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Reproductive Failure and the Major Histocompatibility Complex

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Summary

The association between HLA sharing and recurrent spontaneous abortion (RSA) was tested in 123 couples and the association between HLA sharing, and the outcome of treatment for unexplained infertility by in vitro fertilization (IVF) was tested in 76 couples, by using a new shared-allele test in order to identify more precisely the region of the major histocompatibility complex (MHC) influencing these reproductive defects. The shared-allele test circumvents the problem of rare alleles at HLA loci and at the same time provides a substantial gain in power over the simple χ^2 test. Two statistical methods, a corrected homogeneity test and a bootstrap approach, were developed to compare the allele frequencies at each of the HLA-A, HLA-B, HLA-DR, and HLA-DQ loci; they were not statistically different among the three patient groups and the control group. There was a significant excess of HLA-DR sharing in couples with RSA and a significant excess of HLA-DQ sharing in couples with unexplained infertility who failed treatment by IVF. These findings indicate that genes located in different parts of the class II region of the MHC affect different aspects of reproduction and strongly suggest that the sharing of HLA antigens per se is not the mechanism involved in the reproductive defects. The segment of the MHC that has genes affecting reproduction also has genes associated with different autoimmune diseases, and this juxtaposition may explain the association between reproductive defects and autoimmune diseases.

Introduction

Genes in the region of the major histocompatibility complex (MHC) control many immunological and developmental functions, and defects in these genes are involved in the pathogenesis of a variety of diseases (Gill 1984, 1994; Nelson and Hansen 1990; Sinha et al. 1990; Nepom and Erlich 1991; Lepage et al. 1993). Recurrent spontaneous abortion (RSA) has been associated with the sharing of HLA antigens between husband and wife (Gill 1983, 1984; Kostyu et al. 1993), and recent studies (Ho et al. 1990, 1994) have localized the genetic defects involved to the HLA-B–HLA-DR–HLA-DQ region of the MHC. Sharing of HLA antigens in the same region has also been observed in couples experiencing unexplained infertility (UIF) who failed treatment by in vitro fertilization (IVF) (Ho et al. 1994). These studies did not identify any specific HLA antigen or any combination of HLA antigens that were associated with the reproductive defects. The shared HLA antigens were interpreted as being markers for the sharing of closely linked susceptibility genes or genetic defects.

Experimental studies in the rat (Gill and Kunz 1979; Kanbour et al. 1987; Melham et al. 1993) have identified an MHC-linked region, the growth and reproduction complex, that has recessive genes influencing fertility, growth, and resistance to chemical carcinogens. Deletions of these genes or various combinations thereof (Cortese Hassett et al. 1986; Vardimon et al 1992; Lu et al. 1993) lead to abnormal phenotypes: sterility that is complete in the male and partial in the female, retarded growth in both males and females, and enhanced susceptibility to several different types of chemical carcinogens. These experimental studies have provided the paradigm for our clinical investigations (Ho et al. 1990, 1991, 1994) to test the hypothesis that genes linked to the MHC influence RSA and the failure of IVF to successfully treat UIF.

The present study addresses two questions. First, it employs the shared-allele test to investigate the HLA-sharing data in couples experiencing RSA or UIF, in order to identify more precisely the location of the genetic factors within the HLA-B–HLA-DR–HLA-DQ region involved in their pathogenesis. Second, a corrected homogeneity test and a bootstrap approach were developed to compare the HLA allele frequencies in these couples to those in normally fertile couples to test whether the distribution of HLA alleles was the same in the patient populations as in the normally fertile couples.

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Patients and Methods

Patient Population

All of the patients studied were from a relatively ethnically homogeneous population of Taiwan Chinese who were seen at the Obstetrics Clinic of the National Taiwan University Hospital, and their characteristics have been described in detail elsewhere (Ho et al. 1990, 1994). There were 123 couples in whom the woman experienced RSA (Ho et al. 1990), which is defined as three or more pregnancy losses without any identifiable anatomic, chromosomal, hormonal, or immunological abnormalities; infectious diseases; or seminal abnormalities in the husband. There were no clinical or immunogenetic differences between those women who had no live births before their pregnancy losses (primary aborters, 91 patients) or who had one or more live births before their pregnancy losses (secondary aborters, 32 patients); hence, no distinction was made between these patients.

There were 76 couples with UIF on the basis of a thorough infertility study (Ho et al. 1994): repeated semen analyses, documented ovulation, anatomic evaluation, ultrasonography, endocrine profiles, assay for autoimmune antibodies, and zona-free hamster egg penetration assay. These couples were treated by IVF and tubal embryo transfer; and 34 women had a successful pregnancy (IVF–S), whereas 36 did not (IVF-F).

Fifty-one normally fertile couples were used for controls in all of these studies, and they were matched by age with the patient populations, had two or more live births, and did not have a history of spontaneous or induced abortions (Gill et al. 1990; Ho et al. 1990).

