G_3$-21	γ_3, K	+	+	+
$6C_3$-23	γ_3, K	+	+	+
$3D_6$-G_4	γ_3, K	−	+	−
$6C_7$-5	γ_1, K	−	+	−
$5B_2$-8-7	γ_1, K	+	+	+
$6C_{12}$-5-8	γ_1, K	+	+	+

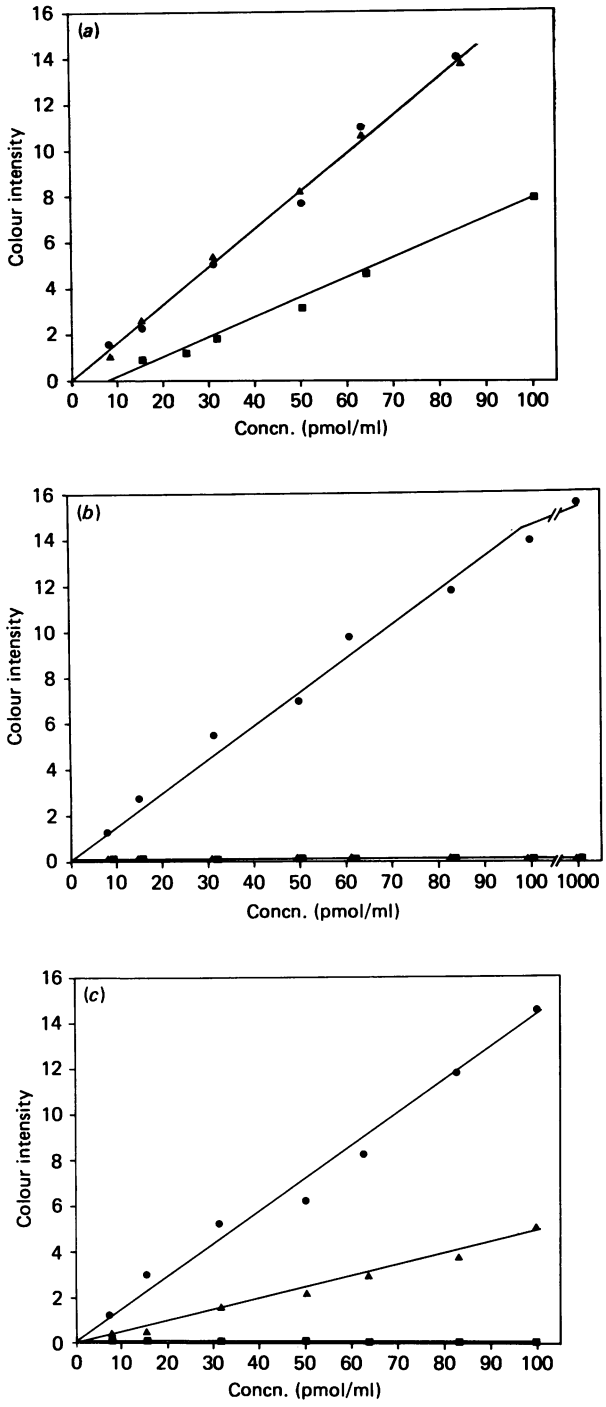

Fig. 2. Relative binding affinities of three specific monoclonal antibodies to three forms of α_1-PI (●, Ox-α_1-PI; ▲, native α_1-PI; ■, α_1-PI*)

Monoclonal antibody $6D_4$-6-18 was used as the primary antibody in (*a*). The antibody was purified on a Protein A–Sepharose column and had a final concentration of 1 mg/ml. A 1:6000 dilution was made before incubation. Monoclonal antibody $6C_7$-5 was used as the primary antibody in (*b*). The culture fluid containing the antibody was used directly in the incubation without prior purification. Monoclonal antibody $5C_{12}$-8-7 was used as the primary antibody in (*c*). The antibody was purified on a Protein A–Sepharose column and had a final concentration of 1 mg/ml. A 1:6000 dilution was made before incubation.

