Existence of an IgG component of naturally occurring HLA class I antibodies that are not directed against self-antigens in human serum

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Abstract

We compared the frequency of IgG type of HLA class I antibodies between patients with systemic lupus erythematosus (SLE) and healthy controls, using a highly sensitive FlowPRA method. Sixteen of 130 normal healthy males and two of 10 normal females without a history of pregnancy (none had ever been transfused) possessed HLA class I antibodies. In SLE, male, but not female patients, showed a significant increase in the frequency of the antibodies, as compared with the corresponding controls. The antibodies did not appear to be involved in the development of SLE because of no substantial relationship to the incidence of cytopenia or SLE disease activity index score. Each individual had 1 to 31 types of HLA class I antibodies. Interestingly, HLA class I antibodies did not correspond to the individual's own HLA antigens. Eight of 32 types of HLA class I antigens detected were rare in the Japanese population. These results suggest that an IgG component of naturally occurring HLA class I antibodies exists in human serum, and that these antibodies are not antibodies against self-antigens.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease affecting multiple organ systems such as the skin, joints, kidneys, and nervous system. Hematologic manifestations are additional typical feature: hemolytic anemia, leukocytopenia, lymphocytopenia, and thrombocytopenia occur in approximately 15% to 45% of the patients (1−3). Earlier studies implied a pathogenic relevance of some specific autoantibodies in SLE. For example, anti-*ds*DNA antibodies are involved in the pathogenesis of immune complex-mediated glomerulonephritis. As a cause of lymphocytopenia, anti-*SSA/Ro* antibodies, anti-ribonucleoprotein antibodies, and anti-*ds*DNA antibodies have been postulated to disturb the cell cycle and lead to apoptosis after penetrating the cell (4). An excessive destruction due to antibodies directed against platelet membrane antigens is considered to be a main cause of thrombocytopenia in SLE in addition to failure of production and abnormal distribution (5, 6).

Neonatal alloimmune thrombocytopenia is caused by maternal alloantibodies transferred via the placenta and directed against paternally inherited antigens located on fetal platelets (7, 8). The antigens usually implicated are human platelet antigens, and occasionally HLA class I antigens. HLA alloimmunization is also important for a poor post-transfusion thrombocyte increment (9−12). Recently, we reported a boy with relapsed leukemia who developed fatal platelet transfusion refractoriness due to the production of HLA class I antibodies after two hematopoietic stem cell transplantations from his HLA-genotypically identical mother (12). The most likely explanation was the increased ability of the mother' cells to respond to HLA antigens, because she had antibodies reactive to multiple HLA class I molecules prior to the transplantation.

SLE occurs predominantly in young women, particularly in females of child-bearing age. Accumulating data have demonstrated that specific HLA haplotypes are implicated as a key genetic element in the susceptibility to SLE (13, 14). Taken together, we speculated on a possible role for antibodies against HLA antigens in the development of SLE. We therefore compared the frequency of HLA class I antibodies between SLE patients and healthy controls, using a highly sensitive technique for the detection and definition of the antibodies.

Methods

Serum Samples

Peripheral blood samples were harvested after obtaining fully informed consent from 47 patients with SLE［31 female patients (mean age: 34, range: 11 to 69 years) ; 16 male patients (31, 9 to 81 years)］, and 149 healthy volunteers［19 healthy females (31, 22 to 53 years) ; 130

healthy control males (35, 20 to 54 years) ］. Blood samples from 17 patients were obtained prior to immunosuppressive therapy, whereas samples from 30 patients were collected after immunosuppressive therapy, including prednisolone treatment. Ten of 17 untreated patients had leukocytopenia (< 4 x 10^9/l), lymphocytopenia (< 1.5 x 10^9/l), hemolytic anemia and thrombocytopenia (< 100 x 10^9/l), alone or in combination. The SLE disease activity index score (15) of 17 untreated patients was 18.9±13.6 (mean±SD, 5 to 59). All sera were heat inactivated by placing in a water bath for 30 minutes at 56°C. Aliquots were frozen at -80°C until use.

HLA typing

HLA-A, -B, and -Cw loci were determined with DNA-based typing (12).