HLA Typing

The typing for HLA-A, HLA-B, HLA-DR, and HLA-DQ was done by the microcytotoxicity test with reagents calibrated against standard antisera (Ho et al. 1990). Because of the high prevalence of the broadly shared HLA-Bw4, Bw6 and HLA-DRw52, DRw53 antigens in the population, these antigens were not used in determining antigen sharing. The antisera used could determine HLA-DR antigens 1–18 and HLA-DQ antigens 1–7, but because of splits in some antigens, the splits were combined with the main antigenic group for analysis (WHO Nomenclature Committee for Factors of the HLA System 1992; Duquesnoy and Marrari 1994). This approach essentially uses an antigen supratype that should reflect the general structure of the DNA in this region and not just the molecular structure of the HLA antigen heavy chain. These antigenic groups and their splits (given in parentheses) are DR1; 2 (15,16); 3 (17,18); 4; 5 (11,12); 6 (13,14); 7; 8; 9; and 10; and DQ1 (5,6); 2; 3 (7); and 4. The HLA-sharing data (Ho et al. 1990, 1994) analyzed in this way are summarized in table 1.

Statistical Methods

Previous studies on the sharing of alleles at various HLA loci (Gill 1994) were done by direct comparison of sharing alleles within couples in patient and control populations using a simple χ^2 test. While this approach can detect differences between the two populations in sharing at several loci, it has low statistical power and cannot identify the effects at each locus. In order to overcome this problem, we employed a shared-allele test, which has a high power to detect excessive sharing among couples (Jin et al., in press). The relative powers of the shared-allele test and the simple χ^2 test are presented in the Results section. This approach circumvents the problem of rare alleles at HLA loci.

Homogeneity test.—The HLA allele frequencies in the three patient groups and the normal population were compared. A simple gene-counting method was used to calculate the allele frequencies for each group, and an ordinary Pearson statistic for testing homogeneity with a $4 \times k$ table, where k is the number of alleles at a given HLA locus, was then calculated. The χ^2 approximation of the test statistic cannot be used because the individual alleles in a $4 \times k$ table are not independent sample units. These alleles are derived from the couples (four alleles each) who are the actual independent sampling units, and they form clustered samples with the couples' genotypes defining the clusters. The distribution of the Pearson test statistic with clustering will not follow the same χ^2 distribution under the null hypothesis as would be the case with simple random samples (Cohen 1976). We propose a corrected homogeneity test statistic for this problem that will adequately approximate the χ^2 under the null hypothesis (see appendix A).

Another way to get around the distributional problem of the Pearson test statistic was a bootstrap method that employed resampling of the couples and that followed the guidelines for nonparametric hypothesis testing described by Hall and Wilson (1991). The bootstrap distribution for the homogeneity test statistic was estimated under the null hypothesis assuming that the population allele frequencies from which the four groups (three patient and one normal) were drawn were the same. These four groups were resampled from the pooled mating genotype sample. Then the bootstrap homogeneity test statistic was calculated. This procedure was repeated 999 times to give an estimate of the distribution of the homogeneity test statistic. The validity of this approach has been established, and the details are presented in appendix A.

Shared-allele test.—The frequencies of sharing alleles at an HLA locus of the couples, in the four populations studied, were compared with the frequencies that would be expected on the assumption of random mating at that locus. A significant deviation at a given locus, from

Table 1

HLA Sharing in the Patient and Control Populations

	Frequency of Sharing Alleles at HLA Loci, with Two, One, or Zero Shared Alleles											
	A			B			DR			DQ		
Group	2	1	0	2	1	0	2	1	0	2	1	0
RSA	16	74	33	1	38	84	10	57	56	35	60	28
IVF-F	6	19	11	1	13	22	1	14	21	18	12	6
IVF-S	0	17	17	0	7	27	1	12	21	7	15	12
Normally fertile	3	25	23	1	13	37	4	17	30	8	23	20

the frequency of sharing expected under the assumption of random mating, indicates a significant association between sharing of alleles at that locus and the criterion that was used to classify the couples, i.e., the patient or normal population into which they fell.

Testing for random mating at an HLA locus is difficult because of the highly polymorphic nature of the HLA complex. Jin et al. (in press) proposed several approaches to this problem, one of which, the shared-allele test, seems particularly suitable in the present context. For this test, couples were classified into three groups according to whether they share zero, one, or two alleles at the locus under consideration. This leads to a set of observed sharing frequencies for the three groups, and these frequencies were compared with those expected under the hypothesis of random mating by a Pearson goodness-of-fit statistic X_s^2. The expected frequencies were calculated by first estimating the genotype probabilities for the male and female in each couple under consideration, multiplying these estimated probabilities to obtain estimates of the probabilities of mating-pair genotypes, and summing over the appropriate mating genotypes of sharing: this procedure gives the estimated probabilities of sharing zero, one, or two alleles under the hypothesis of random mating. The expected frequencies of sharing zero, one, or two alleles were then obtained by multiplying these estimated probabilities by the number of the couples in the group. This test was performed for each of the HLA-A, HLA-B, HLA-DR, and HLA-DQ loci in the three patient populations and the one normal population. The estimates so obtained, of the probabilities of mating-pair genotypes under the hypothesis of random mating, will also be used in the procedure for determining the empirical P value of the shared-allele test.

Because the expected frequencies have been computed using genotype probabilities estimated from the observed data, we can not automatically assume that the Pearson goodness-of-fit statistic follows the χ^2 distribution with 2 df under the null hypothesis, as it would if the genotype probabilities were known. However, the X_s^2 statistic has a null distribution that is close to the standard χ^2 distribution with 2 df (see Zhou et al., in press, for additional discussion). Nonetheless, we determined the P value of the X_s^2 statistic by a simulation method.

