Tumor Necrosis Factor Constellation Polymorphism and Clozapine-Induced Agranulocytosis in Two Different Ethnic Groups

By David Turbay, Jeffrey Lieberman, Chester A. Alper, Julio C. Delgado, Deyanira Corzo, Juan J. Yunis, and Edmond J. Yunis

Genes of the major histocompatibility complex (MHC) are associated with susceptibility to different immune and non-immune mediated diseases. We had reported that the drug adverse reaction, clozapine-induced agranulocytosis (CA), is associated with different HLA types and HSP70 variants in Ashkenazi Jewish and non-Jewish patients, suggesting that a gene within the MHC region is associated with CA. This study was designed to find common genetic markers for this disorder in both ethnic groups. The tumor necrosis factor (TNF) microsatellites d3 and b4 were found in higher frequencies in both Jewish and non-Jewish patients: 51 of 66 (77%) and 48 of 66 (75%), respectively. Comparisons of these frequencies with those of controls, 28 of 66 (42%) and 18 of 66 (27%), were statistically significant (corrected P value = .001 for the d3 allele and .0005 for the b4 allele). On the other hand, the TNF microsatellite b5 was underrepresented in the group of patients, 9 of 66 (14%), when compared with the control subjects, 43 of 66 (65%) (corrected P value = .0005), probably related to protection from CA. Our results show a strong association of some genetic variants of the TNF loci with susceptibility to CA in two different ethnic groups suggesting involvement of TNF and/or associated gene(s) products in the pathogenesis of this hematologic-drug adverse reaction.

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CLOZAPINE (Clozaril; Sandoz Inc, Berne, Switzerland) is a new antipsychotic drug with absolute indications for the treatment of drug-resistant schizophrenia and of patients who are unable to tolerate traditional antipsychotic medications.1 Additionally, clozapine is considered more efficient than traditional antipsychotic drugs and may also be useful in the treatment of other neurologic disorders.1 However, its use has been limited mainly because of the idiosyncratic drug-induced agranulocytosis that occurs in approximately 1% of the population treated with the drug.2,3 Susceptibility to this life-threatening disorder is linked to genetic factors, including: (1) HLA-DR4 and B38 in Ashkenazi Jewish patients and (2) HLA-DR2 in non-Jewish patients.5 Additionally, the high incidence of recurrence of agranulocytosis in patients rechallenged with the drug points toward a genetic predisposition in the pathogenesis of this disorder.5 Different mechanisms are probably involved, but to date there is no clear evidence of an immune or directly toxic effect of the drug or its metabolites on cells of the myeloid lineage.6-13

Studies of the HLA alleles located in the short arm of chromosome 6, region 6p21, have been useful in understanding immune responses, transplantation, and forensic science.14,15 HLA alleles or haplotypes are associated with several immune- and nonimmune-mediated diseases as well as with adverse drug reactions to xenobiotics.16-18 At least one third of haplotypes of unrelated individuals are the product of nonrandom association of alleles of the major histocompatibility complex (MHC) loci. These have been named extended haplotypes and implies that they carry specific alleles in a region defined by their HLA-B, complotype, and DR/DQ variants.16,17 Unrelated individuals with the same extended haplotypes are expected to have common MHC alleles.14,15,19

It is not clear why different alleles are associated with clozapine-induced agranulocytosis (CA) in Jewish and non-Jewish patient populations. It is possible that an abnormal gene product that mediates agranulocytosis is encoded within the HLA-B, DR region, in the intermediate region of the MHC, would be common to patients of several ethnic groups. The association of CA with different HLA types in Jewish and non-Jewish patients with CA could therefore be due to linkage disequilibrium of a common marker for both groups within the MHC region. In the absence of composites and family studies, it is possible to assign known nonrandom associated haplotypes with high delta value that represent linkage disequilibrium.11 In previous studies we described findings consistent with the hypothesis that a dominant gene within the MHC region, marked by HSP70 variants, is associated with CA in the two different ethnic groups studied.12 These findings suggested that a second candidate explanation of the MHC associations are the tumor necrosis factor (TNF) genes. These genes [TNF-α, TNF-β (LTα), and LTβ] are located in a 7-kb span of genomic DNA in the class-III region.15 They are marked by variants that have shown linkage disequilibrium with HLA-B and DR alleles.23,24

Therefore, we have studied the frequencies of the polymorphisms (four dinucleotide microsatellites and two single base variants) of the TNF variants in the intermediate region of the MHC in patients with CA from two different ethnic backgrounds.

