HLA Genotypic Study of Insulin-dependent Diabetes

The Excess of DR3/DR4 Heterozygotes Allows Rejection of the Recessive Hypothesis

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SUMMARY

The genetics of insulin-dependent diabetes mellitus (IDDM) is currently an area of controversy, with some investigators proposing heterogeneity within the HLA region and even the existence of non-HLA-linked susceptibility genes, and others maintaining that a simple autosomal recessive gene linked to HLA with reduced penetrance is an adequate explanation. To resolve this latter question, we report here a simple method of testing whether a single HLA-linked susceptibility gene is inherited in a recessive fashion when it is associated with two different HLA alleles, as is the case for IDDM. It is shown that if the number of DR3/DR4 heterozygotes in a diabetic population exceeds the combined sum of DR3/3 and DR4/4 homozygotes in that same diabetic population, then a recessive mode of inheritance can be rejected. The advantages of the method are that it does not depend on ratios, as do relative risk calculations, nor does it depend on control data, but is based only on studies of the diabetics themselves. With data on 193 genotyped IDDM patients, we can clearly reject the recessive mode of inheritance, since the number of heterozygotes is 68 compared with a maximum of 22 homozygotes ($P < 10^{-4}$). Eight other published studies are in concordance with these results. Therefore, a nonparametric test, independent of the significance of any individual study, rejects equality of heterozygotes and homozygotes ($P < 0.002$) and rejects the simple recessive mode of inheritance as a direct consequence. We conclude that more complex modes of inheritance of HLA-linked IDDM susceptibility, such as two different diabetogenic alleles or multiple loci, must be entertained. **DIABETES *32*:169–174, February 1983.**

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The mode (s) of inheritance of insulin-dependent diabetes mellitus (IDDM, type I) remains the subject of active debate, with proponents for a simple mode of inheritance versus those who have argued for heterogeneity and for more complex modes of inheritance.[1] If we restrict out attention to the HLA region, we and others have argued for the existence of at least two different susceptibility genes linked to the HLA region.[1–7] The principal lines of evidence for this hypothesis are of two general categories. The first is the different clinical and immunologic features of the B8-DR3 and B15-DR4 diabetics in population studies.[1,4–7] The second is the increased relative risk of DR3/DR4 (or B8/B15) compound heterozygotes, compared with that of either homozygote, i.e., that is compared with individuals with either DR3/DR3 or DR4/DR4.[2,4,8] As regards this increased relative risk, it has been claimed that this observation is both consistent and inconsistent with a simple autosomal recessive HLA-linked susceptibility gene.[8–10] Consequently, proponents of a single autosomal recessive mode of inheritance for an HLA-linked diabetes susceptibility gene conclude that since, in their opinion, the above data do not reject a simple mode of inheritance, the recessive model should be accepted.[11]

There are a number of questions or problems with the arguments based on relative risks or odd ratios. First, the statistics of ratios have a number of complexities, e.g., complicated formulations for their variances and standard errors. Second, the concept of relative risk was developed to compare the frequency of a single trait in a disease population with the frequency of that trait in the rest of the population, the control group.[12] Yet IDDM is associated with two or more different alleles at the various HLA loci, e.g., DR3 and DR4 at the HLA DR locus. As a result, there is disagreement on what should constitute the appropriate denominator. Should the denominator be the remainder of the diabetics, i.e., all

diabetics except those with the antigen or antigens whose risk is being calculated?[10] In that case the denominator changes with each relative risk calculation. Or, should the denominator be those diabetics without any of the high risk alleles? In that case the denominator is constant.[8] If we accept the latter method, although we have the benefit of a constant denominator, the number of such diabetics constitutes a relatively small proportion of the IDDM population, especially when dealing with HLA DR alleles, and thus the ratios are subject to potentially large fluctuations based on possible variation in only a few subjects. Third, some of the attempts to characterize the possible range of relative risks for the compound heterozygotes have made certain assumptions regarding the degree of linkage disequilibrium between the diabetes gene and the HLA-associated allele,[10] and these may or may not be accurate.[1,13] Fourth, the relative risk calculation requires accurate control data, and while this is available for phenotypic frequencies, it is often not as adequate for genotypic frequencies. Yet it is the latter information, genotypes rather than phenotypes, that is crucial to any calculations or conclusions regarding the relative risks of homozygotes.