The significance of the X_s^2 value was determined in the following way. For each locus in the group under consideration, a simulated group of the same size, of couples with independent genotype pairs, was generated with the estimates of the probabilities of mating-pair genotypes under the hypothesis of random mating given above. The value of X_s^2 was calculated as described above. This simulation was repeated 999 more times, and the value of X_s^2 for the actual group was assigned a P value equal to the proportion of values in the 1,000 simulated values that exceeded it. The empirical P value was calculated for each group at each HLA locus.

In order to determine whether the significant results obtained with the shared-allele test would be consistent with those obtained by other methods, we used two additional approaches. One is the pooling method, in which the genotype probabilities for the males and females were estimated from the data pooled from the four groups (three patient and one normal) at the locus under consideration. Then the expected frequencies of sharing zero, one, or two alleles were calculated as described above for the four groups at the locus under consideration. The shared-allele test statistic X_s^2 was calculated by comparing the observed frequencies to the expected frequencies. Compared to the method described above, this method will provide more robust estimates of the expected frequencies of sharing. The drawback of this method is that one needs to assume the homogeneity of the genotype frequencies among the four groups. We performed a homogeneity test for the allele frequencies among the four groups; we are not aware of any practical approach for testing the homogeneity of the genotype frequencies for this type of data.

The other approach is the method using random pairing of couples, in which the simulated group of mating genotypes was constructed by randomly pairing every female genotype with a male genotype, other than her own partner, within the same group at the locus under consideration. For each simulated group, the value of X_s^2 was calculated, and the procedure was then repeated 999 times. The P value of the actual X_s^2 was determined as described above.

Simulation power study.—This study was performed to compare the shared-allele test X_s^2 with the commonly used simple χ^2 test X_c^2, as well as to estimate the powers of the shared-allele test at the HLA-DR locus for RSA patients and at the HLA-DQ locus for IVF-F patients. The HLA-DR genotype probabilities were estimated from pooled data from the 123 RSA couples and 51 normal couples, and the HLA-DQ genotype probabilities were from 36 IVF-F couples and 51 normal couples). The mating-pair probabilities for the normal population were calculated by multiplying the relevant genotype probabilities as described above. The mating-pair probabilities of the patient population were created artificially, by systematically changing the probabilities of sharing zero, one, and two alleles from the normal population probabilities toward the observed nonrandom-mating probabilities in the patient groups (RSA or IVF-F). One set of the patient population probabilities will correspond to those probabilities actually observed in patients. The formulation of the mating probabilities is shown in appendix B.

At each locus (HLA-DR or HLA-DQ), a simulated normal group of 51 couples and a simulated patient group (123 couples for RSA and 36 for IVF-F) were generated from the normal and patient population mating probabilities, respectively, and the test statistics X_s^2 and X_c^2 were then calculated for these simulated groups. The entire sampling process was repeated 999 times, and the number of times that the null hypothesis was rejected by each method was counted and expressed as a percentage.

Significance.—The level of significance for each comparison was $P < .01$. Taking into account that each locus being compared has four groups, the overall level of significance (Bonferroni criterion) was $P < .05$.

Table 2

Homogeneity Test Comparing HLA Allele Frequencies in the Three Patient Populations (RSA, IVF-F, IVF-S) and the Normally Fertile Population

	P Values	
HLA Locus	Corrected Homogeneity Test	Bootstrap Approach
A	.828	.689
B	.970	.969
DR	.439	.481
DQ	.158	.147

Table 3

Shared Allele Test on Data from Patients and from Normally Fertile Couples

	Bootstrap Estimate of *P* Values at HLA Loci[a]			
Population	A	B	DR	DQ
RSA	.072	.146	.000[b]	.192
IVF-F	.712	.801	.842	.001[c]
IVF-S	.168	.506	.984	.949
Normally Fertile	.302	.958	.346	.593

[a] The pooling method and the random-pairing method both came to the same conclusion regarding the significance of the results from the shared-allele test.

[b] The corresponding P values for the pooling and the random-pairing methods were .006 and .005, respectively.

[c] The corresponding P values for the pooling and the random-pairing methods were .000. This means that there was no simulated values of X_s^2 that exceeded the observed value of X_s^2.

Results

The allele frequencies of the patients with RSA, IVF-F, and IVF-S, and normally fertile couples were not statistically different at any locus (table 2). Thus, these reproductive defects are not associated with any differences in allele frequencies.

The empirically derived P values of the shared-allele test for the experimental data are shown in table 3. There was a significant excess HLA-DR sharing in couples with RSA; a significant excess HLA-DQ sharing in couples who failed treatment by IVF for UIF; and no significant excess HLA sharing in couples in whom IVF succeeded in the treatment of UIF or in the normally fertile couples. There is a significant excess of sharing of two alleles above that expected for HLA-DR in the RSA group and for HLA-DQ in the UIF group that failed treatment by IVF (table 4). The pooling method and the random-pairing method for four groups at the HLA-DR and HLA-DQ loci (see Patients and Methods) gave conclusions that were consistent with those obtained by the shared-allele test (table 3).

