Molecular-Genetic Approaches to the Study of the HLA System: New Horizons in Basic Research and Applied Studies

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Population studies aimed at identifying genetic markers of diseases and at improving the selection of histocompatible donors for allografting suggest that basic research and applied studies of the system of Human Leukocyte Antigens (HLA) are best performed at the molecular-genetic level (DNA typing).

Key Words: *HLA system; gene typing; polymerase chain reaction*

HLA matching (HLA typing) of the genome of a given individual (HLA genotype) is an integral part of procedures performed during organ and tissue grafting [4].

HLA typing became widely used in experimental and clinical medicine starting in the mid-seventies. This trend, based on the correlation between certain HLA alleles and predisposition toward a number of diseases, including autoimmune pathologies, allergies, infectious and oncological diseases, has been termed "HLA and Diseases." Its development now opens up new avenues for the study of disease pathogenesis and for identification of the genes associated with various pathologies [5].

Two important areas in biology and medicine where HLA typing has found application are population genetics and anthropology. This is due to the fact that the distribution of alleles in the human HLA system [1] has its characteristic features for representatives of different races, populations, and ethnic groups, making it possible to gain insight into the genesis of different human populations [2].

The development of the techniques involving HLA typing has been limited by the following circumstances: viable lymphocytes of the examinee are a prerequisite for "classical" methods of HLA typing. The invention of a new method of typing, making it possible to establish the genotype not by screening HLA expressed in viable lymphocytes, but by directly analyzing the antigen-coding HLA genes themselves, helped overcome this restriction. In this case a minimum amount of biomaterial is required to obtain the DNA necessary for the analysis, and the procedure is not limited by its viability.

Approaches to HLA gene typing have been studied in different research centers by using two polymerase chain reaction (PCR)-based methods, which are now employed in the overwhelming majority of laboratories engaged in HLA DNA typing and which were accordingly recommended by the XI International Histocompatibility Conference [2]. These methods are SSO (sequence-specific oligonucleotide)-PCR and SSP (sequence-specific primer)-PCR [3].

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TABLE 1. DQA1 Allele Frequencies in Various Populations

DQA1 allele	Ukrainians, N=108		Uzbeks, N=184		Kazakhs, N=132		Buryats, N=176	
	n	%	n	%	n	%	n	%
0101	16	14.8	21	11.4	14	10.6	12	6.8
0102	18	16.7	20	10.9	12	9.1	23	13.1
0103	8	7.4	16	8.7	21	15.9	10	5.7
0201	11	10.2	27	14.7	13	9.8	22	12.5
0301*	17	15.7	16	8.7	33	25.0	45	25.6
03012	2	1.9	1	0.5	0	0.0	6	3.4
0401	3	2.8	3	1.6	3	2.3	5	2.8
0501	23	21.3	37	20.1	41	31.1	19	10.8
0601	4	3.7	1	0.5	3	2.3	8	4.5

Note. Here and in Table 2: n is the number of HLA haplotypes containing the given allele; N is the number of HLA haplotypes studied; % is the allele frequency. An asterisk indicates 0301=03011+0302; these alleles are indistinguishable in the amplified region.

The first wide-scale HLA gene typing was performed within the framework of the "anthropological component" of the XI International Conference; 18 populations were examined. The SSO method was chosen for HLA typing by the Organization Committee, since it offers the widest possibilities for identification of HLA alleles, including some not previously identified by other methods.

The present study shows the results of SSO typing with respect to the DQA and DQB genes of four populations from the former USSR: Ukrainian, Uzbek, Kazakh, and Buryat. In addition, a group of 86 patients with insulin-dependent diabetes mellitus (IDDM) was typed for the same genes. The DQ HLA locus was chosen since its alleles have been found to be most closely associated with the predisposition for IDDM.

MATERIALS AND METHODS

Fifty-four Ukrainians (europeoids), 66 Kazakhs and 88 Buryats (mongoloids), and 92 Uzbeks (mixed ethnicity) were involved in the population study. The group of patients with IDDM comprised 86 examinees. Alleles identified by gene typing and their incidence in the test groups are shown in Tables 1 and 2.

DNA was isolated from peripheral blood lymphocytes of examinees by triple treatment with a lysing buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 1% Triton X-100) and centrifugation at 13,000 g for 20-30 sec. The supernatant was discarded, and the pellet was resuspended in 500 μl of buffer for PCR with nonionic detergents and proteinase K (0.6 μl of 10 mg/ml proteinase K per 100 μl of solution). The tubes were then incubated at 60°C for 1 h. Proteinase K was inactivated by heating at 95°C for 10 min. The DNA concentration was assessed from fluorescence in the presence of Hoechst 33258 stain on a Hoefer DNA-minifluorimeter.

