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Human leukocyte antigen matching and fetal loss: results of a 10 year prospective study

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The role that maternal and fetal human leukocyte antigen (HLA) genes play in pregnancy is unknown, but it has been suggested that fetuses whose HLA alleles do not differ from maternal alleles (i.e. histocompatible fetuses) are more likely to be aborted than fetuses with HLA alleles that differ from maternal alleles (i.e. histoincompatible fetuses). To elucidate the role of HLA compatibility in pregnancy, we tested the hypothesis that couples who match for HLA alleles or haplotypes would have reduced fertility because only these couples could produce histocompatible fetuses. We conducted a 10 year prospective study of HLA matching and pregnancy outcome in 111 Hutterite couples, providing information on 251 pregnancies. A logistic regression analysis was performed to determine the effects of HLA matching at HLA regions and loci on pregnancy outcome (fetal loss versus delivery). Significantly increased fetal loss rates were observed among couples matching for the entire 16-locus haplotype ($P = 0.002$). Among the individual loci, loss rates were increased among couples matching for *HLA-B* ($P = 0.019$), *HLA-C* ($P = 0.033$) and the complement component, *C4* ($P = 0.043$). We interpret these results as evidence that matching for the entire 16-locus haplotype and/or alleles at an *HLA-B*-linked locus confers significant risk for fetal loss.

Key words: fetal loss/HLA/Hutterites/maternal–fetal compatibility

Introduction

The survival of the fetal allograft in mammalian pregnancy remains a paradox (Medawar, 1953), although it is clear that a state of mutual tolerance exists between mother and fetus in normal pregnancy. Indeed, the transport of fetal cells into the maternal circulation and their long-term survival (Bianchi *et al.*, 1996) and of maternal cells into fetal tissues (Socie *et al.*, 1994; Piotrowski and Croy, 1996; Bonney and Matzinger, 1997) attest to this state of tolerance. In addition, the lack of responsiveness of adult B cells to non-inherited maternal human leukocyte antigen (HLA) class I antigens (Claas *et al.*, 1988; Bean *et al.*, 1990) demonstrates that pregnancy itself has long-term effects on the immune repertoire of the fetus. Because the HLA genes are important in the rejection of foreign tissues, it is plausible that maternal and fetal HLA genes also play a role in modulating the induction of maternal tolerance in pregnancy.

The Hutterites are an inbred population of European descent that is characterized by both a limited number of HLA haplotypes and a high natural fertility rate (reviewed in Ober, 1995). The limited number of founding haplotypes increases the likelihood that a greater proportion of spouses will match for a haplotype compared with outbred couples. Consequently, maternal–fetal HLA compatibility (i.e. fetuses with a paternally derived HLA haplotype that does not differ from the non-transmitted maternal HLA haplotype) is more common in this population than in outbred populations. To test the hypothesis that maternal–fetal HLA compatibility is disadvantageous during pregnancy (Kirby, 1970; Beer and Billingham, 1976), we studied reproductive outcome in couples matching for and not matching for HLA alleles or haplotypes. In earlier studies, we observed increased intervals from marriage to each birth among Hutterite couples matching for HLA-A, HLA-B or HLA-DR antigens (Ober *et al.*, 1983, 1985, 1988). To determine whether the recognized fetal losses were associated with matching for HLA antigens or haplotypes, we initiated a prospective study of pregnancy outcome in Hutterites. The couples were typed for 11 new HLA region loci, in addition to the five HLA loci typed in previous studies. We present here the results of this 10 year prospective study.

Materials and methods

Subjects

Since 1986 we have been conducting a prospective study of pregnancy outcome in the Hutterites, a communal population of European ancestry that is characterized by a high natural fertility rate and limited use of contraception (reviewed in Ober, 1995). Details of our protocol have been described previously (Ober *et al.*, 1992). Briefly, all married women who were still in their reproductive years were recruited into our study during visits to 31 Hutterite colonies in South Dakota, USA. Participants were provided with a calendar diary and e.p.t.® Pregnancy Test kits (Warner-Lambert Company, Morris Plains, NJ, USA) and instructed to test for pregnancy 1 month after their last period, if menses had not begun. Dates of menses and results of all pregnancy tests were recorded in the diaries, which were collected yearly, along with a short questionnaire eliciting additional information on birth control use and nursing practices during the preceding year. Data were obtained for 251 pregnancies in 111 couples.