The simulation study of the power of the shared-allele test compared to that of the simple χ^2 test is shown in table 5. The top part of the table shows the results of the simulation done to reflect the RSA group at HLA-DR, and

Table 4

Observed and Expected Sharing of Alleles: HLA-DR in RSA and HLA-DQ in IVF-F

	No. of Alleles Shared with Two, One, and Zero Shared Alleles					
	HLA-DR Sharing in RSA			HLA-DQ Sharing in IVF-F		
	2	1	0	2	1	0
Observed	10	57	56	18	12	6
Expected[a]	4.92	47.24	70.84	9.83	16.31	.86

[a] Calculated under the assumption of random mating, as described in Patients and Methods.

the lower part is for the IVF-F group at HLA-DQ. The frequencies of HLA-DR (or HLA-DQ) sharing were chosen to evaluate the power of the tests to detect different levels of sharing. The first level of sharing in each part is that found in the normal population. The rejection of the null hypothesis for the different levels of sharing occurred much more frequently with the shared-allele test. Therefore, the power of the shared-allele test (X_s^2) to detect deviations from random mating resulting in higher-than-normal levels of allele sharing was considerably higher than that of the simple χ^2 test (X_c^2).

At the HLA-DR locus, the level showing the sharing of two, one, and zero alleles with the probabilities of .09, .42, and .49, respectively, corresponds to the observed level of nonrandom mating in RSA patients, and at this level the shared-allele test has a power of 47% rejection, while the simple χ^2 test has a power of only 7%. At the HLA-DQ locus, the level showing the sharing of two, one, and zero alleles with the probabilities of .5, .33, and .17, respectively, corresponds to the observed level of nonrandom mating in IVF-F patients, and at this level the shared-allele test has a power of 90% rejection, while the simple χ^2 test has a power of only 27% rejection.

Discussion

The shared-allele test is a more powerful approach than the simple χ^2 test, since it is designed to detect

Table 5

Comparison of the Power of the Shared-Allele Test (X_s^2) and the Simple χ^2 Test (X_c^2) to Detect Increased Sharing of Alleles

	Probabilities of Sharing Alleles within a Couple			Percentage of the Time Null Hypothesis Rejected[a] (power)	
	Two Alleles	One Allele	Zero Alleles	X_s^2	X_c^2
HLA-DR: RSA	.05	.38	.57[b]	1	1
	.07	.40	.53	10	2
	.09	.42	.49[c]	47	7
	.11	.44	.45	79	13
	.13	.46	.41	97	29
HLA-DQ: UIF	.23	.44	.33[b]	1	1
	.32	.40	.28	13	4
	.41	.37	.22	55	9
	.50	.33	.17[d]	90	27
	.59	.30	.11	99	53

[a] The level of significance is 1%.
[b] Sharing probabilities in the normal populations.
[c] Same nonrandom-mating level as that of the observed RSA.
[d] Same nonrandom-mating level as that of the observed IVF-F.

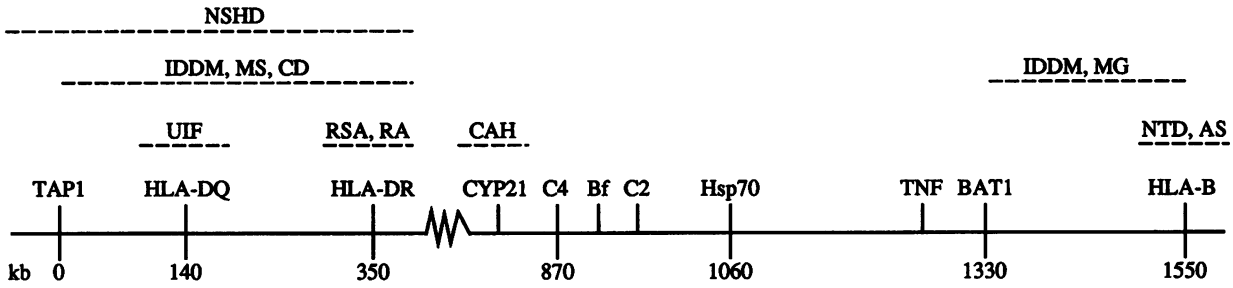

Figure 1 Class II and class III regions of the human MHC (Campbell and Trowsdale 1993) and the location of genes affecting various reproductive and autoimmune diseases. The loci are as follows: HLA-DQ and HLA-DR, class II; TAP1, transporter associated with antigen processing; CYP21, 21-hydroxylase; C4, Bf, and C2, complement components; Hsp70, heat shock protein; TNF, tumor necrosis factor; and BAT1, HLA-B-associated transcripts. A variety of different types of diseases map to this region of the MHC. The autoimmune diseases are as follows: IDDM, insulin-dependent diabetes mellitus; MS, multiple sclerosis; RA, rheumatoid arthritis; AS, ankylosing spondylitis; and MG, myasthenia gravis. Two regions in the MHC contain genes that influence IDDM: TAP1 to HLA-DR (Jackson and Capra 1993) and BAT1 to HLA-B (Degli-Esposti et al. 1992*a*; Marshall et al. 1994). The reproductive disorders are RSA and UIF. The developmental defects are as follows: CAH, congenital adrenal hyperplasia; and NTD, neural tube defects. Nodular sclerosing Hodgkin disease (NSHD) maps to the DR-DQ region (Klitz et al. 1994) and may also include genes in the DP region (Nishimura et al. 1992). A variety of other malignancies (Tiwari and Terasaki 1985; Ho et al. 1991; Gill 1992) and reproductive disorders (Ho et al. 1991; Gill 1992, 1994) are also associated with the MHC but are not more precisely mapped. The approximate distances between loci are from Campbell and Trowsdale (1993).

directly deviations from random mating resulting in increased sharing of alleles at each HLA locus. The simulation study shown in table 5 provides evidence for this conclusion. This method is particularly suitable for studying reproductive failure because it does not require pedigree data for determining genetic similarities or differences at a given locus.