Detection of antibodies against HLA class I antigens

For the HLA class I antibody screening test, we used a panel of FlowPRA beads, which are microparticles (2 to 4 μm in diameter) coated with purified HLA class I molecules (FlowPRA, code FL1-30, One Lambda Inc., Canoga Park, CA), as described previously (12). To identify the specificity of HLA antibodies in serum samples, we used the FlowPRA single-antigen class I HLA antibody detection test (FlowPRA, code FL1HD) according to the procedure described previously (12). For samples with high nonspecific binding, Adsorb Out™ beads were used. FlowPRA control beads were the same microparticles, but were not coated with HLA antigen. Pooled serum collected from five healthy male donors whose blood groups were AB was used as a negative control for the analysis, and serum from a multiply transfused patient who had multiple HLA antibodies detected by anti-human immunoglobulin-lymphocyte cytotoxicity test as a positive control.

In the HLA class I antibody screening test, control beads were used to monitor the background level of nonspecific binding for the test sera. First, we mixed 5 μL of FlowPRA beads and 1μL of control beads with 20 μL of serum, and incubated the mixture for 30 minutes at room temperature with gentle rotation. The beads were washed three times with 1 mL of FlowPRA wash buffer through centrifugation at 9000 x *g* for 2 minutes, and then incubated with 100 μL of a fluorescein isothiocyanate-conjugated $F(ab)_2$ fragment of goat anti-human IgG (Fcγ fragment-specific) for 30 minutes. The beads were washed twice with 1 mL of wash buffer, and then 0.5 mL of a fixing solution (phosphate-buffered saline with 0.5% formaldehyde) was added. The FL1 fluorescence of 10,000 events was analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). In this analysis, the fluorescent intensity of FlowPRA beads reacted with each serum was compared with that of the same beads reacted with a negative control serum. The negative region and histogram marker 1 (M1) were defined as follows: negative region, the region with at least the lowest

99 % of events of the sample sensitized with the negative control; and M1, the remaining region.

In the FlowPRA single-antigen class I HLA antibody detection test, one panel was composed of 32 microbeads coated with purified recombinant HLA Class I single antigens divided into four groups to further identify the specificity for HLA Class I IgG antibody by flow cytometry. Each group contained control bead coated with no antigens. To set the cut-off line before each sample analysis, we ran each group of beads that reacted with the negative and positive control sera. We tested all samples twice with the same results.

Statistical analysis

To determine the significance of differences between two independent groups, we used the unpaired *t*-test or the Mann-Whitney-*U* test when data were not normally distributed. We evaluated the distribution of clinical characteristics between the two groups with the chi-square test.

Results

To examine whether patients with SLE had HLA antibodies in their circulation, we performed a FlowPRA screening test on the serum samples. The results are presented in Figure 1 and Table 1. HLA class I antibodies were detected in sera of 11 (35.5%) of 31 female patients, and in sera of 6 (37.5%) of 16 male patients. On the other hand, of 19 healthy females, 2 without a history of pregnancy as well as 4 with a history of pregnancy had the antibodies. Surprisingly, 16 (12.3%) of 130 healthy control males also possessed HLA antibodies. Statistical evaluation revealed that the frequency of HLA class I antibodies was higher in male SLE patients than in male controls. No substantial difference, however, was found between female patients and controls.

We then attempted to determine the types of HLA class I antibodies in circulation. Figure 2 shows a representative reaction of FlowPRA single-antigen Class I beads with a patient's serum sample. HLA-specific antibodies and HLA types in SLE patients and healthy male controls are shown in Tables 2 and 3. Antibodies against 1 or 2 HLA class I antigens were found in 7 of 17 SLE patients. Sera of the remaining 10 patients responded to 3 to 21 antigens. On the other hand, antibodies against 1 or 2 HLA class I antigens were identified in 10 of 16 healthy male controls. Sera of the remaining 6 controls reacted to 3 to 31 antigens. It is of interest that HLA class I antibodies did not correspond to the individual's own HLA antigens in any of the 12 SLE patients and 16 control males whose HLA antibodies and typing were determined. Additionally, the sera did not cross-react with the FlowPRA beads, different from a post-transplant patient with platelet transfusion refractoriness caused

by HLA alloimmunization (12). Eight types of HLA class I antigens to which serum samples responded were rare in the Japanese population: HLA-A23, A25, A34, B8, B18, B45, B49, and B57 (16).

