Genetic Analysis of Systemic Lupus Erythematosus: 1. Detection of Disease-Associated Variant Proteins by Two-Dimensional Gel Electrophoresis

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Various genetic studies indicate that development of systemic lupus erythematosus (SLE) is regulated by the mode of multifactorial inheritance, i.e., by the overall effect of genes and environmental factors. To elucidate some variant genes involved in the polygenic system responsible for onset of SLE, we resolved and measured the protein components of lymphocytes and sera from inactive-SLE patients, their relatives, and normal controls, using two-dimensional gel electrophoresis. Intercomparison of polypeptide patterns between patients and controls revealed three major variations, two detected in lymphocytes and one in sera. These variations were present in 66–82% of the patients, in 20–36% of the control group, and in 41–64% of the relatives. In addition, nearly half of SLE patients, but only one of 19 normal controls, possessed all three SLE-associate variations, suggesting that these variant proteins may reflect in part the genetic factors contributing to development of SLE.

Systemic lupus erythematosus (SLE) is one of the typical autoimmune diseases characterized by the presence of various autoreactive antibodies and dysfunction of T- and B-lymphocytes. Population and family surveys, including twin studies, suggest a multifactorial mechanism in the development of SLE (1–5). Results of cross- and backcross-experiments in the animal model of SLE support this view (6–10). It is essential and necessary for understanding the pathoetiology to identify each allele of a polygenic system responsible for the onset of the disease, although attempts to detect such a minor effect of each of the polygenes usually meet with much difficulty.

The recent development of high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), originally described by O'Farrell (11), made it possible to detect variant polypeptides according to differences in relative molecular mass (Mr) and isoelectric point (pI) based on changes in amino acid residues in proteins. This systematic procedure has been used to investigate minor genetic alterations, such as abnormalities of protein synthesis reflected in a small change in amino acid sequence. In fact, this technique has been used for the analysis of major gene products in hereditary diseases such as familial polyposis of the colon (12), muscular subaortic stenosis (13), and Bloom syndrome (14), and also for the research on genetic polymorphisms of proteins in human fibroblasts (15, 16) and lymphocytes (17). In the field of rheumatic diseases, Willard et al. (18) analyzed human leukocyte proteins in patients with rheumatoid arthritis by 2D-PAGE and found several disease-associated proteins that appeared to be indicators of disease activity. Recently, lymphocyte proteins from New Zealand Black (NZB) mice that spontaneously develop autoimmune disease similar to human autoimmune hemolytic anemia were analyzed by 2D-PAGE by Leal et al. (19). They found two strain- and age-related variant polypeptides (Mr 12 500 and 10 500) in the cytoplasm of B and null cells from older NZB mice. However, it is unclear whether or not these variations may reflect the genetic change involved in the pathogenesis of the disease, because those described above detected by 2D-PAGE varied according to the activity of the disease.

Genetic alterations responsible for disease may be reflected in specific proteins that could be altered qualitatively and (or) quantitatively. The present study was designed to elucidate some of the variant gene products involved in the polygenic system contributing to development of SLE, by use of 2D-PAGE. For this purpose, we compared some proteins appearing on the 2D-gel in phytohemagglutinin (PHA)-stimulated lymphocytes and in sera from inactive-SLE patients with those from healthy individuals, and detected some proteins showing a qualitative or quantitative difference. Because the relatives of patients with SLE, even without overt onset, frequently show SLE-like immunological abnormalities, we also analyzed these variant proteins in the first-degree relatives of the patients, to study the relation between these variations and genetic susceptibility to the disease.

Materials and Methods

Subjects: The experiments were performed on human peripheral blood lymphocytes and sera from 38 patients with SLE, from 19 healthy individuals as normal controls, and from 38 first-degree relatives of 12 propositi with SLE. Among patients, there were two pairs of sister-onset and one pair of mother- and daughter-onset. The patients, ages 10 to 60 y (mean 30), were all female except for one. All of the patients fulfilled the 1982 revised criteria for the classification of SLE (20). Because the purpose of our study was to detect the genetic variants responsible for the development of the disease, we used only patients that were in remission, to exclude any factors related only to active disease. At the time blood was sampled, the patients were all in remission, none of them showing active nephritis, neurological involvement, or any other signs of active disease. Most of the patients were receiving a low dose of steroid (prednisolone, 5–20 mg/day); the others were receiving no medication. The normal controls, ranging in age from 18 to 25 y (mean 20), consisted of laboratory personnel and medical students, all women but one. The relatives, ages 21 to 77 y (mean 45), were 19 men and 19 women, none with any symptoms.

