Incidence and specificity of HLA antibodies in multitransfused patients with acquired aplastic anemia

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BACKGROUND: This study aimed to establish the prevalence and characteristics of anti-HLA in antibody acquired aplastic anemia patients following cessation of antithymocyte globulin therapy and to characterize antibody in terms of epitope specificity.

STUDY DESIGN AND METHODS: One hundred and fifty multitransfused, untransplanted patients from eight European centers were investigated by serologic methods.

RESULTS: Sixty-two percent were antibody positive. Eighteen HLA-Class-I-specific antibodies (15 IgG, 3 IgM) were identified in 13 patients; 13 antibodies were specific for HLA-A epitopes and 5 for HLA-B. Epitope analysis identified significant correlation between serum reactivity and amino acid substitutions associated with HLA-Class-I epitopes. An excess of antibodies to HLA-A1-associated cross-reactive groups was identified. There was no significant difference in antibody frequency in patients taking cyclosporine compared to those who were not.

CONCLUSION: Data suggested a contribution from B cell memory of alloantigens introduced during pregnancy. In some cases, antibody production continued many years after the last transfusion, and although the target varied between individual patients, the antibody to HLA was focused on a few specific Class I epitopes, the majority of which mapped to the HLA-A molecule.

All components of blood, including WBCs, RBCs, PLTs, and serum, have the potential to elicit antibody to HLA. Exceptions to this rule are the "plasma protein fraction" and albumin.[1] WBCs are implicated as the prime source of sensitization because nonleukofiltered blood is more immunogenic than leukofiltered blood (in which the total number of WBCs is reduced to below $1\text{-}5 \times 10^6$ per unit).[2-10]

In nonsensitized recipients, pure RBCs and PLTs have minimal immunogenicity due to an absence of costimulatory molecules.[11] Nonetheless, pure RBCs have the capacity to reactivate antibody production to HLA in previously sensitized recipients.[12] RBCs do not synthesize HLA per se, but are thought to inherit Class I from their reticulocyte ancestors.[13-15] In individuals sensitized to HLA, the life span of transfused RBCs may be shortened; hemolysis occurs and there is an increased sequestration of transfused RBCs to the spleen.[16,17] Although RBCs express between 40 and 550

ABBREVIATIONS: AAA = acquired aplastic anemia; ABH = ABH blood group antigens; ATG = antithymocyte globulin; CDC = complement-dependent lymphocytotoxicity; CDR3 = complementarity-determining region 3; CREG = cross-reactive groups; CyA = cyclosporine; FC = flow cytometry; HPA = human PLT antigen; LBCL = lymphoblastoid cell line; p_c = probability of association; PR = PLT refractoriness; PRA = panel-reactive antibody; TCR = T-cell receptor.

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HLA Class I molecules per cell, T lymphocytes express 100,000 per cell,[18] and the total HLA Class I content of a blood unit is equally divided between RBC and WBC fractions.[19] However, only the viable WBCs have the opportunity to persist in a microchimearic state after transfusion and continue to synthesize nonself HLA Class I and II.[20,21]

PLTs express ABH blood group antigens, HLA Class I, and human PLT alloantigens (HPA),[22] but like RBCs they are poor inducers of alloantibody in immunologically naïve recipients due to a lack of costimulatory molecules. Nonetheless, prior immunity to PLT alloantigens can cause PLT transfusion refractoriness.[23-29] Immunity to HLA Class I accounts for the majority of refractory patients.[23,30,31] In 30 percent of recipients of nonleukodepleted PLTs, anti-HLA develops. This reduces to 17 to 21 percent after leukodepletion.[3,6,10,32-34] Similar effects are achieved by infusing ABH and Class I–compatible PLTs,[31,35,36] or by neutralizing antibody with intravenous immunoglobulin.[37] PLT cross-matching is advocated as a more pragmatic way to detect antibodies to both HLA and HPA.[38-40] The specificities associated with anti-HLA in patients with PLT transfusion refractoriness tend to conform to the so called "public" antigens shared by "cross-reactive groups" (CREGs) of the HLA Class I alleles.[32,41]

In acquired aplastic anemia (AAA) "leukoagglutinins" were first described by Jean Dausset in the early 1950s.[42] Since then, several studies have confirmed the presence of antibodies to HLA as well as other allo- and autoantigens.[31,43,44] AAA patients also exhibit higher levels of T-cell reactivity to allogeneic HLA, non-HLA, and "autologous" target cells.[45-47] The two major consequences of alloimmunization are PLT refractoriness (PR) and engraftment failure after BMT.[48-50]

PR is defined as failure of a PLT transfusion to stop clinically significant bleeding and/or failure to reach the expected rise in PLT count within 24 hours. Failures associated with massive hemorrhage, disseminated intravascular coagulation, sepsis, and splenomegaly are excluded. Increasing numbers of patients are receiving intensive chemotherapy, necessitating transfusion support over long periods. A significant proportion receive a hemopoietic stem cell transplant[51,52] and require further transfusion support, increasing the number of patients at risk of PR.

