SUPPLEMENTAL MATERIALS

1. MATERIALS and METHODS.

ACE ELISA. 96-well plates (Corning, Corning, NY) were coated with 50 µl of mAbs 9B9 (26) or 1B3 (10 µg/ml) and stored overnight at 4°C. After washing with PBS/0.05% Tween 20, the wells were incubated with 50 µl of diluted (1/10) serum/plasma samples for 2 hours at RT. After washing off unbound ACE, plate-bound ACE was quantified using sheep polyclonal antibodies to human ACE, conjugated with horseradish peroxidase – from ACE ELISA Kit (Chemicon Int, Temecula, CA).

2. RESULTS

ACE activity assay. ACE activity in the individuals carrying Pro1199Leu mutation (30) was roughly 5-fold higher than that in non-affected individuals (Supplemental Data Figure 1). Plasma samples of individuals with normal ACE levels were diluted in PBS 10 times for further analysis in the immunocapture enzyme assay and for the ELISA, whereas samples from affected individuals were diluted 50 times in order to ensure roughly equal ACE-mass in all experiments.

1B3/9B9 binding ratio in patients with elevated blood ACE. The distribution of plasma ACE level is characterized by a large inter-individual variability. Thus, ACE level, determined in 434 plasmas of healthy men by direct radioimmunoassay using polyclonal antibody to ACE, differed more than 5-fold and ranged from 165 to 950 ng/ml with mean value equal to 425 ± 119 ng/ml (J3). ACE concentration determined in 4970 plasma/serum from US, Jamaica and Nigeria by ACE ELISA - based on mAb 9B9 (32) also demonstrated a large variability: within 95% of whole population (mean ± 2SD) ACE level differ up to 5 times (from 150 to 877 ng/ml, Efremov, Cooper and Danilov, unpublished observation). Therefore we tested the stability of this parameter (1B3/9B9 binding ratio) within a wide range (5-fold) of ACE concentration. This range covers the range of ACE elevation observed in various granulomatous diseases (sarcoidosis, tuberculosis), and
in Gaucher’s disease – see (13-18). 1B3/9B9 binding ratio, determined by the immunocapture enzyme assay (0.549 ± 0.096), did not significantly differ for plasma samples of 7 healthy individuals, having ACE level ranged from 264 to 808 ng/ml. We also found that this parameter did not change significantly during further 4-fold stepwise dilution of the serum samples with high ACE activity (700-800 ng/ml) (not shown).

1B3/9B9 binding ratio of the serum of patients with elevated level of blood ACE (sarcoidosis and Gaucher’s disease) is shown on the Supplemental Data Figure 2.

The absolute value of the 1B3/9B9 binding ratio also depends on the type of samples used. Thus this ratio was similar for freshly obtained serum and for plasma samples drawn with heparin and citrate and stored at –80°C, higher in plasma samples drawn with EDTA, whereas it decreased significantly in plasma samples obtained with heparin, and stored for three years at –20°C (25).

In practical terms it means that differences in 1B3/9B9 binding ratios, for example for freshly prepared plasma with EDTA from putative carriers of this mutation and control serum samples that have been stored for years will be less dramatic. But even with different storage times the binding ratio still allows discerning of genetically elevated ACE. In order to increase the sensitivity of the detection of carriers of Pro1199Leu mutation in ACE we recommend that i) sample type of diagnostic and control samples should be the same (serum versus serum, EDTA-prepared plasma versus EDTA-prepared plasma, etc.) and, ii) comparison should be performed between samples stored for approximately the same period.

**Precision.** The precision of the assay is suitable for unambiguous identification of patients with Pro1199Leu mutation of ACE in clinical practice. The intra-assay coefficient of variance (CV) of 1B3/9B9 binding ratio was determined by measuring 12 replicates each of low (for patients with mutation) and normal binding. In three consecutive experiments CV of 1B3/9B9 binding ratio for normal binding (healthy individuals) was 4.3 %, whereas CV of this parameter for patients with a
mutation was 5.4%. The inter-assay precision was derived by evaluating normal and low binding in six replicates in 5 separate assays. CV for normal binding was 5.6%, whereas CV for 1B3/9B9 ratio of ACE precipitation for patients with a mutation was 7.4%.