Lymphocytes preparation for 2D-PAGE: We drew 10 mL of whole blood (heparin anticoagulant) and isolated the lymphocyte-enriched fraction by Ficoll-Paque gradient centrifugation (Pharmacia Fine Chemicals AB, Uppsala, Sweden). After being washed, 3 × 10^8 lymphocytes were suspended in 5 mL of RPMI 1640 medium (Gibco Labs., Grand Island, NY) supplemented with 100 mL of fetal calf serum (Gibco), 100 mg of penicillin, 50 mg of gentamicin, and 20

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1 Nonstandard abbreviations: SLE, systemic lupus erythematosus; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; NZB, New Zealand Black; PHA, phytohemagglutinin.

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mg of PHA-p (Difco, Detroit, MD) per liter. The cells were cultured for four days in a 25 cm² plastic flask (Falcon, Cockeysville, MD) at 37 °C in a humidified atmosphere containing five volumes of CO₂ per 100 volumes.

At the end of this incubation, lymphocytes were harvested and washed once with Hank's balanced salt solution. After pre-incubation in methionine-free minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) for 1 h, the cells were internally labeled with 100 μCi of [³⁵S]methionine (1000–1500 kCi/mol; Amersham International, Amersham, England) per milliliter, in the same medium supplemented with 100 μL of fetal calf serum per liter, for 12 to 15 h. Then the cells were washed three times with phosphate-buffered isotonic saline. Cell pellets were lysed in 100 μL of lysis buffer (per liter: 5 g of Nonident P-40, 150 mmol of NaCl, 5 mmol of EDTA, 1 mmol of phenylmethylsulfonyl fluoride, 50 mmol of Tris HCl at pH 7.0) with vigorous vortex-mixing and incubated for 30 min, on ice. The lysates were stored at −80 °C until used. Radioactivity incorporated into the proteins was determined by measuring the trichloroacetic-acid-precipitable activity. The samples containing 2 × 10⁶ to 1 × 10⁷ counts/min of [³⁵S]-labeled proteins were prepared for 2D-PAGE by dissolving the proteins in a solution containing, per liter, 9.2 mol of urea, 20 g of Nonident P-40 surfactant, 50 mL of 2-mercaptoethanol, and 20 mL of ampholytes (pH range 3.5–9.5; LKB, Bromma, Sweden).

2D-PAGE: The lymphocyte lysates or serum proteins were analyzed by 2D-PAGE according to the method of O'Farrell (11). Briefly, the first-dimension separation was performed with the isoelectric focusing gel containing ampholytes (LKB) with a pH range of 3.5 to 9.5. We determined the pl gradient for isoelectric focusing by checking the pH of the gel with an electrode; the gel was cut into segments 10 mm long. For the second-dimension separation we used a 100 g/L polyacrylamide gel containing 1 g of sodium dodecyl sulfate per liter. Molecular-mass standards (Mr, 13 700 to 94 000) were from Pharmacia Fine Chemicals. We also used actin (Mr, 45 000) as a reference marker on the gel.

Gels were fixed, stained, and destained as described previously (21). For direct autoradiography, the gels were dried and exposed to Kodak XR film at room temperature for two to four weeks. After exposure, the films were developed and fixed according to the manufacturer's instructions.

Analysis of serum proteins: Serum was separated from peripheral blood and stored at −80 °C until used. It then was passed through an affinity column of Blue Sepharose CL-4B (Pharmacia) to remove albumin (22). 2D-PAGE was then performed as described above. Finally, the gels were silver-stained according to a modified method (23).

Statistics: For statistical analysis we used the χ² contingency table method, unless otherwise noted.