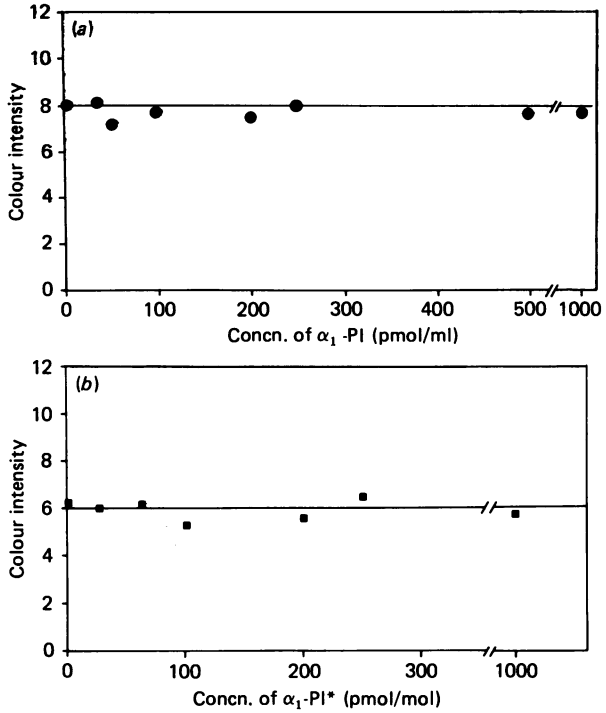

Fig. 3. Competitive binding of specific monoclonal antibodies to different forms of α_1-PI

In (*a*) Ox-α_1-PI at a concentration of 100 pmol/ml was mixed with an equal volume of native α_1-PI solution of increasing concentration as indicated. The mixture (50 μl) was dotted on the nitrocellulose paper. Monoclonal antibody $6C_7$-5 was used as the primary antibody for the Bio-Dot assay. In (*b*) native α_1-PI at a concentration of 100 pmol/ml was mixed with an equal volume of α_1-PI* solution of increasing concentration as indicated. The mixture (50 μl) was dotted on the nitrocellulose paper. Monoclonal antibody $5C_{12}$-8-7 was used as the primary antibody for the Bio-Dot assay.

sample). This factor dictated the absolute upper limit of concentrations to be assayed. It is shown in Fig. 2(*a*) that monoclonal antibody $6D_4$-6-18 binds all three forms of α_1-PI. In Fig. 2(*b*), with monoclonal antibody $6C_7$-5 as the primary antibody, binding to Ox-α_1-PI was strong, but no binding was observed between this antibody and either native α_1-PI or α_1-PI*. Even at an applied concentration of 1 nmol/ml, these latter proteins did not bind to this antibody. In Fig. 2(*c*), with monoclonal antibody $5C_{12}$-8-7 as the primary antibody, both native α_1-PI and Ox-α_1-PI were shown to bind to the antibody, but α_1-PI* did not bind even at an applied concentration of 1 nmol/ml (results not shown). Likewise, when a complex between α_1-PI and trypsin was prepared and its binding to antibody $5C_{12}$-8-7 was determined by the Bio-Dot assay, no binding could be demonstrated at an applied concentration of up to 500 pmol/ml (results not shown). Since α_1-PI* was obtained after dissociation from an α_1-PI–trypsin complex at pH 12, the lack of binding to monoclonal antibody $5C_{12}$-8-7 might be caused by the alkaline treatment. This possibility has been eliminated, since native α_1-PI did not lose the capacity of binding to antibody $5C_{12}$-8-7 after similar treatment at pH 12 (results not shown).