The majority of antibodies produced after multiple transfusions have HLA specificity, but 10 to 30 percent of HLA-matched PLT donations give unsatisfactory post-transfusion increments, possibly due to the presence of PLT-specific antibodies.[53,54] The relationship between HLA antibodies and PR is unclear in that some patients with multiple antibodies remain responsive to randomly selected PLT transfusions, whereas others require HLA-matched PLTs.[54]

The aims of this study were to establish the prevalence of anti-HLA in AAA patients after cessation of antithymocyte globulin (ATG) therapy and to characterize the antibody in terms of its fine specificity. In particular, we wished to know if certain HLA epitopes were immunodominant and could be used as a basis for future immunotherapeutic strategies. Our approach to "epitope analysis" has been described elsewhere.[55] Furthermore, the early identification of patients at high risk from PR would have important consequences for patient and resource management, especially for the provision of costly HLA- and/or HPA-matched PLTs. This strategy requires effective screening to identify "high-risk" patients at diagnosis.

Patient samples and data were generously provided from member centers of the European Blood and Bone Marrow Transplant Group—Aplastic Anemia Working Party. This was a cross-sectional study, with samples taken from patients at various times after diagnosis, but always beyond 3 months after completion of immunosuppressive therapy with ATG.

MATERIALS AND METHODS

Patients

A random group of 150 untransplanted patients with AAA (69 females, 75 males, 6 sex unknown) were submitted to this study (Table 1) from eight European hematology units in England, Germany, Italy, Spain, Sweden, and Switzerland. We do not have information regarding the ethnic origin of the patients. The patients were multitransfused but had not received antithymocyte globulin in the 3 months preceding sampling, although 50 percent (63/127) were known to be on cyclosporine (CyA) therapy at this time. IVIG does not form part of immunosuppressive protocols used for treatment of AAA in Europe. Patients treated by BMT were excluded from the study. The study group was a cross-sectional sample of AAA patients in Europe between 1996 and 2001. Thus, some were recently transfused and others had not been transfused for more than 20 years.

Flow cytometry (FC)

Patient serum was incubated with two pools of eight HLA Class I and Class II phenotyped EBV-transformed lymphoblastoid cell lines (LBCLs). The EBV-LBCL panel was selected to include as many different HLA alleles as possible. After incubation, FITC-conjugated goat antihuman IgG (Sigma-Aldrich, Poole, England) was added. The cells were incubated, washed, and the percentage of positively staining cells assessed using a flow cytometer (FACSCalibur Flow Cytometer, Oxford, England). This procedure facilitated the identification of IgG_1, IgG_2, IgG_3, and IgG_4 antibodies.

TABLE 1. Patient summary

	Male	Female	Sex unknown
Patients	75	69	6
Median age (range)	37 (19-85)	40 (18-83)	
Pregnancy		22 (4 unknown)	
Transfusion history* (units)			
RBCs			
0	2	2	1
1-10	19	19	1
11-100	43	38	1
>100	4	3	0
unknown	7	7	3
PLTs†			
0	3	2	1
1-10	10	15	0
11-100	38	31	1
>100	11	12	1
unknown	13	9	3
Days since last transfusion median (range)	1011 (2-8194)	1029 (3-12105)	NA
Immunosuppression			
ATG ± other	18	12	1
CyA ± other	15	12	2
ATG + CyA ± other	38	41	0
Other	1	0	0
None	2	1	0
Unknown	1	3	3

* Includes transfusion of whole blood, RBCs, and leukoreduced RBCs.
† Includes HLA-matched PLTs, and random and single-donor PLT transfusions.

TABLE 2. Distribution of antilymphocyte antibody detected by flow cytometry and lymphocytotoxicity

	Flow cytometry		
Lymphocytotoxicity	Positive	Negative	Total
Positive	35 (21F/8P, 12M, 2U*)	21 (8F/2P, 13M)	56
Negative	33 (19F/5P, 14M)	55 (20F/7P, 31M, 4U)	88
Total	68	76	144

* F = female; P = pregnant; M = male; U = sex unknown.