To obviate these problems, a simpler method to test whether the observed population data are consistent or inconsistent with an HLA-linked autosomal recessive mode of inheritance for IDDM susceptibility is needed. We report here the development of such a method, applicable to the case where two different HLA alleles are associated with a disease. Its major advantage is that it depends only on the frequency of the alleles, and only needs this information from the diabetic population itself. Thus, it does not depend on ratios, nor on studies in controls. It is most powerful if used with genotypes, but can be applied to phenotypic data as well. Applying this method to our own family study of IDDM, we are able to clearly reject the recessive mode of inheritance because of a significant excess of heterozygotes. Data from eight other published studies provide consistent results, and permit an additional nonparametric rejection of the recessive hypothesis.

METHODS

Methodology. Let us assume the existence of a single autosomal gene, whose locus is tightly linked to the HLA region, and which in the homozygous state provides susceptibility to IDDM. (Tight linkage would be required by necessity to explain the observed population associations.[14] In addition, there is increasing realization that the reports of loose linkage in some of the early analyses probably reflected limitations in existing methodology available at that time.) We assume that only the homozygous state is susceptible to IDDM, i.e., a recessive pattern of inheritance. We also assume that the HLA serologic genes themselves play no role in disease susceptibility, that all diabetes susceptibility is due to the allele at the tightly linked diabetes locus. However, this latter assumption is not necessary for the results that follow; they are true even if the DR alleles themselves are the diabetes susceptibility genes. Note, we are discussing here disease susceptibility; this homozygous state is assumed to be necessary for disease susceptibility but not necessarily sufficient. This accords with the recognized reduced penetrance of the IDDM genotype, as documented by the 50% or less concordance in monozygotic twins.[15] Whether this reduced penetrance is due solely to environmental factors, or whether other genetic factors play a role as well, is irrelevant to our argument. Thus, this formulation for the mode of inheritance of the HLA-linked susceptibility gene holds true even if other non-HLA genes contribute to IDDM genetic susceptibility.

Let us denote the diabetes susceptibility allele as dm. There are, therefore, for the purposes of this discussion, three HLA "haplotypes" that contain the dm allele, dm-DR3, dm-DR4, and dm-DRX, where X is any HLA DR allele other than 3 or 4. Let the frequency of these haplotypes among the diabetic population be q, r, and s, respectively.

Since each individual has two haplotypes, and in our recessive model diabetics must have two haplotypes containing dm, the dm genotypes and their frequencies among the diabetic population will be:

Genotype	Frequency among diabetics	
dm-DR3/dm-DR3	q^2	
dm-DR3/dm-DR4	$2qr$	
dm-DR4/dm-DR4	r^2	
dm-DR3/dm-DRX	$2qs$	(1)
dm-DR4/dm-DRX	$2rs$	
dm-DRX/dm-DRX	s^2	

(Parts of this formulation are similar to Svejgaard and Ryder,[8] and to Greenberg et al.[16] In the former case, the emphasis is on the resultant odds ratios, and in the latter case the emphasis is on estimation of the HLA genotype and phenotype frequencies of affected individuals given different genetic models.) Note that these genotype frequencies are simply the product of $(q + r + s) \cdot (q + r + s)$. If there is any hidden population stratification, this would increase the number of homozygotes, and make the inequalities to follow more pronounced. Also note there is no assumption regarding the magnitude of the linkage disequilibrium with the proposed dm allele of the proposed diabetes locus and the various DR alleles of the DR locus.

Since we cannot type for the diabetic allele, but only the HLA DR alleles, the first group will be homozygous DR3, the second DR3/DR4, the third homozygous DR4, etc. Note that these are the only possible genotypes of the diabetic population, as long as we assume an autosomal recessive mode of inheritance. For what follows, we can ignore the last three genotypes, and concentrate only on those of interest, the first three, that is the DR3/DR3, DR3/DR4, and DR4/DR4 diabetics, respectively.