Isolated DNA was amplified in PCR. The reaction was performed under a layer of mineral oil in 50 μl of amplification mixture containing 200 μM of each deoxynucleotide triphosphate (dNTP), 25 pM of each primer (oligonucleotides used in PCR for DNA polymerase priming), 2.5 IU *T. thermophilus* DNA polymerase, 67 mM Tris-HCl, pH 8.8, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 1 mM 2-mercaptoethanol, 0.1 μg DNA, and 10 μM tetramethylammonium chloride.

Thirty cycles of amplification (using a Cetus-Perkin Elmer Thermal Cycler) were performed, and the amplification products were analyzed by electrophoresis in 12% polyacrylamide gel. Hybridization was performed as follows. One-microliter aliquots of amplified samples were applied to nitrocellulose filters and fixed using ultraviolet radiation (254 nm) for 6 min.

The set of oligonucleotide probes for hybridization was provided by the Organization Committee of the XI International Conference. Seventeen probes were used for gene typing of the DQA locus and 22 for typing of the DQB locus. For hybridization the probes were labeled with [^{32}P]-ATP in the kinase reaction in 25 μl of a mixture of 10 pM of the probe, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM spermidin-HCl, 20 IU T4 polynucleotide kinase, and 60 μCi γ-[^{32}P]-ATP. Preliminary hybridization was performed at 42°C during 2 h in a buffer containing 6×SSPE, 5×Deinhardt solution, 0.5% sodium dodecylsulfate (SDS), and 100 μg/ml denatured DNA from *Salmo* sperm.

TABLE 2. DQB1 Allele Frequencies in Various Populations

DQA1 allele	Ukrainians, N=108		Uzbeks, N=184		Kazakhs, N=132		Buryats, N=176	
	n	%	n	%	n	%	n	%
0201	11	10.2	35	19.0	25	18.9	20	11.4
0301	6	5.6	15	8.2	15	11.4	20	11.4
0302	3	2.8	2	1.1	3	2.3	9	5.1
03031	2	1.9	1	0.5	0	0.0	0	0.0
03032	0	0.0	3	1.6	4	3.0	9	5.1
0401	2	1.9	2	1.1	0	0.0	0	0.0
0501	7	6.5	14	7.6	6	4.5	3	1.7
0502	3	2.8	3	1.6	0	0.0	5	2.8
05031	0	0.0	0	0.0	0	0.0	2	1.1
0504	0	0.0	2	1.1	0	0.0	0	0.0
0601	1	0.9	1	0.5	0	0.0	0	0.0
0602	11	10.2	4	2.2	3	2.3	0	0.0
0603	4	3.7	8	4.3	7	5.3	5	2.8
0604	3	2.8	1	0.5	2	1.5	7	4.0
0605	1	0.9	1	0.5	1	0.8	8	4.5

Subsequent hybridization with labeled oligonucleotide probes was performed at 42°C during 16 h. The filters were washed free of the unhybridized products during 10 min at room temperature, first with 2×SSPE + 0.1% SDS, and then twice with 6×SSPE + 1% SDS at the melting temperature calculated for each probe.

The strength of the hybridization signals was assessed by autoradiography on an RM-1 x-ray film using an arbitrary 9-point scale proposed by the XI International Conference.

The relative risk of the disease (RR) was calculated after Woolf by the formula: $(a \times d)/(b \times c)$, where a is the number of patients with the given genotype, b is the number of patients without the given genotype, c is the number of healthy individuals with the given genotype, and d is the number of healthy individuals without the given genotype. When one of the indexes was 0, RR was calculated by the formula modified by Haldane for small numbers: $[(2a+1)(2d+1)]/[(2b+1)(2c+1)]$.

Statistical analysis of the correlation between certain genotypes and IDDM was performed using the precise Fisher test (unilateral) for four-field tables.

RESULTS

The results of population typing are presented in Tables 1 and 2. Table 1 demonstrates that there are differences in the distribution of the DQA alleles among the examined populations. For instance, in Ukrainians and Uzbeks the frequency of the 0301 allele was reliably lower ($p<0.05$) than in Buryats and Kazakhs. In the latter two populations the frequency of this allele was similar. It was found that the frequency of the 0501 allele was reliably lower ($p<0.02$) in Buryats as compared with the other three populations. The frequency of 0501 in Buryats was 3 times lower than that in Kazakhs. It should

TABLE 3. Comparison of Frequencies of Marker Genotypes of Predisposition for IDDM in Groups of Patients with Diabetes and in Healthy Donors

Genotype	Patients with IDDM, N=86		Healthy controls, N=56		Relative risk
	n	%	n	%	
DQA 0301, 0501	20	23.2	2	3.57	8.18*
DQB 0201, 0302	21	24.4	2	3.57	8.72**
DQA 0301, 0501/DQB 0201, 0302	10	11.6	0	0.00	15.5**

Note. One and two asterisks indicate $p<0.001$ and $p<0.0005$, respectively. N is the number of examinees; n is the number of examinees with the given genotype; % is the genotype frequency.

also be noted that the frequency of the 0101 allele was more than twofold ($p<0.05$) lower in Buryats as compared with Ukrainians. According to our findings, the frequency of the 0103 allele was twice as high in the Kazakh population as in the other populations. The 03012, 0401, and 0601 alleles were rarely encountered in the four populations examined.

