X Chromosome Monosomy: A Common Mechanism for Autoimmune Diseases

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X Chromosome Monosomy: A Common Mechanism for Autoimmune Diseases

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The majority of human autoimmune diseases are characterized by female predominance. Although sex hormone influences have been suggested to explain this phenomenon, the mechanism remains unclear. In contrast to the role of hormones, it has been suggested, based on pilot data in primary biliary cirrhosis, that there is an elevation of monosomy X in autoimmune disease. Using peripheral white blood cells from women with systemic sclerosis (SSc), autoimmune thyroid disease (AITD), or healthy age-matched control women, we studied the presence of monosomy X rates using fluorescence in situ hybridization. We also performed dual-color fluorescence in situ hybridization analysis with a chromosome Y α-satellite probe to determine the presence of the Y chromosome in the monosomic cells. In subsets of patients and controls, we determined X monosomy rates in white blood cell subpopulations. The rates of monosomy X increased with age in all three populations. However, the rate of monosomy X was significantly higher in patients with SSc and AITD when compared with healthy women (6.2 ± 0.3% and 4.3 ± 0.3%, respectively, vs 2.9 ± 0.2% in healthy women, p < 0.0001 in both comparisons). Importantly, X monosomy rate was more frequent in peripheral T and B lymphocytes than in the other cell populations, and there was no evidence for the presence of male fetal microchimerism. These data highlight the thesis that chromosome instability is common to women with SSc and AITD and that haploinsufficiency for X-linked genes may be a critical factor for the female predominance of autoimmune diseases. The Journal of Immunology, 2005, 175: 575–578.

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though sex hormone abnormalities might explain the female predominance in autoimmunity (1), the mechanisms remain enigmatic. Systemic sclerosis (SSc) and autoimmune thyroid disease (AITD) illustrate this paradigm. SSc is a chronic disease characterized by skin and visceral fibrosis, as well as vascular abnormalities (2), with female:male ratios as high as 12:1 (3). AITD, including Graves’ disease (GD) and Hashimoto thyroiditis (HT), also affects more female patients with reported ratios as high as 10:1 (3). Genetic factors play a crucial role in determining susceptibility to both SSc and AITD, as indicated by the increased risk of onset among family members of affected individuals (4, 5) and the concordance rates in monozygotic twins (6).

The persistence of a small population of cells from a genetically distinct individual (i.e., microchimerism, most frequently fetal) has been demonstrated in women with SSc and AITD (7). In contrast, we have recently suggested that primary biliary cirrhosis, an autoimmune liver disease characterized by a high organ specificity of the immune-mediated injury (8), may be induced by sex chromosome defects (9). This thesis is supported by the observation that the X chromosome includes genes that are crucial in determining sex hormone levels and, more importantly, to maintain immune tolerance (10). In addition, alterations in the X chromosome, i.e., 45, X0 Turner’s syndrome, are characterized by a marked susceptibility to develop autoimmune disorders (11), most frequently AITD (12).

We investigated the frequency of X chromosome monosomy in peripheral white blood cells (WBC) in women with SSc, AITD, and healthy women of similar age. To understand whether X monosomic cells are indeed microchimeric (male) cells, we investigated the presence of Y chromosome-specific sequences in monosomic WBC obtained from a nested cohort. We also evaluated the X monosomy rate in the major peripheral blood cell subpopulations. We report herein a significantly higher X monosomy rate in women affected by SSc or AITD compared with healthy women and also rule out that such findings are secondary to fetal microchimerism.