Now, let us examine the quantity $q - r$. The algebraic inequality

$$(q - r)^2 \geq 0 \qquad (2)$$

is true by definition, since the square of any number, positive or negative, must be greater than or equal to zero. And, $(q - r)^2 = 0$ only if $q = r$. Now let us expand equation 2.

$$\begin{aligned} (q - r)^2 &\geq 0 \\ q^2 - 2qr + r^2 &\geq 0 \\ q^2 + r^2 &\geq 2qr \end{aligned} \qquad (3)$$

(This result is not particularly novel, and is well known from basic algebra. It is derived here since it forms the basis of the subsequent nonparametric test.)

But from equation 1: q^2 = the number of DR3/DR3 IDDM diabetics; r^2 = the number of DR4/DR4 IDDM diabetics; and 2qr = the number of DR3/DR4 diabetics.

Therefore, equivalently

$$\Sigma(\text{DR3/DR3's} + \text{DR4/DR4's}) \geq \Sigma\text{DR3/DR4's} \qquad (4)$$

Thus, if the recessive mode of inheritance is true, the number of homozygotes, i.e., the sum of those who are DR3/DR3 plus those who are DR4/DR4, must always be greater or equal to the number of those who are DR3/DR4 heterozygotes. The only time they can even be equal is if q = r, and most of the time the homozygotes should be in excess. Consequently, if the number of heterozygotes is greater than the sum of homozygotes, the recessive model must be rejected.

Statistics. Our own data as well as the combined data of other studies were examined by cell frequencies and the X^2 test of significance with Yates' continuity correction.[17] In each case, the null hypothesis is that the combined number of homozygotes (the sum of those who are DR3/DR3 and those whose are DR4/DR4) is greater or equal to the number of DR3/DR4 heterozygotes. Thus, if that hypothesis is rejected, then so is the recessive hypothesis. In addition, data from eight other studies will be examined by a nonparametric sign test based on the binomial expansion. That is, we will test whether the direction of results of all studies is consistent or inconsistent with the recessive hypothesis, regardless of the significance of an individual study, with the significance a function of the probabilities of the binomial distribution. For example, if two of two studies rejected the recessive hypothesis, the probability would be $(1/2)^2 = 1/4$. If four of five rejected the recessive hypothesis, the probability would be $(1/2)^5 + 5(1/2)^5 = 6/32$.

Data. In the course of an ongoing study of the genetics and etiology of IDDM, HLA A, B, C, and DR typing was performed on 193 diabetics in 108 families, each ascertained through the presence of at least one insulin-dependent diabetic. Three-hundred eighty-eight additional family members were also studied to establish haplotypes and genotypes. HLA-typing was performed by standard microcytotoxicity methods.[18] Specifically, with regard to this report, individuals were typed for nine DR alleles: DR1, -2, -3, -4, -5, -W6, -63, -7, and -W8. Further details of the mode of ascertainment and clinical and immunologic characteristics of the first 77 families are given in Anderson et al.[19,20] All studies were approved by the Human Subjects Protection Committee of the Research and Education Institute of Harbor-UCLA Medical Center.

We have purposely included all diabetic individuals in our sample, even when multiple affected individuals occurred in a single family. If the recessive model is true, sampling from multiple families will not be all that different from sampling from the population at large. This is especially true since these individuals were not ascertained via their HLA types. Thus, this should not bias any conclusions, and increasing the sample size does increase the statistical power of the results. However, we also report the data on one index case per family, with identical conclusions.

In addition, we compared the results of eight other published studies.[21–28] The criterion for inclusion was the reporting of HLA D or DR typing in sufficient detail that the number of diabetics who were DR3/DR4, DR3 alone, and DR4 alone, could be inferred.

