Increased α-Defensins as a Blood Marker for Schizophrenia Susceptibility*

Rachel M. Craddock‡, Jeffrey T. Huang‡, Edmund Jackson§, Nathan Harris¶, E. Fuller Torrey¶¶, Marlis Herbert¶, and Sabine Bahn‡**

Schizophrenia is a severe psychotic illness affecting 1% of the general population. There are no consistent pathological features, and the disorder is defined by a complex symptomatology, which overlaps with other psychiatric illnesses. Diagnosis is based on a clinical interview, relying on the patient meeting criteria according to diagnosis manuals, including Diagnostic and Statistical Manual of Mental Disorders, 4th Ed. and International Statistical Classification of Diseases, 10th Revision. Because of the ambiguous symptoms, the diagnostic process can take many months and often years. Rapid and effective treatment has been shown to impact positively on disease progression and outcome, and it is therefore important to identify disease-associated biomarkers allowing early diagnosis. Reliable biomarkers can be used for the development of diagnostic tests and may also help us understand the underlying pathology of this disorder. In the present study, proteins from anti-CD3 stimulated and unstimulated peripheral blood T cell lysates from 15 minimally medicated and unmedicated patients and 15 age-, sex-, race-, and smoking-matched controls were profiled on cation exchange (CM10) chips using SELDI-TOF. Partial least squares discriminate analysis was used to separate patient and control groups according to the expression of 108 detected peaks, and two peaks of 3,374 and 3,450 Da, corresponding to α-defensins based on masses and cationic properties, were found to contribute significantly to the separation of patient and control groups. Reduction of T cell lysates with DTT resulted in a 6-Da shift in the mass of these peaks consistent with the presence of three cysteine bonds in the structure, confirming them as α-defensins. Quantification of α-defensins in T cell lysates from six patients and 18 healthy controls was carried out by ELISA, which also showed that α-defensin levels were significantly increased in patient lysates when compared with matched controls (p = 0.0197). Plasma from 21 monozygotic twins discordant for schizophrenia and eight healthy unaffected twin pairs was also analyzed for the expression of α-defensins by ELISA. Notably both affected and unaffected twins were found to have significantly elevated α-defensin levels compared with healthy control twin pairs (p = 0.0014 and p = 0.0115, respectively). Increased expression of α-defensins in unaffected as well as affected discordant monozygotic twins is of particular interest as monozygotic twins share genes and usually environmental upbringing. The unaffected twin therefore represents the biological and environmental risk of developing schizophrenia in the absence of overt symptomatology and therapeutic medication. These findings suggest that α-defensins could be an important early indicator of the risk of schizophrenia. Molecular & Cellular Proteomics 7: 1204–1213, 2008.

Schizophrenia is worldwide one of the most severe psychiatric disorders with a prevalence of 1% in the general population. The causes of this devastating disorder are still undetermined, although schizophrenia is widely believed to have a multifactorial etiology with contribution from both heritable and environmental factors. Despite decades of research, we have failed to find consistent pathological features across cases, making laboratory-based diagnosis impossible at the present time. Among the most widely reported findings in schizophrenia are decreased cerebral volume with ventricular enlargement (for a review, see Ref. 1) and a greater prevalence of neurological soft signs (2) as well as hypofrontality (3). There is robust evidence to support a heritable risk to the development of schizophrenia, and individuals with an affected relative have an increased chance of developing schizophrenia. The risk increases with the closeness of the relative but only reaches ~50% concordance in monozygotic twins (4), suggesting that environmental risk factors play an important role. Patterns of inheritance are not straightforward or fully understood, and no individual schizophrenia-associated gene has been identified, although there are several well documented susceptibility genes (5–8). Diagnosis is based purely on subjective clinical symptoms and an interview with diagnosis being confirmed against diagnostic criteria set out in the Diagnostic and Statistical Manual of Mental Disorders,
Biomarkers for Schizophrenia

4th Ed. (DSMIV) and International Statistical Classification of Diseases, 10th Revision. As schizophrenia is associated with a complex spectrum of symptoms that are often shared with other psychiatric conditions, there is often a delay until the correct diagnosis is established.

