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Monoclonal antibody specific for the mutant *PiZ* α_1-antitrypsin and its application in an ELISA procedure for identification of *PiZ* gene carriers

(screening for α_1-antitrypsin deficiency)

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ABSTRACT **The *PiZ* genetic variant of α_1-antitrypsin in its homozygous form is associated with an increased risk for chronic lung and liver disease. About 5% of the population are carriers of one *PiZ* deficiency gene resulting in intermediate levels of plasma α_1-antitrypsin (*PiMZ*, *PiSZ*, *PiPZ*). We report the preparation of a hybridoma cell line, ATZ 11, produced by fusion of spleen cells from BALB/c mice immunized with a purified liver PiZ α_1-antitrypsin and Sp 2/0 Ag 14 mouse myeloma cells. ATZ 11 produces monoclonal antibodies (IgG1 κ) specifically interacting with PiZ α_1-antitrypsin. These antibodies were used in an ELISA procedure permitting easy and accurate identification of *PiZ* gene carriers. The method is especially well-suited for studying large population samples concerning the putative relationship between intermediate α_1-antitrypsin deficiency and disease.**

α_1-Antitrypsin (AAT) is a protective protein that inactivates a variety of proteolytic enzymes by 1:1 complex formation. AAT has several genetic variants resulting from single amino acid substitutions in its polypeptide chain (1). Variants are classified alphabetically according to electrophoretic mobilities at an acid pH into the protease inhibitor (Pi) system (1). Subnormal plasma AAT levels are found in PiZ, PiS, and PiP with ≈15%, 60%, and 80% of normal levels, respectively. Inheritance is autosomal codominant and heterozygotes have intermediate plasma levels—i.e., PiMZ 60%, PiMS 80%, and PiSZ 40%, etc. In Northern Europe, the *PiZ* gene frequency is high (2) and nearly 5% of the population bear the *PiZ* allele with *PiMZ* in 4.7%, *PiSZ* in 0.1%, and *PiPZ* in 0.1% of the population.

The classical AAT deficiency PiZZ predisposes for both lung and liver disease in adults (2, 3). The association of intermediate AAT deficiency (PiMZ, PiSZ, or PiPZ) with lung disease was first suggested in 1968 (4), but it has remained controversial (5, 6). A large study in England has shown an increased frequency of intermediate AAT deficiency among patients with seronegative chronic active hepatitis and cryptogenic cirrhosis (7). Several studies have proposed relationships between the PiMZ intermediate deficiency state and the occurrence of rheumatoid arthritis (8), chronic pancreatitis (9), and hemochromatosis (10).

Identification of heterozygotes for the AAT deficiency has been hampered by plasma levels that frequently lie well within normal concentration ranges and the complexity of the phenotyping procedure. Microheterogeneity due to various degrees of branching and sialylation of the three oligosaccharide side chains normally results in at least five bands in the isoelectric focusing system. Variations in relative intensities occur during inflammation and estrogen influence (11, 12), so that determination of phenotype by this method or by starch gel electrophoresis at an acid pH is intricate. We have, therefore, regarded a simple screening method for the presence of PiZ AAT to be a prerequisite for population studies concerning the intermediate deficiency state as a putative risk factor for the development of chronic disease.

This paper describes a monoclonal antibody raised against AAT purified from the liver of a PiZZ individual. This antibody specifically recognizes the *PiZ* mutation site in AAT (13) regardless of its glycosylation state, and it can be used in an ELISA procedure for screening a large population for carriers of the *Z* gene.

MATERIALS AND METHODS

Animals. BALB/c female mice were obtained from Gl. Bomholtgård Ltd., Ry, Denmark.

Preparation of Normal (PiMM) and Variant (PiZZ) AAT. Normal (PiMM) serum AAT was purchased from Sigma and purified on a Blue-Sepharose CL-6B column. Variant (PiZZ) plasma AAT was a gift from C.-B. Laurell.