Results

We could consistently detect about 500 polypeptide spots in PHA-stimulated lymphocytes from either normal controls (Figure 1) or SLE patients. The number and the location of the spots on the gel showed very high reproducibility when the same individual was tested. When all of these polypeptide spots were carefully compared in quality and quantity between patients and controls, two variant polypeptides, designated Lp 1 (Mr, 70 000/pl 7.0) and Lp 17 (Mr, 41 000/pl 6.0), showed significant differences in their appearance on the gel.

As shown in Figure 2, Lp 1 was a dense spot in most of the SLE patients, whereas normal individuals usually showed only a weak spot. Such an increase in the intensity of this Lp 1 spot was seen in 82% of the patients, but in only 36% of the controls (P < 0.025; Table 1).

Spot Lp 17 was typically seen in normal individuals, but typically not in SLE patients (Figure 2): it could be detected in only 34% of the patients but in 71% of the control group (P < 0.05; Table 1). Also as indicated in Table 1, the frequencies of these variations in the first-degree relatives of SLE patients showed the intermediate values between those in SLE group and in normal control group (Lp 1: 41% and Lp 17: 53%).

2D-PAGE patterns for sera from these populations were analyzed in the same way. Of about 150 spots detectable on the gel, one, designated S1 (Mr, 91 000/pl 5.8), showed a significant difference in appearance between patients and controls. Typically, this spot was apparent in sera from normal individuals, but not in sera from SLE patients.
Table 1. Frequency of SLE-Associated Proteins in PHA-Stimulated Lymphocytes and Sera

<table>
<thead>
<tr>
<th>SLE-associated proteins*</th>
<th>Lymphocytes</th>
<th>SLE patients</th>
<th>Relatives</th>
<th>Normal controls</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M, pl</td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
</tr>
<tr>
<td>Lp 1</td>
<td>70K/7.0</td>
<td>22/27 (82)</td>
<td>12/29 (41)</td>
<td>4/11 (36)</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Lp 17</td>
<td>41K/5.0</td>
<td>10/29 (34)</td>
<td>17/32 (53)</td>
<td>12/17 (71)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sera</td>
<td>31K/5.8</td>
<td>3/17 (18)</td>
<td>9/25 (36)</td>
<td>4/5 (60)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*After careful comparison of all of the polypeptide spots seen on 2D-gel between SLE patients and controls, quantitatively and qualitatively, the three proteins described here were shown to have significant differences between the two groups. A few cases that showed indeterminate pattern on 2D-gel as to each of these three proteins were excluded in this table.

aP-value was estimated from x²-analysis using frequency data of patients and normal controls.

(Figure 3). As shown in Table 1, this polypeptide was detected in only 18% of the patients, but in 80% of the controls (P<0.05). The frequency of this spot in the relatives was intermediate (36%) between that in patients and in controls, as expected (Table 1).

The results, taken together, indicated that these three SLE-associated variations were present in 66–82% of SLE patients, in 20–36% of the controls, and in 41–64% of the relatives. It should be noted that, in Table 1, we excluded some cases that showed an indeterminate pattern with respect to each of these three proteins.

Although the patients analyzed here were in remission, a few of them were also studied while in the active state. However, the same individuals showed no difference in 2D-PAGE pattern between the active and inactive state, suggesting that there is little if any association of these variations with active disease per se (data not shown).

To examine the combined effect of these three traits, we calculated scores for every patient according to the presence or absence of apparently positive Lp 1, negative Lp 17, and negative S1. For example, if a patient was positive for Lp 1, she was given a score of one. If the same patient was negative for Lp 17, she was given another score of one. Finally, if the same patient was negative for S1, she was given an additional score of one. Therefore, her total score would be three. Conversely, if a patient showed none of any of the three traits, she was given a score of zero. It should be noted that there were indeterminate cases in which we could not determine whether the person had or did not have the above traits. In such cases, a score of 0.5 was given. As a result, the average score was 2.3 ± 0.1 (mean ±SEM) for the SLE patients, 0.9 ± 0.2 for the normal controls, and 1.6 ± 0.1 for relatives (Figure 4). The difference in average score between patients and controls was statistically significant (P <0.001; Student’s t-test). Figure 4 indicates also that 47% of SLE patients had full scores (score 3), and that more than half of the normal individuals had a total score of less than 1.0. However, there were a few discrepancies; that is, a few patients had low scores and a few normal individuals had high scores.