Identifying disease-related pathological mechanisms is difficult in a disorder that largely affects brain-related functions such as cognition, thought, and mood because the brain is not readily accessible. Various studies have been carried out investigating gene and protein profiles in postmortem brain tissue (9, 10), although these are subject to postmortem effect and can only provide molecular information from the time of death. Much of the recent research in our laboratory has therefore been focused on screening peripheral patient and control samples such as serum and cerebrospinal fluid for altered expression of proteins and metabolites that could serve as a schizophrenia biomarker or “fingerprint” (11, 12).

SELDI-TOF MS allows the profiling of protein expression in biological samples and has proved a useful tool in the search for biomarkers. SELDI technology involves mass spectrometry of proteins and peptides captured from a biological sample according to charge or hydrophobicity, depending upon the surface chemistry of the SELDI chip used. Patterns of protein expression between two test groups can be identified by cluster analyses, such as partial least squares discriminate analysis (PLS-DA) and principal component analysis (PCA), allowing profiling of patient samples in comparison with healthy controls. This can be used to develop a diagnostic fingerprint of protein and peptide expression in body fluids to detect and monitor disease and drug therapy.

Serum, plasma, and blood cells are ideally suited to the development of a diagnostic test as they are readily accessible in the living patient. There is increasing evidence, both from the literature and from studies carried out within our own group, to suggest that disease-related changes can be detected outside the brain (11, 13, 14). Various immune alterations have been reported previously in schizophrenia (15–18), and disease-related changes are detectable in T cells (19). In the present study we extended our search for biomarkers to proteomics profiling of peripheral blood CD3+ T cells. T cells can be stimulated in vitro, allowing a representation of cell function, giving us another dimension of biomarker discovery as some subtle disease-related changes may only be detectable upon cell challenge.

MATERIALS AND METHODS

Collection of T Cell Samples—SELDI protein profiling was carried out on T cell lysates prepared from a cohort of patients and controls.

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Demographic details of patients and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SEDLI-TOF profiling</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>34.1 ± 9.6</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
</tr>
<tr>
<td>Race</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>15</td>
</tr>
<tr>
<td>Black</td>
<td>0</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
</tr>
<tr>
<td>Oriental</td>
<td>0</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>5</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
</tr>
</tbody>
</table>

Lysates from an independent cohort of unmedicated patients and controls were used for confirmatory ELISA (Table I). Blood was collected and prepared from patients and their corresponding controls at the same time, and all samples were handled and treated in the same way. Peripheral blood was taken from minimally medicated patients with a confirmed diagnosis of schizophrenia who had either received less than 4 weeks of therapy or were non-compliant with drug therapy. Blood was also taken from drug-naive patients with first onset psychosis who presented with clinical symptoms consistent with a diagnosis of schizophrenia (DSMIV). For each patient sample, blood was taken from corresponding age-, sex-, and race-matched controls. Controls were also matched as far as possible for smoking. Patients and controls were excluded from the study if they had any comorbidity such as diabetes, heart disease, thyroid disease, autoimmune disease, or any recent infections, and patients with a history of substance abuse were excluded. Written consent was obtained from subjects in accordance with local ethics committee approval.

Collection of Plasma Samples from Discordant Monozygotic Twins—Plasma samples from 21 pairs of monozygotic twins discordant for schizophrenia and 16 matched control twins were collected under standardized conditions by Dr. Fuller Torrey, Stanley Medical Research Institute, Bethesda, MD. All study participants gave their written informed consent, and the original study was approved by an Institutional Review Board. The global assessment of functioning and structured clinical interview for DSMIV axis II personality disorders for each individual were derived by consensus between two psychiatrists. Plasma was obtained from both twins simultaneously as part of a lymphocyte collection apheresis procedure carried out at midmorning with both twins having been on similar diets and residing in a hotel together. Twin samples were divided into aliquots and stored at −80°C.