RESULTS

The HLA DR phenotype and genotype data on the 193 diabetics studied by us are given in Table 1. Of the 193 diabetics, 175 or 90.7% typed positive for one or more of the high risk HLA DR alleles, DR3 and/or DR4. Among these 193 individuals there were 386 HLA haplotypes. Of these, 100 (28.5%) contained DR3, 145 (37.6%) had DR4, 71 (18.4%) had some other recognized DR allele, and 50 (13.0%) were blank, i.e., null alleles. In some 10 (2.6%) of the haplotypes, the DR allele was indeterminate, either due to typing difficulties, or because not enough relatives were studied to enable a firm decision to be made between homozygosity or the existence of a null allele.

Seventeen individuals typed for HLA DR3 alone. However, the family genotype studies revealed that only six of these individuals were unequivocally homozygous DR3/DR3. Of the remaining 11, 3 were DR3/DR indeterminate, and 8 were DR3/DR blank. The former may or may not have been DR3/DR3 homozygous, the latter are definitely not. Therefore, the largest possible number of DR3/DR3 homozygotes was nine, and it may have been as small as six. Forty-seven percent (8/17) and possibly as many as 65% (11/17) of individuals who by phenotype alone would have been designated DR3 homozygotes, were shown not to be so by these family studies. Similarly, 37 individuals typed for HLA DR4 alone, but the genotype studies revealed that only six were definitely homozygous DR4/DR4, 7 were DR4/DR indeterminate, and 24 were DR4/DR blank. The largest possible number of DR4/DR4 homozygotes was 13, and it may have been as small as 6. Sixty-five percent (24/37), and possibly as many as 84% (31/37), of those typing for DR4 were shown not to be homozygous.

Thus, of 54 diabetics who appeared to be homozygous

TABLE 1
HLA DR genotypes in IDDM: current study

Phenotypes	Genotypes	Number of* diabetics	Index† diabetics
DR3	DR3/DR blank‡	8	4
DR3	DR3/DR indeterminate§	3	1
DR3	DR3/DR3	6	5
DR3, DR4	DR3/DR4	68	39
DR4	DR4/DR4	6	5
DR4	DR4/DR indeterminate	7	4
DR4	DR4/DR blank	24	13
DR3/DRX‖	DR3/DRX	19	9
DR4/DRX	DR4/DRX	34	18
Other¶	Other	18	10
Total		193	108

*All the diabetics in the study population.
†One diabetic per family.
‡DR blank denotes a null allele.
§DR indeterminate denotes that the DR allele on this haplotype was not fully clarified. Such individuals may or may not be homozygous.
‖DRX denotes any other DR allele except 3 and 4.
¶Other includes DRX/DR blank, DRX/DR indeterminate, DR blank/DR blank, DRX/DRX, etc.

3/3 or 4/4 by HLA phenotype, at most 22, and possibly as few as 12, were homozygous by family genotype studies. In sharp contrast, 68 of the diabetics were HLA DR3/DR4, both by phenotype and family studies. This excess of heterozygotes over the combined sum of homozygotes is highly significant (68 versus 22, $X^2 = 22.5$, 1 df, $P < 10^{-4}$). (Note: this is the least possible significance, since the number of homozygotes may be considerably less than 22, i.e., as few as 12, and the significance of the difference would increase accordingly.) Since this excess is in direct contrast to the results predicted by the recessive model, that model must be rejected. If we restrict ourselves to one index case per family, we still observe a significant excess of heterozygotes (39 versus 15, $X^2 = 9.8$, 1 df, $P < 0.005$).

Further evidence requiring us to reject the recessive model comes from an examination of all published studies of HLA D and DR typing in IDDM diabetics that provide data in enough detail that an upper bound to the number of homozygotes can be determined (Table 2). Two principal observations can be derived from Table 2. First, when the results of all studies are totaled, there is a highly significant excess of DR3/DR4 heterozygous over the combined sum of both homozygotes (254 versus 104, $X^2 = 55.2$, 1 df, $P < 10^{-8}$), leading us again to reject the recessive model. The actual excess (and hence significance) is almost certainly greater, since in many of these studies the individuals were not genotyped, only phenotyped. Thus, some of those typing for DR3 or DR4 alone were only "possible" homozygotes, and undoubtedly some have only one DR3 or only one DR4 allele and a null allele, as was found in a minimum of 59% (32/54) of our "possible" homozygotes. The second observation is that in each of these studies individually, the data are in the same direction, i.e., there is an excess of heterozygotes in each study. Thus, even if none of the studies individually had a significant excess of heterozygotes, the observation that all nine are in the opposite direction predicted by the recessive model would allow us to reject the recessive model by a nonparametric sign test [$(1/2)^9 = 1/512$; therefore, $P < 0.002$].