Complement-dependent lymphocytotoxicity (CDC) assay

A standard two-color fluorescence CDC antibody screening protocol incorporating acridine orange (0.2 mg/mL, Sigma-Aldrich) and ethidium bromide (0.1 mg/mL, Sigma-Aldrich) was followed.[55] Essentially, each patient's serum sample was screened for antibody activity against a panel of between 40 and 100 HLA Class I phenotyped normal blood donors. This procedure facilitated the detection of complement-fixing IgG_1, IgG_3, and IgM antibodies.

DTT sensitivity assay

CDC screening was repeated using 0.01 mol/L DTT-treated patient serum (Sigma-Aldrich). Ablation or reduction in lymphocytotoxic activity indicated the presence of IgM antibody.

Assessment of panel-reactive antibody (PRA)

The extent of PRA is a measure of the antibody activity in a patient's serum and is expressed as a percentage of reactive donors in a random population. In this study, the PRA could not be accurately established because of the selected nature of the donor panels used; however, it provided a reliable indication of the relative extent of alloimmunization.

Definition of antibody specificity and identification of putative target epitopes

A correlation between AAA serum antibody activity and variations in HLA Class I primary sequences was sought by manual comparison.[55] It was assumed that, whereas AAA sera may react with more than one public epitope, the model containing the least number of postulated antibody specificities was the most acceptable explanation of serum activity. The definition of the target epitope allowed the prediction of cross-match–negative blood donors.[55]

Statistics

The significance of observed associations between antibody activity and amino acid residue was tested using the chi-squared test for 2×2 comparisons. The probability of association (p_c) was corrected for the number of critical (variable) amino acid residues in the Class I molecule, which total 57 in the HLA-A molecule, 56 in HLA-B molecule, and 43 in the HLA-C molecule (total 156).[56]

RESULTS

Interpretation of antibody activity

Of 144 evaluable patients 89 (62%) were antibody positive (Table 2). Patients were classified into four groups on the basis of FC and CDC antibody activity.

FC+ CDC+. Thirty-five patients (12 male, 21 female, 2 sex unknown) demonstrated antibody activity associated with complement-fixing IgM, IgG_1, IgG_3, and possibly IgG_2 antibodies or a combination of these. Eighteen patients were found to have both IgG and IgM antibody components. Thirteen patients had lymphocytotoxic antibodies

of definable HLA specificity (Table 3). Patient AA177, a nulliparous female 7 days after transfusion reacted positively against all donors by both FC and CDC and was effectively nontransfusable with random donor blood products at the time of sampling. Antibody specificity in the remaining 21 patients could not be evaluated.

FC+ CDC–. IgG antibodies detected by FC alone include low-titer HLA Class I antibodies, which are too weak to be detected by CDC; noncomplement-fixing IgG_4 and possibly IgG_2 antibodies; HLA Class II antibodies and antibodies directed toward non-HLA targets. Thirty-three patients (14 male, 19 female) were included in this group.

FC– CDC+. Twenty-one patients (13 male, 8 female) demonstrated serum reactivity due to weak IgM antibody (DTT sensitive). Such antibodies may demonstrate discrete HLA specificity (Table 3, patient SI168), be autoreactive, or be directed toward non-HLA targets.

FC– CDC–. Fifty-five patients (31 male, 20 female, 4 sex unknown) were antibody negative.

Identification of HLA-Class-I–specific antibodies

Eighteen HLA-Class-I–specific antibodies (15 IgG, 3 IgM) were identified in 13 patients (Table 3). There were 13 specific for HLA-A epitopes and 5 specific for HLA-B epitopes. Seven (6 IgG, 1 IgM) had specificity for HLA-A1 or HLA-A1–associated CREG epitopes. One patient, FM113, had antibody reactivity associated with the presence of two IgG antibodies directed against HLA-A25 + A26 + A34 + A43 + A66 and against HLA-A11. MB227 was the only patient with coexisting HLA-A and HLA-B antibodies, namely A1 + A11 + A36 and B44 + B45. Two patients (RK147, FR122) had IgG antibodies associated with HLA-A2, one of which (FR122) reacted with homozygous HLA-A2 donors only. An IgM antibody identified in LE201 had specificity for A9 and was weakly reactive with HLA-A2. Two patients (WC136 and RM189) had IgG antibodies specific for HLA-B8, and one patient (SI168) had a weakly reactive IgM anti-HLA-B17 + B50. The HLA antibody-positive patients (4 male, 8 female, 1 sex unknown) were sampled from 27 to 3058 days after last transfusion (median, 831 days). The PRA of those patients with HLA antibody ranged from 9 to 86 percent (median, 43%).