Variant (PiZZ) liver AAT was purified as described (14). Pellets containing aggregated AAT corresponding to 10 g of liver tissue were dissolved in 8 ml of 6 M guanidine·HCl/0.05 M 2-mercaptoethanol, with agitation for 16 hr. After centrifugation, the clear supernatant was applied to a Sepharose CL-6B column. AAT in effluent fractions was detected by electroimmunoassay. The purified liver AAT gave one band on $NaDodSO_4$/polyacrylamide gels and had a single NH_2-terminal amino acid.

Phenotyping of plasma samples was carried out according to Pierce *et al.* (15). Protein concentration was determined according to Hartree (16). Gradient $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out with 20 × 30 cm slab gels containing a 10%–15% polyacrylamide gradient with a 2-cm spacing gel according to Blobel and Dobberstein (17), using the gel system of Maizel (18).

Immunization, Cell Fusion, and Growth of Clones. PiZZ AAT isolated from liver tissue was used as immunogen. One hundred micrograms of this preparation was emulsified in 0.1 ml of BCG-vaccin (Statens Seruminstitut, Copenhagen) and injected subcutaneously into 3-month-old BALB/c mice. After 31 days, the mice received subcutaneous booster injections of 100 μg of protein without BCG. A final intraperitoneal injection of 100 μg was given 122 days after priming. Four days later, mouse spleen cells were suspended and fused with Sp 2/0-Ag 14 mouse myeloma cells (19) using 50% polyethylene glycol (M_r, 1500) principally as described by Galfre *et al.* (20). After fusion, the cells were suspended in RPMI 1640 medium (containing 10 mM Hepes/4 mM L-glutamine/1 mM sodium puruvate/antibiotics/20% fetal calf serum) supplemented with HAT medium (13.6 μg of hypoxan-

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Abbreviations: AAT, α_1-antitrypsin; ABTS, 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid); Pi, protease inhibitor.

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thine per ml/0.176 μg of aminopterin per ml/3.88 μg of thymidine per ml) and seeded in 96-well culture plates (Titertek tissue culture plate, Flow Laboratories, Stockholm) with 2×10^7 spleen cells, 5×10^6 myeloma cells, and 6×10^7 feeder cells (rat thymocytes) per plate. Culture supernatants were screened 14 days after fusion for specific antibodies. Selected positive cultures were cloned 4 times by the limiting-dilution technique (21), expanded and frozen in liquid nitrogen, and grown as ascites tumors in BALB/c mice.

Assay of Antibodies Against AAT. The screening for specific hybridomas was carried out by a solid-phase ELISA. Liver AAT PiZZ or serum AAT PiMM were coated to the wells of polystyrene microtiter plates (Nunc-Immuno plate II F) by incubation of 50 μl (2 μg/ml in coating buffer/0.05 M sodium carbonate, pH 9.6) overnight at room temperature. After washing 5 times with washing buffer (10 mM sodium phosphate, pH 8.0/0.5 M NaCl/0.1% Tween 20), the plastic was saturated with 200 μl of medium (containing 10% fetal calf serum) per well. After overnight incubation at 37°C, the medium was aspirated, the wells were washed as described above, and 50 μl of hybridoma supernatant was added. After incubation for 1 hr at 37°C, the wells were again washed and 50 μl of peroxidase-conjugated rabbit anti-mouse Ig antiserum (Dakopatts a/s, Denmark) diluted 1:1000 in washing buffer was added. The last incubation and washings were repeated; 200 μl of 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) (Sigma), 160 μM in 0.05 M citrate buffer containing 2.5 mM H_2O_2 at pH 4.0 was added, and 15 min later the absorbance at 405 nm was measured in a Titertek Multiskan. The IgG subclasses and the light chains of the monoclonal anti-AAT antibody were determined by exchanging the third layer (enzyme-labeled anti-Ig) with 50 μl of rabbit anti-mouse antiserum to IgG1, IgG2a, IgG2b, IgG3, κ or λ chains (diluted 1:500 in washing buffer) (Miles). After 1 hr of incubation at 37°C, the wells were washed as before and 50 μl of peroxidase-conjugated goat anti-rabbit Ig antiserum, diluted 1:1000 in washing buffer, was added. After incubation for 1 hr at 37°C, the procedure was carried out as described above.