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Genetic typing

Serologically defined haplotypes (*HLA-A*, *HLA-C*, *HLA-B*, *HLA-DR*, *HLA-DQ*) have been described previously (Kostyu *et al.*, 1989). In this study, extended haplotypes were defined by alleles at 16 HLA region loci (Figure 1). Methods for genotyping individuals at the *HLA-G*, *HLA-E*, *HLA-C*, tumour necrosis factor a (TNFa), properdin factor B (*BF*), *C4A*, *C4B*, *HLA-DRB1*, HLA-*DQA1*, HLA-*DQB1*, *TAP1*, *LMP2*, *LMP7* and HLA-*DPB1* loci and for assigning alleles to the serologically defined haplotypes are described in Table I. Couples were classified as to whether they shared no alleles (or haplotypes or antigens) or one or more alleles (or haplotypes or antigens) at each of the following loci — *HLA-A*, *HLA-C*, *HLA-B*, TNFa, *C4A*, *C4B*, *HLA-DRB1*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DPB1* — and for each of the HLA regions. Class I region haplotypes included *HLA-G*, *HLA-A*, *HLA-E*, *HLA-C* and *HLA-B* alleles; class III region haplotypes included *TNFa*, *BF*, *C4A* and *C4B* alleles; and class II region haplotypes included *HLA-DRB1*, *HLA-DQA1*, *HLA-DQB1*, *LMP2*, *TAP1*, *LMP7* and *HLA-DPB1* alleles. The effects of sharing at loci with few alleles (*HLA-E*, *HLA-G*, *BF*, *LMP2*, *TAP1*, *LMP7*) were not examined individually, but these loci were included in the construction of haplotypes.

Fetal loss analysis

As in our previous study, we included in our analyses all fetal losses (spontaneous abortions and ectopic pregnancies) that occurred at <20 weeks gestation based on the dates of last menses (Ober *et al.*, 1992). The date of the loss was determined as either the first sign of bleeding, per vagina, preceding a spontaneous abortion or the date of diagnosis of an ectopic pregnancy or intrauterine demise, as determined by ultrasound examination. Analyses of pregnancy outcomes (fetal loss versus delivery) included all couples who achieved a pregnancy that was or could have been followed at least 20 weeks past the last menses for which genetic typing was complete.

The generalized estimating equation approach of Liang and Zeger (1986) was used to obtain SE for the logistic regression analyses of the effects of HLA matching, maternal age and wife's inbreeding on pregnancy outcome. [Previous studies have determined that gravidity, husband's inbreeding and the couple's kinship coefficients did not influence pregnancy outcome, so these covariates were not included in the model (unpublished data).] This method is preferable to standard logistic regression SE because it allows for repeated observations (multiple pregnancies) per couple (Hauck and Ober, 1991).

Analysis of surviving offspring

If couples matching for alleles or haplotypes had increased fetal loss rates, then certain fetal HLA genotypes should be associated with loss. To determine whether there was a selective loss of fetuses with specific genotypes, we examined the HLA types among the living children of couples matching for loci or haplotypes that showed significant effects on fetal loss rates. (It is not possible to collect

