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

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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 nonJewish patients: 51 of 66 (77%) and 48 of 66 (57%), 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.[4] 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. Unrelated individuals with the same extended haplotypes are expected to have common MHC alleles.[15,19,20]

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 complotypes and family studies, it is possible to assign known nonrandom associated haplotypes with high delta value that represent linkage disequilibrium.[21] 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.[22] 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

physicians.[25] 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 $500/\mu L$ 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.[25] 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.[25] 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.[4]

TNF *Constellation Polymorphism Typing*

All patients and controls had previously been typed for class I and II *HLA* alleles,[4] and were also typed for the *TNF* polymorphisms as described before.[23,24,26-28]

TNF *microsatellites* a-b, d-e.   The primers and polymerase chain reaction (PCR) conditions for the *TNF* microsatellite typing were those reported previously in the literature.[23] For the first round of amplification, different primer combinations were used. For the *TNF* microsatellites *a-b*, the primers were sense: 5′ GCCTCTAGATTT-CATCCAGCCACA 3′ and antisense: 5′ CCTCTCTCCCCTGCA-ACACACA 3′. For the *TNF* microsatellites *d-e*, the primers were
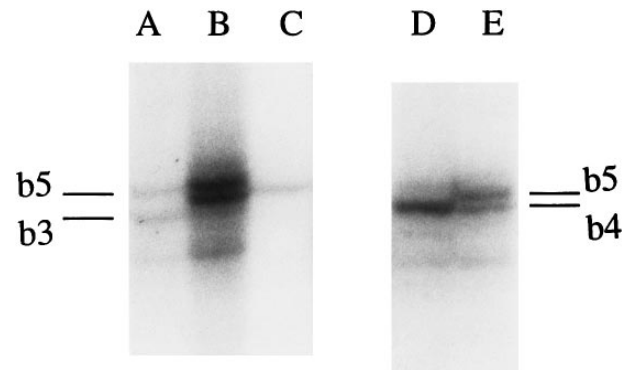
Fig 1.   Autoradiography of the electrophoresis of PCR products after amplification of CA samples. A 6% polyacrylamide gel was used as described in Materials and Methods. (A) Sample heterozygous for alleles *b3* and *b5*. (B) Sample heterozygous for the *b4* and *b5* alleles. (C) Sample homozygous for the *b4* allele. (D) Sample homozygous for the *b4* allele. (E) Sample heterozygous for the *b4* and *b5* alleles.

sense: 5′ AGATCCTTCCCTGTGAGTTCTGCT 3′ and antisense: 5′ TGAGACAGAGGATAGGAGAGACAG 3′. For these amplifications, 200 ng of genomic DNA were processed in a 20-μL final 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′ GCACTCCAGCCTAGGGAGA 3′; for *TNFb*, sense: 5′ GTGTGT-GTTGCAGGGGAGAGAG 3′; for *TNFd*, antisense: 5′ CATAGT-GGGACTCTGTCTCCAAAG 3′; and for *TNFe*, sense: 5′ GTG-CCTGGTTCTGGAGCCTCTC 3′ using conditions as described for the first PCR amplification but using 5 cycles with 0.1 μL of $\alpha^{32}P$ 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 (GIBCO-BRL 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 homozygous cells (Tenth International Histocompatibility Workshop),[28a] 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 *TNFd1*, VAVY; for *TNFd3*, BTB; for *TNFd4*, JHAF; for *TNFd5*, TISI; for *TNFd7*, TUBO 89; for *TNFe1*, LWAGS; and for *TNFe3*, 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 homozygous cell lines with known *TNF* microsatellite alleles had been characterized in the past by other investigators[23,24] and was confirmed by our experiments. New

