Comparison between hybridoma and Fab/phage anti-RhD: Their V gene usage and pairings

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Our 11 anti-RhD's in conjunction with 37 previously published RhD antibodies, produced by hybridoma technology were analysed for germline gene usage and restriction in V_H and V_L pairings. The 17 V_H germline genes used by the hybridoma anti-RhD IgG were derived from 4 V_H families (VH1, VH2, VH3 and VH4). Eighteen kappa chains were restricted to only 5 germline genes from only 2 V_K families (V_K1 and V_K3). However, the 13 lambda chains were not as restricted, using 10 V_λ germline genes from 4 families ($V_\lambda1$, $V_\lambda2$, $V_\lambda3$ and $V_\lambda8$). Fifty six unique Fab/phage anti-RhD were also analysed. In all cases the Fab/phage V_H germline genes were derived from the VH3 family (41/41). The 29 kappa chains were restricted to 4 germline genes primarily from V_K1 (97%) and the 24 lambda chains used 10 V_λ germline genes from 5 families ($V_\lambda1$, $V_\lambda2$, $V_\lambda3$, $V_\lambda4$ and $V_\lambda7$). The V_H germline genes of the Fab/phage were restricted to 4 of the 17 used by the hybridoma anti-RhD IgG (DP46, DP49, DP50 and DP77). Ninety percent of the Fab/phage were restricted to 1 of the 5 V_K germline genes used by the IgG (DPK9). However, the repertoire of the V_λ germline genes used in these two systems is different, with analysis showing greater diversity in V_λ gene usage with 8 unique germline genes used by 76% Fab/phage compared to 4 unique genes used by 46% of the IgG hybridoma anti-RhD.

Keywords: Anti-RhD, germline gene, canonical structure, V_H and V_L pairings

1. Introduction

The RhD antigen is a highly immunogenic human red cell antigen. Alloimmunisation against the RhD antigen produces high affinity antibodies that cause haemolytic transfusion reactions (HTR) and haemolytic disease of the newborn (HDN) [1]. Currently, plasma-derived polyclonal anti-RhD is used for prophylaxis of RhD HDN although, human monoclonal RhD antibodies are being evaluated for possible replacement. The biochemistry and physiological function of the RhD antigen is not yet fully understood. Cloning and sequence analysis predicted a 417 amino acid protein of 45.5 kDa [2], with 12 hydrophobic transmembrane domains and cytoplasmic N and C termini resulting in 6 extracellular loops [3,4]. Epitope mapping of the RhD antigen, investigated by site directed mutagenesis indicated the presence of six external, distinct, non-overlapping epitopes [5].

Diversity of the antibody repertoire is a consequence of the recombination of the germline gene segments (V, D and J) during B cell development [6]. The rearranged V(D)J gene segments of the heavy and light chains are derived from functional germline genes consisting of 51 V_H , 25 D, 6 J_H and 31 V_λ , 4 J_λ or 40 V_K and 5 J_K genes [7–11]. Diversity is further increased when boundaries between the V(D)J gene segments undergo additions or deletions [12] and somatic hypermutation at the mature B cell stage.

In recent years a large number of hybridoma and Fab/phage anti-RhD have been sequenced in an attempt to identify usage of V_H and V_L germline genes, somatic hypermutations and affinity maturation. With construction of a database consisting of 48 hybridoma and 56 Fab/phage anti-RhD, restriction of the V_H and V_L gene repertoire and their pairings can be confidently compared and determined.

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Table 1

Compilation of V genes for hybridoma IgG and Fab/phage anti-RhD

Publications	Year	No of Anti-RhD	Donors
Hybridoma anti-RhD			
Bye et al.	1992	10x IgG; 4x IgM	8 donors
Chouchane et al.	1992	1x IgG	1 donor
Dziegel et al.	1995	1x IgG	1 donor
Borretzen et al.	1995	9x IgM	1 donor
Boucher et al.	1997	10x IgG	2 donors
Edelman et al.	1997	1x IgM	1 donor
Paterson et al.	1998	1x IgG	1 donors
Perera et al.	2000	10x IgG; 1x IgM	8 donors
Fab/Phage anti-RhD			
Chang et al.	1998	43x Fab/Phage	1 donor
Miescher et al.	1998	13x Fab/Phage	1 donor

The compiled IgG anti-RhD are represented by V_H : $n = 33$, V_K : $n = 18$ and V_λ : $n = 13$ (λ chain of MD03 and the light chain of Oak-3 [14] was not determined) [13–20]. Fab/phage anti-RhD are represented by V_H : $n = 41$, V_K : $n = 29$ and V_λ : $n = 24$ [21,22].