T Cell Isolation and Stimulation—CD3+ T cells were isolated from the peripheral blood of schizophrenia patients and age-, sex-, and race-matched controls. In brief, peripheral blood was taken using S-Monovette blood collection system containing EDTA (Sarstedt). Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll-Paque (Amersham Biosciences), and CD3+ pan-T cells were then purified from these by negative selection using the MACS

1 The abbreviations used are: DSMIV, Diagnostic and Statistical Manual of Mental Disorders, 4th Ed.; ACTH, adrenocorticotropic hormone; PCA, principal component analysis; PLS-DA, partial least squares discriminate analysis; PLS, partial least squares; ANOVA, analysis of variance.
human pan-T cell isolation kit in association with LS separation columns (Miltenyi Biotec). T cell purity was routinely above 99% when analyzed for CD3-κ expression by flow cytometry (FACSCalibur, BD Biosciences). Cells were cultured for 48 h at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin, streptomycin, and glutamine (Sigma) in 96-well flat bottomed plates precoated for 1 h at 37 °C with either 1 μl/ml anti-CD3 (clone OKT-3), hereby referred to as stimulated, or PBS, hereby referred to as unstimulated. Following stimulation, cells were washed in PBS, counted, and frozen at −80 °C as cell pellets of 5 × 10⁶ cells until experimentation.

Preparation of T Cell Lysates for SELDI-TOF—Frozen pellets of 5 × 10⁶ stimulated and unstimulated T cells from patients and matched controls were lysed in 250 μl of binding buffer (9 M urea, 2% CHAPS, 200 mM Tris (Sigma), pH 7) containing protease inhibitor mixture (Complete Mini protease mixture, Roche Applied Science), 1 mM sodium orthovannadate, 1 mM sodium pyrophosphate, 10 μM β-glycerophosphate, and 50 mM sodium fluoride (all from Sigma) on ice for 15 min with periodical vortexing. Samples were centrifuged at 15,000 × g for 10 min at 4 °C to remove cell debris. Supernatants were removed and transferred to a separate microcentrifuge tube. T cell lysates had been profiled previously by SELDI-TOF, trying a variety of surface chemistries with differing pH conditions (data not shown), to optimize binding conditions for the most informative protein profiling for patient and control populations. CM10 weak cation exchanger chips with pH 7 buffer were selected for profiling of T cell lysates from schizophrenia patients based on the number and separation of peaks resolved.

Array spots were prepared by two 10-min incubations with binding buffer at room temperature on a plate shaker. 50 μl of binding buffer was added to each spot before the addition of 60 μl of T cell lysate. Samples were incubated for 1 h at room temperature on a plate shaker, then washed twice with binding buffer to remove unbound proteins, and then washed twice with distilled water to remove salts from the binding buffer. Spots were air-dried before the addition of 100% saturated sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) in 50% acetonitrile and 0.5% trifluoroacetic acid in two 0.6-μl volumes (Ciphergen Biosystems). The arrays were analyzed with the Ciphergen ProteinChip Reader (Ciphergen ProteinChip System Series 4000). Each sample was analyzed twice to confirm reproducibility in identifying the differentially expressed proteins.

SELDI-TOF MS Analysis—The arrays were analyzed with the Ciphergen ProteinChip System Series 4000 (Ciphergen Biosystems). Mass spectra of proteins were generated by using an average of 254 laser shots at a laser intensity of 1,800 nJ. For data acquisition, the detection size range was between 3 and 200 kDa. The laser was focused at 10 kDa. The m/z of each of the proteins captured on the array surface was determined relative to the following external calibration standards (Ciphergen Biosystems): bovine insulin (5,733.6 Da), human ubiquitin (8,564.8 Da), bovine cytochrome c (12,230.9 Da), bovine superoxide dismutase (15,591.4 Da), horseradish peroxidase (43,240 Da), and BSA (66,410 Da). The data were analyzed with ProteinChip data analysis software version 3.0 and Ciphergen Express Software 3.0 ( Ciphergen Biosystems). The Ciphergen Express Software 3.0 was used to compile all spectra and autodetect quantified mass peaks. Peak labeling was completed by using second pass peak selection with 0.2% of the mass window. Peak information for all spectra was exported for further statistical analyses.

