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In vitro removal of anti-HLA IgG antibodies from highly sensitized transplant recipients by immunoadsorption with protein A and protein G sepharose columns: a comparison

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Abstract. In the present study we compared the capabilities of sepharose-bound protein A versus protein G columns to remove in vitro lymphocytotoxic anti-HLA antibodies from sera of four highly sensitized renal transplant recipients (PRA ≥ 70%). In none of the patients were protein A sepharose columns capable of completely removing anti-HLA antibodies, as demonstrated by the presence of residual alloreactive lymphocytotoxic activity in IgG 3 antibodies containing fractions eluted at pH 7. In contrast, no residual anti-HLA lymphocytotoxic antibody activity was found in fractions eluted at pH 7 from protein G columns. These data demonstrate that: (1) IgG 3 antibodies can be partly responsible for lymphocytotoxic anti-HLA reactivity in some sensitized renal transplant recipients and (2) at least in this patient category, in vitro immunoadsorption with protein G is more efficient than protein A sepharose columns in completely removing anti-HLA IgG antibodies from sera.

Key words: Highly sensitized recipients – Immunoadsorption – Anti-HLA IgG antibodies

One of the major problems in renal transplantation is represented by the growing number of highly sensitized renal transplant recipients (HSRTR) on the waiting list. These are patients whose sera generally react against more than 70% of a random panel of allogeneic peripheral blood lymphocytes. These patients have little chance of receiving a compatible kidney graft from a negative crossmatch donor because their sera contain alloreactive lymphocytotoxic antibodies directed to a large number of public and private HLA specificities. Over the past years, a number of strategies, such as plasma exchange [8, 12] and extracorporeal immunoadsorption with sepharose-bound staphylococcal protein A columns [6, 10, 11] prior to transplantation, have been used in order to remove anti-HLA IgG antibodies from the sera of HSRTR. These procedures, however, were found to be effective only in some HSRTR and, up until the present, their usefulness has not been unequivocally proven. In this study, the capability of protein A sepharose CL-4B columns to adsorb anti-HLA cytotoxic IgG antibodies from sera of four HSRTR on the waiting list for a first or second kidney transplant in our center has been analyzed by means of affinity chromatography and compared with antibody removal achieved by protein G sepharose 4FF columns.

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Materials and methods

Patients

Four transfused dialysis patients who were on the waiting list for a first or second kidney transplant and whose sera showed, at serial determinations during a 2-year period, a consistently high reactivity (PRA ≥ 70%) against an allogeneic HLA-typed peripheral blood lymphocyte panel (PBLP) were selected for this study. The patients' clinical and immunological characteristics are shown in Table 1.

Serological tests

No allo-or autoreactive IgM antibodies were detected in the above patients' sera following pretreatment with the reducing agent dithiothreitol (DTT) [3, 5] and testing against autologous and allogeneic PBL by means of an antihuman/globulin (AHG)-augmented complement-dependent cytotoxicity (CDC) assay [7]. When analyzed by means of serial dilution experiments against our PBLP [10, 13], sera from all the patients showed the presence of type I anti-HLA antibodies, as their antibody specificity was limited to two class I HLA antigens with a broad crossreactivity [12]. In fact, in all cases, after diluting the patients' sera, the broad crossreactivity was lost, thus allowing us to identify the antigen specificity of the lymphocytotoxic antibodies [10, 13]. The antigen specificity of the lymphocytotoxic antibodies was A28, B12 for patient 1; A1, B5 for patient 2; A2, B5 for patient 3; and A1, B5 for patient 4.

Immunoglobulin precipitation

Two-milliliter aliquots of six serum samples collected at different intervals and displaying a PRA greater than or equal to 70% were taken for each patient, pooled, and sterilized by 0.2 μm filtration

Table 1. Clinical and immunological characteristics of patients under study. CAD, Cadaveric; NA, not applicable

Patient Number	Age	Sex	Number of transfusions	Pregnancies	Prior transplantation/ type/duration of function	Time on cadaveric transplant waiting list (years)	PRA[a]	Antigen specificity of patient's HLA antibodies
1	22	F	>50	None	Yes/CAD/2 years	6	85%	A28, B12
2	18	F	>50	None	Yes/CAD/4 years	8	90%	A1, B5
3	52	F	4	2	None	3	80%	A2, B5
4	44	M	5	NA	Yes/CAD/5 years	3	70%	A1, B5

[a] Patient PRA was evaluated against a peripheral blood lymphocyte panel of 20 allogenic HLA-typed healthy subjects