Genes in the HLA-B to HLA-DQ region are associated with a variety of different types of diseases: autoimmune, reproductive, developmental, and malignant (fig. 1). We wish to raise the possibility that there are genes in tight linkage disequilibrium with the HLA loci in this region, particularly with the HLA-DR and HLA-DQ loci, that are critical etiological factors in these diseases and that the identification of such genes and of their mode of action will provide important insight into a unifying element in the pathogenesis of these different types of diseases. Rheumatoid arthritis (Harris 1990; Winchester et al. 1992), insulin-dependent diabetes mellitus (IDDM) (Todd et al. 1988; Dorman et al. 1990; Kockum et al. 1993), multiple sclerosis (Francis et al. 1991; Allen et al. 1994), myasthenia gravis (Degli-Esposti 1992*a*, 1992*b*), and celiac disease (Nishimura et al. 1992) have been associated with HLA-DR and/or HLA-DQ antigens, and ankylosing spondylitis (Benjamin and Parham 1990) has been associated with HLA-B. The data presented in this paper demonstrate the association between RSA and HLA-DR and between UIF and HLA-DQ. The developmental defects congenital adrenal hyperplasia (White et al. 1984, 1987*a*, 1987*b*; Partanen et al. 1989) and neural tube abnormalities (Schacter et al. 1979; Weitkamp and Schacter 1985) have been associated with CYP21 and HLA-B, respectively. Nodular sclerosing Hodgkin disease is associated with genes in the HLA-DR and HLA-DQ regions (Klitz et al. 1994) and possibly also with genes in the HLA-DP region (Nishimura et al. 1992). Other malignant (Tiwari and Terasaki 1985; Ho et al. 1991; Gill 1992) and reproductive (Ho et al. 1991; Gill 1992, 1994) disorders are also associated with genes in the HLA region, but are less well defined.

Immunological mechanisms have been postulated to explain the role of the HLA antigens in the pathogenesis of autoimmune diseases by the presentation of self-peptides (Sinha et al. 1990; Nepom and Erlich 1991; Lepage et al. 1993) and of reproductive disorders by not eliciting an immune response in couples sharing HLA antigens (Beer et al. 1984; Mowbray et al. 1985; Clark 1991). However, recent statistical analyses of the association between HLA-DR and rheumatoid arthritis (Dizier et al. 1993) and between HLA-DR and HLA-DQ and IDDM (Clerget-Darpoux et al. 1991; Dizier et al. 1994) by using the marker association segregation χ^2 method have raised questions about the role of the HLA antigens themselves in these diseases. The analyses indicate that a susceptibility locus (or loci) tightly linked to the HLA-DR and/or HLA-DQ loci best explains the association between rheumatoid arthritis or IDDM and the MHC. Analysis of the MHC association with multiple sclerosis (Francis et al. 1991) is also consistent with this model. Extensive studies on the relationship between RSA and the sharing of HLA antigens between father and mother (summarized in Gill 1994) and the work presented here on RSA and UIF also strongly suggest that the HLA antigens themselves are not the etiological agents but rather are in linkage disequilibrium with the gene or genetic defect responsible for embryonic loss.

Whatever the specific mechanisms are—the HLA

genes themselves or genes in tight linkage disequilibrium with them—it is clear that genes in the HLA-B to HLA-DQ region are involved in the pathogenesis of the variety of different types of diseases. There is the possibility that defects such as mutations or deletions in this region may affect different genetic mechanisms simultaneously and thereby present clinically as an association between two diseases. One example of this possibility is the association between rheumatoid arthritis and decreased fecundity (Nelson et al. 1993) and RSA (Shelton et al. 1994): the genes influencing rheumatoid arthritis and those influencing the reproductive defects may be closely linked in the HLA-DR region.

The hypothesis that MHC-linked genes are critical in the etiology of reproductive defects, growth defects, and susceptibility to cancer (Gill 1984) receives strong support from extensive experimental studies in rats. The MHC-linked growth and reproduction complex (*grc*) contains recessive genes that affect resistance to chemical carcinogenesis, fertility, and body size (Gill and Kunz 1979; Kanbour et al. 1987; Melham et al. 1993). Deletions in this region are associated with the various phenotypic defects (Cortese Hassett et al. 1986; Vardimon et al. 1992), and the *grc* maps to the position homologous to the HLA-B to HLA-DR region in the human. Since the defects in growth, development, and resistance to chemical carcinogens in the rat are associated with deletions of genes that are not related to those encoding histocompatibility antigens, but are closely linked to them, it is reasonable to postulate that the reproductive and developmental disorders associated with the HLA-B to HLA-DQ region in humans are also associated with the deletion of genes involved in the normal control of growth. Such a pathogenetic deletion has already been demonstrated in the case of congenital adrenal hyperplasia (White et al. 1984, 1987*a*, 1987*b*; Partanen et al. 1989). The possibility that larger segments of DNA, e.g., ancestral haplotypes (Degli-Esposti et al. 1992*a*, 1992*b*; Marshall et al. 1994), might contain the genes that play a role in the genetic basis of the different types of MHC-associated diseases has yet to be explored fully.