We did not include the latest review of data from the Danish group[29] for two reasons. First, the majority of the data was given in Christy et al.[21] Second, and more important, the data in Christy et al.[21] were genotype data, while the slightly larger data in Platz et al.[29] were phenotype data. For the interested reader, however, Platz et al. reported 151 diabetics studied by HLA D typing of which 38 were D3/4, 21 were D3 alone, and 28 were D4 alone (and, therefore, a total of 49 possible homozygotes). They also report a subset of 93 diabetics studied by HLA DR typing, 32 of which were DR3/DR4, 9 DR3 alone, and 21 DR4 alone (and, therefore, a total of 30 possible homozygotes). If we include each of these results as an independent study (we would then have to include the D and DR results of Barbosa et al.[28] separately as well), there is still as great a significant excess of DR3/DR4 heterozygotes, and 11 of 12 "studies" would have results discordant with the recessive hypothesis [the latter would have a significance of $(1/2)^{12} + 12(1/2)^{12} = 13/4096$ by the binomial expansion, i.e., $P < 0.003$ by the nonparametric test].

We come to conclusions similar to those of Svegaard and Ryder,[8] i.e., that the excess of DR3/DR4 heterozygotes is inconsistent with the recessive model. They used the methods of odds ratio for relative risk and thus kept a constant denominator. In addition, they applied their analysis to only one data set,[27] and while they felt the data were against the recessive hypothesis, they did not feel they could fully reject it. But the method reported here does not require ratios or control data. As a result, our method requires both fewer variables and less assumptions.

It should be noted that the results that are closest to being compatible with the recessive model are the report of Suciu-Foca et al.[23] These investigators remain among the most active proponents of simple recessive inheritance.[11] How-

TABLE 2
HLA D or DR in IDDM: summary of studies

				Homozygotes				
				3/3		4/4		Total§ homozygotes (N)
	D or DR	N*	3/4 (N)	Def.†	Pos.‡	Def.	Pos.	
Christy et al. (1979)[21]	D	98	23	5		5		10
Farid et al. (1979)[22]	DR	40	10	1	1		2	4
Suciu-Foca et al. (1979)[23]	DR	57	14	9		4		13
Sachs et al. (1980)[24]	D	58	29		9		0	9
Deschamps et al. (1980)[25]	DR	57	17	1		1		2
Serjeantson et al. (1980)[26]	DR	23	12	0		3		3
Svejgaard et al. (1980)[27]	DR	112	46	4	8	4	7	23
Barbosa et al. (1982)[28]	DR	134	35	7		11		18
	(D	81	13	0		1		1)¶
Current study	DR	193	68	6	3	6	7	22
Total			254					104

*Number of diabetics.
†Definitely homozygous.
‡Possible homozygotes; genotypes were not determined in study or not capable of being inferred from published report.
§Total possible number of homozygotes: the sum of columns 4, 5, 6, and 7. This is the maximum number of homozygotes, and the actual number is probably less.
¶This row omitted from totals, since included in row above.

ever, even in their data, the heterozygote versus homozygote results are in the opposite direction of those predicted by the recessive model.

DISCUSSION

We have shown in this report that the autosomal recessive model of an IDDM susceptibility gene linked to HLA necessarily predicts that the combined number of DR3/DR3 and DR4/DR4 homozygotes must at least equal, and in most cases exceed, the number of DR3/DR4 homozygotes. We have then demonstrated that in our data reported here, and in practically all studies reported in the literature, the results are in exactly the opposite direction. Instead of an excess of homozygotes, which the recessive model requires, there is a large, consistent, and significant excess of heterozygotes. This is true both in the study reported here, and in all the reported data. It is true whether the data are pooled or examined nonparametrically by individual study. We feel the only conclusion that can be drawn is that the autosomal recessive model is firmly and unequivocally rejected.