Table 2. IgG subclass distribution (mg/dl) in pooled serum samples and in ammonium sulphate-precipitated serum proteins eluted from protein A and protein G columns. ND, Not detectable

		Pooled serum samples[a]	Protein A fraction pools[b,d]		Protein G fraction pools[c,d]	
			pH7	pH3	pH7	pH2.7
Patient 1	IgG 1	1200	ND	307	ND	312
	IgG 2	100	ND	25	ND	9
	IgG 3	25	4.5	0.05	ND	2.7
	IgG 4	18	ND	1.5	ND	1.5
Patient 2	IgG 1	1230	ND	373	ND	414
	IgG 2	190	ND	9	ND	18.5
	IgG 3	16	3	ND	ND	1.8
	IgG 4	13	ND	0.6	ND	0.6
Patient 3	IgG 1	670	ND	271	ND	284
	IgG 2	210	ND	24	ND	31
	IgG 3	60	8	ND	ND	64
	IgG 4	50	ND	1.2	ND	1.1
Patient 4	IgG 1	930	ND	257	ND	461
	IgG 2	440	ND	22	ND	55
	IgG 3	130	14.5	ND	ND	17
	IgG 4	40	ND	6	ND	4.3

[a] Pooled serum samples were analyzed for IgG subclass distribution before ammonium sulphate precipitation and column adsorption

[b] A 10-ml sample of saturated ammonium sulphate-precipitated serum proteins was applied to a protein A sepharose CL-4B column with a bed volume of 1 × 21 cm. One-milliliter fractions were collected by a fraction collector at pH7 and pH3 as previously described [7]. Fractions collected at each pH were mixed together in order to obtain two pools: pH7 pool, which constituted fractions 1–30 and pH3 pool, which constituted fractions 31–54

[c] A 10-ml sample of saturated ammonium sulphate-precipitated serum proteins was applied to a protein G sepharose 4 FF column with a bed volume of 1 × 12 cm. One-milliliter fractions were collected by a fraction collector at pH7 and pH2.7 as previously described [13]. Fractions collected at each pH were mixed together in order to obtain two pools: pH7 pool, which constituted fractions 1–32 and pH2.7 pool, which constituted fractions 33–54

[d] After mixing both pH7 and pH3/pH2.7, pools were concentrated ten times by means of Centricon 30 microconcentrators (Amicon Division, Danvers, Mass., USA)

(Nalgene, Rochester, N.Y., USA). One volume of saturated ammonium sulphate was added to one volume of pooled serum samples and stirred for 1 h at room temperature. Precipitated serum proteins were recovered by centrifugation (30 min at 12000 rpm at 4 °C) and dissolved in 0.15 M NaCl phosphate-buffered saline (PBS), pH 7.2, 0.02 M sodium azide. Precipitation was repeated three times to achieve a better yield. The final precipitate was dissolved in PBS and exhaustively dialyzed against phosphate buffer 0.02 M pH 7.0.

Protein A and protein G immunoadsorption procedures

Ammonium sulphate-precipitated pooled serum samples were subsequently immunoadsorbed on protein A sepharose CL-4B and protein G sepharose 4FF columns (Pharmacia-LKB Biotechnology, Uppsala, Sweden) at pH 7 [1, 9]. IgG antibodies adsorbed on both columns were eluted at acid pH (protein A, pH 3; protein G, pH 2.7) with glycine-HCl buffer 0.1 M, collected in 1-ml fractions by a fraction collector and pooled in two sets (Tables 2, 3). In order to protect column-eluted IgG from acid pH denaturation, 100 µl TRIS-HCl 1 M pH 8.7 were immediately added to each fraction. Nonabsorbed pooled serum samples, as well as immunosorbent-eluted, concentrated (ten times), protein pooled fractions, were subsequently analyzed for IgG subclass content, using commercial ELISA kits (ICN Immunobiologicals, Lisle, IL, USA), and tested by means of a CDC assay against a panel ranging from eight to ten HLA-typed, Epstein-Barr virus (EBV)-transformed allogeneic lymphoblastoid B cell lines (LBCL) [2, 3] expressing the HLA class I antigen specificities recognized by patients' antibodies and some related public cross-reactive group antigens (Tables 2, 3).

Cell lines

EBV-LBCL were obtained following transformation of HLA-typed allogeneic PBL with EBV containing supernatants prepared from the B95-8 marmoset cell line, as previously described [4].