Statistical Analysis of SELDI Profiles—Multivariate statistical analyses including PCA to reduce data dimensionality, PLS-DA to maximize separation between stimulated and unstimulated patient and control groups, and PLS were used to summarize the data output from Ciphergen Express. PLS-DA score plots summarize patient and control groups and how they relate to each other, and loading plots indicate how variables contribute to the relationships in the score plots.

Holdout cross-validation was performed three times to estimate the sensitivity and specificity of the PLS model. In each of the three rounds of holdout cross-validation, one-third of the samples were randomly selected to form the validation data, and the remaining samples were used as the training data. All multivariate analyses were performed using SIMCA-P+ 10 (Umetrics AB). Where appropriate, a test was performed using the Statistical Package for Social Scientists (SPSS/PC+; SPSS, Chicago, IL).

On-chip Reduction of 3,374- and 3,450-Da Peaks—Pooled T cell lysates from SELDI profiling experiments described above were used as a representation of the sample set. These were added to four spots on CM10 chips exactly as described earlier. Before the addition of matrix, chips were heated to 70 °C on a heating block after the addition of 5 μl of 10 mM DTT, 50 μM NH₄CO₃ to two of the lyse spots and 50 μM NH₄CO₃ only to the other two spots as negative controls. Chips were allowed to dry, and another 5 μl of DTT/NH₄CO₃ or NH₄CO₃ alone was added. Chips were once again allowed to dry, and matrix was added before analysis on the Ciphergen ProteinChip Reader using the same run protocol as for profiling.

Quantitative Analysis of α-Defensin in T Cell Lysates and Plasma from Monozygotic Twins Discordant for Schizophrenia by ELISA—α-Defensins were quantified in T cell lysates from six patients and 18 matched controls (Table I) and in plasma from 21 pairs of monozygotic twins discordant for schizophrenia and eight pairs of healthy unaffected monozygotic twins (Table II) using a commercially available kit (Hycult Biotechnology).

T cells were isolated as described above. Pellets of 2 × 10⁶ cells were washed in PBS and frozen at −80 °C until use. Cell pellets were lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100, 0.1% SDS (all from Sigma), and Complete Mini protease inhibitor mixture (Roche Applied Science) for 20 min on ice with periodical vortexing. Lysates were centrifuged at 10,000 × g for 15 min at 4 °C to remove cell debris. α-Defensins were assayed according to the manufacturer’s protocol. In brief, standards were prepared by reconstitution of lyophilized standard in distilled water followed by dilution in complete medium for T cell lysate analysis and dilution buffer for plasma. A 2.5 dilution series was made with a top standard of 10,000 pg/ml down to 41 pg/ml.

Plasma samples were diluted 1:2 in dilution buffer and incubated for 1 h at room temperature to reduce interactions between α-defensins and immunoglobulins present in plasma. 100 μl of each standard dilution, precleared T cell lysate, and diluted plasma was added to the assay and incubated for 1 h at room temperature. Wells were washed four times, and biotinylated detection antibody tracer solution was added for 1 h at room temperature. Wells were washed four times before the addition of streptavidin-peroxidase conjugate. This was incubated for 1 h at room temperature, and plates were washed before the addition of tetramethylbenzidine (TMB) substrate solution. The reaction was stopped with stop solution, and plates were read at 450 nm on a plate reader (Bio-Rad). Data were analyzed in GraphPad Prism, and statistical significance was determined using a non-parametric Mann-Whitney U test.

RESULTS

Differential Expression of T Cell Protein/Peptide Profiles in Schizophrenia Patients—Protein/peptide profiles of stimulated and unstimulated CD3⁺ T cell lysates from 15 patients with schizophrenia and 15 age-, sex, and race-matched healthy controls were systematically analyzed, and the re-
In total, there were 108 peaks detected from one schizophrenia patient and one healthy control chip at pH 7.0 and analyzed using SELDI MS. Typical protein/peptide content within patient and control groups was much lower before stimulation, and the loading plots (lower panels) show the peaks contributing the most to separation between stimulated and unstimulated T cell samples for both patients and controls. Expression of peaks at 13,791 and 6,700 Da changed the most following stimulation for both patient and control groups; however, there were differences in response to stimulation between patients and controls demonstrated by expression changes in a peak at 12,337 Da in controls following stimulation and changes to a peak at 10,918 Da in patients following stimulation. These differences were further investigated by PLS-DA of patient and control responses to stimulation, i.e., the differences between unstimulated and stimulated samples for patients and controls (Fig. 2). The PLS-DA score plot revealed that responses within the healthy control group were very similar as they clustered together closely, whereas responses within the patient group were comparatively very diffuse (Fig. 2A). Healthy controls showed higher responses to stimulation in peaks at m/z = 3,374, 3,242, 3,450, 5,870, 6,839, and 6,700, whereas schizophrenia patients had higher responses to stimulation in peaks at m/z = 8,160, 8,173, 8,394, and 10,918 (Fig. 2B).