We examined whether the presence of HLA class I antibodies was related to cytopenia in SLE, using sera obtained from 17 SLE patients prior to immunosuppressive therapy, including prednisolone treatment. Two (33.3%) of 6 HLA antibody-positive patients and eight (72.7%) of 11 antibody-negative patients had leukocytopenia, lymphocytopenia, hemolytic anemia and thrombocytopenia, alone or in combination (p =0.162). Furthermore, there was no significant relation between the existence of HLA antibodies and the SLE disease activity index score (15): 21.5±20.4 (mean±SD, 7 to 59) in the HLA antibody-positive group vs. 17.5±9.1 (mean±SD, 5 to 36) in the HLA antibody-negative group (p =0.575). We then compared the positivity of HLA class I antibodies before and after immunosuppressive treatment. Six (35.3%) of 17 patients with no history of treatment were positive for HLA antibodies, whereas 11 (36.7%) of 30 patients during immunosuppressive therapy had the antibodies (p = 0.925). In addition, there was no difference in the numbers of HLA antibodies per patient between the two groups: 3.2±1.9 (mean±SD, 1 to 6) in the non-treatment group vs. 5.3±5.6 (mean±SD, 1 to 21) in the post-treatment group (p =0.394).

Since it was possible that our observation was just due to higher levels of IgG commonly found in SLE patients, we compared the IgG values of HLA antibody-positive groups and those of HLA antibody-negative groups. There was no significant difference between the two groups: 1938.7±614.7 mg/dl in positive SLE patients vs. 1750.1±807.3 mg/dl in negative patients (p=0.462), and 1306.3±276.8 mg/dl in positive controls vs. 1361.4±310.4 mg/dl in negative controls (p=0.502).

We finally examined the possibility that HLA antibodies were generated through an immune response, including feto-maternal immunization. The results for a healthy family with no history of transfusion are presented in Table 4. The mother's sera responded to HLA-B8 antigen, which neither the husband nor son carried. The HLA-B35 antibody in serum from the husband and HLA-A31 antibody in serum from the son were antibodies against the relevant antigens of the mother, respectively; however, the other HLA antibodies of the father and son did not correspond to the maternal antigens.

Discussion

In the present study, the frequency of HLA class I antibodies in female SLE patients was similar to the value of the corresponding controls, while a substantial difference between male

SLE patients and controls was observed. Additionally, the existence of HLA antibodies was not significantly related to the incidence of cytopenia or the SLE disease activity index score (15) before the initiation of immunosuppressive treatment. Accordingly, HLA class I antibodies do not appear to be associated with the development of SLE, although the involvement of HLA class II antibody remains to be determined.

An unexpected finding in the current study was that 16 of 130 normal healthy males and 2 of 10 normal females without a history of pregnancy possessed HLA class I antibodies in their circulation. The individuals with circulating HLA antibodies had never been transfused and had never been pregnant with a fetus carrying the relevant antigen. Several investigators (17−19) have reported that antibodies against HLA-A2, A3, B7, B8, and B13 were detected as natural antibodies by the classic lymphocytotoxicity test, and that the activity of the natural antibodies could be found in the IgM fraction, whereas the activity of the immune antibodies generated with pregnancy was in the IgG fraction. In our study, 32 types of IgG HLA class I antibodies were detected in 16 healthy male controls, and included antibodies against five types of HLA antigens described above. Coupled with no pathogenic significance of the antibodies in SLE, the IgG component of HLA class I antibodies may be naturally occurring.

There are several possibilities for the production of naturally occurring HLA class I antibodies. (1) Feto-maternal immunization. In the results of family analysis, the son carried 4 types of HLA antibodies that did not correspond to the antigens of the mother as well as one type of antibody against the relevant maternal antigen. (2) Antibody response arising through sexual contact. Eight types of HLA class I antigens to which the serum samples responded were rare in the Japanese population. Thus, antibody generation may not be completely explained by the possibility of (1) and (2). (3) Autoantibodies. Autoantibodies of IgM, IgG and IgA classes, reactive with a variety of serum proteins, cell surface structures and intracellular structures, are naturally found in all normal individuals (20, 21). In the present study, HLA class I antibodies did not correspond to the individual’s own HLA antigens in any of the SLE patients or normal control males analyzed. These results imply that naturally occurring antibodies are not always produced as an immune response to individual self-antigens.