Table 3. Complement-dependent cytotoxicity (CDC)[a] activity of pooled serum samples and ammonium sulphate-precipitated serum proteins eluted from protein A and protein G sepharose columns. EBV, Epstein-Barr virus; LBCL, lymphoblastoid B cell lines

Patient 1					
Cell targets EBV-LBCL[b]	Nonabsorbed, pooled serum samples[c]	Fraction pools			
		Protein A[g]		Protein G[h]	
		pH7	pH3	pH7	pH2.7
1. HLA-A2, 9; B21, 40	8	8	1	1	6
2. HLA-A2, 30; B5, 40	4	1	2	1	2
3. HLA-A3, 28; B7, 35	8	4	1	1	4
4. HLA-A2, -; w22, 40	6	1	2	1	2
5. HLA-A28, 32; B15, 16	8	4	1	1	4
6. HLA-A2, 3; B12, 17	8	6	1	1	4
7. HLA-A1, 29; B8, 12	8	6	4	1	4
8. HLA-A30, w33; B13, 14	8	4	4	1	4
Patient 2					
Cell targets EBV-LBCL[b]	Nonabsorbed, pooled serum samples[d]	Fraction pools			
		Protein A[g]		Protein G[h]	
		pH7	pH3	pH7	pH2.7
1. HLA-A2, 9; B21, 40	8	4	1	1	4
2. HLA-A2, 10; B16, 17	4	1	1	1	1
3. HLA-A1, 32; B5, w22	8	4	6	1	4
4. HLA-A3, 28; B7, 35	8	4	1	1	2
5. HLA-A1, 31; B5, 18	8	4	4	1	4
6. HLA-A1, 11; B35, 37	8	2	4	1	6
7. HLA-A1, 29; B8, 12	8	4	4	1	6
8. HLA-A30, w33; B13, 14	6	2	1	1	2
9. HLA-A2, -; B5, 8	8	4	4	1	6
Patient 3					
Cell targets EBV-LBCL[b]	Nonabsorbed, pooled serum samples[e]	Fraction pools			
		Protein A[g]		Protein G[h]	
		pH7	pH3	pH7	pH2.7
1. HLA-A2, -; B5, 8	8	1	2	1	2
2. HLA-A3, -; B5, 7	8	1	2	1	2
3. HLA-A2, 9; B21, 40	8	2	8	1	6
4. HLA-A1, 11; B17, 35	8	2	1	1	2
5. HLA-A1, 2; B5, 27	8	4	2	1	4
6. HLA-A1, 29; B12, 37	1	1	1	1	1
7. HLA-A1, 10; B16, 17	1	1	1	1	1
8. HLA-A28, 10; B18, w22	8	2	1	1	2
9. HLA-A1, 32; B8, 15	4	1	1	1	1
10. HLA-A30, w33; B13, 14	1	1	1	1	1
Patient 4					
Cell targets EBV-LBCL[b]	Nonabsorbed, pooled serum samples[f]	Fraction pools			
		Protein A[g]		Protein G[h]	
		pH7	pH3	pH7	pH2.7
1. HLA-A2, -; B5, 8	8	1	1	1	4
2. HLA-A3, -; B5, 7	8	1	1	1	2
3. HLA-A2, 9; B21, 40	4	1	1	1	1
4. HLA-A1, 11; B17, 35	8	2	2	1	2
5. HLA-A1, 2; B5, 27	8	2	2	1	2
6. HLA-A1, 29; B12, 37	8	2	2	1	4
7. HLA-A1, 10; B16, 17	8	8	4	1	4
8. HLA-A10, 28; B18, w22	4	2	2	1	2
9. HLA-A1, 32; B8, 15	6	1	2	1	2
10. HLA-A30, w33; B13, 14	1	1	1	1	1

[a] CDC on EBV-LBCL was performed as previously described [3, 4] and scored as follows: % dead cells 0–19, score 1; % dead cells 20–39, score 2; % dead cells 40–59, score 4; % dead cells 60–79, score 6; % dead cells 80–100, score 8

[b] EBV-LBCL were obtained as described in [4]

[c] For this pool the dilution still giving at least a 50 % killing above the background was 1:32 against target 7; 1:16 against targets 3, 5, 6; 1:8 against target 1; 1:4 against targets 4, 8; and 1:2 against target 2

[d] For this pool the dilution still giving at least a 50 % killing above the background was 1:64 against targets 3, 5, 6, 7, 9; 1:32 against targets 1, 4; 1:16 against target 2, and 1:8 against target 8