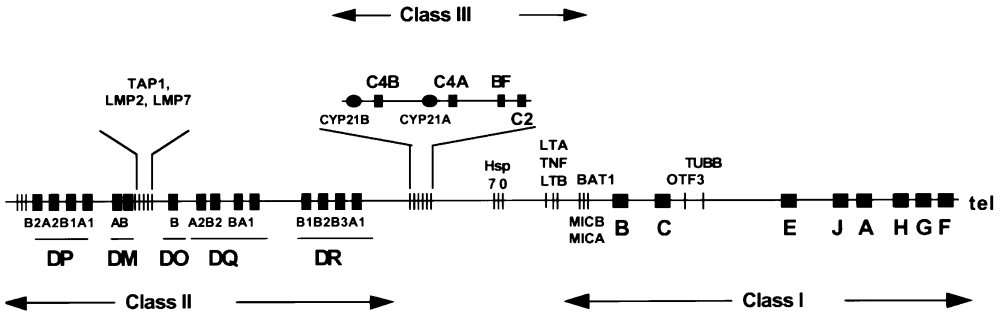

Figure 1. The human leukocyte antigen (HLA) region on chromosome 6p. Modified from Ober and van der Ven (1996). HLA loci are shown below the line and non-HLA loci are shown above the line. The number of antigens (*HLA-A* and *HLA-B*) or alleles (all other loci) present in the Hutterites are as follows: *HLA-G* = 3; *HLA-A* = 12; *HLA-E* = 2; *HLA-C* = 13; *HLA-B* = 16; *TNF*α = 11; *BF* = 3; *C4A/C4B* = 12; *DRB1* = 14; *DQA1* = 7; *DQB1* = 9; *LMP2* = 2; *TAP1* = 4; *LMP7* = 2; *DPB1* = 9; HLA haplotypes = 68.

Table I. Number of alleles and detection method used for 14 major histocompatibility complex loci in the Hutterites

Locus	No. of alleles	Detection method	Reference
G	3	Sequencing	Ober *et al.* (1996)
E	2	SSCP	Grimsley and Ober (1997)
C	13	SSOP with sequencing	Bunce *et al.* (1994), Bunce and Welsh (1994)
TNFa	11	Microsatellite	Udalova *et al.* (1993)
BF	3	EIP	Alper *et al.* (1972)
C4A-C4B	12	EIP	Zhang *et al.* (1988)
DRB1	14	Sequencing[a]	Tilanus and Eliaou (1995)
DQA1	7	RFLP, SS-PCR	Inoko and Masao (1993), Olerup *et al.* (1993)
DQB1	9	Sequencing[a]	Tilanus and Eliaou (1995)
LMP2	2	RFLP	Deng *et al.* (1995)
TAP1	4	Microsatellite	Carrington and Dean (1994)
LMP7	2	RFLP	Deng *et al.* (1995)
DPB1	9	Sequencing[a]	Tilanus and Eliaou (1995)

All sequencing was performed in solid phase using an automated sequencer (A.L.F.; Pharmacia Biotech, Piscataway, NJ, USA). SSCP = single strand conformational polymorphism; SSOP = sequence-specific oligonucleotide probes; RFLP = restriction fragment length polymorphism; EIP = electrophoresis with immunoprecipitation; SS-PCR = sequence-specific polymerase chain reaction; TNF = tumour necrosis factor.
[a]Allele assignments were made by SBTyper (Pharmacia Biotech) (Tilanus and Eliaou, 1995).

abortus tissue in this population.) The children were categorized into three groups: homozygous for the shared antigen or haplotype, heterozygous and identical to the mother, or heterozygous and different from the mother. The first two groups would be histocompatible with the mother (i.e. they would not have a paternal antigen or haplotype that differed from a maternal haplotype), whereas the third group of children would be HLA incompatible with the mother. Observations were compared with expectations based on Mendelian segregation ratios (Figure 2).

Results

At the time of the first study pregnancy the mean ± SD age of the 111 wives was 28.7 ± 4.8 years (range 20–42), the couples had a mean ± SD of 2.9 ± 2.2 prior pregnancies (range 0–9) and 19 couples (17.1%) had experienced a total of 29 fetal losses. The 111 couples provided information on 251 study pregnancies that were or could have been followed at least 20 weeks past the last menses.