Materials and Methods

Study population and design

Following informed consent, we consecutively enrolled newly diagnosed patients including 44 women with SSc and 44 with AITD between June and December 2003. Seventy-three healthy women of similar age were
used as controls, chosen from a larger group of healthy subjects (HS) by randomly selecting women within 10 five-year age classes ranging from 24 or younger to 75 years or older. All patients were Italians and were followed by secondary and tertiary referral centers. The diagnosis of SSc was made according to internationally accepted criteria (13) and patients were defined according to their clinical features. Patients were considered to have SSc with limited cutaneous (lSSc) involvement if, throughout their illness, skin thickening was either absent or restricted to the distal extremities (not proximal to the elbows or knees) (13) (Table I). Patients were classified as having SSc with diffuse cutaneous (diffuse SSc) involvement if, at any time during the course of their illness, they had skin thickening proximal to the elbows or knees (upper arms, thighs, anterior chest, abdomen) (13). Patients with CREST syndrome (3) (i.e., characterized by the presence of calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, and telangectasias) were also included in the group with lSSc (14). The diagnosis of AITD was similarly based on internationally accepted criteria (15) (Table II). In particular, the diagnosis of GD was assigned on the basis of clinical data (hyperthyroidism with diffuse goiter at ultrasound and/or scintiscan) and laboratory findings (suppressed thyroid-stimulating hormone (TSH) values <0.05 mU/L and high thyroid peroxidase Abs (TPOAb), and TSH Receptor Abs (TRAb)). The diagnosis of HT was also defined on the basis of clinical (hyperthyroidism associated with a hypoechogenic pattern at thyroid ultrasound) and laboratory evidence (elevated TSH >6.0 mU/L and TPOAb). Patients with AITD were then subdivided according to the degree of thyroid dysfunction; circulating levels of free thyroid hormones (FT4 and/or FT3) within the normal range were indicators of mild forms of AITD, whereas abnormal FT4/FT3 (FT4 < 9.0 pM in HT or FT3 > 8.0 pM in GD) indicated overt/severe disease (15). The presence of thyroid-associated ophthalmopathy and other autoimmune manifestations was also assessed (Table II). Patients with SSc or AITD were not receiving any medical treatment at the time of blood sampling.

Identification of autoantibodies

Autoantibodies were studied in serum samples collected at the time of enrollment and stored at −20°C until assayed. The presence of anti-centromere Abs was investigated by indirect immunofluorescence. The identification of other SSc-associated (anti-topoisomerase I) autoantibodies was performed by immunoblotting. TPOAb (normal values <10 kU/L) and TRAb (n.v. <4 U/L) levels were measured by ELISA.

Chromosome preparations and fluorescence in situ hybridization (FISH) analysis

WBC were obtained as previously described (9). All preparations were cultured for 72 h using chromosome medium P (Euromedex) and added mitogens. In a preliminary test (and in agreement with data from others (16)), we found that the X monosomy rates in blood cells cultured for up to 72 h were not different from those measured in directly isolated cells. All samples were blindly analyzed in relation to disease group and subject age using a digoxigenin-labeled chromosome Y (DYZ3; Qbiogene) (17). Dual-color FISH with a fluorescein-labeled DXZ1 and rhodamine-labeled chromosome Y α-satellite (DYZ3; Qbiogene) probes was conducted in a nested study. Similarly, dual-color FISH with digoxigenin-labeled DXZ1 and biotin-labeled D15Z probes was performed in a nested study on six patients with autoimmune disease and five healthy controls. The slides were counterstained with 4’,6’-diamidine-2-phenylindole and then visualized using a Leitz Diaplan microscope equipped with 4’,6’-diamidine-2-phenylindole and FITC-tetramethylrhodamine isothiocyanate epifluorescence optics and a digital camera. For each sample, at least 500 nuclei were scored. No monosomic nuclei for chromosome 15 were found. Blinded replicate analysis was done in eight subjects and consistent data were obtained (data not shown).

Cell subpopulation analysis

FISH analysis of cell subpopulations was conducted using a subset of 6 patients with SSc, 6 patients with AITD, and 12 healthy women (with high whole blood X monosomy rates). A magnetic cell sorting method (MACS; Miltenyi Biotec) was used to isolate CD3+ (T lymphocytes), CD56+ (NK cells), CD14+ (monocytes/macrophages), and CD19+ cells (B lymphocytes). The purity of the sorted fractions was measured by means of flow cytometry (18) and was found to be >95–98% in all experiments. FISH was conducted using the chromosome X α-satellite probe used for the cultured whole peripheral blood cells.