Table 1. TNF Gene Constellation in White Extended Haplotypes

HLA-DR	Complotype				HSP70 −2, −1	TNF						HLA-B	Haplotype No.
	C4B	C4A	BF	C2		e	d	α-308	βn	a	b		
3	1	0	S	C	8.5 − C	3	1	2	1	2	3	8	10
2	1	3	S	C	9 − A	3	3	1	2	11	4	7	12
7	1	3	F	C	9 − A	3	3	1	2	7, 8	4	44	4
4	0	3	S	C	9 − A	3	3	1	1	6, 7	5	44	4
7	1	6	S	C	9 − A	3	4	1	2	2	5	57	4
5	1	3	S	C	9 − A	3	3	1	2	5	5	35	4
1	1, 2	2	S	C	9 − A	1	4	1	2	2	1	14	4
4	1	3	S	C	9 − A	3	3	1	2	10	4	35	3
4	1	2	S	C	9 − A	3	3	1	2	10	4	38	10
1	0	3, 2	F	C	9 − A	1	4	1	2	5	5	35	1
4	3	3	S	C	9 − A	1	4	1	2	2	1	62	4
2	2	4	S	0	9 − A	3	3	1	2	10	4	18	2
3	0	3	F1	C	8.5 − C	3	4	1	2	1	5	18	8
7	1	3	S	C	9 − A	3	3	1	2	7	4	13	2

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.[26,28] The primers used for the analysis of the *TNFα* promoter (-308) A/G polymorphism were sense: 5′ AGGCAATAGGTTTTGAGGGCCAT 3′ and antisense: 5′ TCC-TCCCTGCTCCGATTCCG 3′. The primers used for the analysis of the *TNFβ* second intron (A/G) polymorphism were sense: 5′ CCGTGCTTCGTGCTTTGGACTA 3′ and antisense: 5′ AGAGCT-GGTGGGACATGTCTG 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 $MgCl_2$ 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 *α1 and of 107 bp for allele *α2, 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.[18]

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, 9 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 *TNFa* and *TNFe* microsatellite alleles or the *TNFα*(-308) and *TNFβ*n(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 *TNFa, 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 *TNFa, b,* and *c* to extended haplotypes has been described before.[23,24] Table 1 shows assignments of *HLA-DR,* complotype, *HSP70-1, -2; 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 *TNFa10, 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

	Allele or Haplotype	Genotype Frequencies		Pc Value
		CA* Patients	Controls	
Ashkenazi	*HLA-DRB1*0402*	11/24	6/54	NS†
Jewish	*HLA-DQB1*0302*	11/24	8/54	NS†
	*HLA-DQA1*0301*	12/24	13/54	NS†
	TNFd3	17/24	11/36	.02
	TNFb4	19/24	11/36	.002
	TNFb5	1/24	21/36	.0005
	[*HLA-DRB1*0402, DRB4*0101, DQB1*0302, DQA1*0301, HSP70-2*A, HSP70-1*9, TNFe3, TNFd3, TNFα(−308)*1, TNFβn(A/G)*2, TNFa10, TNFb4, HLA-B38*]	12/24	3/36	NS†
Non-Jewish	*HLA-DRB1*02*	14/40	4/32	NS†
	*HLA-DQB1*0502*	10/40	1/32	NS†
	*HLA-DQA1*0102*	15/40	3/32	.04
	TNFd3	34/42	17/30	NS
	TNFb4	29/42	6/30	.0005
	TNFb5	8/42	22/30	.0005
	[*HLA-DRB1*02+, DRB5*02, DQB1*0502, DQA1*0102, HSP70-2*A, HSP70-1*9, TNFe3, TNFd3, TNFα(−308)*1, TNFβn(A/G)*2, TNFa11, TNFb4*]	10/40	0/32	NS†
Combined	*TNFd3*	51/66	28/66	.001
	TNFb4	48/66	17/66	.0005
	TNFb5	9/66	43/66	.0005
	[*HLA-DRB1*0402, DRB4*0101, DQB1*0302, DQA1*0301, HSP70-2*A, HSP70-1*9, TNFe3, TNFd3, TNFα(−308)*1, TNFβn(A/G)*2, TNFa10, TNFb4, HLA-B38*]	12/64	4/66	NS
	[*HLA-DRB1*02#, DRB5*02, DQB1*0502, DQA1*0102, HSP70-2*A, HSP70-1*9, TNFe3, TNFd3, TNFα(−308)*1, TNFβn(A/G)*2, TNFa11, TNFb4*]	10/64	0/66	NS

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.

* Clozapine-induced agranulocytosis.