MATERIALS AND METHODS

Patients

We arranged with the Clozaril Monitoring System to notify us of nationwide agranulocytosis cases and to identify the attending

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Submitted October 8, 1996; accepted January 21, 1997.

Supported by Grant No. MH-47029 (E.J.Y.) from the National Institute of Mental Health, HL-29583 (C.A.A. and E.J.Y.), and HL-14157 (C.A.A.) from the National Institutes of Health.

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Blood, Vol 89, No 11 (June 1), 1997: pp 4167-4174
physicians. To maintain confidentiality, patient identities were not disclosed. Clinical case information was reviewed to determine the patient’s suitability for the study’s criteria for agranulocytosis, ie, an absolute neutrophil count less than 500/μL in the course of treatment by clozapine. The treating doctors of patients meeting these criteria were sent letters explaining the purpose and nature of the study and requesting assistance in facilitating access to the patient for the purpose of obtaining blood samples. With a patient’s consent, additional information was obtained about the case history of the disease, including indication for clozapine, duration and dose of clozapine treatment, concomitant medication, and medical history. A total of 33 schizophrenic patients (12 Jewish and 21 non-Jewish) who had agranulocytosis during treatment with clozapine were included in the study. Ashkenazi Jewish or non-Jewish ethnicity was determined by historical evidence of the patient’s four grandparents. Patients were not related; the patients and controls included in this study were not from inbred communities. All patients were white and of European origin. The patients included in this study did not have relatives available at the time of the collection of blood samples, and therefore phenotypes only were ascertained. The mean age of the patients who developed agranulocytosis was 39 years and the mean age in the group of controls was 33 years (P corrected [Pc] = not significant). The percentage of men was 83% (10 of 12) in the Ashkenazi Jewish group of patients and 38% (8 of 21) in the non-Jewish group of patients. Overall, the sex distribution in the group of patients was 65% (18 of 33) men and 35% (15 of 33) women.

**Controls**

The control group consisted of 33 genetically unrelated, white schizophrenic patients of European ancestry (18 Ashkenazi Jewish and 15 non-Jewish) who were treated with clozapine for at least 52 weeks but did not develop agranulocytosis. Control patients were ascertained from the clinical services of Hillside Hospital, Long Island Jewish Medical Center (Long Island, NY), where they were receiving treatment with clozapine. The clozapine dose was titrated gradually, on the basis of clinical judgment, to the level that produced the best clinical response. No difference was found in the dose administered to the patients who developed agranulocytosis and those who did not. Because all patients (agranulocytosis and controls) received clozapine in the context of the Clozaril Monitoring System (white blood cell counts [WBCs]), they underwent clinical and hematologic assessment on a weekly basis. Similarly, probands and controls had treatment-resistant schizophrenia, intolerant schizophrenia, or schizoaffective disorder, which was a requirement for eligibility for clozapine treatment. Thus, insofar as frequency of assessment and form of illness, probands and control groups did not differ. There were 78% (14 of 18) males in the Ashkenazi Jewish control group and 73% (11 of 15) males in the non-Jewish control population. Overall, the sex distribution in the control group was 76% (25 of 33) males and 24% (8 of 33) females. All these subjects had previously been typed for class I and II HLA alleles.