**ELISA.** In order to explore the potential for large-scale studies (with automation of processing) we developed an ELISA for ACE with mAbs 1B3 and 9B9. Diluted plasma samples were incubated in wells of microtiter plates coated directly by mAbs 1B3 and 9B9. The amount of ACE precipitated from plasma of affected individuals and their non-affected relatives by different mAbs was quantified using polyclonal sheep anti-ACE antibodies conjugated with peroxidase (32). As in the case of plate precipitation assay the absolute values of ACE precipitated by mAb 1B3 did not allow to classify patients as mutation carriers (Supplemental Data Figure 3A). However calculation of 1B3/9B9 binding ratio allows to distinguish unambiguously the mutation carriers from non-affected individuals (Supplemental Data Figure 3C). The difference in 1B3/9B9 binding ratio between affected individual and patients with high level of ACE was more than 3-fold and gave very high level of confidence (p<0.00008) without any overlap (Supplemental Data Figure 3D).

Therefore the analysis of plasma samples with a simple variant of ACE ELISA (which is easily automated) allows to identify an individuals with autosomal dominant elevation of ACE activity in the blood in the large-scale format.
3. LEGENDS to SUPPLEMENTAL FIGURES.

Supplemental Data Figure 1. Plasma ACE activity. ACE activity in heparinized plasma from 4 patients with Pro1199Leu mutations (19) and their non-affected relatives was measured fluorimetrically with Hip-His-Leu as a substrate. Each bar denotes ACE activity of one individual (mean ± SD) measured in triplicates. As an independent control we also used plasma samples from 8 unrelated volunteers.

Supplemental Data Figure 2. Precipitation of ACE activity from serum of patients with elevated ACE. (A) ACE level determination. ACE level in the serum of these groups was determined by ACE ELISA (32). Serum of patients with sarcoidosis (n=7), Gaucher’s disease (n=18), individuals with Pro1199Leu mutation of ACE (n=5) and serum of healthy individuals (n=6) were diluted with PBS (1/10 for healthy individuals and 1/50 for other groups) and were incubated in wells on microtiter plate covered by mAb 9B9. Data presented are mean ± SD of triplicates for each serum sample. * -p < 0.05 for comparison between groups and controls (healthy individuals).

(B) Immunocapture enzyme assay. Serum of the above mentioned groups of patients was incubated in wells on microtiter plates, covered by mAb 1B3 and 9B9 as in Fig.1. * -p < 0.01 for comparison between groups and controls (healthy individuals). & -p < 0.01 for comparison between patients with Gaucher’s disease and individuals with Pro1199Leu mutation.

Supplemental Data Figure 3. Precipitation of ACE protein from plasma by mAb to ACE (ELISA). Plasma of indicated patients was diluted with PBS (1/10 for patients with normal ACE and 1/50 for patients with high ACE) and incubated with a wells on the microtiter plate directly covered by mAb 1B3 (A), 9B9 (B) (32). Precipitated ACE protein was quantified by polyclonal sheep antibodies to human ACE, conjugated with peroxidase (ACE ELISA kit, Chemicon Inc. Temecula, CA). Data presented are mean ± SD of triplicates. Ratio of ACE precipitation by mAb 1B3 to that by mAb 9B9 is presented for individual patients on C and for the groups on D.
Supplemental Data Figure 1.
Supplemental Data Figure 2.

**A**

![Bar chart showing ACE level in ng/ml for Normal, Sarcoidosis, Gaucher's disease, and High ACE. The x-axis represents different disease states, and the y-axis represents the ACE level in ng/ml.](chartA)

**B**

![Bar chart showing Plate precipitation assay 1B3/9B9 ratio for Normal, Sarcoidosis, Gaucher's disease, and High ACE. The x-axis represents different disease states, and the y-axis represents the Plate precipitation assay 1B3/9B9 ratio.](chartB)
Supplemental Data Figure 3.