Further analyses of the genes in the HLA-B to HLA-DQ region of the human and of the genes in the homologous *grc* region of the rat will continue to provide new insight into the function of the MHC and its linked genes and into its remarkable evolutionary conservation. This work will also allow the development of unique approaches to assessing more accurately the risk of reproductive failure, and possibility of other MHC-associated diseases, and to predicting the utility of IVF in the treatment of UIF.

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Appendix A

Comparison of Allele Frequencies at HLA Loci

As we pointed out in the paper, the homogeneity test statistic for comparing allele frequencies among the RSA, IVF-F, IVF-S, and normal groups does not follow the standard χ^2 distribution under the null hypothesis. Here, we propose a corrected homogeneity test statistic for comparing allele frequencies under such circumstances and discuss the bootstrap approach.

Corrected Homogeneity Test

Suppose there are c target populations of mating couples. Let $p_{s,i}$ be the allele frequencies of allele i in population s at a given locus with r alleles ($s = 1, \ldots, c$, $i = 1, \ldots, r$). To assess whether the allele frequencies at a given locus are the same among these populations, we test the homogeneity hypothesis

$$H_0: p_{1,i} = \ldots = p_{c,i}, (i = 1, \ldots, r) .$$

Let $(ij, kl)^s$ be the simple random sample of mating genotypes of size n_s from the population s. Samples from different population are independent, and m_{si} is the frequency of allele i in the sample $(ij, kl)^s$. Then $p_{s,i}$ can be estimated by $\hat{p}_{s,i} = m_{si}/4n_s$, and the ordinary Pearson homogeneity test statistic for H_0 would be given by $X^2 = \sum_{s=1}^{c} \sum_{i=1}^{r} (m_{si} - 4n_s\hat{p}_{.i})^2/4n_s\hat{p}_{.i}$, where $\hat{p}_{.i} = \sum_{s=1}^{c} m_{si}/4\sum_{s=1}^{c} n_s$. If the individual alleles were sampled independently, X^2 would follow asymptotically the χ^2 distribution with $(c - 1)(r - 1)$ df under H_0. However, the alleles that were analyzed were cluster samples, so the deviation from random mating and from Hardy-Weinberg equilibrium among the mating-pair genotypes can severely affect the distribution of X^2 (see the description of the simulation study, below). To get around this problem, we offer a *corrected* homogeneity test statistic.

Change the form of X^2 to $X^2 = 4\sum_{s=1}^{c} n_s \sum_{i=1}^{r} (\hat{p}_{s,i} - \hat{p}_{.i})^2/\hat{p}_{.i}$. Under simple random sampling, an estimate of the variance of $\hat{p}_{s,i}$ would be $\hat{p}_{s,i}(1 - \hat{p}_{s,i})/4n_s$, but the clustering changes the variances and the associated covariances. Define $\hat{\mathbf{p}}^s = (\hat{p}_{s,1}, \ldots, \hat{p}_{s,r})$; we derive the correct covariance matrix $V(\hat{\mathbf{p}}^s)$ that reflects the clustering, and then "corrects" X^2 on the basis of these $V(\hat{\mathbf{p}}^s)$, $(s = 1, \ldots, c)$. Let $n^s_{ij,kl}$ be the observed count of mating-pair genotypes $(ij, kl)^s$ and $p^s_{ij,kl}$ be the probability of observing mating-pair genotype $(ij, kl)^s$. We also define the following vectors of probabilities and frequencies:

$$\mathbf{q}^s = (p^s_{11,11}, p^s_{11,12}, \ldots, p^s_{11,rr}, p^s_{12,11}, \ldots, p^s_{12,rr}, \ldots, p^s_{rr,11}, \ldots, p^s_{rr,rr}),$$

and

$$\mathbf{n}^s = (n^s_{11,11}, n^s_{11,12}, \ldots, n^s_{11,rr}, n^s_{12,11}, \ldots, n^s_{12,rr}, \ldots, n^s_{rr,11}, \ldots, n^s_{rr,rr}).$$

The vectors $\mathbf{q}^s$ and $\mathbf{n}^s$ are of length $H = r^2(r+1)^2/4$. For simplicity, we will use $h = 1, \ldots, H$ as the index for these vectors and use $g(h) = (ij, kl)$ to indicate the mapping from the index h to (ij, kl).

Under the assumption that observed mating genotypes $(ij, kl)^s$ are simple random samples from the target population s, the covariance matrix $V(\mathbf{n}^s)$ of $\mathbf{n}^s$ has entries $v^s_{hh'} = nq^s_h(1 - q^s_h)$, $(h = h')$, and $-nq^s_h q^s_{h'}$, $(h \neq h')$. An estimate of $\hat{V}(\mathbf{n}^s)$ can be obtained by replacing $\mathbf{q}^s$ with $\hat{\mathbf{q}}^s = \mathbf{n}^s/n_s$. There is a linear relationship between the marginal totals $m_{s,i}$ and $\mathbf{n}^s$, i.e., $m_{s,i} = T_i\mathbf{n}^s$, where $T_i = (t_{i1}, \ldots, t_{iH})$, with t_{ih} being the number of alleles i in $g(h)$, and $i = 1, \ldots, r$, and $h = 1, \ldots, H$. Since $\hat{p}_{s,i} = m_{s,i}/4n_s$, $(\hat{\mathbf{p}}^s)^t = T^s\mathbf{n}^s$, where T^s is an $r \times H$ matrix whose ith row is $T_i/4n_s$. Therefore, the covariance matrix of $V(\hat{\mathbf{p}}^s)$ can be estimated by $\hat{V}(\hat{\mathbf{p}}^s) = T^s\hat{V}(\mathbf{n}^s)(T^s)^t$.