Statistical power and analysis

The number of subjects included in the present study was based on the observed differences and SDs between patients and controls from our previous work (9). Briefly, we used the sample size calculation for Dunnett’s method for mean comparisons that allows estimates when two study populations are compared with a control population (19), and then calculated that 44 subjects in each study group allowed a statistical power of 0.8037 on 5% size in our comparisons.

For the frequency of X chromosome monosomy, ANOVA was used to compare groups of patients. If the results of the overall F test in an ANOVA were significant, pairwise comparisons were made to identify which groups differed from the others. The joint effects of subject group (SSc vs HS or between groups of patients with different clinical features and controls) and age on X monosomy rate were assessed by analysis of covariance methodology (analysis of covariance model). The statistical comparisons were made using Stata Statistical software (Stata) or SAS (SAS). All analyses were two-tailed.

Results

Monosomy X in WBC

We determined the frequency of monosomy X in WBC from women with SSc, AITD, and healthy women of similar age. In each case, a minimum of 500 nuclei were analyzed and the monosomy rate was expressed as (monosomic nuclei/total number of scored nuclei) × 100. Table III and Fig. 1 illustrate the data. In all groups, the rate of monosomy X was found to increase with age (Fig. 1), with similar angular coefficients (0.04421 in SSc, 0.04480 in AITD, 0.03784 in HS; p = NS) and women with SSc presented significantly older age compared with the other two groups (Table III). For these reasons, age was included in all models for the comparison of monosomy X rates. Monosomy X was observed in AITD in 10 patients, primary biliary cirrhosis in 2 patients (in both cases coexisting with AITD), polymyositis in 2 patients (in one case coexisting with AITD), and rheumatoid arthritis in 2 patients.

<table>
<thead>
<tr>
<th>Table II. Clinical features of women with HT and GD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Abs</td>
</tr>
<tr>
<td>TPOAb</td>
</tr>
<tr>
<td>TRAb</td>
</tr>
<tr>
<td>Thyroid dysfunction</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Associated autoimmune manifestations*</td>
</tr>
<tr>
<td>* Normal TSH values, 0.3–4.0 mU/L</td>
</tr>
<tr>
<td>* Normal ranges, FT4, 9–20 pM; FT3, 4–8 pM</td>
</tr>
</tbody>
</table>

* Normal TSH values, 0.3–4.0 mU/L
* Normal ranges, FT4, 9–20 pM; FT3, 4–8 pM

Thyroid-associated ophthalmopathy with clinical activity scores ranging from 4–5 (31) in 6 GD patients, atrophic gastritis in 2 HT, vitiligo in 2 (1 HT and 1 GD), polyglandular autoimmune disease type 3 in 1 HT, and diabetes mellitus in 1 GD.

| Table I. Clinical features of women with SSc

<table>
<thead>
<tr>
<th>SSc type</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limited</td>
<td>34 (77%)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>10 (23%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum autoantibodies</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-topoisomerase I positive</td>
<td>15 (34%)</td>
</tr>
<tr>
<td>Anti-centromere positive</td>
<td>20 (45%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ involvement (13)</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid</td>
<td>16 (36%)</td>
</tr>
<tr>
<td>Digestive tract</td>
<td>32 (73%)</td>
</tr>
<tr>
<td>Heart</td>
<td>33 (75%)</td>
</tr>
<tr>
<td>Lung</td>
<td>22 (50%)</td>
</tr>
<tr>
<td>Kidney</td>
<td>23 (52%)</td>
</tr>
<tr>
<td>Associated autoimmune conditions*</td>
<td>13 (30%)</td>
</tr>
</tbody>
</table>