Preparation of Monoclonal Anti-AAT Antibody from Ascites. Ascites was induced by intraperitoneal inoculation of $5–10 \times 10^6$ hybridoma cells per animal. After 1–2 weeks, the mice were sacrificed and ascitic fluid was washed out with 3×8 ml of phosphate-buffered saline (pH 7.4). Immunoglobulin from 81 ml of ascitic fluid was precipitated by adding ammonium sulfate to 50% saturation. After centrifugation, the pellet was dissolved in 6 ml of 20 mM Tris·HCl/20 mM NaCl, pH 7.9, and dialyzed overnight against the same buffer. The turbid solution was then centrifuged and the clear supernate (7.0 ml, 85 mg of protein) was chromatographed on a DEAE-Sephacel (Pharmacia) column (1.6×15 cm) equilibrated in the same buffer. Monoclonal antibody was then eluted with a linear gradient of NaCl (0.02–0.40 M, 150 ml per vessel) in the buffer described above. The flow rate was 38 ml/hr, and 7.5-min fractions were collected. The fractions were then subjected to agarose gel electrophoresis and screened for anti-AAT activity. Those containing anti-AAT were pooled and stored at −20°C in the presence of 0.02% sodium azide. The purity was confirmed on gradient $NaDodSO_4$/polyacrylamide gels.

ELISA of Plasma Samples. Plasma AAT PiZ was semiquantitated by a double sandwich ELISA. The wells of polystyrene microtiter plates were coated with 100 μl of monospecific polyclonal rabbit anti-AAT antiserum (available at the laboratory) diluted 1:200 in coating buffer overnight at room temperature. The wells were then washed 5 times with washing buffer, and 100 μl of plasma (diluted 1:25 in washing buffer) was added prior to overnight incubation at 4°C. After washings as described above, 100 μl of monoclonal anti-AAT (diluted 1:300 in washing buffer) was added. After incubation overnight at 4°C, the washing procedure was repeated and 100 μl of peroxidase-conjugated rabbit anti-mouse Ig antiserum (diluted 1:1000 in washing buffer) was added prior to overnight incubation at 4°C. Finally, the wells were washed as before, 200 μl of ABTS (see above) was added, and 15 min later the absorbance at 405 nm was estimated in a Titertek Multiskan.

All plasma samples were analyzed as duplicates with individual blanks. In the blanks, the monoclonal antibody was substituted by washing buffer. To evaluate the interplate variation, plasma from one single PiZZ individual was included on every plate.

RESULTS

Preparation of Monoclonal Antibodies. Hybridoma growth was initially seen in 672 microtiter wells. All supernatants were screened against normal serum AAT PiMM and variant liver AAT PiZZ. Of 130 initial positive clones, 62 different clones producing antibodies against either antigen remained after repeated testing. They were expanded in 25-cm^2 culture bottles and then frozen in liquid nitrogen. Two hybridomas (ATZ 3 and ATZ 11) were recloned 4 times and inoculated intraperitoneally in BALB/c mice ($5–10 \times 10^6$ cells/animal) for ascites production.

Table 1 shows the reactivity of the polyclonal mouse anti-AAT (collected at the time of fusion), diluted 1:500, and the two hybridomas against AAT PiZ and PiM. ATZ 11 reacts specifically with PiZ antigen in contrast to ATZ 3, which reacts with both AAT types. Monoclonal antibodies prepared from ascitic fluid were eluted with 0.02–0.40 M NaCl from the DEAE-Sephacel column, and the purity of the fractions was confirmed on gradient $NaDodSO_4$/polyacrylamide gels. Protein content in the pooled fractions was 0.65 mg/ml, and the class and subtype of ATZ 11 was IgG1 κ. ATZ 3 had a low titer and was not studied further.