Identification of Two Biomarker Peptides at m/z = 3,347 and 3,450 as \( \alpha \)-Defensins—The masses of two candidate biomarkers at m/z = 3,347 and 3,450 were searched against literature and protein databases (Swiss-Prot and National Center for Biotechnology Information (NCBI)) and were found most likely to represent \( \alpha \)-defensin peptides (expression of these peaks in stimulated and unstimulated patient and control samples can be seen in Fig. 3A) (20). Defensins are relatively resistant to proteolysis and therefore cannot always be reliably identified using MS/MS sequencing. On-spot reduction of bound proteins/peptides was therefore carried out using DTT to detect cysteine bonds within the structure of these peptides. \( \alpha \)-Defensins are characterized by the pres-

---

**TABLE II**

Demographic details of monozygotic twins

<table>
<thead>
<tr>
<th>Total</th>
<th>Age</th>
<th>Drug treatment</th>
<th>Duration of illness</th>
<th>DSMIV (Axis V)</th>
<th>Gender (male/female)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>yr</td>
<td></td>
<td>yr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twins discordant for schizophrenia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affected</td>
<td>21</td>
<td>33.0 ± 6.1</td>
<td>26,757 ± 27,320</td>
<td>12.4 ± 7.0</td>
<td>40.1 ± 13.7^b</td>
</tr>
<tr>
<td>Unaffected</td>
<td>21</td>
<td>33.0 ± 6.1</td>
<td>0</td>
<td>0</td>
<td>82.5 ± 5.0^c</td>
</tr>
<tr>
<td>Control twins</td>
<td>16</td>
<td>32.1 ± 7.5</td>
<td>0</td>
<td>0</td>
<td>86.8 ± 4.5</td>
</tr>
<tr>
<td>Male Twins discordant for schizophrenia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affected</td>
<td>13</td>
<td>32.5 ± 6.2</td>
<td>27,430 ± 32,607</td>
<td>13.4 ± 6.9</td>
<td>43.4 ± 11.9^a</td>
</tr>
<tr>
<td>Unaffected</td>
<td>13</td>
<td>32.5 ± 6.2</td>
<td>0</td>
<td>0</td>
<td>82.1 ± 4.8^c</td>
</tr>
<tr>
<td>Control twins</td>
<td>6</td>
<td>38.7 ± 6.7^d</td>
<td>0</td>
<td>0</td>
<td>88.7 ± 1.5</td>
</tr>
<tr>
<td>Female Twins discordant for schizophrenia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affected</td>
<td>8</td>
<td>33.9 ± 6.4</td>
<td>25,662 ± 17,537</td>
<td>11.8 ± 7.3</td>
<td>34.8 ± 15.5^o</td>
</tr>
<tr>
<td>Unaffected</td>
<td>8</td>
<td>33.9 ± 6.4</td>
<td>0</td>
<td>0</td>
<td>83.3 ± 5.4</td>
</tr>
<tr>
<td>Control twins</td>
<td>10</td>
<td>29.3 ± 6.4</td>
<td>0</td>
<td>0</td>
<td>85.6 ± 5.4</td>
</tr>
</tbody>
</table>

^a Fluphenazine equivalent; these patients only received typical antipsychotics at time of testing.

^b \( p < 0.01 \) versus the unaffected and control twins, one-way ANOVA.

^c \( p < 0.05 \) versus control twins, one-way ANOVA.