† These *P* values were significant because they confirmed previous hypothesis based on pilot studies.[45]

Data from Yunis et al[4] and Corzo et al.[22]

TNFa2, b1, βn2, 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, βn2, 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, βn2, 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 nonrandom 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 *TNFa10, b4* or *TNFa11, b4*. Therefore, the overrepresentation of the *TNFb4* and *d3* 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.[16] 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.[4] 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-
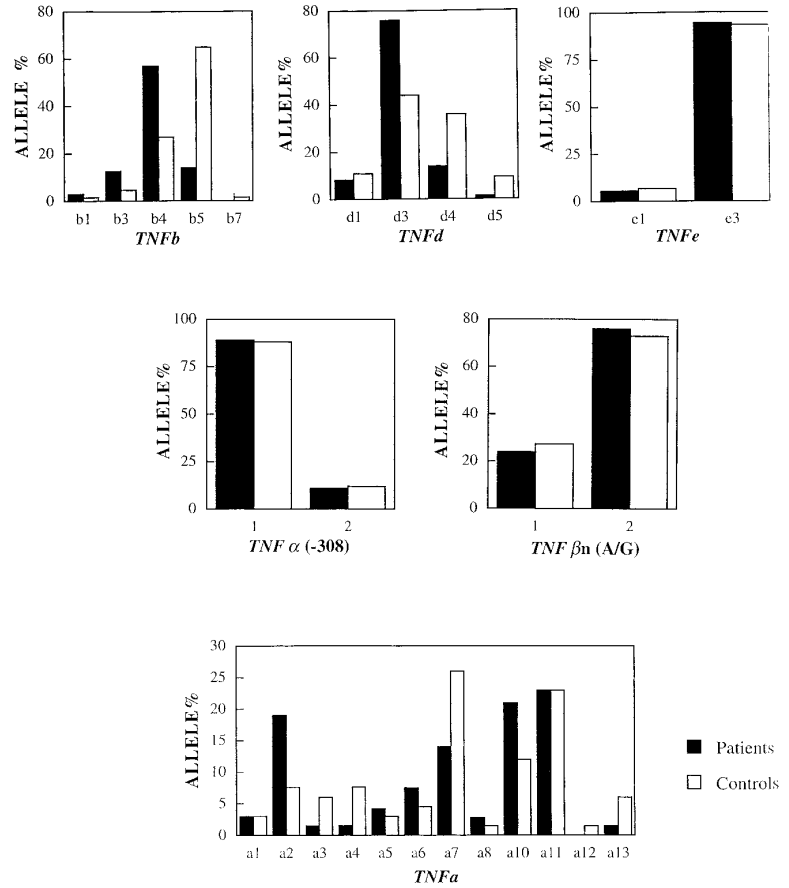
Fig 2. The distribution of (microsatellite) alleles in CA patients versus control individuals. Horizontal axis shows the number (n) of tandem repeats. Vertical axis shows the frequency of the alleles in the populations studied. The frequencies of the *d3* and *b4* alleles were significantly higher in the patients whereas the frequency of the *b5* allele was significantly higher in the controls (see text for details).

tion of patients. A possibility that non-*HLA* alleles in linkage disequilibrium with *HLA* were involved was raised based on the findings that variants of *HSP70* were shared by the two haplotypes associated with CA.[4,22]

In the present study, we compared the frequencies of polymorphisms of the *TNF* genes in the group of CA patients with those in a population of 33 white schizophrenic patients who were treated with clozapine without developing agranulocytosis. Susceptibility to CA was associated with a high

frequency of the *TNF b4* and *d3* microsatellite alleles in two different ethnic groups, whereas protection was associated with the microsatellite *b5*. This is consistent with the hypothesis that susceptibility to this drug-induced reaction is partly due to the linkage disequilibrium of these alleles with *HLA-B* and *DR* specificities.[4,22-24] The significant association of *TNFb4* and *d3* in CA patients of Jewish origin could have been predicted as they are part of the extended haplotype *HLA-DRB1*0402, DRB4*0101, DQB1*0302, DQA1*0301,*