Epitope analysis

Using the approach described previously,[55] we have identified significant correlation between serum reactivity in AAA patients and amino acid substitutions associated with HLA Class I public epitopes. To illustrate this, we describe fully our observations in patients KG186 and SR145.

Patient KG186. KG186 was a 36-year-old multiparous female with a PRA of 86 percent. She had received up to 100 units of WBC-poor RBCs. Her antibody sample was taken 1455 days after transfusion. Extensive screening by CDC revealed a "window of negativity" that included HLA-A3, alleles of the HLA-A19 CREG, and all HLA-B and HLA-C locus alleles. The HLA-A1 + A10 + A11 and HLA-A2 CREGs were conspicuously absent from the compatible screening donors. Comparison of serum reactivity with HLA Class I primary amino acid sequences (Table 3) revealed a significant association ($p_c = 1.8 \times 10^{-7}$) with two postulated target residues, 163R and 127K, and a complex epitope involving one or more of the residues 44K, 150V, and 158V in the A locus product (Fig. 1).

The identification of the probable target epitopes allowed us to determine that patient KG186 would be transfusion compatible with all permutations of HLA-A3, -A29, -A30, -A31, -A32, -A33, -A34, and all HLA-B and HLA-C locus alleles. Significantly, this includes HLA-A3 and B7, and all transfusion centers have regular access to donors homozygous for this haplotype.

We have shown in previous studies that this approach can be confirmed in retrospective cross-matching.[55]

Patient SR145. Patient SR145 was a 61-year-old, PLT refractory male with a PRA of 53 percent. He had been transfused with up to 100 units of WBC-poor RBCs and had more than 100 donor exposures to HLA-compatible PLTs or PLTs isolated by single-donor apheresis.

Extensive antibody screening of a serum sample acquired 27 days after transfusion revealed a well-defined "window of negativity," which included all HLA-B and HLA-C alleles. The HLA-A1 CREG-associated alleles HLA-A1, -A11, -A25, -A26, -A34, -A36, -A43, -A66, and -A80 were conspicuously absent from this window. DTT pretreatment of the serum confirmed the presence of an IgM antibody and reduced the PRA from 53 to 23 percent. Comparison of pre- and post-DTT serum activity with Class I primary amino acid sequences revealed that these observations were exquisitely explained by postulating two target epitopes, a 90D substitution found in all HLA-A1, -A11, -A25, -A26, -A34, -A36, -A43, -A6601, and -A80 alleles defined by an IgM antibody and the 44K/150V/158V epitope uniquely associated with HLA-A1 + A36 as described in patient KG186 (Fig. 2). Given these observations, we determined a window of acceptable mismatching for this patient; significantly this included HLA-A3, B7 and HLA-A2, B44, two of the most common HLA Class I haplotypes found in Caucasians. Antibodies with specificity for the 44K/150V/158V epitope were also observed in two further patients, GE159 and CH191.

HLA Class I phenotypes

HLA Class I phenotyping data was available in 91 patients. HLA allele frequencies were in agreement with previous studies in European populations.[57]

TABLE 3. 2 × 2 contingency table correlation between predicted target residues and antibody in 13 acquired aplastic anaemia (AAA) patients