^d \( p = 0.04 \), control twins versus discordant twins with schizophrenia, one-way ANOVA.
Protein/peptide profiling of T cell samples shows greater variation in schizophrenia patients before and after OKT-3 stimulation compared with controls. A, typical protein/peptide spectra of T cells with or without OKT-3 treatment using a cation exchanger chip (CM10; 50 mM HEPES, pH 7.0) showing the m/z range of 3,000–15,000 from a schizophrenia patient and a healthy volunteer. Differences in peaks across spectra reflect biological variation between individuals. B, protein/peptide profiling of T cell samples shows a higher variation in schizophrenia patients after OKT-3 stimulation compared with controls. The peak intensity of protein/peptide peaks from SELDI spectra were analyzed using PCA. The PCA plot for unstimulated samples demonstrates variation among both patients (blue triangles) and healthy controls (gray dots) with the greatest variation among the patient group. After stimulation healthy controls (black dots) and patients (red triangles) cluster more closely. C and D, PLS-DA of OKT-3 stimulation in healthy controls and schizophrenia patients. The score plot (top panel) summarizes the separation between stimulated and unstimulated samples for healthy controls (C) and patients (D). The loading plot (bottom panel) indicates the key protein/peptide peaks contributing the most toward the separation. Expression of peaks at 6,700 and 13,791 Da were among the major changes caused by OKT-3 stimulation in both patients and controls. Additionally expression of a peak of 12,337 Da was considerably altered in controls alone following stimulation, and a peak of 10,918 Da was considerably altered in patients alone. w * c, the coefficient weighting, the weight of each SELDI peak in the transformation vector.
ence of three intramolecular cysteine bonds (20), and reduction with DTT should result in a 6-Da increase in mass. T cell lysates were prepared on CM10 chips as described above except for the addition of DTT. An increase of 6 Da was observed in chip spots to which DTT had been added compared with control spots to which only ammonium bicarbonate was added (Fig. 3B). This is consistent with the addition of a hydrogen atom to each end of a broken cysteine bond with the formation of two free sulfhydryl groups for each cysteine bond within the structure. This result confirms the presence of three cysteine bonds in the structure of these proteins.

Defensins were also immunodepleted from pooled patient and control samples. They were eluted and run on normal phase (NP20) SELDI chips, resulting in peaks of appropriate masses (data not shown).

To confirm that α-defensins were significantly overexpressed in patient T cells compared with matched healthy controls, ELISA was used for quantification of defensins in an independent sample set using a different platform (Fig. 4A). Lysates of freshly isolated, unstimulated T cells were prepared from six minimally medicated patients and 18 age-, sex-, and race-matched controls (Table I). Patients were found to have a significantly higher concentration of α-defensins compared with healthy controls (p = 0.0197).

Expression Levels of α-Defensins in Plasma from Discordant Monozygotic Twin Pairs and Healthy Unaffected Twin Pairs—Having demonstrated increased expression of α-defensins in T cell lysates from two independent sample sets of schizophrenia patients, we next investigated expression in plasma, which is more readily accessible and thus is a more suitable body fluid for diagnostic purposes. Plasma samples from 21 pairs of monozygotic twins discordant for schizophrenia and eight pairs of healthy twins were investigated. This experimental design has the advantage of providing an estimation of biomarkers in unmedicated individuals at risk of developing schizophrenia as well as providing a comparison of α-defensin concentrations in control and schizophrenia patient plasma. Plasma was assayed by ELISA, and schizophrenic twins had significantly higher levels of α-defensins compared with the control twin pairs (p = 0.0115). Plasma from the unaffected discordant twin also showed significantly higher α-defensin levels (p = 0.0014) at a level intermediate to schizophrenic twins and controls. This was particularly notable because the unaffected dis-
cordant twins did not display overt clinical symptoms of schizophrenia but share susceptibility genes as well as risk-associated environmental factors with their affected co-twins. Increased expression of \( \alpha \)-defensins may therefore provide an indicator of the risk of developing schizophrenia. Importantly these data also imply that increased levels of \( \alpha \)-defensins in schizophrenia are not a consequence of disease process or drug effect.

