Detection of Mutated Angiotensin I-Converting Enzyme by Serum/Plasma Analysis Using a Pair of Monoclonal Antibodies, Sergei M. Danilov,1* Jaap Deinum,2 Irina V. Balayaskoova,1 Zhu-Li Sun,1 Cornelis Kramers,2,3,4 Carla E.M. Hollak,5 and Ronald F. Albrecht1 (1 Department of Anesthesiology, University of Illinois at Chicago, Chicago, IL; Departments of 2 Medicine, 3 Pharmacology/Toxicology, and 4 Internal Medicine, University Medical Center, Nijmegen, The Netherlands; 5 Department of Hematology, Academic Medical Center, Amsterdam, The Netherlands; * address correspondence to this author at: Anesthesiology Research Center, University of Illinois at Chicago, 1819 W. Polk St. (M/C 519), Chicago, IL 60612; fax 312-996-9680, e-mail danilov@uic.edu)

Angiotensin I-converting enzyme (ACE; CD143) is a Zn2+-carboxypeptidase that plays a key role in the regulation of blood pressure and in the development of vascular pathology and remodeling (1–3). ACE is constitutively expressed on the surface of endothelial cells, macrophages, dendritic cells, and various other cell types (4, 5). Somatic ACE contains two homologous domains, N- and C-terminal, each with a catalytic center (2, 6). ACE has been accepted as a CD marker, CD143 (4, 6).

Soluble serum ACE originates from endothelial cells by proteolytic cleavage by an unidentified protease of the Arg1203–Ser1204 peptide bond in the stalk region near the C-terminal transmembrane sequence of the ACE molecule (7–11). At physiologic conditions, the concentration of ACE in blood is very stable (12), whereas the ACE concentration in serum is often significantly increased in granulomatous diseases (in particular, sarcoidosis) or Gaucher disease (13–18).

We described a Pro1199Leu mutation, located in the juxtamembrane stalk region of ACE (19, 20), that explained a considerable familial increase in blood ACE activity in individuals from several Dutch families (19). The same phenotype and autosomal-dominant inheritance pattern have been described in Japan (21) and Italy (22). Despite the fact that patients with this mutation at first scrutiny do not have clinical abnormalities (19), the finding of increased ACE has led to confusion for treating physicians (23, 24).

We recently observed reduced binding of soluble ACE in Dutch patients with a Pro1199Leu substitution detected by a new monoclonal antibody (mAb), 1B3, which recognizes a Pro1199-containing epitope in the C-terminal region of soluble ACE (25). We therefore set out to develop a method that would use mAb 1B3 in combination with mAb 9B9 to the central part of the N-domain of ACE (26–28), which would enable us to distinguish persons with the Pro1199Leu mutation from patients with increased ACE attributable to other diseases, such as sarcoidosis and Gaucher disease.

We used sera from 7 persons with an ~5-fold increase in ACE activity in blood attributable to the Pro1199Leu mutation, designated “hyperACE” (19), and sera from 10 first-degree relatives without the mutation. All individuals gave permission to use their blood and samples. The Medical Ethical Committee of the University Medical Center in Nijmegen, The Netherlands, approved the sampling protocol. As controls, we used sera and citrated plasmas from 32 members of the Department of Anesthesiology, University of Illinois at Chicago (5 women and 27 men; age range, 30–75 years). All were apparently healthy and not on medication.

We also obtained sera from 7 patients with active sarcoidosis (4 women and 3 men, age range, 29–54 years). The serum samples had been kept at ~80 °C for 1–6 years. The diagnosis of sarcoidosis was based on clinical and radiographic findings and was supported by a tissue biopsy showing characteristic histologic features.

Sera from 17 patients with Gaucher disease (10 men and 7 women; age range, 30–62 years) had been stored at ~20 °C at the Department of Hematology, Academic Medical Center (Amsterdam, The Netherlands) for 8–13 years. These sera had been obtained just after the start of treatment with enzyme supplementation (placental or recombinant glucocerebrosidase). In all patients with Gaucher disease, the diagnosis was confirmed by defi-
icient glucocerebrosidase activity in leukocytes (29) and by genotyping.

For immunocapture studies, the following mAbs to human ACE were used: mAb 1B3, which recognizes a C-terminal part of soluble ACE (25), and mAbs 9B9 (26–28) and 2B11, which recognize epitopes in the N- and C-domains of ACE, respectively. ACE activity in human serum or plasma was measured by a fluorometric assay (30, 31).

For the immunocapture enzyme assay (ICEA), we coated 96-well plates (Corning) with anti-ACE mAbs (5 mg/L) via a bridge of affinity-purified goat anti-mouse IgG (26). We then incubated the wells with 50 μL of diluted (1:10) serum/plasma and measured plate-bound ACE activity by adding a substrate for ACE directly into the wells (26).