Specificity of Monoclonal Antibody ATZ 11. Serial dilution curves of plasma samples were constructed from individuals belonging to phenotypes PiZ, PiS, and PiM. As shown in Fig. 1, there was no overlapping of OD_{405} given by PiZZ, PiMM, and PiMZ plasma samples in dilutions from 1:2 to 1:1000. The phenotypes PiMM, PiMS, and PiSS behaved as a separate group with low absorbances. Differentiation between various phenotypes within this subgroup was not possible. The specific ability of PiZ-plasma, but not PiM-plasma, to inhibit binding of the monoclonal ATZ 11 antibody to immobilized PiZ antigen was also demonstrated by competitive experiments, as illustrated in Fig. 2. The experiment demonstrated that PiZ plasma was an effective competitor, even in dilutions close to 1:1000 and that PiM-plasma lacks this competitive ability.

Identification of *PiZ* Gene Carriers Using Monoclonal Antibody ATZ 11. A large number of plasma samples from *PiZ*

Table 1. Reactivity of monoclonal antibodies with PiZZ and PiMM AAT

Antibody tested	Immobilized antigen	
	PiZZ AAT	PiMM AAT
Mouse polyclonal anti-AAT (1:500)	0.63	0.37
Hybridoma supernatant		
ATZ 3	0.83	0.40
	0.71	0.27
ATZ 11	0.84	0.08
	0.86	0.02
Culture medium	0.08	0
	0	0

The data are expressed as OD_{405} measured in the ELISA test 15 min after addition of substrate to the assay.

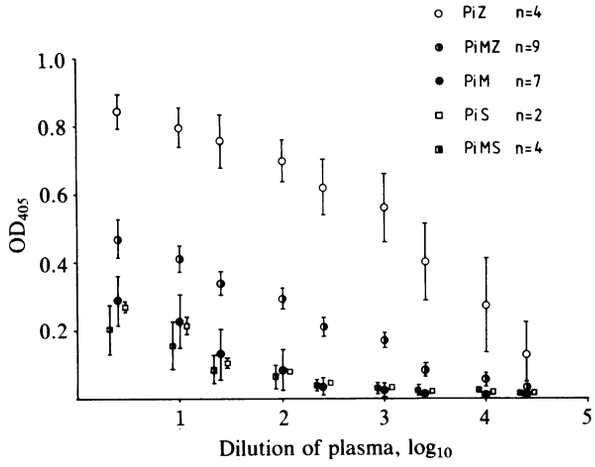

FIG. 1. Dilution curves showing enzyme immunoassay of various AAT phenotypes, using monoclonal ATZ 11 antibody. Dilutions of plasma samples were analyzed according to the standard procedure described in the text. Mean value ± SD are given.

gene carriers (PiZZ, PiFZ, PiMZ, and PiSZ) and other phenotypes were screened by the ELISA procedure described. The net absorbances (blank values subtracted) at 405 nm given by the first 103 samples analyzed are depicted in Fig. 3. The mean plasma blank value was 0.20 ± 0.18 (SD). The interplate variation coefficient was 8%. Plasma from homozygous *PiZZ* individuals had the highest absorbance (0.46–0.97). Heterozygotes (*PiMZ*, *PiSZ*) had intermediate values (0.15–0.63) and non-PiZ phenotypes (MM, MS, S, FM, MP) had low absorbances (0–0.10). The rare phenotypes PiBM, EM, GM, IM, MV, MW, and MX also had $OD_{405} < 0.10$ (not shown). The ranges of OD_{405} for homo- and heterozygotes partially overlap when $OD_{405} > 0.40$. In contrast, no overlapping between heterozygotes and non-*PiZ* gene carriers was seen when using differentiation OD_{405} of 0.10 (Fig. 3).

