

Cutting Edge: Persistent Fetal Microchimerism in T Lymphocytes Is Associated with HLA-DQA1*0501: Implications in Autoimmunity¹

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The host's MHC genotype plays a critical role in susceptibility to autoimmune diseases. We previously proposed that persistent fetal microchimerism from pregnancy contributes to the pathogenesis of autoimmune diseases such as scleroderma. In the current study, we investigated whether the specific host MHC genotype is associated with persistent microchimerism among T lymphocytes in women with scleroderma and in healthy women. Fetal microchimerism among T lymphocytes was strongly associated with HLA DQA1*0501 of the mother (odds ratio (OR) = 13.5, $p = 0.007$, p corrected (pc) = 0.06) and even more strongly with DQA1*0501 of the son (OR = ∞ ; $p = 0.00002$, $pc = 0.0002$). This is the first description of an association between persistent fetal microchimerism in maternal T lymphocytes and specific HLA class II alleles. Although the association was observed in both healthy women and in women with scleroderma, the finding suggests an additional route by which HLA genes might contribute to susceptibility to autoimmune disease. *The Journal of Immunology*, 2000, 164: 5545–5548.

The full spectrum of factors influencing the pathogenesis of autoimmune disease is not known, but two striking characteristics are the predilection for women and the association of specific HLA class II alleles with disease. The host genotype undoubtedly plays a role in determining susceptibility to autoimmune diseases. The HLA DRB1*03 genotype has been found to be increased among Caucasians in numerous autoimmune

diseases (1, 2). Additionally, healthy DRB1*03-positive individuals exhibit a number of immune abnormalities, including defective apoptosis pathway (3) and a modified pattern of cytokine production (4), abnormalities that could increase the risk of developing autoimmune disease.

Autoimmune diseases, such as scleroderma (SSc),³ exhibit an increased incidence in women following childbearing years (5). It is now recognized that during pregnancy trafficking of cells occurs in both directions across the placenta (6); moreover, these microchimeric cells persist for decades in maternal peripheral blood (7). SSc has clinical similarities to chronic graft-vs-host disease, a known condition of chimerism (8, 9). We therefore proposed that microchimerism arising from pregnancy contributes to the pathogenesis of autoimmune diseases such as SSc (10). This hypothesis was supported by the finding of a significant difference in levels of microchimerism between women with SSc compared with healthy women when peripheral blood samples were tested using a quantitative assay (11). Other investigators have described a significant difference of microchimerism in patients with SSc and controls in skin biopsy specimens (12). However, the presence of fetal microchimerism per se is also common in healthy individuals (7, 13).

Because HLA class II associations are found with autoimmune diseases (14), including SSc, and because T cells are thought to be intimately involved in SSc (15, 16), we investigated whether specific HLA class II alleles could influence the persistence of fetal T lymphocyte microchimerism in women. Among Caucasians, DRB1*11 has most consistently been reported in association with diffuse disease SSc (17–19), although other reports have described an increase of DRB1*03 (19, 20). Both DRB1*11 and DRB1*03 are in strong linkage disequilibrium with DQA1*0501. This raises the question as to whether DRB1*11, DRB1*03, or DQA1*0501 alleles are associated with the persistence of fetal microchimerism in the T lymphocyte population. To answer this question, we targeted healthy women and women with SSc with a history of at least one male birth, sorted PBMC by FACS on the basis of the CD3 surface marker, and analyzed for the presence of male DNA. Microchimerism among T lymphocytes was examined for correlation with specific HLA class II alleles of the mother and/or the child.

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³ Abbreviations used in this paper: SSc, scleroderma; SSOP, sequence-specific oligonucleotide probe; OR, odds ratio; pc , p corrected.

Patients and Methods

Subjects

Thirty-seven women were studied: 19 healthy women and 12 women with SSc who previously gave birth to at least one son, 3 healthy nulligravid women, and 3 healthy women who gave birth only to daughters were included as negative controls for Y-chromosome PCR. Of the women with sons, the majority were Caucasian (26/31) and the others were Asian (1/31), African American (2/31), or Native American (2/31). At the time of blood draw, women ranged from 26.5 to 65 years of age (mean age: 48.3 years old) and their youngest sons ranged from 4 mo to 37 years (mean age: 20.7 years old). All SSc patients satisfied the American College of Rheumatology criteria for SSc; nine had diffuse and three had limited cutaneous disease.