**TNF Constellation Polymorphism Typing**

All patients and controls had previously been typed for class I and II HLA alleles, and were also typed for the TNF polymorphisms as described before. The primers and polymerase chain reaction (PCR) conditions for the TNF microsatellite typing were those reported previously in the literature. The first round of amplification, different primer combinations were used. For the TNF microsatellites a-b, the primers were sense: 5’ AGATCCCTCCCTGTTGATTTGCT 3’ and antisense: 5’ TGAGACAGGGAGATGGAGACAG 3’. For these amplifications, 200 ng of genomic DNA were processed in a 20-μL reaction volume containing 2 μL of 10× PCR buffer, 2 μL of 10× dNTPs mix, 1 μL of each primer (10 pmol/L stock) and 0.2 μL of Taq polymerase (Perkin-Elmer Cetus, Cambridge, MA). The PCR reaction was performed in a thermal cycler (9600, Perkin-Elmer Cetus) under the following conditions: 3 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C, followed by a final extension for 10 minutes at 72°C. After the first PCR amplification, an aliquot (2 μL) was used as a template for the second PCR amplification round using the “heminested” PCR strategy with the following primers: for TNFa, antisense: 5’ GCACCTCCAGCCTAGGGAGA 3’; for TNfb, sense: 5’ GAGCGGTTG-GTTGCAAGGGAGAGACAG 3’; for TNFb, antisense: 5’ CATACTGTGGCACCTGTCTCTCCACAG 3’ and for TNFe, sense: 5’ GTGCGCTGTTCTGAGCTCCTC 3’ using conditions as described for the first PCR amplification but using 5 cycles with 0.1 μL of α-32P dCTP per each PCR reaction. After the final PCR amplification, 5 μL of the PCR products were added to 5 μL of a formamide-containing stop solution (US Biochemicals, Cleveland, OH) heated at 94°C for 2 minutes and analyzed by electrophoresis in a 0.4-mm thick, 6% polyacrylamide sequencing gel (GIUSCO-RL Life Technologies, Gaithersburg, MD) at 30 W for 4 hours. For autoradiography, the gels were dried and exposed for 2 to 12 hours using Kodak X-OMAT film (Eastman Kodak, Rochester, NY) at ~70°C with an intensifying screen.

The microsatellites were assigned using reference homoygous cells (Tenth International Histocompatibility Workshop), as follows: for TNFa1, BTB; for TNFa2, VAVY; for TNFa3, COX; for TNFa4, SLE005; for TNFa5, TISI; for TNFa6, HOM2; for TNFa7, LBUF; for TNFa8, MOU; for TNFa10, BM16; for TNFa11, HHKB; for TNFa12, TUBO; for TNfb1, TUBO; for TNfb3, VAVY; for TNfb4, LBUF; for TNfb5, TISI; for TNfb6, TISI; for TNfb7, VAVY; for TNfb8, TUBO; for TNfb9, TUBO; for TNfc1, LWAGS; and for TNfc3, LBUF. In addition, several heterozygous samples were typed in the CA population. To demonstrate that it is possible to distinguish microsatellite variants, including those that have close electrophoretic migration, an example is shown in Fig 1. The panel of homoygous cell lines with known TNF microsatellite alleles had been characterized in the past by other investigators and was confirmed by our experiments.

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![Fig 1. Autoradiography of the electrophoresis of PCR products after amplification of CA samples. A 6% polyacrylamide gel was used as described in Materials and Methods. (A) Sample heterozygous for alleles b5 and b5. (B) Sample heterozygous for the b4 and b5 alleles. (C) Sample homozygous for the b4 allele. (D) Sample homozygous for the b4 and b5 alleles.](image-url)
information added in these studies was the assignment of variants of the TNFα(-308) A/G polymorphism (see Table 1).