Shown in Fig. 1 is the ACE activity captured from plasma of affected individuals vs healthy controls by mAb 1B3 (directed to a C-terminal epitope; Fig. 1A), mAb 9B9 (directed to the central part of the N-domain of ACE; Fig. 1B), and mAb 2B11 (directed to the central part of the C-domain of ACE; Fig. 1C). Because of variations in ACE concentrations in the tested patients, the absolute amounts of ACE captured by mAb 1B3 were not visibly lower in individuals with hyperACE compared with healthy controls. However, hyperACE individuals could be clearly separated from healthy controls and from patients with increased plasma ACE by calculation of the ratio of the amounts of ACE captured by mAbs 1B3 and 9B9 (or mAb 2B11). The 1B3/9B9 binding ratio was not influenced by ACE concentration in individuals with low-normal or high-normal ACE concentrations (for details, see the online Data Supplement). Dilution of samples also did not significantly affect the ratio (see the online Data Supplement). The intra- and interassay CVs for the 1B3/9B9 binding ratio were 4.3% and 5.6%, respectively, in healthy controls and 5.4% and 7.4%, respectively, in patients with high ACE activity (details provided in the online Data Supplement).

To validate this assay for use in clinical practice, we simultaneously determined the serum 1B3/9B9 binding ratio of patients with sarcoidosis and Gaucher disease vs that of healthy individuals and patients with the Pro1199Leu mutation. Patients with sarcoidosis (n = 7) or Gaucher disease (n = 17), and hyperACE patients (n = 5) had 3- to 4-fold increased serum ACE activity compared with healthy individuals. In the individuals with the Pro1199Leu mutation, however, the 1B3/9B9 binding ratio was 3-fold lower than in healthy individuals or patients with sarcoidosis (Fig. 2 in the online Data Supplement). We observed no overlap of 1B3/9B9 binding ratio between carriers of the Pro1199Leu mutation and other patients. We should note that despite the fact that the mean (SD) 1B3/9B9 binding ratio of patients with Gaucher disease [0.435 (0.065)] was dramatically higher (2.7-fold) than in carriers of the ACE mutation, the absolute value of the ratio was significantly lower than in healthy individuals or patients with sarcoidosis.

The absolute value of the 1B3/9B9 binding ratio depends to some extent on assay configuration (ICEA or ELISA, duration of incubation of serum/plasma samples with mAb-coated plate, source of bridge antibodies) and ranged between 0.4 and 0.7, as described by Balyasnikova et al. (25) and in the online Data Supplement.

The absolute value of the 1B3/9B9 binding ratio also
depends on duration of storage. The ratio was somewhat decreased in sera from patients with Gaucher disease that had been stored for 8–13 years at −20 °C compared with sera from sarcoidosis patients that had been stored for shorter times. This suggests that the decrease in IB3/9B9 binding ratio during long-term storage reflects proteolytic cleavage of the C-terminal end of soluble ACE. Nevertheless, even with different storage times, the binding ratio still allows differentiation of genetically increased ACE.

From the reduced binding of mAb IB3 to the soluble ACE from heterozygous individuals with the Pro1199Leu mutation, we conclude that the epitope that is recognized by mAb IB3 is either disrupted or severely altered by the mutation. We do not know whether mAb IB3 binds at all to mutated ACE because at this time we do not have pure, mutated ACE or sera from homozygous carriers.

The differential binding characteristics of mAbs 9B9 and IB3 allowed us to develop an easily applied ELISA to clearly differentiate individuals with increased ACE attributable to the Pro1199Leu mutation from persons with increased ACE attributable to other causes. This ELISA is described in detail in Fig. 3 of the online Data Supplement.

The issue of the impact of mutated ACE on the assay is relevant for the following reasons. The occurrence of increased ACE activity attributable to the Pro1199Leu mutation (hyperACE) is fairly common; more than 30 apparently unrelated index patients are currently known in The Netherlands, with one-half of their family members harboring the mutation (C. Kramers and J. Deinum, personal observations). These persons have come to light in almost all instances because their physicians ordered ACE tests when the probands presented with nonspecific complaints. In none of the patients could the diagnosis of sarcoidosis (and other granulomatous disease) or Gaucher disease be made. Often these patients had undergone extensive diagnostic evaluation (23). The genetically determined increase in blood ACE may lead to incorrect diagnosis of (neuro)sarcoidosis and unwarranted treatment with immunosuppressants (24). With the assays we propose here, it is possible that, in the case of increased ACE activity, hyperACE can be diagnosed straightforwardly, without need for further evaluation. If the IB3/9B9 ratio is within the value for a reference population, further evaluation is necessary. We are not certain whether the strategy we propose will apply to the situation elsewhere in the world, but the mutation has been described recently in Germany as well, and previous reports from Italy and Japan (21, 22) suggest that hyperACE may occur worldwide.