[e] For this pool the dilution still giving at least a 50 % killing above the background was 1:8 against target 5; 1:4 against targets 1, 2, 3; and 1:1 against targets 4, 8, and 9

[f] For this pool the dilution still giving at least a 50 % killing above the background was 1:8 against target 6; 1:4 against targets 4, 5, 9; 1:2 against targets 1, 2, 8; and 1:1 against target 3

[g] See legends [b] and [d] of Table 2

[h] See legends [c] and [d] of Table 2

Results

Immunoadsorption experiments

IgG subclass distribution. As shown in Table 2, IgG 3 antibody content in pooled fractions eluted at pH 7 from protein A columns was well represented in all four patients. In contrast, the corresponding pools, eluted at pH 7 from protein G columns, did not contain detectable amounts of any IgG subclass. Moreover, pooled fractions eluted at pH 3 from protein A columns were found to contain only IgG 1, 2, and 4 subclasses, as opposed to protein G fractions eluted at pH 2.7 that displayed a complete spectrum of IgG subclasses (Table 2). These data were also confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) experiments on both protein A and protein G column-eluted fractions whose analysis demonstrated the simultaneous presence of 25 and 50 kDa bands in all the samples in which IgG of any subclass were detected by ELISA. In addition, the same analysis showed, in the corresponding pH 7-eluted pools, the presence of several bands in the 118–14 kDa molecular weight range corresponding to non-IgG contaminant serum proteins (data not shown).

Complement-dependent cytotoxicity assays against EBV-LBCL. The results are shown in Table 3. When protein A column-eluted fractions were tested by means of the CDC assay against various EBV-LBCL target cell panels, it was found in all four patients that cytotoxic activity was expressed not only by pH 3-eluted pools containing IgG 1, 2, and 4 subclasses but also by pH 7-eluted pools in which IgG 3 antibodies were present, together with other non-IgG serum proteins. It is worth mentioning here that in patient 1 (Table 3), CDC activity of protein A pH 7-eluted fractions was even greater than that expressed by the corresponding pH 3-eluted fractions, whereas in the other three patients the cytotoxic activity displayed by the two protein A-eluted pools appeared almost superimposable. In contrast, no cytotoxic activity was present in pooled fractions eluted at pH 7 from the protein G columns, i.e., in those fractions that were shown to contain only non-IgG serum proteins. In this case, as expected, all the cytotoxic activity was found in pH 2.7-eluted fractions. We could, therefore, conclude that immunoadsorption by protein A was not able to completely eliminate the cytotoxic activity of patients' sera whose residual activity could be ascribed to unabsorbed IgG 3 antibody content.

Discussion

All of these results enabled us to conclude that IgG 3 antibodies were partly responsible for anti-HLA antibody reactivity in the four HSRTR studied. We are currently extending this kind of analysis to a larger number of HSRTR on the waiting list at our center in order to see whether IgG 3 antibodies, endowed with anti-HLA specificity, are present in all sensitized patients or in a selected percentage of them. Preliminary observations, carried out in another HSRTR (PRA = 100%) having antibodies in high titers directed against more than two HLA antigens (type II anti-HLA antibodies [12]), have demonstrated the presence of IgG 3 antibodies endowed with anti-HLA lymphocytoxicity in this type of sensitized patient as well (data not shown).

In addition, protein G columns were able to remove the IgG 3 subclass to a greater degree than protein A columns, thus leading to a complete disappearance of cytotoxicity in the PRA analysis of our four patients. To date, all previously published immunoadsorption studies [6, 10, 11], carried out in sensitized patients with type I anti-HLA antibodies, have shown that the failure of immunoadsorption to increase the transplantation chances for HSRTR is not due to inadequate IgG removal but rather to a rapid IgG resynthesis. In all the protocols, this resynthesis needed a mandatory concomitant immunosuppression. Our in vitro immunoadsorption experiments may, in our opinion, give further insight into this problem by indicating that at least in patients with a documented anti-HLA type I antibody also due to IgG 3 subclass, better results might be obtained using immunosorbents such as sepharose-bound streptococcal protein G columns. Finally, the experimental approach reported here, dealing only with small aliquots of serum, suggests that sera from HSRTR might be screened in vitro beforehand with fast procedures utilizing commercially available, prepacked, disposable protein G and protein A sepharose minicolumns. Such a screening could evaluate the efficacy of adsorption procedures in removing lymphocytotoxic anti-HLA IgG antibodies before any in vivo adsorption treatment is begun.

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