Since monoclonal antibody $6C_7$-5 binds only to

Ox-α_1-PI but not to native α_1-PI, and monoclonal antibody $5C_{12}$-8-7 binds to native α_1-PI but not to α_1-PI*, these antibodies were respectively used to measure the concentrations of Ox-α_1-PI in the presence of native α_1-PI and of native α_1-PI in the presence of α_1-PI*. The results of these experiments are illustrated in Fig. 3. Increasing concentrations of native α_1-PI to 1 nmol/ml (Fig. 3*a*) and increasing concentrations of α_1-PI* to 1 nmol/ml (Fig. 3*b*) did not alter the intensity of the colour yield given by a sample containing respectively 5 pmol of Ox-α_1-PI and native α_1-PI/ml.

DISCUSSION

The purpose of our investigation was to develop monoclonal antibodies that could distinguish the three forms of α_1-PI: native α_1-PI, Ox-α_1-PI and α_1-PI*. The rationale of our approach was based on three considerations. First, if oxidation does create new epitopic sites in Ox-α_1-PI, some monoclonal antibodies elicited in response to Ox-α_1-PI as the immunogen would exclusively react only with Ox-α_1-PI, but not with α_1-PI. Thus we could use such a monoclonal antibody to detect the presence and measure the concentration of Ox-α_1-PI in biological fluid. Secondly, since crystallographic studies (Löbermann *et al.*, 1984) amply demonstrated that the conformation of α_1-PI* must be considerably different from that of native α_1-PI, it was postulated that some epitopic site(s) present in native α_1-PI and Ox-α_1-PI might not be accessible in α_1-PI* and thus monoclonal antibodies elicited in response to these sites would not bind α_1-PI*. Thirdly, in spite of differences in conformation among the three forms of α_1-PI, some epitopic sites would not be affected. Thus a monoclonal antibody might be found that may bind all three forms of α_1-PI. In that case a 'universal' antibody would be available.

As described in the Results section, monoclonal antibody $6D_4$-6-18 bound all three forms of α_1-PI. This finding indicated that the epitopic site recognized by this monoclonal antibody is present in all three forms of α_1-PI. This monoclonal antibody appears to be a 'universal' antibody from a qualitative point of view because it binds to all three forms of α_1-PI.

Monoclonal antibody $6C_7$-5 was found to bind Ox-α_1-PI, but not native α_1-PI or α_1-PI*. No binding was demonstrated even when the concentration was increased to 1 nmol/ml. This antibody should be useful for determining the presence and concentration of Ox-α_1-PI in a sample. Difficulty may be encountered if the concentration of Ox-α_1-PI is only a few per cent of that of native α_1-PI. However, results shown in Fig. 2(*a*) showed that the presence of native α_1-PI does not seem to interfere with the measurement of Ox-α_1-PI in the same sample.

Monoclonal antibody $5C_{12}$-8-7 bound Ox-α_1-PI and native α_1-PI, but not α_1-PI* at all. The lack of binding between this antibody and α_1-PI* was absolute, as no binding could be noted even when the α_1-PI* applied to the paper was increased to 1 nmol/ml. Likewise, monoclonal antibody $5C_{12}$-8-7 also does not bind the α_1-PI–trypsin complex in the Bio-Dot assay. These data demonstrated that the epitopic site specific to this monoclonal antibody must be inaccessible in α_1-PI* and in the complex and thus not be amenable to binding with this monoclonal antibody. Thus we now have available two monoclonal antibodies: antibody $5C_{12}$-8-7 does not bind α_1-PI*, whereas antibody $6D_4$-6-18 does. The combined use of these two antibodies should detect the presence and estimate the concentration of α_1-PI* in a sample.

On the basis of the data presented above, it is possible to use monoclonal antibodies with different epitopic specificities to determine the concentration of the three forms of α_1-PI. However, it must be emphasized that Ox-α_1-PI and α_1-PI* used in our experiments are chemically prepared in the laboratory (for instance Ox-α_1-PI is the product of oxidation of native α_1-PI by *N*-chlorosuccinimide).

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