The overall fetal loss rate was 15.6% (27 couples experienced 38 fetal losses), similar to loss rates in outbred couples (reviewed in Bulletti *et al.*, 1996). Among the 26 couples with loss, 26 had at least one prior liveborn child and 21 had at least two prior liveborn children; none had more than two consecutive losses. The mean ± SD gestational age at the time of the loss was 9.5 ± 3.5 weeks (range 4–19).

Of the 68 haplotypes present in the Hutterite population, 37 were present in the couples experiencing a fetal loss; the relative frequencies of these 37 haplotypes did not differ from the relative frequencies of the same 37 haplotypes in the Hutterite population (haplotypes whose frequency was <0.01 were pooled; $\chi^2 = 24.08$, 30 df, $P_{exact} = 0.760$). This indicated that the Hutterite haplotypes, *per se*, did not carry abortion-susceptibility alleles (Christiansen *et al.*, 1989) or other associated alleles that may predispose the women to fetal loss (Christiansen *et al.*, 1994b).

The loss rates for couples matching and not matching for alleles at HLA loci and regions and results of the logistic regression analysis are shown in Table II. Fetal loss rates were greatest among couples matching for alleles at all 16 loci in the extended HLA haplotype [odds ratio (OR) 4.39, confidence interval (CI) 1.74–11.06, $P = 0.002$]. Among the individual loci, matching for *HLA-B* (OR 2.54, CI 1.16–5.53, $P = 0.019$), *HLA-C* (OR 2.20, CI 1.07–4.55, $P = 0.033$) or *C4* (OR 2.11, CI 1.03–4.35, $P = 0.043$) alleles was a statistically significant predictor of fetal loss. When the 30 couples that matched for the entire haplotype were removed from the analysis, the increased loss rate associated with *HLA-B* matching became non-significant (loss rates = 0.019 among couples matching and 0.014 among couples not matching for *HLA-B*; $P = 0.622$). Matching for class I, class III and class II region haplotypes defined by multiple alleles was also associated with significant risk for fetal loss (class I, OR 3.52, CI 1.30–9.53, $P = 0.013$; class III, OR 3.22, CI 1.21–8.55, $P = 0.019$; class II, OR 2.99, CI 1.12–7.99, $P = 0.029$). Too few couples matched for a region that did not match for the entire haplotype to determine whether the risks associated with matching for a region were independent of the risk associated with matching for the haplotype.