Patient	Sex/ Pregnancy y/n	Days after transfusion	PRA (%)	Specificity			2×2 contingency table						
				Serologic	Epitope	Ig class	++	+–	–+	––	n	Chi-square	r
GE159	NA/NA	NA	70	Includes A1,A36 +	44K/150V/158V + ?	IgM/IgG	17	1	25	18	61	6.20	0.319
				A1,A36	44K/150V/158V	IgG	17	1	0	43	61	51.70	0.921
CH191	F/y	365	52	Includes A1,A36 +	44K/150V/158V + ?	IgM/IgG	21	2	10	46	79	33.87	0.655
				A1,A36	44K/150V/158V	IgG	16	2	2	40	60	38.55	0.802
SR145	M	27	53	A1,A11,A25,A26,A34,A36,A43,A6601,A8001	90D	IgM	39	1	2	36	78	62.84	0.898
				A1,A36	44K/150V/158V	IgG	9	0	0	30	39	33.57	1.000
KG186	F/y	1455	86	A1,A36	44K/150V/158V	IgG	23	0	46	10	79	3.23	0.202
				A1,A11,A25,A26,A43,A6601	163R	IgG	18	0	28	10	56	4.11	0.271
				A2,A23,A24,A68,A69	127K	IgG	28	1	0	9	38	28.23	0.862
					combined		69	1	0	9	79	61.45	0.882
FM113	F/y	1377	29	A25,A26,A34,A43,A66	149T	IgG	16	1	13	70	100	38.46	0.620
				A11	??	IgG	8	0	5	70	83	40.87	0.702
					combined		24	1	5	70	100	68.40	0.827
MB227	M	NA	48	A1,A11,A36	152A	IgG	8	1	4	14	27	8.27	0.484
				B44,B45	167S	IgG	3	0	1	14	18	7.80	0.549
					combined		11	1	1	14	27	26.99	0.635
RK147	F/y	1168	43	A2	74H/107W	IgG	26	0	0	34	60	56.00	1.000
FR122	F/n	3058	9	A2	74H/107W	IgG	9	31	0	60	100	12.22	0.349
					homozygous 74H/107W		7	0	2	91	100	64.63	0.804
LE201	F/y	1190	47	A9, weak A2	66K	IgM	19	10	2	14	45	9.61	0.420
RR206	M	NA	19	B7, B60, B48	178K	IgG	10	2	1	31	44	25.82	0.608
WC136	M	27	18	B8	9D/11A/12M	IgG	15	0	3	82	100	73.99	0.860
RM189	F/n	493	11	B8	9D/11A/12M	IgG	9	0	0	70	79	69.40	1.000
SI168	F/n	463	18	B17, B50	??	IgM	8	1	3	48	60	29.88	0.706

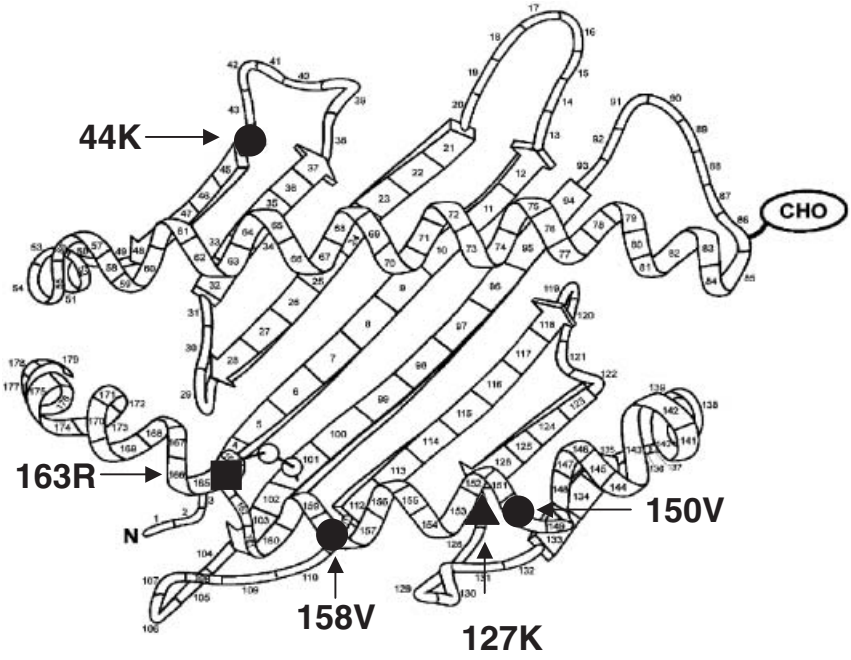

Fig. 1. Amino acid substitutions associated with antibody activity in patient KG186.163R (■) is an alpha helix (alpha 2 domain) substitution shared by all HLA-A1, -A11, -A25, -A26, -A43, and -A6601 alleles, and antibodies with this specificity have previously been observed.[70] Residue 163 represents one of the constituent amino acids of the A pocket of the peptide-binding groove.[97] 127K (▲) is an alpha 2 domain substitution shared by HLA-A2, -A23, -A24, -A68, and -A69 alleles.[70] The 44K/150V/158V (●) complex epitope has been postulated as a third target because the KG186 antibody is strongly positive with HLA-A36 donor cells but negative with HLA-A34. Adapted from Bjorkman et al.[98] and Parham et al.[99] K = lysine; R = arginine; V = valine.

Antibody incidence and patient gender

There was an excess of females in the antibody-positive group (Table 2), but this was not statistically significant. However, the association observed between sex and the presence of IgG antibody in AAA was significant (RR = 1.58, CI = 1.10-2.28, $p < 0.02$), as was the association of pregnancy in the HLA-defined antibody group (RR = 2.11, CI 1.22-3.66, $p = 0.00002$).