2. Compilation of hybridoma and Fab/phage anti-RhD database

A hybridoma database was created with our 11 anti-RhD [13] and 37 previously published antibodies [14–20] (see Table 1). Each of these 48 anti-RhD had been positively selected against the RhD antigen and derived from 23 immunised donors. Using the phage library technique, two groups [21,22] (see Table 1) have described usage of 41 heavy chain and 53 light chain germline genes (29 kappa and 24 lambda), generating 56 unique Fab/phage anti-RhD.

3. Comparison of V gene usage and pairing between hybridoma IgG and Fab/phage anti-RhD

It has been recognised that biased expression of V_H and V_L families or particular V genes occurs in non-immunised individuals. Bias for the VH3 and $V_{\lambda 2}$ family have been described in IgG and IgM peripheral blood B cells from normal healthy donors [23,24], although there is no evidence for preferential V_H and V_L pairings. In the case of autoantibodies there is evidence that there may be preferential use of certain V_H genes. The anti-I/i cold agglutinins use DP-63 (VH4.21) but demonstrate different light chain usage with anti-I preferring VK3 family light chains [25–28]. Antibodies against gp120 of HIV-1 [29] showed preferential usage of the VH1, VH3 and VH5 families. Similarly, a limited set of VH3 family members have been found in the immune response to *H. influenzae* type b [30].

The favoured V_H germline gene segments used by the 48 hybridoma anti-RhDs (33 IgG and 15 IgM) were

mainly from the VH4 (44%, 21/48), VH3 (40%, 19/48) and VH1 (15%, 7/48) families. The V_H germline gene segment preferred by the 15 IgM was DP-63 (14/15) and in all cases where the confirmed light chain was lambda, the V gene DPL16 (9/9) was preferred. This data suggests that the pairing of DP-63 with DPL16 is the most common primary immune response to the RhD antigen.

The 17 V_H germline genes used by the 33 hybridoma anti-RhD IgGs were derived from the VH1 (7/33), VH2 (1/33), VH3 (18/33) and VH4 (7/33) families. The 18 kappa chain anti-RhD (9 IgG1 and 9 IgG3) favoured V_H germline gene segments from the VH3 family (13/18). Furthermore, these kappa chains were restricted to a total of only 5 germline genes from the VK1 (13/18) and VK3 (5/18) families, particularly DPK9 and DPK22 (Table 2). In 6 instances the DPK9 germline gene was paired with a member of the “VH3-33 superspecies” [21]. In contrast to the IgG/ κ anti-RhD, germline gene usage by the 14 IgG/ λ antibodies was not as restricted. These antibodies used 6 V_λ germline genes from the $V_{\lambda 1}$ (4/13), $V_{\lambda 2}$ (1/13), $V_{\lambda 3}$ (7/13) and $V_{\lambda 8}$ (1/13) (λ chain of MD03 [13] was not determined) families which paired with V_H germline genes from the VH4 (6/14), VH1 (4/14) and VH3 (4/14) families (Table 2). However, preferential usage of the D6 (9/14) and JH6 (9/14) gene segments was recognised, with 8 of 9 anti-RhD using the D6-JH6 pairing.

The 41 V_H germline genes used by the 56 unique Fab/phage anti-RhD were derived in all cases from the VH3 family and the kappa chains were restricted to only 4 germline genes from the VK1 (28/29) and VK2 (1/29) families. The vast majority of these Fab/phage preferred the DPK9 germline gene for kappa chain and the “VH3-33 superspecies” gene for the heavy chain. The lambda chains however used 10 V_L germline genes from the $V_{\lambda 1}$ (13/24), $V_{\lambda 2}$ (2/24), $V_{\lambda 3}$ (5/24), $V_{\lambda 4}$ (1/24) and $V_{\lambda 7}$ (3/24) families. Table 2 compares the usage and frequency of the germline genes used in both the hybridoma and phage library systems. Only 4 out of the 17 V_H germline genes used by hybridoma IgG were observed for Fab/phage. These 4 V_H germline genes accounted for 100% of the Fab/phage but only 43% of the IgG. Ninety percent of the Fab/phage were restricted to 1 of the 5 V_K germline genes used by the hybridoma anti-RhD IgG. However, the repertoire of the V_λ germline genes used between these two systems is different and with greater diversity. Two germline genes, DPL5 and DPL16 were common to both systems but only 24% of the Fab/phage and 54% of the anti-RhD IgG used these 2 genes. An additional 8 unique

Table 2
Usage and frequency of germline V genes for hybridoma IgG and Fab/phage anti-RhD