The natural estimate of the standard error of $\hat{p}_{s,i}$ is v^s_{ii}, the diagonal element of $\hat{V}(\hat{\mathbf{p}}^s)$. We propose to "correct" the X^2 by employing a method commonly used in sample survey literature (see Fellegi 1980, Rao and Scott 1981, and also Jin et al. [in press] for similar approaches). The correction factor for cell s, i is $b_{s,i} = 4n_s v^s_{ii}/[\hat{p}_{s,i}(1 - \hat{p}_{s,i})]$, and the overall correction factor is $\bar{b} = \sum_{s=1}^{c}\sum_{i=1}^{r} b_{s,i}/rc$. The corrected homogeneity test statistic is then defined as $X^2_{CH} = X^2/\bar{b}$.

Bootstrap Approach

An alternative approach to the distributional problem of the homogeneity test is the bootstrap method. Following the guidelines for nonparametric hypothesis testing (Hall and Wilson 1991), we estimate the empirical distribution of the homogeneity test statistic under the null hypothesis H_0. To do this, we create a population of mating-pair genotypes by pooling the samples $(ij, kl)^s$, $(s = 1, \ldots, c)$, and resampling c groups of mating couples with sizes n_s, $(s = 1, \ldots, c)$. A bootstrap homogeneity test statistic can then be calculated, and after B replications, a bootstrap estimate of the distribution of the homogeneity test statistic can be obtained.

A population of mating-pair genotypes is properly described by the mating-pair probabilities $(p_{ij,kl})$: the marginal allele frequencies do not fully specify the population. Thus the resampling can not be done assuming only the null hypothesis of homogeneity of the allele frequencies. Assumptions or information concerning mating-pair probabilities must be provided in order to perform the resampling. The method described above, in which the four observed mating-pair sets are pooled, is one relatively simple approach, but it imposes a strong requirement on the samples, namely, that there is homogeneity of the mating pair frequencies among the three patient groups and one normal group. However, our empirical study (see below) suggests that heterogeneity in the mating-pair frequencies has little effect on the null bootstrap distribution of the homogeneity test statistic. This interesting fact deserves further theoretical investigation.

Simulation Study

In order to evaluate the performance of these tests, we conducted a small simulation study. The mating pair samples (ij, kl) in the simulation were generated from the following probabilities:

$$p_{ij,kl;\delta_{HW},\delta_R} = \begin{cases} (1 - \delta_R)p^2_{ij,\delta_{HW}} + \delta_R p_{ij,\delta_{HW}} & \text{if } i = k \text{ and } j = l; \\ (1 - \delta_R)p_{ij,\delta_{HW}}p_{kl,\delta_{HW}} & \text{otherwise,} \end{cases}$$

where

$$p_{ij,\delta_{HW}} = \begin{cases} p_i^2 + \delta_{HW}p_i(1 - p_i) & \text{if } i = j; \\ 2p_ip_j(1 - \delta_{HW}) & \text{if } i \neq j. \end{cases}$$

Here, δ_{HW} is a parameter representing the deviation from Hardy-Weinberg proportions, δ_R is a parameter representing deviation from random mating, and (p_i) is the allele frequency. A positive value of δ_{HW} indicates a deficiency of heterozygotes compared to Hardy-Weinberg proportions; see Thompson (1986) for further discussion of this model. Similarly, a positive value of δ_R indicates a deficiency of mating couples with different genotypes, that is, an excess of mating couples with similar genotypes compared to the mating-pair genotypes obtained under random mating. A reference population of mating-pair couples was taken with $(p_{ij,kl;0,0})$, and a deviant population was taken with probabilities $(p_{ij,kl;.3,.3})$. Both the random-mating assumption and the Hardy-Weinberg equilibrium hold for the reference population but not for the deviant population.

In our simulation, we generated four groups of mating pair genotypes at a locus with 10 alleles, with a sample size of 100 for each group. The allele frequencies under H_0 are assumed to be equal in all groups; that is $p_{s,i} = 1/10(s = 1, \ldots, 4; i = 1, \ldots, 10)$.

Comparison of sizes of homogeneity, bootstrap, and corrected homogeneity tests.—To compare the uncorrected homoge-

Table A1

Number of Times, of 1,000 Simulations, that the Uncorrected, Bootstrap, and Corrected Homogeneity Test Statistics Exceeded the Critical Value Corresponding to a Nominal Significance Level of .05.