Discussion

Although there have been great advances in our knowledge of the etiology of SLE, its fundamental mechanism concerning the development of the disease and production of autoantibodies has not yet been well elucidated. However, it is well known that some genetic factors are associated with the etiology of SLE, for the following reasons: (a) its familial occurrence (1–3); (b) the increased incidence of immunological abnormalities such as hypergammaglobulinemia and the presence of autoantibodies in relatives of patients (1, 2); and (c) the high rate of concordance (63%) of SLE in monozygotic twins, much higher than that (13%) reported in

Fig. 3. Comparison of serum protein patterns from SLE patient and normal control
When all of the spots were compared between SLE patients (upper left) and normal controls (upper right), the spot indicated as S1 was shown to have a significant difference between the two groups. Enlarged fields including this spot are shown in the lower part. Alb: albumin, AT: α1-antitrypsin, ACT: α1-antichymotrypsin, HS: α2-glycoprotein, HAP: haptoglobin β-chain

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Fig. 4. Distribution of three SLE-associated protein patterns in each individual in the SLE patient group, relative group, and normal control group

In an individual, each of SLE-associated protein patterns (apparent presence of Lp 1, absence of Lp 17, and absence of S1) gives each a score of 1, and normal pattern gives score 0, and intermediate pattern gives score 0.5. So, individual score expresses additive number of SLE-associated protein patterns in each person, ranging from 0 (having none of SLE-associated protein patterns) to 3 (having all of three SLE-associated protein patterns). One circle expresses one person, square with bar expresses mean ± SEM

dizygotic twins (4, 5). In addition, there have been many reports of experiments on animal models showing SLE-like autoimmune features that suggest there is an essential role of genetic factors in the pathogenesis of SLE (6, 10). In fact, some experiments on recombinant inbred mice established from these animal models indicate that expression of various autoimmune features like those seen in SLE patients requires the involvement of multiple, at least six, different genetic loci (10). Moreover, an extensive population survey on the development of SLE revealed that the results calculated from Edwards’ simulation model was more likely to be compatible with a multifactorial mechanism (5). Thus the evidence suggests that multiple genetic factors (polygenes) in cooperation with some environmental factors may well be responsible for development of SLE. In the theory of multifactorial inheritance (24), the disease becomes overt in each individual when the sum of those factors exceeds a “threshold.”

Usually the effect of each of the polygenes is too weak to be recognized, but recent progress in the method of high-resolution 2D-PAGE may make it possible to detect the products of such minor genes. In the present study, we have used 2D-PAGE to look for genetic factors associated with the onset of SLE. We found three SLE-associated polypeptides that may be gene products related to the development of SLE, because of significant differences in the frequencies between the patient group and the normal control group and because the frequencies of these proteins in the relatives was intermediate between those in the patients and in the controls. The relative risk of the disease developing in persons with SLE-associated variation of three polypeptides, Lp 1, Lp 17, and S1, was 7.7, 4.5, and 18.6, respectively. The relative risk with respect to the spot S1 seems to be very high and may be overestimated, because only a small number of normal controls were analyzed (Table 1). Although these polypeptides remain to be characterized functionally, we believe that these three SLE-associated proteins may be the products of a significant part of the polygenic system contributing to the development of SLE.

However, in our study there were several contradictory cases, that is, patients with a low score and normal individuals with a high score. We presume that additional factors, known or unknown, may exist and play some role in the development of the disease. Recent progress in immunogenetics has revealed several genetic markers considered to be associated with the pathogenesis of SLE, such as major histocompatibility complex (25), immunoglobulin allotype (26), and C3b receptor expression (27). These may be the candidates for the additional factors, because chemical characteristics of Lp 1, Lp 17, and S1 were supposed to be different from those of such immunogenetic products. Now, we are studying the combined relation of three variant polypeptides and an additional three immunogenetic markers to the development of SLE.

References