To put these results in perspective, it may be useful to briefly review the evolving knowledge regarding the genetics of IDDM. First, familial aggregation of this disorder or group of disorders is well recognized, but no definitive conclusions regarding mode of inheritance could be decided from these observations alone.[30] The discovery of the HLA antigen population associations, first with B locus antigens and then stronger with D or DR locus antigens, and the observation of disturbed HLA haplotype sharing in families suggesting linkage, led to the hope that the mode(s) of inheritance might quickly be resolved.[1] Instead, every mode of inheritance has developed its champions, from single diabetogenic allele models (i.e., two alleles, one normal, and one diabetogenic)—autosomal dominant, autosomal recessive, or the more general gene dosage model—to recessive and dominant forms, to three-allele models (two diabetogenic alleles), and to two-locus models. Based on currently available evidence and the data presented in this paper, it would appear that all single diabetogenic allele models can be rejected.

Where does this lead? If all the simpler genetic models have been rejected, we must proceed to more complex ones. Early on, we and others were convinced by various lines of clinical and immunologic evidence that there was phenotypic heterogeneity within IDDM, and that this occurred on a genetic basis.[2–7] The principal lines of evidence are (1) the excess of DR3/4 (or B8/B15) heterozygotes among the diabetics[2,4,6] and (2) the different immunologic and clinical features of the B8-DR3 and B15-DR4 diabetics, with B8-DR3 being characterized by persistent pancreatic autoimmunity and B15-DR4 with higher insulin antibody levels.[1,4,5,7] These observations suggested that there were two different HLA-linked genes that predispose to IDDM, and that when they occurred together the susceptibility to IDDM was greatly increased. Additional support for this concept of heterogeneity has come from the ability of a mathematical genetic model based on this heterogeneity concept to make accurate predictions confirmed by subsequent studies.[13] This has included the prediction that autoimmunity would be less in U.S. IDDM blacks than in U.S. IDDM Caucasians, and that the relative increase in DR4 would be greater in U.S. blacks with IDDM.[31–33] It also anticipated the reported linkage heterogeneity within IDDM.[13,34,35]

There is evidence for further complexity to the problem of IDDM genetics.[1] First, there is evidence for a third HLA-linked diabetogenic allele, or high risk haplotype, one containing BfF1, B18, and DR3, having a young age of onset and associated with a predisposition to form lymphocytotoxic antibodies.[36,37] Second, there is accumulating evidence for other, non-HLA-linked genetic loci predisposing to IDDM. This includes both theoretical mathematical considerations,[38,39] theoretical biologic considerations,[1] and direct, albeit tentative, evidence by either linkage or association studies for genes at or near the linked Kidd blood group and Km (immunoglobulin light chain) loci on chromosome 2,[40–42] the acetylator locus,[43] and the Gm (immunoglobulin heavy chain) locus on chromosome 14.[41,44]

Thus, the still imperfect picture emerging regarding the genetics of IDDM appears as follows. It appears that there are at least two, and possibly three, diabetes susceptibility genes tightly linked to the HLA complex on chromosome 6. These are preferentially associated with the B8-DR3, B15-DR4, and BfF1-B18 haplotypes, respectively. At least two of the diabetogenic genes, those preferentially associated with B8-DR3 and B15-DR4, act synergistically when they occur together, increasing the susceptibility to IDDM. While the HLA-linked diabetes genes on chromosome 6 probably provide the majority of disease susceptibility to IDDM, there is tentative evidence for the effect of an additional gene or genes at other loci not on chromosome 6, namely the Kidd blood group and Km immunoglobulin loci on chromosome 2, the Gm locus on chromosome 14, and the acetylator locus. It seems clear that the genetics of IDDM remains an area of great complexity. This complexity is gradually being resolved by approaches that emphasize the many differences between patients with the IDDM phenotype.

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