Preformed natural IgG antibodies against AB antigens are present at birth as a result of diaplacental transport of maternal antibodies, and disappear from the neonate after 2 weeks. At approximately 8-12 weeks, the newborn infant starts producing IgM and IgG of its own. Adult levels are reached by the age of 5-10 years. One commonly held hypothesis is that the production of antibodies to A and/or B determinants is a response to the presence of A and/or B saccharides on bacteria or other microorganisms that colonize the infant gastrointestinal

tract. Scofield *et al.* (22) demonstrated that HLA-B27 shares short amino acid sequences with proteins from enteric bacteria. In addition, segment 807-816 of the EB virus glycoprotein gp110 has been shown to contain a six-amino acid stretch (EQKRAA) that perfectly matches HLA Dw4 (23). A Japanese study of the EB virus seroprevalence in recent years revealed that the positivity rate of viral capsid antigen-IgG was 86% at 10 -12 years old, 96% at 20 - 22 years old, and reached 100% at 25 - 27 years of age (24). Actually, IgG antibody against EB-virus capsid antigen was positive in all 8 patients (24 - 81 years old) who had HLA class I antibodies. Taken together, it is likely that naturally occurring IgG HLA class I antibodies are generated as a result of an immune response to environmental agents such as bacteria and viruses, which are antigenetically identical with or similar to HLA antigens. These antibodies may be an important part of the first line of defense against hematological spreading infections.

HLA class I antibodies are important for a poor post-transfusion thrombocyte increment. The present study revealed that approximately 10-20% of individuals possessed circulating HLA class I antibodies despite no history of transfusion in FlowPRA determinations. In our patient with relapsed leukemia who received peripheral blood stem cell transplantation from the mother, the FlowPRA assay, but not anti-human immunoglobulin lymphocyte cytotoxicity test, could detect the generation of low titer of multiple HLA class I antibodies, which might cause the fetal platelet refractoriness (12). Additionally, Karpinski et al. (25) reported that in 18 (12.6%) of 143 renal transplant recipients, anti-HLA antibodies were not detected by anti-human globulin enhanced cytotoxicity techniques, but were positive in FlowPRA determinations, and that 6 (33.3%) of 18 patients experienced early graft loss. Taken together, more sensitive techniques for detecting anti-HLA antibodies may be useful for early and definitive diagnosis of platelet transfusion refractoriness.

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Figure legends

Figure 1. Reactions of FlowPRA beads coated with 30 purified HLA class I molecules to sera of SLE patients and a control

For the screening of HLA class I antibodies in sera, we used a panel of FlowPRA beads, which are microparticles coated with 30 purified HLA class I molecules. Horizontal axis, fluorescence intensity; vertical axis, number of beads. (A) Control; (B) patient No.4; (C) patient No.9; (D) patient No.13.

Figure 2. Reactions of FlowPRA single-antigen class I beads to the patient's serum

To identify the specificity of HLA Class I IgG antibody, we used a panel composed of 32 microbeads coated with purified single HLA Class I antigens. The results show the reaction of the sera of patient No. 2 with 4 groups of FlowPRA single-antigen class I beads. (A) Group 1-beads; (B) Group 2-beads; (C) Group 3-beads; (D) Group 4-beads. The horizontal axis shows fluorescence intensity. FlowPRA single-antigen beads consist of a mixture of different colored beads with different fluorescent properties. These beads can be excited at 488 nm, generating a maximum emission of approximately 580 nm, similar to phycoerythrin, which can be detected by the FL2 channel. Because different beads generate different FL2 channel shifts, different colored beads in a group can be separated by a flow cytometer on the FL2 channel.