TNF A/G single base variants polymorphisms (α-308, βn). The A/G single-base polymorphisms in the TNFα promoter (-308) and the TNFβ second intron were analyzed by the PCR-restriction fragment length polymorphism method using the Nco I restriction enzyme as described before.26,27 The primers used for the analysis of the TNFα promoter (-308) A/G polymorphism were sense: 5’ AGGCATAGTTTGGGACATGTCTG 3’ and antisense: 5’ TCCCTGCTCCGATTCCG 3’. The primers used for the analysis of the TNFβ second intron (A/G) polymorphism were sense: 5’ CCGTGCTCTGGTTTGAGGGCAT 3’ and antisense: 5’ AGAGCTGGTGGGACATGTCTG 3’. For these amplifications, 200 ng of genomic DNA were added to 20 µL of PCR reaction mixture containing 0.5 pmol/L of each primer, 0.25 mmol/L of each dNTP, 1.5 mmol/L MgCl2, and 1 U of Taq polymerase (Perkin-Elmer Cetus). The cycling conditions for these amplifications were as follows: 1 cycle of 94°C for 3 minutes, 60°C for 1 minute, 72°C for 1 minute; 35 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute; 1 cycle of 94°C for 1 minute, 60°C for 1 minute, 72°C for 5 minutes. Restriction digests of the PCR products were generated in a 25-µL final volume using 10 to 20 U of the restriction enzyme Nco I (GIBCO-BRL, New York, NY) for 16 to 24 hours. The TNFα promoter (-308) A/G restriction digests generated products of 87 and 20 bp for allele *a1 and of 107 bp for allele *a2, and were analyzed on a 4% agarose gel (2% NuSieve, 2% GIBCO-BRL). The TNFβ second intron (A/G) restriction digests generated a 740-bp fragment for the allele *β1 and a 555-bp plus 185-bp fragment for the allele *β2, and were analyzed on a 1% agarose gel (GIBCO-BRL).26,28

Statistical Analysis

All data analysis was performed with the aid of the Instat software system (Graphpad, San Diego, CA). P and odds ratio values were determined by the Fisher’s exact test and all P values were corrected for the number of comparisons to obtain the Pc value.15

RESULTS

The data demonstrating significant association of HLA class II alleles with CA are summarized in Table 2. This table shows that in Ashkenazi Jewish patients, HLA-DRB1*0402, HLA-DQB1*0302, and HLA-DQA1*0301 independently or together, presumably as a haplotype, were increased in patients with CA when compared with those without it. In non-Jewish patients, the HLA-DRB1*02, DQB1*0502, and DQA1*0102 alleles, independently or together, presumably as a haplotype, were increased in patients with CA when compared with those without it.

Table 2 shows the frequencies of the TNF polymorphisms in the 33 patients and 33 controls. In the group of patients, the frequencies of the d3 and b4 alleles were 77% and 72%. In the control group, the same alleles were present with frequencies of 42% and 27%. The frequencies of the d3 and b4 alleles in CA patients were significantly higher than those of the controls, for the b4 marker (odds ratio ~8, Pc = .0005) and for the d3 marker (odds ratio ~4, Pc = .001). On the other hand, the microsatellite allele b5 was underrepresented in the patients, of 66 patient alleles (14%) against 43 of 66 control alleles (65%). This difference was statistically significant (Pc = .0005). There was no significant difference in the frequency of the TNFα and TNFβ microsatellite alleles or the TNFα(-308) and TNFβn(A/G) polymorphisms between patients and controls. It is clear that the TNF variants have different frequencies in CA patients and controls, independent of the ethnic origin of the patients and controls tested (see Fig 2).

The microsatellites TNFα, b, d, e, as well as the TNFα promoter (-308) A/G and TNFβ second intron (A/G) polymorphisms were studied in a panel of homozygous cells previously typed for HLA-B, HLA-DR, HSP70-2, -1, and complotype. The homozygous cells used had been previously reported from family studies corresponding to individuals homozygous for extended haplotypes.15 The assignments of variants of microsatellites TNFα, b, and c to extended haplotypes has been described before.23,24 Table 1 shows assignments of HLA-DR, complotype, HSP70-2, -1; TNF constellation and HLA-B in 14 white extended haplotypes. There are 7 of the 14 extended haplotypes listed with unique and different TNF constellations. The remaining 7 are marked by shared TNF constellations; B38, DR4 and B35, DR4 share the TNFα010, b4, βn2, α(-308)1, d3, e3, B35, DR1 and B62, DR4 share...
Table 2. Associations of TNF Variants, MHC Class II Alleles, and MHC Haplotypes in Patients With CA