Antibody duration

The median antibody duration (date of last transfusion to date of sampling) was 1168 days. There was no statistical difference between the sexes (females 1159 days, males 1308 days).

DISCUSSION

In this study, the principal findings are limited to a "snapshot" of AAA patients at different times after treatment with immunosuppression including ATG. All samples were taken at least 3 months after completion of ATG therapy at a time when heterologous antibody would have disappeared from the circulation. Forty-one of 79 patients with antibody and 22 of 48 patients without antibody remained on CyA at the time of sampling. The study yielded several fascinating results. Firstly, more females than males produced IgG anti-HLA, suggesting a contribution from B-cell memory of alloantigens introduced during pregnancy. Secondly, in some cases anti-HLA production continued many years after the last transfusion, 8 years in one case (FR122). Thirdly, although the target varied between individual patients, the antibody to HLA was focused on a few specific Class I epitopes. The majority of these mapped to the HLA-A molecule. In one individual (AA177), the antibody specificity could not be analyzed due to the absence of negative reactions against the reference panel. Many of these findings have parallels in pregnancy, transfusion, and transplantation, but the questions they raise are nonetheless important to our understanding of the underlying mechanisms in AAA and in our attempts to design future therapeutic strategies.

How can these responses to HLA be interpreted? Immunosuppression with ATG is reported to eliminate PR and antibody to HLA in AAA patients, but in some it persists.[58] The present study shows that PR and HLA antibody were not completely obliterated by immunosuppressive therapy. ATG cannot be assumed primarily to affect T cells because it contains antibodies to a wide range of tissues.[59,60] Cyclosporin has been reported to suppress antibody to HLA in a proportion of cases treated in vivo.[61,62] In our study, 8 of 11 patients with definable HLA antibody were undergoing CyA therapy at the time of sampling. Paradoxically, in vitro CyA appears to enhance immunoglobulin synthesis by preactivated B cells.[63] Many experimental models designed to study B-cell maturation, antibody responses, and immunologic memory involve the use of synthetic short-lived nonreplicating antigens.[64,65] The transfusion model offered by AAA patients may be more akin to models involving chronic antigenic stimulation (e.g., by EBV), where transfused antigen persists in the recipient and is secreted by sequestered viable cells, thereby reactivating memory after cessation of immunosuppressive therapy.

Overall, 89 of 144 patients (62%) produced antibody to lymphocytes. Antibodies to RBCs and PLTs or other allotypes were not the subject of this study. Antibody detected

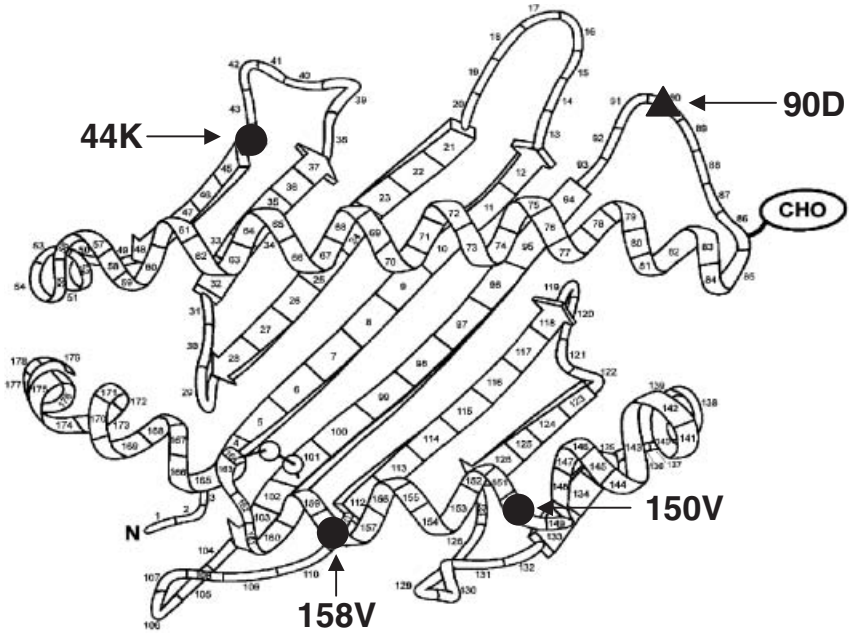

Fig. 2. Amino acid substitutions associated with antibody activity in patient SR145. An IgM antibody reacted with an epitope characterized by the 90D (▲) amino acid substitution found in all HLA-A1, -A11, -A25, -A26, -A34, -A36, -A43, -A6601, and -A80 alleles. Treatment with DTT revealed an IgG antibody with HLA-A1 + A36 specificity ($p_c = 5.3 \times 10^{-5}$). The 44K/150V/158V epitope (●) uniquely associated with this specificity is described in Fig. 1.