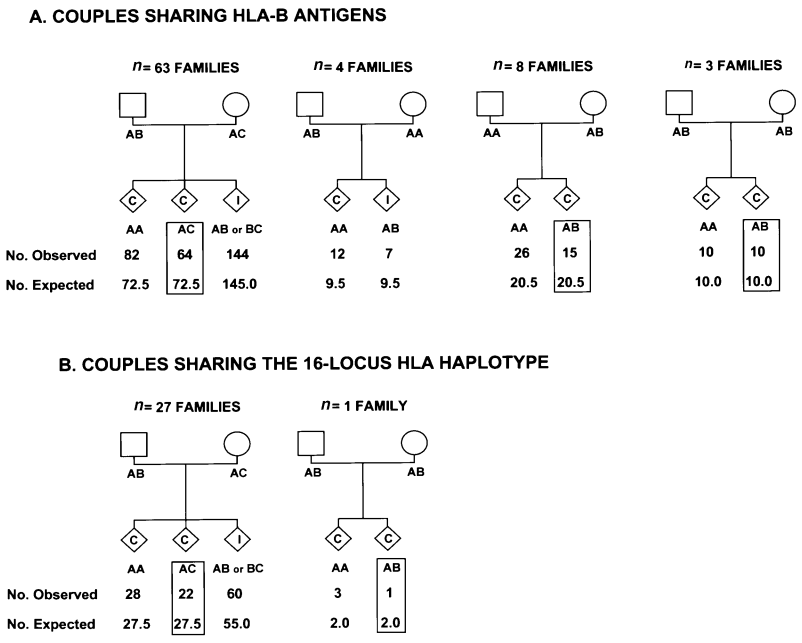

Figure 2. Family structures of couples sharing human leukocyte antigen (HLA)-*B* antigens (**A**) and HLA haplotypes (**B**). The letters under each symbol represent genotypes for *HLA-B* antigens (A) or HLA haplotypes (B). The letters in the children's symbols designate the compatible (C) and incompatible (I) children. The heterozygous-compatible children are shown in a box. (**A**) *HLA-B* types were known for 370 children in 78 families. Among these children, 130 were homozygous (112.5 expected), 89 were heterozygous-compatible (identical to the mother; 103.0 expected), and 151 were heterozygous-incompatible (154.5 expected) ($\chi^2_{(2\ df)} = 4.7$; $P = 0.095$). When families of couples sharing a haplotype were removed from the analysis, the significance level remained unchanged despite the smaller sample size ($\chi^2_{(2\ df)} = 4.7$; $P = 0.095$.). (**B**) HLA haplotypes were known for 114 children in 28 families. Among these children, 31 were homozygous (29.5 expected), 23 were heterozygous and identical to the mother (29.5 expected), and 60 were heterozygous and incompatible with the mother (55.0 expected) ($\chi^2_{(2\ df)} = 1.96$; $P = 0.376$).

Table II. Fetal loss rates by human leukocyte antigen matching[a]

HLA locus/region	No. of alleles/haplotypes shared			Odds ratio	Confidence intervals
	0	1	*P* value		
A	0.12 (13/106)	0.17 (25/145)	0.537	1.289	0.576–2.884
C	**0.12 (19/156)**	**0.20 (19/95)**	**0.033**	**2.203**	**1.067–4.549**
B	**0.10 (16/157)**	**0.23 (22/94)**	**0.019**	**2.539**	**1.163–5.534**
TNFa[b]	0.09 (9/100)	0.18 (27/149)	0.078	2.138	0.917–4.986
C4[b]	**0.10 (13/125)**	**0.19 (23/124)**	**0.043**	**2.112**	**1.025–4.353**
DRB1	0.15 (20/136)	0.16 (18/115)	0.649	1.200	0.547–2.633
DQA1	0.14 (14/97)	0.16 (24/154)	0.757	1.137	0.533–2.374
DQB1[b]	0.13 (19/145)	0.18 (18/101)	0.344	1.522	0.683–3.635
DPB1	0.13 (12/93)	0.16 (26/158)	0.444	1.322	0.647–2.704
Class I[b]	**0.13 (27/207)**	**0.28 (10/36)**	**0.013**	**3.521**	**1.300–9.530**
Class III[b]	**0.12 (25/208)**	**0.28 (10/36)**	**0.019**	**3.222**	**1.214–8.552**
Class II[b]	**0.13 (26/205)**	**0.27 (11/41)**	**0.029**	**2.991**	**1.120–7.985**
Haplotype	**0.13 (28/221)**	**0.33 (10/30)**	**0.002**	**4.386**	**1.739–11.061**

Numbers in parentheses are the number of losses/the number of pregnancies. All *P* values were adjusted for wife's age, wife's inbreeding coefficient and multiple pregnancies per couple. Significant results are shown in bold type.

[a]Two women each experienced an ectopic pregnancy. In one, the couple shared alleles at the *HLA-A*, *HLA-B* and *TNF* loci; in the other, the couple shared alleles at the *HLA-A*, *TNF* and *HLA-DPB1* loci. Neither couple shared all alleles across an HLA region or an entire haplotype.

[b]The numbers of pregnancies included in these analyses were <251 because alleles at these loci could not be assigned to all haplotypes. (No data were missing for alleles on matched haplotypes.)

To determine whether there was preferential loss of fetuses with particular genotypes, the genotype and compatibility statuses of the living children of couples matching at the *HLA-B* locus or for the haplotype were examined (Figure 2). Among the 370 children of couples matching for *HLA-B*, there were 13.6% fewer than expected living children who were heterozygous and identical to the mother ($P = 0.095$); among the 114 offspring of couples matching for the haplotype, there were 21% fewer than expected liveborn offspring that were heterozygous and identical to the mother ($P = 0.376$). There was no deficiency of homozygous children.

