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 VH families (VH1, VH2, VH3 and VH4). Eighteen kappa chains were restricted to only 5 germline genes from only 2 V_K families (VK1 and VK3). However, the 13 lambda chains were not as restricted, using 10 V_L germline genes from 4 families (VL1, VL2, VL3 and VL8). 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 VK1 (97%) and the 24 lambda chains used 10 V_L germline genes from 5 families (VL1, VL2, VL3, VL4 and VL7). 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_L germline genes used in these two systems is different, with analysis showing greater diversity in V_L 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_L, 4 J_L 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.
immune response to
ited set of VH3 family members have been found in the
against gp120 of HIV-1 [29] showed preferential usage
ferring VK3 family light chains [25–28]. Antibodies
demonstrate different light chain usage with anti-I pre-
that there may be preferential use of certain V
pairings. In the case of autoantibodies there is evidence
and V_H genes.

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|
| Choucane et al.     | 1992 | 1x IgG         | 1 donor |
| Dziegel et al.      | 1995 | 1x IgG         | 1 donor |
| Borrezen 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_L: n = 18 and V_L: n = 13 (\lambda 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_L: n = 29 and V_L: 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_L2 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-Ig cold agglutinins use DP-63 (VH4-21) but
demonstrate different light chain usage with anti-I pre-
ferring 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 limi-
ted 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/IgM anti-
RhD, germline gene usage by the 14 IgG/\lambda antibod-
ies was not as restricted. These antibodies used 6 V_L
germline genes from the V_L1 (4/13), V_L2 (1/13), V_L3
(7/13) and V_L8 (1/13) (\lambda 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 recog-
nised, 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_L1 (13/24), V_L2 (2/24), V_L3 (5/24), V_L4
(1/24) and V_L7 (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_L germline genes used by the
hybridoma anti-RhD IgG. However, the repertoire of the
V_L 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                              |
|        | DP-77           | 1 (3%)                            | 2 (5%)                         |
| VH4    | DP-63           | 3 (9%)                            | 0                              |
|        | DP-71           | 1 (3%)                            | 0                              |
|        | DP-79           | 1 (3%)                            | 0                              |
|        | VH5             | 1 (3%)                            | 0                              |
|        | V2-1            | 1 (3%)                            | 0                              |
| VA1    | DPL2            | 0                                 | 4 (17%)                        |
|        | DPL3            | 0                                 | 4 (17%)                        |
|        | DPL5            | 3 (23%)                           | 2 (8%)                         |
|        | DPL6            | 1 (8%)                            | 0                              |
|        | DPL7            | 0                                 | 3 (13%)                        |
| VA2    | DPL10           | 0                                 | 1 (4%)                         |
|        | DPL11           | 1 (8%)                            | 0                              |
|        | 2c.118D9        | 0                                 | 1 (4%)                         |
| VA3    | DPL16           | 4 (31%)                           | 4 (17%)                        |
|        | DPL23           | 3 (23%)                           | 0                              |
|        | 3p.81A4         | 0                                 | 1 (4%)                         |
| VA4    | 4b.68B6         | 0                                 | 1 (4%)                         |
| VA7    | DPL18           | 0                                 | 3 (13%)                        |
| VA8    | DPL21           | 1 (8%)                            | 0                              |
| Vh1    | DPK3            | 2 (11%)                           | 0                              |
|        | DPK8            | 0                                 | 1 (3%)                         |
|        | DPK9            | 9 (50%)                           | 26 (90%)                       |
|        | A30             | 0                                 | 1 (3%)                         |
|        | L12             | 2 (11%)                           | 0                              |
| Vh2    | DPK15           | 0                                 | 1 (3%)                         |
| Vh3    | DPK21           | 1 (6%)                            | 0                              |
|        | DPK22           | 4 (22%)                           | 0                              |

Sequence data was available for 33 V<sub>H</sub>; 18 V<sub>h</sub> and 13 V<sub>λ</sub> hybridoma IgG and 41 V<sub>H</sub>; 29 V<sub>h</sub> and 24 V<sub>λ</sub> for Fab/phage anti-RhD.

V<sub>λ</sub> germline genes used by 76% Fab/phage were absent from hybridoma IgG. Thus the Fab/phage germline gene usage was restricted for the V<sub>H</sub> and V<sub>h</sub> but different for the V<sub>λ</sub> 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<sub>H</sub> and V<sub>L</sub> provides a better understanding of the conformation of the paratope which is responsible for binding of antigens. Here we describe and compare the canonical combination between hybridoma IgG and Fab/phage anti-RhD.