**DISCUSSION**

One of the key social problems facing schizophrenia patients is the time it takes to reach a correct diagnosis and to find the most efficacious therapeutic regime. Many cases remain undiagnosed, resulting in homelessness or imprisonment for violent or criminal behavior. The identification of diagnostic markers or molecular profiles associated with schizophrenia will greatly improve this shortcoming and the quality of life of patients and their families. There is strong evidence to suggest that early intervention can improve the prognosis of schizophrenia (12), highlighting the necessity for rapid diagnosis and correct treatment of schizophrenia.

It is likely that disorders, such as in schizophrenia, with complex etiologies associated with a range of molecular abnormalities are defined by panels of diagnostic biomarkers rather than just one. Ideally biomarkers should be detectable in accessible tissues such as blood or cerebrospinal fluid, and the use of stimulated and unstimulated peripheral blood T cell lysates allows us to use T cell responses as another level of biomarker discovery while also examining cell function.

Various approaches are commonly used in the hunt for biomarkers in a diverse range of disorders and infectious diseases, involving measurement of gene, protein, or metabolite expression. In the present study we used SELDI to investigate differential expression of proteins and peptides in schizophrenia. One of the key problems in identifying protein biomarkers in any given sample is the complexity of the protein mixture that can interfere with MS/MS sequencing. SELDI uses chips with diverse surface chemistries to decrease the complexity of a sample by enrichment of protein subfractions, according to the type of chip used, such as cation exchange or anion exchange, with time of flight mass spectrometry resulting in peaks indicating the molecular masses of proteins and peptides detected.

Protein identification using SELDI is only possible for molecules of less than 4 Da when interfacing a tandem mass spectrometer. Identification of individual peaks of in-
One of the major drawbacks of using SELDI to identify the masses of differentially expressed proteins is that in many cases peaks may not be suitable for identification because of low peak magnitude, reflecting a poor relative abundance of that protein. There may also be several peaks of similar mass that can be resolved by SELDI-TOF but that are not resolved by electrophoresis, resulting in a protein mixture that is too complex for reliable MS/MS identification.

Here proteins were profiled in T cell lysates from 15 minimally and untreated schizophrenia patients and 15 age-, sex-, and race-matched controls. Multivariate analyses were used to assess differences in protein profiles and to identify protein peaks contributing the most to the separation between patient and control groups. PCA based on the 108 peaks detected using SELDI-TOF was used to assess the distribution and separation of patients and control T cell lysates both before and after stimulation with anti-CD3 (Fig. 1B). Control samples clustered together before stimulation and even more so following 48-h culture in OKT-3-coated plates. This suggests that control samples are similar to each other in protein composition and responded similarly to stimulation. In contrast, protein expression profiles of unstimulated T cell lysates from schizophrenia patients were very diffuse but clustered closely together following stimulation with anti-CD3 although much less than stimulated healthy control lysates. This indicates that patient cells are capable of responding to stimulation and behave more similarly to control samples following stimulation, suggesting that the greatest differences in protein composition between patients and controls occurs before stimulation. The dispersion of unstimulated lysates suggests that T cells from patients differ in protein composition and are dissimilar both to each other and to their healthy control counterparts.

PLS-DA of the protein peaks of stimulated and unstimulated groups for patients and controls (Fig. 1, C and D) identified several proteins changing independently in both groups. A peak of 12,337 Da was among those contributing most to the separation between stimulated and unstimulated control samples, and patient samples showed changes in the expression of a peak at 10,918 Da following stimulation. Further PLS-DA comparing response to stimulation (i.e., differences between samples before and after stimulation) between patients and controls identified peaks at 3,242, 3,374, 3,450, 5,870, and 10,918 Da as the key differences between patient and control responses. Because of the obstacles of protein identification associated with SELDI profiling outlined above, many of these have proved unsuitable for reliable identification.