For clinical practice, we propose that a sizeable increase in ACE activity (more than 2-fold higher than the mean activity in the general population) should lead to a request for IB3/9B9 IAEA or ELISA testing (see the online Data Supplement). For higher sensitivity, we would recommend diluting samples with high ACE activities to the mean value of a control sample cohort.

In summary, we have developed an immunoassay-based strategy to detect the presence of mutated ACE in plasma. The assay could be a valuable tool in the exploration of the differential diagnosis of increased ACE.
Combination of His-Tagged T4 Endonuclease VII with Microplate Array Diagonal Gel Electrophoresis for High-Throughput Mutation Scanning, Matt J. Smith,1 Gabriella Pante-de-Sousa,1,2 Khalid K. Alharbi,3 Xiao-he Chen,1 Ian N.M. Day,1* and Keith R. Fox3 1Human Genetics Division, School of Medicine, Southampton University Hospital, Southampton, UK; 2Department of Physiology, Federal University of Para, Belem-Para, Brazil; 3School of Biological Sciences, University of Southampton, Southampton, UK; * address correspondence to this author at: Human Genetics Division, Duthie Building (Mp808), School of Medicine, Southampton University Hospital, Tremona Road, Southampton SO16 6DY, UK; fax 44-(0)23-80794264, e-mail inmd@soton.ac.uk

Various physical mutation-scanning methods have been developed to avoid unnecessary resequencing of long stretches of DNA (1–6). Protein-based mutation-scanning techniques include enzymatic digestion [reviewed in Ref. (7)], protein binding to a DNA duplex, and direct analyses of the in vivo or in vitro gene product. One such enzyme is T4 endonuclease VII (endoVII), the product of gene 49 of bacteriophage T4 (8). Radiolabel replacement with fluorescent tags has facilitated automated analysis (9). EndoVII recognizes heteroduplex structural distortions, nicking 2–6 bp3 to the distortion, with efficiency dependent on sequence context (10) and mismatch type (11). Perfectly matched DNA undergoes some background digestion, which produces a highly reproducible pattern (12). Mutation detection sensitivity obtained with endoVII digestion was found to be similar to that for denaturing HPLC and direct sequencing (13).

Microplate array diagonal gel electrophoresis (MADGE) (14) provides an open-faced 96-well gel format for polyacrylamide gels. Recently, non-denaturing 192–384– and 768-well formats of MADGE for high-throughput checking of PCR and post-PCR reactions (15) have been developed. We have combined, in proof-of-principle experiments, the mismatch digestion properties of endoVII with the high-throughput capabilities of MADGE and a newly developed denaturing MADGE format to create a simple mutation-scanning technique that can screen ~1000 PCR samples during a single 35-min electrophoretic run.

Plasmid pRB210 (T4 endonuclease VII in pET11a) was a kind gift from Professor B. Kemper (Institute for Genetics, University of Cologne, Germany). The PCR primers used to amplify the endoVII gene from pRB210 were as follows: forward, 5′-GGCCCATATGATGTATTGAC-3′; reverse, 5′-CAGCCGGATCCCATTTAAAATCT-3′. After trimming was performed with BamHI and Ndel (New England Biolabs), pETendoVII was generated by ligation into pET15b (Novagen). Expressed N-terminal His-tagged endoVII was then purified by affinity chromatography.

We used a single colony from pETendoVII-transfected BL21 (DE3) Gold cells (Strategene) to inoculate a 1-L Luria broth culture containing 100 μg/L carbenicillin. After overnight culture at 30 °C, an identical fresh 500 mL passage was made, and at mid-log phase of growth (absorbance at 600 nm, 0.6–0.8), protein expression was confirmed by 2-h centrifugation at 5000g for 10 min, and then lysed by sonication (10 cycles of 30 s on and 30 s off at a probe amplitude of 10–15 μm in a MSE Soniprep 150). Cell debris and intact cells were removed by centrifugation at 10 000g for 40 min. All steps were carried out at 4 °C. The cell lysate was passed through a Schleicher & Schuell 0.2 μm single-use filter.

EndoVII was purified by use of 1-mL Hitrap columns in conjunction with the ΔAKTA™ FPLC™ chromatography system (Amersham Bioscience), according to the manufacturer’s instructions. Protein purity was assessed by sodium dodecyl sulfate gel electrophoresis (Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue6/), enzyme activity was confirmed (without His-tag removal) with digests of synthetic heteroduplex substrates (data not shown), and protein quantification was by Bradford assay. Storage was in 50 mmol/L Tris-HCl (pH 8) with 1 mmol/L dithiothreitol and 500 mL/L glycerol at −80 °C.