Peculiarities in each of the populations examined were also found in the distribution of the DQB alleles (Table 2). The 0201 allele was the one most frequently encountered in all the examined populations, but its frequency varied among them. For instance, the frequency of the 0201 allele, which was similar in Uzbeks and Kazakhs, exceeded that in Ukrainians and Buryats more than 1.5-fold. The frequency of the 0605 allele was reliably higher in Buryats, and that of the 0602 allele in Ukrainians, than in the other populations, the latter allele not being found at all in the Buryat population. No reliable differences in the frequency of other DQB alleles was revealed among the populations.

Thus, our findings provide evidence that each of the four populations exhibits a specific pattern of allele distribution with respect to the class II HLA genes. On the other hand, common features characteristic of the majority of the populations (for example, an increased frequency of the 0501 allele of the DQA gene in the Ukrainian, Uzbek, and Kazakh populations) were observed as well as differences in each individual population. The results obtained in studies of the correlation between the DQA and DQB genotypes and IDDM are presented in Table 3.

It should be mentioned that the level of RR (15.5, $p<0.0005$) for the genotype comprising the marker alleles for predisposition for IDDM (DQA 0301, 0501/DQB 0201, 0302) markedly surpasses that detected in studies where the HLA-IDDM association was studied using serological typing methods.

It is worth noting that the frequency of the allele markers of predisposition for IDDM in the Russian population differed from that in healthy individuals of different races from the examined populations. These differences cannot but affect the correlation between the above-mentioned alleles and IDDM in the test ethnic groups.

Thus, the new methodological plane of studies of the HLA system - gene typing - has not only yielded new data on the allelic polymorphism of the HLA system, but also shed new light on the problem of "HLA and Diseases," the development of which will surely require gene typing studies of different "healthy" groups of people and persons with different pathologies.

The use of SSO gene typing in clinical transplantology has been limited until now, since it is a lengthy and laborious procedure. Other methods of PCR gene typing, including the SSP method which is currently used in nonurgent transplant procedures (bone marrow transplants, organ grafts between relatives), have the same disadvantages. The recently developed SSPM (sequence-specific primer mixture) method, which is a modification of the "classical" SSP method, makes it possible to combat the above-mentioned disadvantages. This method is a less labor-intensive variant of SSP. It consists in using primer mixtures in specific amplifications. In this case the product of a certain length corresponds to each specificity. This helps greatly reduce the number of tubes and the number of tracks on the gel, because the possible products can be easily identified according to their length along one track.

The developed set of primers amplifies all well-known DRB1 alleles, which then allows them to be classified among the following DRB1 groups: DR1, DR2, DR3, DR4, DR5(11), DR5(12), DR6(13), DR6(14), DR7, DR8, DR9, and DR10. The typing procedure consists of two steps. The first step is standard amplification of the DRB gene. The second step is group-specific amplification of the DRB gene. The groups are identified according to the length of fragments in two corresponding tracks of polyacrylamide gel. The use of mixture 1 discloses the following DR specificities on the first of them: DR1(46), DR10(52), DR4(60), DR7(69), DR9(101), and DR2(119). On the second track the use of mixture 2 reveals the presence of the specificities DR6.14(50), DR3(58), DR5.12(86), DR8(98), and DR5.11(118). The length of identified fragments in base pairs is shown in parentheses. With the new variant the typing procedure altogether (after isolating DNA) took no more than 3 h.

Our findings call attention to fundamentally new possibilities in the study of the HLA system by introducing biotechnological methods, notably PCR. The results may find wide application in different fields of biomedical science.

REFERENCES

1. R. Ceppellini, E. S. Curtoni, P. L. Mattius, *et al.*, in: *Histocompatibility Testing,* Eds. E. S. Curtoni *et al.*, Copenhagen (1967), pp. 149-187.
2. T. I. Imanishi, A. Wakisaka, and T. Gojobori, in: *Proc. XI International Histocompatibility Workshop and Conference,* Eds. K. Tsuji *et al.*, Vol. 1, Oxford (1992), pp. 627-632.
3. T. Kimura and T. Sasazuki, in: *IX International Histocompatibility Workshop Reference Protocol for the HLA DNA Typing Technique,* Eds. K. Tsuji *et al.*, Vol. 1, Oxford (1992), pp. 397-419.
4. G. Opelz, *Lancet,* № 11, 61-64 (1988).
5. A. Svejgaard, C. Iersild, L. S. Nicolson, and W. F. Bodmer, *Tiss. Antigens,* № 4, 95-105 (1974).