in the CDC assay is complement-fixing IgG_1, IgG_3, or IgM and is generally directed to HLA Class I antigens. Antibodies detected by our EBV-LBCL flow-cytometry technique included all the IgG subclasses (IgG_{1-4}). The EBV-LBCL were selected to include all the main HLA alleles.[66-69] This antibody group would have included antibody to HLA Class I, Class II, and possibly non-HLA antigens (including EBV-derived antigens). The 35/144 (24%) FC+/CDC– sera, probably included noncomplement-fixing IgG_2 or IgG_4 antibodies to HLA products together with weak or sublytic levels of IgG_1 or IgG_3 antibodies.

The epitope specificity of the antibody was deduced from an informed analysis of the lymphocytotoxic reactions against the reference panel.[55] Thus, the analysis was confined to IgG_1, IgG_3, and IgM antibodies to HLA Class I epitopes. Serum AA177, which reacted with 100 percent of the reference panel, may have been analyzable after dilution and absorption, but this was beyond the scope of the study. Overall 56/144 (39%) of sera were positive in the lymphocytotoxicity test. Our observations confirmed that the precise HLA Class I amino acid substitutions associated with antibodies identified in sensitized patients can be predicted from the results of lymphocytotoxicity screening and a knowledge of the peptide sequence of each allele.

Strikingly, the majority (13/18, 72%) of the HLA-defined antibodies recognized HLA-A epitopes, and of these 7 (54%) were specific for epitopes associated with the HLA-A1 CREG. There are several epitopes associated with HLA-A1 CREGs.[70] These include 44K/150V/158V (shared by HLA-A1 and HLA-A36 alleles); 62Q (HLA-A1, -A3, -A11, -A30, -A31, -A32, -A36, -A74); 90D (HLA-A1, -A11, -A25, -A26, -A34, -A36, -A43, -A6601, -A80); 144K (HLA-A1, -A2, -A68, -A69, -A3, -A11, -A24, -A36, -A80); 152A (HLA-A1, -A11, -A36); 163R (HLA-A1, -A25, -A26, -A11, -A43, -A6601), and 166D/167G (HLA-A1, -A23, -A24). Four HLA-A1 CREG epitopes were identified by antibodies defined in this patient group, namely 44K/150V/158V (in four patients), 90D, 163R, and 152A (each in a single patient). The observation that the majority of antibodies were HLA-A associated has been previously reported in sensitized renal transplant recipients,[71,72] but this is the first report of such an association in multiply transfused AAA patients. Our observation that HLA-A1 CREG epitopes appear to be dominant targets in the alloresponse to multiple transfusions may also be true in other multitransfused patient groups, although there is no published epitope analysis data to that effect at present. Antibodies with specificity for HLA-A1 CREGs associated with HLA-A2 and alleles of the HLA-A19 complex[70] were not identified in this study. HLA-C is not considered to play a significant role in PR[73] and our data support this. Data regarding possible "passive" transfer of antibodies through transfusion were not available. However, the strong reactivity and specificity of the antibody-defined group make passive transfer an unlikely explanation.

Dobbe et al.[74] have reported poor renal graft survival in non-CyA-treated HLA-A1-negative recipients transplanted with HLA-A1-positive kidneys. Poor survival was not associated with the HLA-A1, B8, DR3 haplotype and occurred irrespective of HLA-DR match grade. Sequence analysis of HLA-A1 reveals the highest number of amino acid substitutions in the α1 and particularly the α2 domains of the HLA Class I molecule. This may be reflected in the unique patterns of reactivity of HLA-A1 with MoAbs[75] and the uniqueness of the peptide binding motifs associated with HLA-A1.[76] Furthermore, incompatibility at the amino acid level of the α1 and α2 domains is significantly correlated with FC cross-match outcome.[77] The extensive sequence variation of the HLA-A1 molecule makes it potentially highly immunogenic. Thus, proteolytic degradation would produce a number of unique

peptides that could be presented by the recipient's antigen-presenting cells and T cells.