T lymphocyte isolation by FACS

Samples of heparinized whole blood were processed by Ficoll-Hypaque density centrifugation. PBMC were filtered on nylon wool (DuPont Biotechnologies, Boston, MA) to avoid cell aggregation and resuspended in PBS/1% FCS. Staining was performed as previously described (13) on $10\text{--}20 \times 10^6$ cells with anti-CD3-FITC (Becton Dickinson, Mountain View, CA) at 3 μl /million of cells. After staining incubation, DNase (Boehringer Mannheim, Indianapolis, IN or Life Technologies, Burlington, Ontario, Canada) was added (30 U/million cells) after the last wash to avoid cell aggregation during the FACS sorting. Cells were sorted by a single laser on a FACS (Becton Dickinson) on the basis of the marker described above. An aliquot of the sorted cells was run and the percentage of sorted correctly gated cells calculated to assess the purity. Purity was consistently $\geq 99\%$. Cells were collected in RPMI and 20% FCS. Cell sorting was followed immediately by centrifugation and two washes to remove all remaining DNase.

DNA extraction on T lymphocytes and qualitative analysis

DNA from at least 200,000 cells was extracted using an Isoquick Nucleic Acid Extraction Kit (ORCA Research, Bothell, WA) according to manufacturer's instructions. The purified DNA was resuspended in 20 μl of 10 mM Tris-HCl (pH 9.0). The quality of the DNA extraction was tested by PCR for a human growth hormone sequence. Primers amplifying a 573-bp segment were combined in a 50- μl reaction mixture of 250 pM primer 1 (sense: 5'-GCCTTCCCAACCATTCCTTA-3'), 250 pM primer 2 (antisense: 5'-TCACGGATTTCTGTTGTGTTT-3'), 1.5 mM MgCl_2 , 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 5 U AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). The PCR consisted of 10 cycles at 94°C for 20 s and 65°C for 1 min immediately followed by 30 cycles at 94°C for 20 s, 61°C for 50 s, and 72°C for 30 s.

Nested PCR for Y-chromosome-specific DNA

The presence of fetal microchimerism was detected by nested PCR for a Y-chromosome-specific sequence according to a method modified from Lo et al. (21) as previously described (13). Extremely strict measures were taken to prevent contamination during DNA preparation, reaction mix preparation, and transfer of primary product after step I, and all experiments were conducted by females. The PCR was performed on 330 ng of DNA (~50,000 cells) in two steps. The first PCR included 40 cycles (94°C for 1 min, 67°C for 1 min, and 72°C for 2 min) yielding a product of 238 bp. The second reaction had 25 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) yielding a product of 197 bp. A positive control of male DNA and a negative control (water and reagent) were included in each

experiment. DNA from nulligravid women and healthy women who gave birth only to daughters were included as negative controls for Y-chromosome PCR. Final reaction products were visualized on agarose gels, and all samples were tested from four separate aliquots. Results were considered positive if at least two of four repeated experiments displayed a positive band.

The sensitivity of the nested Y-chromosome sequence-specific PCR was determined by adding serially diluted male DNA to aliquots of female DNA equivalent to 50,000 cells. The DNA equivalent of 1 male cell in 50,000 female cells could be detected at the first step and the DNA equivalent of 0.1 male cell in 50,000 female cells at the second step of the nested PCR.

HLA genotyping

Sequence-specific oligonucleotide probe (SSOP) typing was used to determine DQA1, DQB1, and DRB1 alleles of healthy controls, SSc patients, and their children. DQA1 and DQB1 alleles were determined by SSOP typing with methods similar to those previously described (22) to which other probes were added to detect newly identified alleles of DQA1 and DQB1 (23). For DRB1, a low-resolution assay was used that detects the DRB1 families DRB1*01 through DRB1*14 as previously described (11).

Statistical analysis

To investigate whether maternal HLA genotype influences microchimerism, the analysis considered women with a specific allele compared with women without this allele. The two categories were compared for the presence or absence of fetal microchimerism in the T lymphocyte population. Similarly, to investigate whether the son's HLA genotype influences microchimerism of the mother, the analysis considered women who had at least one son with a specific allele compared with women for whom the sons did not have the allele. The odds ratios were calculated as the ratio of the proportion of women who had fetal cells detected to the proportion of women who did not. Exact methods were used to compute 95% confidence intervals and two-sided *p* values associated with the odds ratios (StatXact 3 for Windows). The *p* values were also calculated after correction for multiple comparisons, considering the number of alleles detectable by the SSOP method. Therefore, *p* values were corrected by the number of DRB1 families (*n* = 12), DQA1 alleles (*n* = 8), or DQB1 alleles (*n* = 26) and are regarded as significant evidence of an association when < 0.05 .