* AITD in 10 patients, primary biliary cirrhosis in 2 patients (in both cases coexisting with AITD), polymyositis in 2 patients (in one case coexisting with AITD), and rheumatoid arthritis in 2 patients.
with significantly higher frequency in WBC of women with SSc and in women with AITD when compared with healthy controls (6.2 ± 0.3% in SSc and 4.3 ± 0.3% in AITD vs 2.9 ± 0.2% in healthy women, p < 0.0001 in both comparisons; Table III). No significant difference in monosomy rates was observed in patients with different types of SSc (6.3 ± 1.9% in ISSc vs 5.7 ± 2.5% in diffuse SSc; p = NS). When subgroups of AITD were analyzed, no significant differences in monosomy X rates were observed between patients with GD and HT (3.9 ± 0.5 vs 4.4 ± 0.5; p = NS), with mild or severe AITD (4.8 ± 0.4 vs 3.7 ± 0.3; p = NS), or with or without autoimmune-associated manifestations (4.0 ± 0.5 vs 4.4 ± 0.3; p = NS). Similarly, we note that no significant differences were found in age between patients with GD or HT (41 ± 4 years vs 50 ± 3 years; p = NS) and mild or severe AITD (50 ± 3 vs 46 ± 3; p = NS).

**Cell subpopulation analysis**

The presence of monosomy X in the major blood subpopulations was assessed in a subpopulation of women with SSc and AITD and in HS. Monosomy X was more frequent in all of the peripheral blood cell subpopulations of patients with autoimmune diseases than in those of the HS (Table IV). Furthermore, monosomy X was more frequent in the cells constituting the adaptive immune system (T and B lymphocytes) than in monocytes/macrophages, polymorphonuclear, or NK cells in both SSc (4.9 ± 0.6 vs 2.2 ± 0.6; p < 0.0001) and AITD (5.4 ± 0.6 vs 2.4 ± 0.5; p < 0.0001).

**Fetal microchimerism in monosomic WBC**

To understand whether X monosomic cells are indeed microchimeric (male) cells, we also investigated the presence of Y chromosome-specific sequences in monosomic WBC obtained from a nested cohort. The presence of Y chromosome-specific sequences in cells presenting monosomy X was assessed on a subpopulation of 11 women with male offspring (6 patients with autoimmune disease and 5 healthy controls). Cells with monosomy X did not have evidence for the presence of Y chromosome and thus were not microchimeric.

**Discussion**

We submit that genetic defects of the X chromosome may constitute the common mechanism leading to female predominance in autoimmune. Despite distinct features, autoimmune diseases share characteristics that might provide clues in the search for a common etiopathogenesis. For example, genetic factors are crucial in conferring susceptibility to autoimmune diseases, as indicated by the higher concordance rates in monozygotic compared with dizygotic twins. Some autoimmune diseases, such as the syndrome of autoimmune polyglandular endocrinopathy with candidiasis and ectodermal dysplasia (20), are characterized by well-defined mutations that lead to disease. However, most autoimmune diseases are considered to be polygenic, with multiple susceptibility genes contributing to produce the abnormal phenotype in concert with epigenetic or environmental factors. The clinical observation that several autoimmune diseases may coexist within the same subject or family suggests the existence of generic genetic factors that predispose individuals to lose tolerance. In general, it is now accepted that the onset of autoimmune disease represents the result of an unbalance between predisposing and protective genes.

The X chromosome contains a considerable number of sex- and immune-related genes which are essential in determining sex hormone levels and, more importantly, immune tolerance (9, 21). We also note that alterations of the X chromosome, such as monosomy or structural abnormalities, are known determinants of genetic disorders such as Turner’s syndrome and premature ovarian failure, commonly characterized by autoimmune features (11, 22). Furthermore, mutations in specific X-linked genes are known to cause a number of immunodeficiencies (23). Genome-wide linkage searches of autoimmune and inflammatory/immune disorders have identified a number of non-MHC loci that collectively contribute to disease susceptibility (24). Interestingly, a number of the published human positive linkages map nonrandomly into clusters on the X chromosome (25, 26). Similar to data on primary biliary cirrhosis (9), we report herein that women with other autoimmune diseases, represented by SSc and AITD, also have significantly higher X monosomy rates than healthy women of similar age. We note that these two autoimmune conditions have very different organ specificity, thus indicating once again that major defects of the X chromosome may be a common trait to autoimmunity. The monosomy rates in healthy women observed in the present study are slightly higher than those observed in our previous work (9). However, such differences were not statistically significant and do not reflect undiagnosed autoimmune diseases in this population; the clinical criteria for exclusion of autoimmunity were uniform in both studies. Finally, we note that the higher rates in patients with AITD or SSc are not secondary to medical treatment since patients were not receiving any medical treatment at the time of blood sampling.