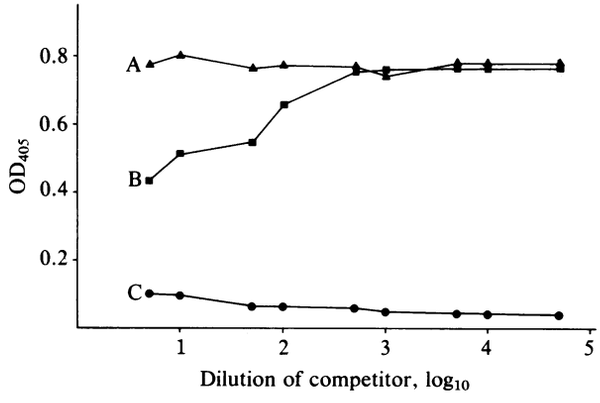

FIG. 2. Competitive assay illustrating the binding (expressed as OD_{405}) of monoclonal antibody ATZ 11 to immobilized PiZ antigen (*y* axis) in the presence of different PiM or PiZ plasma dilutions (*x* axis). The test was carried out by mixing a fixed dilution (1:300) of monoclonal antibody ATZ 11 with a dilution series of PiM or PiZ plasma. After overnight incubation, 100 μl of the mixtures was added to PiZ antigen-coated microtiter wells. Immobilization of PiZ antigen to polyclonal rabbit anti-AAT had been carried out by incubating 100 μl of PiZ plasma (diluted 1:2) with antibody-coated microtiter wells (see text). After overnight incubation, the ELISA was developed with ABTS according to standard procedure. Curves A and B show the results obtained from dilution series of PiM and PiZ plasma, respectively. Curve C represents the results obtained from dilution series of PiM plasma when the immobilized PiZ antigen was substituted by buffer.

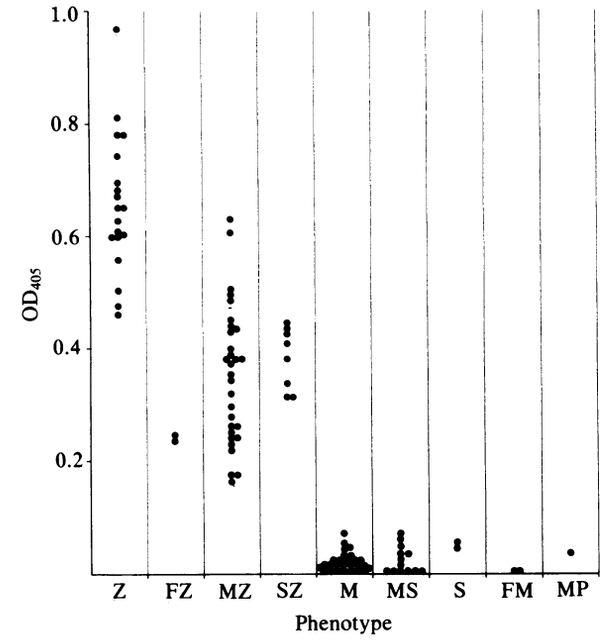

FIG. 3. Immunoassay of plasma AAT phenotypes using monoclonal antibody ATZ 11. The test was carried out as described in the text. Each dot represents the mean value.

Validation of the *PiZ*-Specific ELISA. The hypothesis that the mean absorbance is the same for *PiZ* homozygotes, *PiZ* heterozygotes, and non-PiZ phenotypes was tested by one-way analysis of variance. To determine which groups significantly differed, the Scheffé or the Tukey multiple-comparison procedures (22) were used at the $\alpha = 0.10$ level and the $\alpha = 0.05$ level, respectively. Variation coefficients were calculated on double samples from the respective subgroups. The results based on the whole material (207 samples) are given in Table 2. The mean absorbances of the three groups differed significantly ($F_{2/204} = 890.2$, $P < 0.001$). The multiple-comparison methods showed significant differences between all pairs of means. Variation coefficients varied between 2.2% and 4.1%. In a blind procedure comparing the ELISA technique described with isoelectric focusing, all 122 non-*PiZ* gene carriers were correctly identified by the ELISA method. Of 63 *PiZ* heterozygotes, all were correctly identified by the ELISA procedure, but only 57 were identified by the first isoelectric focusing. The remaining six samples were correctly reclassified after a second isoelectric focusing.

DISCUSSION

We have produced an antibody against AAT isolated from the liver of a PiZZ individual. In addition to the specific amino acid substitution at position 342 (Glu → Lys), this protein has a carbohydrate structure in the core-glycosylated, high-mannose form totally lacking sialic acid. This relatively insoluble substance has previously been shown to be an excellent immunogen (14).