Table 1. Frequency of HLA class I antibodies in SLE patients and healthy individuals

(1) Whole patients and controls

	Female			Male		
	Patients (n=31)	Controls (n=19)		Patients (n=16)	Control (n=130)	
HLA antibodies (+)	11 (35.5%)	6 (31.6%)	*p*=0.777	6 (37.5%)	16 (12.3%)	*p*=0.017
HLA antibodies (–)	20 (64.5%)	13 (68.4%)		10 (62.5%)	114 (87.7%)	

(2) Female patients and controls with or without pregnancy history

	with pregnancy history			without pregnancy history		
	Patients (n=18)	Controls (n=9)		Patients (n=13)	Control (n=10)	
HLA antibodies (+)	10 (55.6%)	4 (44.4%)	*p*=0.695	1 (7.7%)	2 (20%)	*p*=0.560
HLA antibodies (–)	8 (44.4%)	5 (55.6 %)		12 (92.3%)	8 (80%)	

Table 2. HLA class I antibodies and HLA types in SLE patients

Patients Group	Patient I.D.	HLA class I antibodies	HLA type					
			A		B		Cw	
Female	1	B18, B35, B51, B52, B62	2402	3303	4403	5401	0102	1403
	2	B7,B13, B27, B60	ND*		ND		ND	
	3	A3, A25,A26, A32, A33, A34, A68, B7,B13, B14, B18, B27, B35, B38, B44, B45, B49, B51, B52, B55, B62	ND		ND		ND	
	4	A30, A31, B7, B13, B27, B60	0206	3303	1518	4403	0704	1403
	5	B7, B27	1101	2402	5101	6701	0702	1402
	6	B13, B35, B49, B51, B52, B57, B62	ND		ND		ND	
	7	A2, B7, B27, B55, B60	ND		ND		ND	
	8	B49, B51, B52	2402	2601	0702	–	0702	–
	9	B13, B44, B45 , B49, B60	2402	–	5201	–	1202	–
	10	B7, B13, B27, B60, B62	1101	2402	3901	–	0702	–
	11	B57, B62	0206	2601	3901	5502	0102	0702
Male	12	B8, B45	0201	1101	1501	4801	0401	0803
	13	A3	0201	0206	1301	4006	0304	0801
	14	B8	0207	2402	4601	5502	0102	–
	15	B45	0206	2601	4002	5101	0304	1402
	16	B18, B35, B45,B51	1101	–	1501	4001	0401	0702
	17	B44, B45	ND		ND		ND	

Underline, rare HLA antigens in the Japanese population. *ND, not determined.

Table 3. HLA class I antibodies and HLA types in male controls

Control I.D.	HLA class I antibodies	HLA type					
		A		B		Cw	
1	B7, B8	2402	3303	5101	4403	1402	1403
2	A25, A26, A34, A68	2402	3101	5901	0702	0102	0702
3	B35, B44, B45, B51, B52,	0101	0210	5504	4006	0801	0303
4	B8, B35	2602	3303	4002	4006	0801	0304
5	A3, A31, A34, A68, B44	0201	2402	4601	5201	0102	1202
6	B35, B45	0207	—	4601	—	0102	—
7	B35	2402	3101	5201	4001	0304	1201
8	B45	2402	1101	4801	1501	0401	0803
9	B8	2402	—	5201	—	1202	—
10	A68	0206	2601	3501	4002	0303	0304
11	B8	2402	3101	5401	4002	0102	0304
12	B8	2402	2601	5101	1501	0303	1402
13	A23, A24, B45	0207	—	4601	4801	0102	0801
14	A2, B7, B18, B55, B57	2402	3303	5201	1502	0801	1202
15	B44, B45	0207	3101	3501	4601	0102	0401
16	A1, A3, A11, A23, A24, A25, A26, A29, A30, A31, A32, A33, A34, A68, B7, B8, B13, B14, B18, B27, B35, B38, B44, B45, B49, B51, B52, B55, B57, B60, B62	0201	0206	4801	4006	0801	0803

Underline, rare HLA antigens in the Japanese population.

Table 4. Familial analysis of HLA class I antibodies and HLA types

	HLA class I antibodies	HLA type					
		A		B		Cw	
Father	B35, B45	0207	–	4601	–	0102	–
Mother	B8	3101	2402	3501	5201	0401	1202
Son	A3, A31, A34, A68, B44	0207	2402	4601	5201	0102	1202

Son is healthy control No.5 in Table 3. No family member had a history of transfusion.