Results and Discussion

Persistent fetal microchimerism was evaluated by Y-chromosome-specific PCR in T lymphocytes sorted by FACS to a purity of $\geq 99\%$ for 37 women. Four separate aliquots, each with the DNA equivalent of 50,000 T lymphocytes, were tested for each subject. No evidence of PCR contamination was observed in any experiment, and three nulligravid women and three parous women who only gave birth to daughter(s), tested as negative controls, were consistently negative. Among the 31 women who had given birth to at least one son, 14 (45%) had male DNA including 9 of 19 (47%) healthy controls and 5 of 12 (42%) SSc patients. These results indicate that microchimerism was present in T lymphocytes whether the subject was healthy or had SSc. In prior studies we also found that microchimerism is common in healthy women (11, 13). The current results are not inconsistent with our prior findings in that the nested PCR assay provides a qualitative result and we

Table I. Influence of maternal genotype on T lymphocyte microchimerism

HLA Allele	Mothers Positive for Microchimerism in T Cells (%) (<i>n</i> = 14)	Mothers Negative for Microchimerism in T Cells (%) (<i>n</i> = 17)	OR, <i>p</i> Value
DRB1*11	29	0	∞ , <i>p</i> = 0.03 ^a
DRB1*03	21	12	NS ^b
DQA1*0501	64	12	13.5, <i>p</i> = 0.007 ^c
DQB1*0301	43	12	NS
DQB1*0201	36	29	NS

^a The *p* value corrected by the number of DRB1 families = 0.36.

^b NS, not significant.

^c The *p* value corrected by the number of DQA1 families = 0.06.

Table II. Influence of the son's genotype on T lymphocyte microchimerism of the mother

HLA Allele	Mothers Positive for Microchimerism in T Cells (%) (n = 14)	Mothers Negative for Microchimerism in T Cells (%) (n = 15)	OR, p Value
DRB1*11	29	7	NS ^a
DRB1*03	57	7	18.7, p = 0.005 ^b
DQA1*0501	100	13	∞ , p = 0.00002 ^b
DQB1*0301	57	13	8.7, p = 0.02 ^c
DQB1*0201	57	40	NS

^a NS, not significant.

^b The p values corrected by the number of DRB1 families = 0.06.

^c The p value corrected by the number of DQA1 families = 0.0002.

^d The p value corrected by the number of DQB1 families = 0.52.

described a significant quantitative difference of microchimerism in SSc patients in a previous study (11).

The host genotype clearly plays a role in determining susceptibility to autoimmune diseases. An increase of the DRB1*03 genotype has been described in a number of autoimmune diseases and may predispose the host to immune dysfunction (3, 4). Because T lymphocytes are implicated in the pathogenesis of autoimmune diseases such as SSc and because specific HLA class II alleles are associated with increased disease risk, we asked whether mothers carrying SSc-associated HLA alleles (DRB1*11 and DRB1*03) were predisposed to fetal microchimerism in the T lymphocyte population. As shown in Table I, among the women positive for fetal microchimerism in T lymphocytes, only 29% had DRB1*11 and 21% had DRB1*03. Thus, neither DRB1*03 nor DRB1*11 alone was significantly associated with persistent fetal T lymphocyte microchimerism, although the odds ratio for DRB1*11 was infinity (OR = ∞ , p = 0.03, p corrected (pc) = 0.36). However, DQA1*0501, which is in linkage disequilibrium with both DRB1*03 and DRB1*11, was carried by 64% of women positive for fetal T lymphocyte microchimerism. Women with the DQA1*0501 allele were 13.5 times more likely to have microchimerism among T lymphocytes than if they did not have this allele, (OR = 13.5, 95% CI = 1.71–136.73, p = 0.007, pc = 0.06).

We next examined whether the HLA genotype of the son(s) correlated with persistent T-lymphocyte microchimerism in the mother (Table II). Every woman with T lymphocyte microchimerism had a DQA1*0501-positive son. Conversely all women who did not have a DQA1*0501-positive son were negative for T lymphocyte microchimerism (OR = ∞ , p = 0.00002, pc = 0.0002). Surprisingly, although the host's MHC genotype correlated with T lymphocyte microchimerism, the child's genotype was an even stronger factor in determining persistent T lymphocyte microchimerism of the mother (p = 0.03 according to McNemar's test). The mean years since last birth was 18 years in women with T lymphocyte microchimerism and 22 years in women without, and the mean number of children was 1.2 and 2, respectively. Two mothers (one healthy and one patient) were negative for microchimerism in T lymphocytes but had a DQA1*0501-positive son, suggesting either this allele is not the only requirement for T lymphocyte microchimerism or that the level of detection was too low in these patients.