<table>
<thead>
<tr>
<th>Allele or Haplotype</th>
<th>CA* Patients</th>
<th>Controls</th>
<th>P&lt; Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ashkenazi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1*0402</td>
<td>11/24</td>
<td>6/54</td>
<td>NS†</td>
</tr>
<tr>
<td>Jewish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1*0302</td>
<td>11/24</td>
<td>8/54</td>
<td>NS†</td>
</tr>
<tr>
<td>HLA-DQA1*0301</td>
<td>12/24</td>
<td>13/54</td>
<td>NS†</td>
</tr>
<tr>
<td>TNFd3</td>
<td>17/24</td>
<td>11/36</td>
<td>.02</td>
</tr>
<tr>
<td>TNFb4</td>
<td>19/24</td>
<td>11/36</td>
<td>.002</td>
</tr>
<tr>
<td>TNFb5</td>
<td>1/24</td>
<td>21/36</td>
<td>.0005</td>
</tr>
<tr>
<td>[HLA-DRB1<em>0402, DRB4</em>0101, DQB1<em>0302, DQA1</em>0301, HSP70-2<em>A, HSP70-1</em>9, TNFe3, TNFd3, TNF(-308)*1, TNF[lin(A/G)]*2]</td>
<td>12/24</td>
<td>3/36</td>
<td>NS†</td>
</tr>
<tr>
<td>Non-Jewish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DRB1*02</td>
<td>14/40</td>
<td>4/32</td>
<td>NS†</td>
</tr>
<tr>
<td>HLA-DQB1*0502</td>
<td>10/40</td>
<td>1/32</td>
<td>NS†</td>
</tr>
<tr>
<td>HLA-DQA1*0102</td>
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<td>.04</td>
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<tr>
<td>TNFd3</td>
<td>34/42</td>
<td>17/30</td>
<td>NS</td>
</tr>
<tr>
<td>TNFb4</td>
<td>29/42</td>
<td>6/30</td>
<td>.0005</td>
</tr>
<tr>
<td>TNFb5</td>
<td>8/42</td>
<td>22/30</td>
<td>.0005</td>
</tr>
<tr>
<td>[HLA-DRB1<em>02</em>, DRB5<em>02, DQB1</em>0502, DQA1<em>0102, HSP70-2</em>A, HSP70-1*9, TNFe3, TNFd3, TNF(-308)*1, TNF[lin(A/G)]*2, TNFa11, TNFb4]</td>
<td>10/40</td>
<td>0/32</td>
<td>NS†</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFd3</td>
<td>51/66</td>
<td>28/66</td>
<td>.001</td>
</tr>
<tr>
<td>TNFb4</td>
<td>48/66</td>
<td>17/66</td>
<td>.0005</td>
</tr>
<tr>
<td>TNFb5</td>
<td>9/66</td>
<td>43/66</td>
<td>.0005</td>
</tr>
<tr>
<td>[HLA-DRB1<em>0402, DRB4</em>0101, DQB1<em>0302, DQA1</em>0301, HSP70-2<em>A, HSP70-1</em>9, TNFe3, TNFd3, TNF(-308)*1, TNF[lin(A/G)]*2, TNFa11, TNFb4]</td>
<td>12/64</td>
<td>4/66</td>
<td>NS</td>
</tr>
<tr>
<td>[HLA-DRB1<em>02</em>, DRB5<em>02, DQB1</em>0502, DQA1<em>0102, HSP70-2</em>A, HSP70-1*9, TNFe3, TNFd3, TNF(-308)*1, TNF[lin(A/G)]*2, TNFa11, TNFb4]</td>
<td>10/64</td>
<td>0/66</td>
<td>NS</td>
</tr>
</tbody>
</table>

The P values not corrected were as follows: Jewish: .02 for DRB1*0402, 0.008 for DQB1*0302, 0.003 for DQA1*0301, .02 for the haplotype; Non-Jewish: .03 for DRB1*02, .02 for DQB1*0502, .007 for DQA1*0102, .002 for the haplotype.