So far we have identified the peaks at 3,374 and 3,450 Da as α-defensins, a family of small cationic peptides with antibacterial, antiviral, and immunomodulatory capabilities that represent a major arm of innate immunity (for reviews, see Refs. 20 and 21). They are structurally characterized by their triple stranded β-sheet configuration that is stabilized by di-
sulfide bonds formed by six invariant cysteines thought to be crucial for their antimicrobial effect (20). α-Defensins were first found to be produced by neutrophils (22) but have since been found to be produced by a variety of leukocytes, including αβ T cells (23), natural killer cells (24), γδ T cells, B cells, and monocytes (25). Defensins exert their effect through use of electrostatic interactions with negatively charged molecules to permeabilize cell membranes but can also act as opsonins, targeting microbes for phagocytosis, and are able to inhibit protein kinase C. They have also been found to bind to ACTH receptors to block steroidogenesis (26), and defensins can also act as chemooattractants for monocytes (27). Defensins have been shown in vitro to be effective at concentrations of 10–100 μg/ml, although host tissue damage can be modulated by sequestration with binding proteins present in blood such as α2-macroglobulin (28) before disposal by macrophages (29). Cytotoxicity to host tissues by defensins is also controlled by the requirement for processing of inactive precursors that are synthesized as prepro-α-defensins that display very little microbicidal activity in vitro (30). At this stage it is difficult to speculate about the reason for increased α-defensins in schizophrenia. There are various hypotheses of infectious etiology in schizophrenia and reports of immune alteration, although the specific nature of these alterations remains a matter of great debate. Immune involvement in schizophrenia could contribute to the pathology, could result from pathology, or imply an infective etiology of schizophrenia. Increased amounts of α-defensins could also provide a compensatory mechanism for diminished adaptive immunity. For example, there is evidence for abnormal cellular responses and adaptive immunity in schizophrenia (18) that could result in reduced cellular immunity, and this could be compensated for by increased efforts from innate immunity as seen by increased levels of acute phase proteins reported previously in the literature (31, 32) and increased concentrations of defensins as demonstrated in the present study.

The discovery of increased α-defensins in both schizophrenic and unaffected discordant monozygotic twins compared with healthy control twin pairs is particularly intriguing. The hereditary component to schizophrenia is well documented with the involvement of various susceptibility genes but does not fully explain the etiology of this disorder. It is expected that identical twins share the same susceptibility genes for schizophrenia, and in most cases monozygotic twins have similar lifestyles during their formative years and therefore share environmental risk factors associated with schizophrenia. Our results demonstrate increased α-defensins in the unaffected discordant twin in the absence of overt schizophrenia-associated pathology. Thus α-defensins appear to represent a blood marker associated with the risk of developing schizophrenia rather than a consequence of pathology. Biomarkers associated with disease risk could be crucial in developing a presymptomatic diagnostic test, which in turn could facilitate early therapeutic intervention, which recent evidence suggests can improve the outcome of schizophrenia (12).

Candidate biomarkers for schizophrenia require extensive screening of independent cohorts and across other related psychiatric disorders to assess sensitivity and specificity. The sensitivity and specificity of α-defensins as an indicator of schizophrenia requires further testing, and it must be noted that these antimicrobial peptides are likely to be increased in a variety of inflammatory and infectious diseases including human immunodeficiency virus (23) and herpes simplex virus (33) and have also been shown to promote adaptive responses to tumor cells. However, we found increased levels of defensins in three independent sample sets and in two different samples types, namely T cell lysates and haperinized plasma, and across two platforms, SELDI-TOF and ELISA. Further investigation into underlying causes and consequences of increased α-defensin expression in schizophrenia may further our understanding of pathological mechanisms in schizophrenia and may also aid in the diagnosis and monitoring of this disorder.

Acknowledgments—We thank all volunteer patient and control blood donors and Dr. Rashid Zaman, Dr. Samir Shah, Dr. Babu Sandilyan Mani, and Dr. Zahoor Syeed for help in recruiting volunteers for this study. We also thank Dr. Philip Chapman of CipherGen Biosystems for advice with protein identification.

REFERENCES


14. Prabakaran, S., Wengenroth, M., Lockstone, H. E., Lilley, K., Leweke, F. M., and Bahn, S. (2007) 2-D DIGE analysis of liver and red blood cells provides further evidence for oxidative stress in schizophrenia. J. Proteome Res. 6, 141–149