The observed longevity of antibody production and its fine specificity were remarkable and inconsistent with notions of maintenance of diversity in memory B-cell populations developed in experimental models.[65,78] In one case (FR122), more than 8 years had elapsed since the last blood transfusion, suggesting that hypermutated IgG memory B cells had continued to replicate and give progeny specifically focused on a single Class I epitope, characterized by 74H and 107W (Table 3). Similar observations have been made in patients receiving multiple PLT transfusions and in patients with end-stage renal disease.[79,80] It appears that memory B cell or plasma cell clones responsible for these antibodies have become dominant compared to other alloantibody-producing clones. These dominant clones could be replicating in the absence of exposure to antigen, a scenario that might have evolved if they had undergone in vivo EBV transformation. Our previous finding of an IgM anti-HLA Class II secreting memory B-cell clone in a renal transplant recipient who had been treated with several courses of immunosuppressive therapy are consistent with this notion.[81] Alternatively, if antibody-producing cells are deemed to require persistent antigen to drive them,[82] viable cells originating from the transfusion donor and establishing long-term multilineage chimaerism in the recipient would offer an obvious source.[21,83,84]

What are the implications for T-cell memory? Allosensitized cytotoxic and helper T cells reactive in mixed lymphocyte reactions are well documented in multiply transfused AAA. Their reactions are directed to HLA, non-HLA, and autologous antigens.[45-47] This heightened T-cell reactivity appears to be resistant to ATG treatment, yet one of the known properties of ATG is to wipe out immunologic memory.[85] Autoreactive CD8+ T cells directed to HPCs may play a role in the pathogenesis of AAA, and these appear to be susceptible, at least in the short term, to ATG treatment.[86] In mouse models, evidence is emerging that CD4+ T-cell memory is dependent on B-cell sensitization, but CD8+ T-cell memory is independent of B cells.[87,88] In multiply transfused patients, HLA molecules could be taken up via epitope-specific B-cell receptors and after intracellular digestion be presented as peptides to CD4+ T-cell clones, thereby maintaining T-cell memory. Thus, memory B cells in AAA could play a crucial role in maintaining immunologic memory and accounting for transfusion refractoriness and the high risk of nonengraftment after stem cell transplantation.

What are the implications for understanding the pathogenesis of AAA? Although AAA is associated with defective stem cell function with both qualitative and quantitative deficiencies of $CD34^+$ hemopoietic precursor cells documented,[89] the response to therapy suggests a strong autoimmune overlay. Humoral and cellular responses to alloantigens may therefore be a paradigm for responses to the auto or neo-antigens on abnormal stem cells that lead to aplasia. Humoral inhibitors in AAA suppress colony-forming cells and, as with antibodies to HLA, they disappear after ATG treatment in some cases.[90,91] Equally, colony-forming cells from AAA patients are suppressed by CD8+ T cells, and these disappear after ATG therapy in some cases.[86] Thus, individual susceptibility to ATG and CyA therapy of responses to allo- and autoantigens may be similar. In patients who respond initially to immunosuppressive therapy but then relapse, there may be a resurgence of neo-antigen positive stem cells causing reawakening of memory B and T cells.

The extent and pattern of skewing of the variable region of the beta-chain (V β), T-cell receptor (TCR) repertoire has been studied in AA patients through analysis of the polymorphisms within the complementarity-determining region 3 (CDR3) of the TCR-V β chain.[92-95] Evidence suggested that particular V β families were more likely to show specific patterns of CDR3 skewing and these were clearly different from those seen in diseases associated with monoclonal expansion.[94] Thus, many clones appear to be involved in the immune response in AAA. CDR3 size polymorphism has been shown to be significantly skewed in HLA-DRB*1501-positive CyA-dependent AAA patients,[93] whereas there was no significant deviation observed in patients who had received ATG.[94]

What are the practical implications of our findings? Firstly, the current strategy to avoid transfusion refractoriness involves the recruitment of HLA-Class-I–compatible PLT donors. This is both expensive and labor intensive. Where refractoriness is attributable to antibodies to a few epitopes, a strategy of "epitope avoidance" could be invoked that would be more cost-effective. Antibodies would be analyzed with respect to their actual HLA specificity, and only donors that typed positive for the relevant epitope(s) would be excluded from the panel. This would substantially increase the proportion of acceptable donations. A predictive approach utilizing the "HLAMatchmaker" algorithm facilitates the determination of HLA Class I compatibility at the molecular level.[96] Our proposed strategy of epitope analysis followed by epitope avoidance is testable in a prospective multicenter trial.

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