Abbreviations: NS, not significant; *, can be either *1501 or *1601.
† These P values were significant because they confirmed previous hypothesis based on pilot studies.45
Data from Yunis et al4 and Corzo et al.22

TNFa2, b1, b2, α(-308)1, e1, d4. Also, the extended haplotypes marked by B44 can be distinguished by their TNF polymorphisms. However, the HLA-B44, DR7 haplotype shares a TNF constellation with HLA-B13, DR7 haplotype. Of interest, the known association of HLA-B38, TNFb4, a10, b2, α(-308)1, e3, d3 with DRB1*0402 was useful in assigning haplotypes in CA patients of Jewish ancestry as shown in Table 3.23,24 Additionally, the B7, DR2 haplotype and the DR2 carrying haplotypes associated with TNF b4, a11, b2, α(-308)1, e3, d3, DRB1*1501 were useful to a lesser degree in non-Jewish CA patients as shown in Table 4. The assignment of high delta haplotypes was based on well-known non-random association of HLA specificities and alleles.21 It is clear that the haplotype assignment was easier in Jewish patients. In non-Jewish patients it was possible in 10 of 21 patients. In addition, 7 of 8 patients with DRB1*1601 were assumed to carry TNF constellations marked by TNFα10, b4 or TNFα11, b4. Therefore, the overrepresentation of the TNFβ4 and δ3 alleles is because of the presence of haplotypes carrying B44, DR7, or DR2 in non-Jewish patients and B38, DR4 in Jewish patients as shown in Fig 3.

DISCUSSION

Several associations of HLA alleles with drug-induced adverse reactions have been reported in the literature. For example, hydralazine-induced systemic lupus erythematosus (SLE) is associated with HLA-DR4 and the presence of null alleles at the C4 locus.36 Additionally, penicillamine-induced proteinuria and toxicity to chlorpromazine are associated with certain HLA types.29,30

In previous studies we had reported significant association of HLA alleles and haplotypes with CA.7 Additionally, common HSP70 variants were found in CA patients regardless of the ethnic background, suggesting that the genetic susceptibility to develop CA was located in the intermediate portion of the MHC region between the class I and class II loci.22 The associations of HLA alleles and the HSP70 variants were due primarily to the association of two MHC haplotypes: HLA-DRB1*0402, DRB4*0101, DQB1*0302, DQA1*0301, HSP70-2 A, HSP70-1 9.0 in Jewish patients and HLA-DRB1*02, DRB5*02, DQB1*0502, DQA1*0102, HSP70-2 A, HSP70-1 9.0 in non-Jewish patients. However, these haplotypes were not increased significantly in the total popula-
Fig 2. The distribution of (microsatellite) alleles in CA patients versus control individuals. Horizontal axis shows the number \( n \) of tandem repeats. Vertical axis shows the frequency of the alleles in the populations studied. The frequencies of the \( d2 \) and \( b4 \) alleles were significantly higher in the patients whereas the frequency of the \( b5 \) allele was significantly higher in the controls (see text for details).

Haplotypes or generic types with high delta values are in italics. Statistically significant alleles are written in bold.

Data from Udalova et al.\textsuperscript{23} and Garcia-Merino.\textsuperscript{24}
Table 4. TNF Constellation Polymorphism in Non-Jewish CA Patients

<table>
<thead>
<tr>
<th>First Haplotype</th>
<th>Second Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a10, b4, HLA-B27</td>
</tr>
<tr>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a2, b4, HLA-B7</td>
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<tr>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1504 (DR4), TNFε3, d3, α=−3081, βn1, a2, b3, HLA-B8</td>
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<td>HLA-DRB1*1302 (DR13), TNFε3, d3, α=−3081, βn1, a4, b5, HLA-B7</td>
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<tr>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1503 (DR3), TNFε3, d3, α=−3081, βn1, a2, b4, HLA-B39</td>
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<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1502 (DR5), TNFε3, d3, α=−3081, βn1, a2, b4, HLA-B51</td>
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<td>HLA-DRB1*1301 (DR3), TNFε3, d3, α=−3081, βn1, a2, b3, HLA-B8</td>
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<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1101 (DR5), TNFε3, d3, α=−3081, βn2, a6, b5, HLA-B44</td>
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<td>HLA-DRB1*1401 (DR4), TNFε3, d3, α=−3081, βn1, a6, b5, HLA-B44</td>
</tr>
<tr>
<td>HLA-DRB1*1501 (DR2), TNFε3, d3, α=−3081, βn2, a11, b4, HLA-B7</td>
<td>HLA-DRB1*1301 (DR3), TNFε3, d3, α=−3081, βn1, a2, b3, HLA-B8</td>
</tr>
</tbody>
</table>

Haplotypes or generic types with high delta values are in italics. Statistically significant alleles are written in bold. Haplotypes that cannot be assigned are written in brackets.