Discussion

We report here the results of the first prospective study of HLA matching and pregnancy outcome. The subjects in this study were unselected with respect to either pregnancy history or HLA matching status, although they are members of a population that is characterized by a relatively large proportion of couples matching for HLA and a high natural fertility rate (Ober, 1995). Previous retrospective studies in couples with recurrent spontaneous abortion (RSA) have been discrepant with respect to the effects of HLA sharing on fetal loss. For example, only approximately half of published studies found significantly increased HLA sharing among couples with RSA, and these studies differ with respect to the specific *HLA* gene or region that is associated with RSA (reviewed in Ober and van der Ven, 1996). Discrepancies between retrospective studies are difficult to reconcile but could be explained in part by differences in the definition of RSA and control couples, in HLA typing methodology or in HLA antigen frequencies between study populations. We initiated prospective studies in the Hutterites to avoid the methodological limitations inherent in retrospective studies and to minimize biases due to sample selection and poorly matched controls.

The results of these studies indicate that couples matching for the extended HLA haplotype have a significantly increased risk for fetal loss (OR 4.386; $P = 0.002$). Among the individual loci, *HLA-B* matching was also associated with increased risk (OR 2.539; $P = 0.019$). Often a 'disease' is associated with a marker locus because of linkage disequilibrium between alleles at the marker and 'disease' loci. This could account for the observed effects of *HLA-B* matching on fetal loss rates in the Hutterites. If true, there should be evidence of associations with fetal loss at loci that are most closely linked to *HLA-B*. In fact, matching for alleles at the neighbouring loci (*HLA-C*, *TNFa* and *C4*) is also associated with increased loss rates in this population, suggesting that an as yet unknown locus that is in linkage disequilibrium with *HLA-B* could be the primary susceptibility locus. At this time it is not possible to determine whether primary risk for fetal loss is associated with the *HLA-B* locus *per se*, with an *HLA-B*-linked locus or with the extended haplotype. This study considered only serologically defined HLA-B antigens, which are heterogeneous at the molecular level. For example, the two most common HLA-B antigens in the Hutterites, B51 and B35, have at least three and six alleles, respectively, defined at the molecular level (So, 1994). Furthermore, B51 is present on seven and B35 is present on eight Hutterite haplotypes. Therefore, although Hutterite spouses who match for the entire haplotype will match for the same *B*-locus allele, spouses matching for serologically defined HLA-B antigens on different haplotypes may not match for the same allele at the molecular level. This could explain why the significance of the *HLA-B* matching effect on fetal loss was reduced when couples matching for the haplotype were removed from the analysis, and matching for *HLA-B* alleles defined at the molecular level could explain the observed haplotype matching effect if *HLA-B* is the primary susceptibility locus. Alternatively, a separate locus that is in linkage disequilibrium with *HLA-B* may have a stronger

association with fetal loss than *HLA-B per se*, and this locus could also account for the effect of haplotype matching (because all couples matching for a haplotype would also match alleles at this locus). We are currently exploring these alternative hypotheses in the Hutterites.