Table 2. Mean absorbance (±SD) and variation coefficients in the different subgroups

	Phenotype		
	PiZZ (n = 22)	PiMZ, -SZ (n = 63)	non-PiZ (n = 122)
OD_{405}	0.64 ± 0.12	0.34 ± 0.11	0.02 ± 0.02
Variation coefficient, %	2.2	2.5	4.1

The antibody produced was judged to be monoclonal according to the following criteria: (*i*) The hybridoma line ATZ 11 was recloned 4 times before intraperitoneal injection, (*ii*) the isolated antibody represents one defined subtype, IgG1 κ, and (*iii*) it migrates as a single band on $NaDodSO_4$/polyacrylamide gels (Fig. 4).

The antibody recognizes plasma PiZ AAT, which contains complex glycosylated oligosaccharide side chains, terminating in sialic acid, but it does not recognize plasma PiM, S, or P AAT. This antibody can thus be regarded as specific for the AAT polypeptide domain containing the specific Glu → Lys substitution at position 342. Specificity was further established by blocking experiments (Fig. 2).

Whereas the antibody ATZ 11 recognizes a polypeptide domain, it is possible that others of the 60 clones obtained but not yet investigated may react specifically for the core-glycosylated oligosaccharide and be useful in cell studies to distinguish intracellular AAT forms.

A double-sandwich technique was found to be advantageous in the ELISA procedure. An initial coating of the polystyrene microtiter wells with an ordinary polyclonal anti-AAT antiserum was necessary to bind antigen from plasma samples and expose the critical AAT antigen determinant to the secondary monoclonal antibody. Quantitatively, *PiZ* heterozygotes had lower (but partially overlapping when $OD_{405} > 0.40$) absorbances than *PiZZ* homozygotes and distinctly higher absorbances (no overlapping) than plasma from patients lacking the Z allele (Fig. 3). *PiZ* heterozygotes (MZ, SZ, and PZ) behaved similarly in this respect. All non-Z phenotypes had low absorbances and were indistinguishable in this system.

Screening of a large number of plasma samples with a monoclonal antibody using the ELISA procedure allows rapid and easy identification of individuals with intermediate AAT deficiency PiMZ, SZ, and PZ. There is no overlapping between *PiZ* heterozygotes and plasma from subjects lacking the *Z* allele (Fig. 3), permitting immediate classification of a sample as PiZ positive or negative. The variation of absorbances among *PiZ* heterozygous samples should theoretically reflect only the amount of the PiZ AAT present in each plasma sample. We cannot, however, exclude the possibility of other amino acid substitutions in the polypeptide chain with no net charge difference but with varying affinities for the antibody in some plasma samples. Similar substitutions have been reported within the PiM phenotype (position 376, Glu → Asp (23)]. It is not possible at present to differentiate *PiZZ* from *PiZ* heterozygotes in the absorbance range >0.40 without subsequent electroimmunoassay or isoelectric focusing.

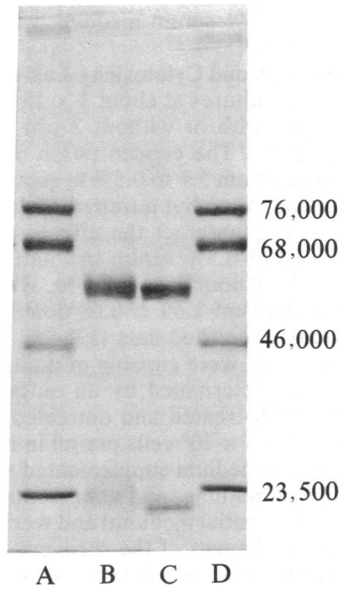

FIG. 4. Gradient $NaDodSO_4$/polyacrylamide gel electrophoresis (slab) of purified monoclonal anti-AAT antibody. Lanes A and D represent molecular weight markers; lane B, human IgG; lane C, ATZ 11.

We have used the ATZ 11 antibody and ELISA procedure to screen 860 frozen plasma samples from patients with liver disease and have been impressed by its efficiency and specificity compared to that of isoelectric focusing in identifying bearers of the *Z* gene (unpublished results). This antibody also offers the unique possibility of histochemical differentiation between PiZ-related AAT globular inclusions and nonspecific protein aggregates occasionally seen in liver tissue.

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