Table 3. TNF Constellation Polymorphism in Jewish CA Patients

First Haplotype	Second Haplotype
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B38*	*HLA-DRB1*0701 (DR7),* TNFe3, d7, $\alpha(-308)1$, βn2, a7, **b4,** *HLA-B44*
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B38*	HLA-BRB1*0701 (DR7), TNFe1, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** HLA-B37
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	*HLA-DRB1*0402 (DR4),* TNFe3, **d3,** $\alpha(-308)1$, βn2, a7, **b4,** HLA-B44
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	HLA-DRB1*1501 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a7, **b4,** HLA-B55
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	HLA-DRB1*1502 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a13, b5, HLA-B5
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	HLA-DRB1*1001 (DR10), TNFe3, **d3,** $\alpha(-308)1$, βn2, a2, b5, HLA-Bx
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	HLA-DRB1*1401 (DR6), TNFe3, d5, $\alpha(-308)1$, βn2, a2, b5, HLA-B44
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	HLA-DRB1*1401 (DR6), TNFe3, d5, $\alpha(-308)1$, βn2, a7, b1, HLA-B41
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	HLA-DRB1*1501 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a2, b3, HLA-B8
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	*HLA-DRB1*0301 (DR3),* TNFe3, d1, $\alpha(-308)2$, βn1, a2, b3, *HLA-B8*
HLA-DRB1*0402 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** *HLA-B38*	*HLA-DRB1*0301 (DR3),* TNFe3, d1, $\alpha(-308)2$, βn1, a2, b3, *HLA-B8*
HLA-DRB1*1301 (DR6), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** HLA-B45	*HLA-DRB1*0301 (DR3),* TNFe3, d1, $\alpha(-308)2$, βn1, a2, b3, *HLA-B8*

Haplotypes or generic types with high delta values are in italics. Statistically significant alleles are written in bold.
Data from Udalova et al[23] and Garcia-Merino.[24]

Table 4. TNF Constellation Polymorphism in Non-Jewish CA Patients

First Haplotype	Second Haplotype
HLA-DRB1*1501 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B7*	HLA-DRB1*1501 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** HLA-B27
HLA-DRB1*1501 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B7*	HLA-DRB1*0101 (DR1), TNFe3, **d3,** $\alpha(-308)1$, βn2, a2, **b4,** HLA-B7
HLA-DRB1*1501 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B7*	HLA-DRB1*0404 (DR4), TNFe3, d4, $\alpha(-308)2$, βn1, a2, b3, HLA-B8
HLA-DRB1*1601 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B7*	HLA-DRB1*0101 (DR1), TNFe3, **d3,** $\alpha(-308)2$, βn1, a5, b5, HLA-B37
HLA-DRB1*1601 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B7*	HLA-DRB1*1302 (DR13), TNFe3, **d3,** $\alpha(-308)1$, βn1, a4, b5, HLA-B7
HLA-DRB1*1601 (DR2), TNFe3, d4, $\alpha(-308)1$, βn2, a11, **b4,** *HLA-B27*	*HLA-DRB1*0401 (DR4),* TNFe3, d4, $\alpha(-308)1$, βn2, a3, b3, *HLA-B62*
HLA-DRB1*1601 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** HLA-B44	HLA-DRB1*0102 (DR1), TNFe3, **d3,** $\alpha(-308)1$, βn2, a1, **b4,** HLA-B44
HLA-DRB1*1601 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** HLA-B35	HLA-DRB1*0405 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** HLA-B60
HLA-DRB1*1601 (DR2), TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4,** [HLA-B14]	HLA-DRB1*1502 (DR6), TNFe3, **d3,** $\alpha(-308)1$, βn1, α2, **b4,** [HLA-B39]
HLA-DRB1*1601(DR2), TNFe3, d4, $\alpha(-308)1$, βn2, a11, **b4,** HLA-B35	*HLA-DRB1*0701 (DR7),* TNFe3, **d3,** $\alpha(-308)2$, βn1, a7, **b4,** *HLA-B13*
HLA-DRB1*1601 (DR2), [TNFe3, **d3,** $\alpha(-308)1$, βn2, a3, **b4,** HLA-B51]	HLA-DRB1*0402 (DR4), [TNFe3, **d3,** $\alpha(-308)1$, βn2, a6, **b4,** HLA-B51]
HLA-DRB1*1101 (DR11), TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4,** HLA-B27	*HLA-DRB1*0301 (DR3),* TNFe3, d1, $\alpha(-308)2$, βn1, a2, b3, *HLA-B8*
HLA-DRB1*0701 (DR7), TNFe3, d4, $\alpha(-308)1$, βn2, a6, **b4,** *HLA-13*	HLA-DRB1*1101 (DR5), TNFe3, **d3,** $\alpha(-308)1$, βn2, a6, b5, HLA-B44
HLA-DRB1*0901 (DR9), TNFe3, **d3,** $\alpha(-308)1$, βn1, a5, **b4,** HLA-B7	*HLA-DRB1*0401 (DR4),* TNFe3, **d3,** $\alpha(-308)2$, βn1, a6, b5, *HLA-B44*
HLA-DRB1*0401 (DR4), TNFe3, **d3,** $\alpha(-308)1$, βn2, a6, b5, *HLA-B44*	*HLA-DRB1*0301 (DR3),* TNFe3, d1, $\alpha(-308)2$, βn1, a2, b3, *HLA-B8*
TNFe3, d4, $\alpha(-308)1$, βn2, a7, **b4**	TNFe3, **d3,** $\alpha(-308)1$, βn2, a7, **b4**
TNFe3, **d3,** $\alpha(-308)1$, βn2, a7, **b4**	TNFe3, d4, $\alpha(-308)1$, βn2, a8, **b4**
TNFe3, **d3,** $\alpha(-308)1$, βn2, a11, **b4**	TNFe3, d4, $\alpha(-308)1$, βn1, a7, b5
TNFe3, d4, $\alpha(-308)1$, βn2, a10, **b4**	TNFe3, d4, $\alpha(-308)1$, βn1, a5, b5
TNFe3, **d3,** $\alpha(-308)1$, βn2, a10, **b4**	TNFe3, d4, $\alpha(-308)1$, βn2, a2, **b4**
TNFe3, **d3,** $\alpha(-308)1$, βn2, a1, **b4**	TNFe3, **d3,** $\alpha(-308)1$, βn1, a4, **b4**