Test	No. of Deviant Populations				
	0	1	2	3	4
Uncorrected Homogeneity	51	171	306	491	628
Bootstrap Homogeneity	52	62	53	51	40
Corrected Homogeneity	52	72	60	59	44

neity, bootstrap, and corrected homogeneity tests, we simulated five comparisons in which the four groups of mating couples were generated from $4 - i$ reference populations and i deviant populations, where $i = 0, \ldots, 4$ indicates the comparison conducted. The cases in which $i = 0$ and 4 represent two extreme situations: all four groups come from the reference population, or all four groups come from the deviant population. Although the mating-pair probabilities are different for the two populations, the allele frequencies are identical in every case. The uncorrected and corrected homogeneity test statistics were calculated for the four simulated groups of mating genotypes. The procedure was repeated 999 more times, and the sizes of the tests were estimated as described earlier, using the 95% point of χ^2_{27} as the critical value. To determine the size of the bootstrap method, we resampled with $B = 500$. The bootstrap test rejects H_0 if the observed homogeneity test statistic is greater than the 95th percentile of the order statistics of the bootstrap homogeneity test statistics. The proportion of the rejections in 1,000 runs gives the size of the bootstrap method. The results are shown in table A1.

The sizes for the uncorrected homogeneity test increased as the number of deviant populations increased. The sizes for the bootstrap and the corrected homogeneity tests, however, remained around the nominal value of .05, regardless of the number of deviant populations in the comparison. While the populations with $i = 1, 2, 3$ are heterogeneous in regard to mating-pair genotypes, the bootstrap method based on the resampling from a pooled sample still provides an adequate approximation to the null distribution of the test statistic.

Comparison of powers of bootstrap and corrected homogeneity tests.—Here, we present a power study on the comparison of the bootstrap and the corrected homogeneity test statistics. The alternative allele frequencies ($p^A_{s,i}$) deviate from H_0: $p_{s,i} = 1/10$, $(s = 1, \ldots, 4; i = 1, \ldots, 10)$ in such a way that $\sqrt{\Sigma_{s,i}\,(p^A_{s,i} - p_{s,i})^2/40}$ is 0, 5, 7.5, 10, and 15 percent of $p_{s,i} = 1/10$, consecutively. For each set of $(p^A_{s,i})$, we formulate three sets of mating-pair probabilities: all four reference populations, two reference and two deviant populations with $\delta_{HW} = .3$, $\delta_R = .3$, and all four deviant populations with $\delta_{HW} = .3$, $\delta_R = .3$. Note that δ_{HW}, δ_R are nuisance parameters. The powers of the tests were calculated in the same way as the sizes in the section above: there were 1,000 replications, and for each one of the runs, the bootstrap resampling was done with $B = 500$. The results are shown in table A2. Again, we see that the bootstrap and corrected homoge-

Table A2

Number of Times, of 1,000 Simulations, That the Bootstrap and Corrected Homogeneity Test Statistics Exceeded the Critical Value Corresponding to a Nominal Significance Level of .05.

Test	No. of Deviant Populations	Deviation from H_0				
		0%	5%	7.5%	10%	15%
Bootstrap homogeneity	0	52	131	289	569	954
	2	53	119	229	420	853
	4	40	94	189	336	726
Corrected homogeneity	0	52	134	302	581	962
	2	60	120	239	433	859
	4	44	93	200	348	733

neity tests behave very similarly. The deviation from Hardy-Weinberg equilibrium and random mating has a considerable impact on the powers of the tests.

Appendix B

Generation of Mating Populations for the Simulation Study

Let p_{ij} be the probability of parental genotype ij and $p_{ij,kl}$ be the probability of a male with genotype ij mating with a female with genotype kl. The probabilities of sharing two, one, and no alleles in the population will be

$$P_2 = \sum_{i=k,j=l} p_{ij,kl};$$

$$P_1 = \sum_{\substack{\{i,j\}\cap\{k,l\}=\{i\} \text{ or } \{j\},\\ i\neq j \text{ or } k\neq l}} p_{ij,kl};$$

and

$$P_0 = \sum_{\{i,j\}\cap\{k,l\}=\phi} p_{ij,kl},$$

respectively.

Using estimated genotype probability p_{ij} at the HLA-DR (or HLA-DQ) locus from the pooled RSA (or IVF-F) and normal couples, we calculated mating probabilities for the normal population under the assumption of random mating, $p^0_{ij,kl} = p_{ij}p_{kl}$ and then the associated sharing probabilities. The results are $P_2^0 = .05$, $P_1^0 = .38$, and $P_0^0 = .57$ at HLA-DR and $P_2^0 = .23$, $P_1^0 = .44$, and $P_0^0 = .33$ at HLA-DQ, respectively. Since P_2 and P_1 increase and P_0 decreases among RSA patients, we formulated nonrandom mating probabilities $p'_{ij,kl}$ such that $P'_2 > .05$, $P'_1 > .38$, and $P'_0 < .57$, respectively, at HLA-DR. For IVF-F patients, P_2 increases and P_1 and P_0 decrease, and we formulated $p'_{ij,kl}$ such that $P'_2 > .23$, $P'_1 < .44$, and $P'_0 < .33$, respectively, at HLA-DQ. It was also necessary for $p'_{ij,kl}$ to be subject to the following constraints: $p'_{ij,kl} \geq 0$, $\Sigma\, p'_{ij,kl} = 1$, $\Sigma_{i,j}\, p'_{ij,kl} = p_{kl}$, and $\Sigma_{k,l}\, p'_{ij,kl} = p_{ij}$. An algorithm was developed to calculate the values of $p'_{ij,kl}$ from a given set of (p_{ij}) leading to the designed probabilities P'_2, P'_1, and P'_0.

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