In the three populations studied, we observed an increase of monosomy with age, similar to findings in our previous study (9). In the three groups studied, a time period of 25 years would be expected to produce only a 1% increase in monosomy rates, as indicated by the angular coefficients observed (Fig. 1). For these reasons, our analysis took into account the age differences between populations. Nevertheless, the differences observed remained statistically significant (Table III). In addition, similar to data in primary biliary cirrhosis, we note that X monosomy was found more

**Table III.** Age and X chromosome monosomy in women with SSc, AITD, and HSA

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Monosomy X (%)</th>
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<tbody>
<tr>
<td>SSc (n = 44)</td>
<td>58 ± 2*</td>
</tr>
<tr>
<td>AITD (n = 44)</td>
<td>48 ± 2*</td>
</tr>
<tr>
<td>HS (n = 73)</td>
<td>50 ± 2*</td>
</tr>
</tbody>
</table>

* Values are expressed as age-adjusted mean ± SE.
* *p = 0.0021 for SSc vs AITD and p = 0.0005 for SSc vs HS;
** *p < 0.0001 for all three comparisons.

![FIGURE 1. Correlation between peripheral white blood cell monosomy X rate and age in women with SSc and AITD and in HS.](http://www.jimmunol.org/Downloadedfrom/)

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commonly in cells from the adaptive immune system in both SSc andAITD. In contrast, prompted by the hypothesis that the mono-
somic cells might in fact be circulating male (microchimeric) cells (21), we also investigated the presence of Y chromosome-specific sequences. We emphasize that no cell with X monosomy was found positive for a Y chromosome, thus ruling out the possible confounding effect of the enhanced fetal microchimerism previously reported in several autoimmune diseases (7) including SSc. In the case of SSc (27), however, male microchimerism was determined in large volume in whole blood samples and the frequency of Y-specific sequences observed (11 microchimeric cells per 16 ml of whole blood) is consistent with our FISH analysis of peripheral WBC. Interestingly, male cells were also found in sites that are specific targets of autoimmunity in SSc, such as lung, kidney, and skin (28). Accordingly, our observation that male microchimerism is not the cause of X monosomy is particularly im-
portant (21).

Based on our findings, we hypothesize two genetic models pro-
viding a connection between autoimmune disorders and candidate genes. First, we propose a polygenic model with an X-linked major locus of susceptibility, with genes escaping X chromosome inac-
tivation as the major candidates. We also note that the sex ratios observed in autoimmunity could imply that the genes responsible are located on the Y chromosome. Further studies should empha-
size the study of sex chromosome defects in male patients with female predominant autoimmune diseases and in patients with male-predominant autoimmune diseases (such as type 1 diabetes).

Alternatively, we hypothesize a multigenic complex inheritance model in which Y chromosome genes play a protective role. In this scenario, genes located on the X chromosome will also explain the female predisposition as part of either hypothesis. A progressively acquired haploinsufficiency for specific X-linked genes in peripher-
al lymphocytes may be a common mechanism for immunose-
nescence, the state of dysregulated immune function of the elderly that contributes to the increased susceptibility to infection (29) and, possibly, to the appearance of autoantibodies (30). Overall, our data may constitute a bridge across several characteristics of autoimmune diseases, including genetic susceptibility, microor-
ganisms and molecular mimicry, and female predominance.

Disclosures
The authors have no financial conflict of interest.

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Infect. Dis. 31: 578–585.