Family	Germline V gene	Frequency of usage by anti-RhD IgG	Frequency of usage by Fab/phage
VH1	DP-7	2 (6%)	0
	DP-8	2 (6%)	0
	DP-25	1 (3%)	0
	DP-75	1 (3%)	0
	DP-88	1 (3%)	0
VH2	DP-26	1 (3%)	0
VH3	COS-3	1 (3%)	0
	DP-46	1 (3%)	1 (2%)
	DP-49	3 (9%)	6 (15%)
	DP-50	10 (30%)	32 (78%)
	DP-53	2 (6%)	0
VH4	DP-77	1 (3%)	2 (5%)
	DP-63	3 (9%)	0
	DP-71	1 (3%)	0
	DP-79	1 (3%)	0
	VH5	1 (3%)	0
Vλ1	V2-1	1 (3%)	0
	DPL2	0	4 (17%)
	DPL3	0	4 (17%)
	DPL5	3 (23%)	2 (8%)
	DPL6	1 (8%)	0
Vλ2	DPL7	0	3 (13%)
	DPL10	0	1 (4%)
	DPL11	1 (8%)	0
	2c.118D9	0	1 (4%)
	DPL16	4 (31%)	4 (17%)
Vλ3	DPL23	3 (23%)	0
	3p.81A4	0	1 (4%)
	4b.68B6	0	1 (4%)
Vλ4	DPL18	0	3 (13%)
Vλ7	DPL21	1 (8%)	0
Vκ1	DPK3	2 (11%)	0
	DPK8	0	1 (3%)
	DPK9	9 (50%)	26 (90%)
	A30	0	1 (3%)
	L12	2 (11%)	0
Vκ2	DPK15	0	1 (3%)
Vκ3	DPK21	1 (6%)	0
	DPK22	4 (22%)	0

Sequence data was available for 33 V_H; 18 V_κ and 13 V_λ hybridoma IgG and 41 V_H; 29 V_κ and 24 V_λ for Fab/phage anti-RhD.

V_λ germline genes used by 76% Fab/phage were absent from hybridoma IgG. Thus the Fab/phage germline gene usage was restricted for the V_H and V_κ but different for the V_λ when compared with the hybridoma IgG anti-RhD's.

4. Comparison of canonical combinations between hybridoma IgG and Fab/phage anti-RhD

Canonical combinations of the V_H and V_L provides a better understanding of the conformation of the paratope which is responsible for binding of antigens. Here we describe and compare the canonical combina-

tion between hybridoma IgG and Fab/phage anti-RhD. Twenty four V_H and V_L gene pairings were identified for the hybridoma IgG compared to only 18 for the Fab/phage anti-RhD, resulting in 10 and 7 canonical combinations, respectively. The most commonly used combination by the kappa IgG anti-RhD was V_H1-3 : V_κ2-1 (67%, 12/18). This combination was found almost exclusively for the kappa Fab/phage (97%, 29/30). A further 3 canonical structures were unique to kappa IgG but not Fab/phage. The V_H1-3 : V_κ2-1 structure has been reported most heavily used (21%) by randomly paired V gene segments from an non-immunised source [31]. Usage of this canonical combination was

Table 3
Canonical combinations for hybridoma IgG and Fab/phage anti-RhD

(A) $V_H - V_\kappa$	$V_H - V_\kappa$		(B) $V_H - V_\lambda$	$V_H - V_\lambda$	
	18 IgG	30 Fab/phage		13 IgG	26 Fab/phage
3-1 : 2-1	1	0	3-1 : 13-7A	2	
1-1 : 2-1	1	0	1-3 : 14-7A	2	5
1-3 : 2-1	12	29	1-3 : 13-7A		11
1-3 : 6-1	4	0	1-3 : 11-7	4	6
1-3 : 4-1	0	1	1-3 : 14-7B	1	3
			1-1 : 11-7	3	
			1-2 : 13-7A	1	
			1-3 : 12-11		1

The comparison of (A) V_H and V_κ and (B) V_H and V_λ canonical combinations between hybridoma IgG and Fab/phage anti-RhD. The V_H , V_κ and V_λ loop structures were determined using the V-BASE Directory of Human V gene Sequences [33].

recently reported for antibodies raised against a second antigen (E) of the Rh system [32].

Two canonical combinations, $V_H1-3 : V_\lambda11-7$ and $V_H1-1 : V_\lambda11-7$ were used by the majority of the lambda IgG hybridomas although a different and unique structure ($V_H1-3 : V_\lambda13-7A$) was preferred by the Fab/phage (see Table 3). Hybridoma IgM anti-RhD were exclusive to the $V_H1-1 : V_\lambda11-7$ structure which was not favoured by Fab/phage and used by only one IgG. The $V_H1-3 : V_\lambda14-7A$ (10%), $V_H1-3 : V_\lambda11-7$ (7%) and $V_H1-3 : V_\lambda13-7A$ (5.5%) are the three most heavily used canonical structures by B cells from a non-immunised source [31].

It is apparent from this analysis that the V_H and V_L germline genes for Fab/phage are restricted only for V_H and V_κ but different for the V_λ when compared with hybridoma IgG. This was further apparent when their pairing and canonical combinations were considered. In general, when creating phage libraries for a particular specificity, the germline gene usage and pairing for kappa libraries may be more restricted compared to lambda libraries.

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