Increased risk for fetal loss among couples matching for HLA-B antigens or for the haplotype suggest that certain fetal HLA genotypes are 'high risk' genotypes with respect to loss. If so, these 'high risk' genotypes should be under-represented in the surviving children of couples matching for *HLA-B* alleles or for HLA haplotypes. Although the HLA types of the living children of these couples were not significantly different from random expectations in these relatively small samples (Figure 2), the numbers of heterozygous-incompatible children and of homozygous children were equal to or greater than expected. In contrast, a decrease (albeit non-significant) in heterozygous-compatible offspring (i.e. those with identical genotypes to their mother) was observed in these children (Figure 2). The deficiency of heterozygous-compatible children but not homozygous-compatible children suggests a possible novel mechanism for fetal loss in these couples. HLA homozygous fetuses and HLA heterozygous fetuses that are identical to the mother would both represent compatible pregnancies from the mother's perspective, i.e. neither would carry paternally derived HLA that differ from maternal HLA. On the other hand, HLA homozygous fetuses would recognize cells from HLA heterozygous mothers as non-self, but heterozygous identical fetuses would not. Thus these data suggest that fetal recognition of maternal tissues as non-self may be beneficial in pregnancy, and not maternal recognition of fetal tissues as thought previously (Kirby, 1970; Beer and Billingham, 1976). If this hypothesis is true, then mothers who were homozygous for *HLA-B* should have increased fetal loss rates because all fetuses of homozygous mothers would be unable to recognize maternal cells as non-self. Consistent with this hypothesis is the observation that fetal loss rates were 0.30 among 33 mothers who were homozygous for HLA-B antigens and 0.13 among 212 mothers who were heterozygous for HLA-B antigens ($P = 0.02$) (there were no mothers in our study who were homozygous for the extended haplotype). Consistent with our finding is the report of increased homozygosity for HLA-B antigens in outbred couples with RSA (Bolis *et al.*, 1984; Coulam *et al.*, 1987; Johnson *et al.*, 1988). Lastly, it is unlikely that losses were due to the effects of deleterious recessive genes in the HLA region because there was no deficiency of offspring who were homozygous for HLA in these families.

This study demonstrates that matching for HLA-B antigens or for the extended haplotype is associated with sporadic fetal loss ($P = 0.019$ and 0.002 respectively), and suggests that fetuses who are HLA identical to their mother are at increased risk for loss. Although it is less likely that outbred couples will share alleles at HLA loci as often as Hutterite couples, the actual risks associated with HLA matching may be similar in outbred couples and could explain the increased HLA sharing observed in outbred couples with RSA (Komlos *et al.*, 1977; Gerencer *et al.*, 1979; Gerencer and Kastelan, 1983; Bolis *et al.*, 1984; Schacter *et al.*, 1984; Beer *et al.*, 1985; McIntyre *et al.*, 1986; Takakuwa *et al.*, 1986; Coulam *et al.*, 1987; Johnson *et al.*, 1988; Ho *et al.*, 1990; Reznikoff-Etievant *et al.*, 1991; Koyama *et al.*, 1992; Ober *et al.*, 1993) and in non-human primates experiencing fetal wastage (Knapp *et al.*, 1996). Similar to results in the Hutterites, Knapp *et al.* (1996) reported significantly increased sporadic fetal loss in captive pigtailed macaque pairs matching for class I major histocompatibility complex (MHC) antigens.

The relative paucity of independent genomes represented in the current Hutterite population enhances the ability to detect the effects of unknown genes that are in linkage disequilibrium with known MHC loci and makes it probable that identical haplotypes are likely to be identical by descent from a common ancestor. The high-resolution typing used to define haplotypes in this study also increases the probability that the composition of the haplotypes is, in fact, identical at all loci. As a result, Hutterite couples who share a haplotype will share all alleles on that haplotype, including unknown (or untyped) loci. In outbred couples it is less likely that two 'identical' haplotypes are identical by descent, and haplotypes are rarely defined at the same level of resolution as that used in this study. Therefore outbred couples who share HLA loci, or even a 'haplotype', may not share alleles at the loci that actually confer risk for fetal loss. Thus it is not surprising that HLA sharing is not a significant predictor of pregnancy outcome among outbred couples with RSA (Mowbray *et al.*, 1983; Smith and Cowchock, 1988; Cowchock *et al.*, 1990; Cowchock and Smith, 1992; Christiansen *et al.*, 1994a). Because data on HLA status offer little information about the cause of the miscarriages in any particular couple, do not identify a subgroup of couples that will benefit from a particular treatment and provide no information on the likelihood of another abortion (reviewed by Christiansen, 1996), HLA typing should not be included as part of the clinical evaluation of couples with recurrent miscarriage. However, once the loci that confer risk for fetal loss in the Hutterites are identified and the mechanism clearly defined, the risk associated with these loci in outbred couples can be assessed directly.

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