HSP70-2 A, HSP70-1 9.0 found in Ashkenazi Jews.35 More informative was the association of TNFb4 and d3 with CA in patients of non-Jewish origin. In 10 of 11 non-Jewish DR2 positive patients and in the remaining 11 patients, there was an association of TNFb4, d3 with CA. This suggests that in non-Jewish patients, the TNF variants are more informative in assigning genetic associations in CA not due to linkage disequilibrium with class I or II alleles. This form of genetic mapping using extended haplotypes or fragments of them (or high delta value haplotypes) has been used successfully in studies of MHC associations with disease.15,21,31

As there is no genetic linkage between the HLA region genes and schizophrenia or resistance to conventional antipsychotic treatment, any alteration of HLA region gene(s) frequencies could not be attributed to these factors. Our findings of an increased frequency of the b4 and d3 TNF microsatellite alleles in the patients suggest that one of these, or a trait determined by another closely linked gene in the intermediate region of the MHC, such as HSP70, is associated with CA. Furthermore, once the disease-causing gene in these ethnic groups is identified, a single direct test can be designed to predict the genetic susceptibility to this disorder. Therefore, the prevention of this adverse drug reaction will allow safer clinical use.

In vitro data on the effects of various cytokines (eg, TNF-α and Interferon-γ) on human hematopoiesis have shown inhibition of the myeloid lineage precursor cells, both slowing their differentiation and inducing their programmed cell death.32-34 In addition, there is in vivo evidence of increased expression of different cytokines in several bone marrow-failure disorders,35 and neutropenia has been reported after the administration of TNFa to healthy individuals.36 There may be a certain mechanism, either immune or toxic, by which clozapine or its metabolites stimulate the production or decrease the clearance of products of the TNF locus in certain genetically susceptible individuals, producing neutropenia or agranulocytosis, depending on the severity of the insult.2,3 However, there are no data at present to support the direct involvement of TNF in the pathogenesis of CA.

It has been established that some HLA haplotypes are associated with a higher production of both TNFa and TNFβ.24,37,38 and it has been suggested that these effects may be mediated by both transcriptional and posttranscriptional regulatory mechanisms.40,42 Additionally, it has been re-
ported that TNFα secretion is influenced either by the TNF microsatellites\textsuperscript{37,40} or by the HLA-DR generic type\textsuperscript{37,38,43}. In this regard, studies of neutrophils and of their survival rate in the presence of clozapine or its metabolites could elucidate the role of the TNF constellation polymorphism in TNFα production.

However, it is possible that in individuals carrying risk-associated MHC markers clozapine or its metabolites could induce the expression of HSP70 and/or TNFα and TNFβ, which act as a signal to decrease the proliferative capacity or induce apoptosis in granulocyte precursors or circulating neutrophils.\textsuperscript{32-34,44,45} Additionally, it may be that certain genetic variants of the different cytokines and their receptors (ie, GM-CSF, IL-2, IL-3) involved in neutrophil development are associated with a different secretion pattern after exposure to clozapine and its metabolites.\textsuperscript{46}

Given the MHC associations found in CA, it would be reasonable to perform HLA phenotyping studies in different idiopathic diseases associated with bone marrow suppression. An illustration of this is the finding that there is a similar genetic background of patients with idiopathic SLE and hydralazine-induced SLE.\textsuperscript{16} Alternatively, the approach described here could be used by others to find common susceptibility markers on chromosome 6p21 in diseases with different HLA associations.\textsuperscript{14,15,18}

**ACKNOWLEDGMENT**

We thank Elizabeth Morton and Alison Angel for editorial assistance.

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