Because of linkage disequilibrium among DRB1, DQA1, and DQB1 genes, correction for the number of alleles tested at all loci is excessively conservative. However, even if this correction is applied, the results remain significant for the DQA1*0501 allele from the son (pc = 0.0009; see *Patients and Methods*), although not for DQA1*0501 of the mother (pc = 0.32). The likelihood of having fetal cells in the T lymphocyte population correlated less strongly with DQB1*0301 of the son, (OR = 8.7, p = 0.02) and

with DRB1*03 of the son (OR = 18.7, p = 0.005), both of which are in linkage disequilibrium with DQA1*0501. These results were not significant after correction by the number of DRB1 families and DQB1 alleles.

Two SSc patients had a history of blood transfusion, which could be an alternative source of microchimerism (24); however, if these patients are excluded, the association of T lymphocyte microchimerism with DQA1*0501 of the son remains significant (OR = ∞ , p = 0.00004) even after correction for alleles at all loci (pc = 0.002).

When healthy women and women with SSc were analyzed separately, a maternal genotype of DQA1*0501 was more strongly correlated with T lymphocyte microchimerism in women with SSc, but the difference between the two groups was not significant (OR = 24 p = 0.07 vs OR = 11.3 p = 0.06). With respect to HLA-DQA1*0501 of the son, the correlation was not different in women with SSc and healthy women (OR = ∞ , p = 0.05 vs OR = ∞ , p = 0.0001). Thus these results indicate that the DQA1*0501 allele, whether in patient or healthy control populations, was associated with persistent fetal microchimerism in T lymphocytes. However, this study is only qualitative and there could be quantitative differences between SSc patients and controls. In a prior study we utilized a quantitative assay that was developed and standardized to testing whole peripheral blood samples and found a significant quantitative difference of microchimerism in SSc patients compared with controls (11).

A very recent report described maternal microchimerism in children with juvenile dermatomyositis but also in their healthy siblings when they were carrying the disease-susceptibility allele DQA1*0501.⁴ Considered together with the current findings, this supports the hypothesis that individuals with DQA1*0501 may have a predisposition for persistent microchimerism and could be at increased risk for autoimmune disease. The DQA1*0501 allele is in linkage disequilibrium with DQB1*0301, DQB1*0201, and with DRB1*11 and DRB1*0301, and has been implicated in a number of autoimmune diseases in addition to SSc, including juvenile dermatomyositis (25), celiac disease (26) and Sjögren's syndrome (27). In studies of juvenile dermatomyositis it has been suggested that the DQA1*0501-DQB1*0301 heterodimer might have access to peptides earlier in the processing pathway, thus encountering novel peptides conducive to autoimmunity (28).

Our description of HLA-DQA1*0501 association with long-term microchimerism in T lymphocytes derives from a Caucasian population where this allele is also common (>40% in the U.S. Caucasian population). It is also noteworthy that the DQA1*0501-DQB1*0301-DRB1*11 or DRB1*03 haplotypes are haplotypes

⁴ A. M. Reed, L. P. Shock, and Y. J. Picornell. Microchimerism in children with juvenile dermatomyositis. Submitted for publication.

associated with SSc in Caucasian populations (29–32). Of further interest, an extremely high prevalence of SSc has recently been described in a Native American population in association with another haplotype that also carries DQA1*0501, i.e., DRB1*1602-DQA1*0501-DQB1*0301 (33). However, in the Japanese population, SSc is not associated with the DQA1*0501 allele but with the haplotype DRB1*1502, DQA1*0103, DQB1*0601 (34), one of the most common haplotypes in this population. Among the DRB1*1502 negative patients, the haplotype DRB1*0802, DQA1*0401, DQB1*0402 is increased (34). DQA1*0103 and DQA1*0401 alleles have a common amino acid with DQA1*0501 at position 34. Similar studies of microchimerism in Japanese will be informative to determine whether there is an amino acid sequence that is common across racial/ethnic groups.

In conclusion, DQA1*0501-positive women and/or women with a DQA1*0501-positive son have a greater chance to maintain persistent fetal microchimerism in their T lymphocyte population. The greater magnitude of association when the son had DQA1*0501 suggests that patients with autoimmune diseases who lack disease-specific HLA molecules could be investigated for persistent microchimerism as an alternative source of HLA disease-associated molecules or peptides. In addition to persistent fetal cells in women with children, alternative sources of microchimerism that could affect men and women who have never been pregnant include from a blood transfusion (24), from a twin (35) or from the mother (36).

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