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.
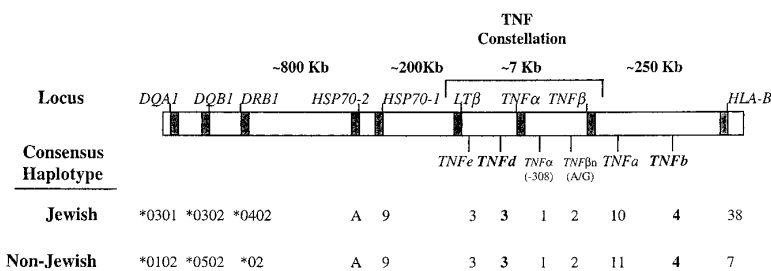
HSP70-2 A, HSP70-1 9.0 found in Ashkenazi Jews.[15] More informative was the association of *TNFb4* and *d3* with CA in patients of non-Jewish origin. In 10 of 11 non-Jewish DR2 positive patients and in the remaining 11 patients, there was an association of *TNFb4, d3* with CA. This suggests that in 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 TNFα 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* loci 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 TNFα and TNFβ,[24,37-39] 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-

Locus	DQA1	DQB1	DRB1	HSP70-2	HSP70-1	LTβ	TNFα	TNFβ			HLA-B
Consensus Haplotype						TNFe	TNFd	TNFα (-308)	TNFβn (A/G)	TNFα TNFb	
Jewish	*0301	*0302	*0402	A	9	3	**3**	1	2	10 **4**	38
Non-Jewish	*0102	*0502	*02	A	9	3	**3**	1	2	11 **4**	7

Fig 3. Partial approximate map of the human MHC region with the consensus haplotypes in Jewish and non-Jewish CA patients. *TNF* alleles with statistically significant differences in frequencies in patients compared with controls are written in bold. The borders for the class-III region are defined by the *HLA* class II (*DRB1* locus) and *HLA* class I (*B* locus). The allele assignment for the *HSP70-1, -2,* and the *HLA-DR* and *HLA-B* loci was summarized from our previous publications[4,21] (see text for details).

ported that TNFα secretion is influenced either by the *TNF* microsatellites[38,40] or by the *HLA-DR* generic type.[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.[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.[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.[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.[14,15,18]

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