Twenty four V<sub>H</sub> and V<sub>L</sub> 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<sub>H</sub>1-3 : V<sub>κ</sub>2-1 (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<sub>H</sub>1-3 : V<sub>κ</sub>2-1 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
recently reported for antibodies raised against a second antigen (E) of the Rh system [32].

Two canonical combinations, $V_H1-3 : V_L11-7$ and $V_H1-1 : V_L11-7$ were used by the majority of the lambda IgG hybridomas although a different and unique structure ($V_H1-3 : V_L13-7A$) was preferred by the Fab/phage (see Table 3). Hybridoma IgM anti-RhD were exclusive to the $V_H1-1 : V_L11-7$ structure which was not favoured by Fab/phage and used by only one IgG. The $V_H1-3 : V_L14-7A (10\%)$, $V_H1-3 : V_L11-7 (7\%)$ and $V_H1-3 : V_L13-7A (5.5\%)$ 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 $V_K$ but different for the $V_L$ 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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References

[1] P.L. Mollison, C.P. Engelfriet and M. Contreras, Blood transfusion in clinical medicine, (10th ed.), Blackwell Science, Oxford, 1996.

[2] C. Le Van Kim, I. Mouro and B. Cherif-Zahar et al., Molecular cloning and primary structure of the human blood group RhD polypeptide, Proc Natl Acad Sci USA 89 (1992), 10925–10929.

[3] D.J. Anstee and M.J. Tanner, Biochemical aspects of the blood group Rh (rhesus) antigens, Baillieres Clinical Haematology 6 (1993), 401–422.

[4] N.D. Avent, W. Liu and K.M. Warner et al., Immunochromatographic analysis of the human erythrocyte Rh polypeptides, J Biol Chem 271 (1996), 14233–14239.

[5] W. Liu, J.S. Smythe and M.L. Scott et al., Site-directed mutagenesis of the human D antigen: definition of D epitopes on the sixth external domain of the D protein expressed on K562 cells, Transfusion 39 (1999), 17–25.

[6] G. Winter and C. Milstein, Man-made antibodies, Nature 349 (1991), 293–395.

[7] L.J. Fanning, A.M. Connor and G.E. Wu, Development of the immunoglobulin repertoire, Clin Immunol & Immunopath 79 (1996), 401–422.

[8] G.P. Cook, I.M. Tomlinson, G. Walter, H. Riethman, N.P. Carter, L. Buluwela, G. Winter and T.H. Rabbitts, A map of the human immunoglobulin $V_L$ locus completed by analysis of the telomeric region of chromosome 14q, Nat Genet 7 (1994), 162–169.

[9] J.P. Cox, I.M. Tomlinson and G. Winter, A directory of human germ-line V kappa segments reveals a strong bias in their usage, Eur J Immunol 24 (1994), 827–835.

[10] I.M. Tomlinson, G.P. Cook, G. Walter, N.P. Carter, H. Riethman, L. Buluwela, T.H. Rabbitts and G. Winter, A complete map of the human immunoglobulin $V_H$ locus, Ann NY Acad Sci 764 (1995), 43–50.

[11] J.P. Frappiat, S.C. Williams, I.M. Tomlinson, G.P. Cook, D. Cherif, D. Le Paslier, J.E. Collins, I. Dunham, G. Winter and M.P. Lefranc, Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2, Hum Mol Genet 4 (1995), 983–996.

[12] G.H. Gauss and M.R. Lieber, Mechanistic constraints on diversity in human V(D)J recombination, Mol & Cell Biol 16 (1996), 258–263.

[13] W.S. Perera, M.T. Moss and S.J. Urbaniai, V(D)J germline gene repertoire analysis of RhD antibodies and their implication to RhD epitope specificity, Transfusion 40 (2000), 1–10.

[14] J.M. Bye, C. Carter and Y. Cui et al., Germline variable region gene segment derivation of human monoclonal anti-Rh(D) antibodies. Evidence for affinity maturation by somatic hypermutation and repertoire shift, J Clin Invest 90 (1992), 2481–2490.

[15] G. Boucher, H. Broly and R. Lemieux, Restricted use of cationic germline $V(H)$ gene segments in human Rh(D) red cell antibodies, Blood 89 (1997), 3277–3286.
[16] L. Chouchane, A. Van Spronsen and J. Breyer et al., Molecular characterization of a human anti-Rh(D) antibody with a DH segment encoded by a germ-line sequence, *Eur J Biochem* **207** (1992), 1115–1121.

[17] J.D. Marks, W.H. Ouwehand and J.M. Bye et al., Human antibody fragments specific for human blood group antigens from a phage display library, *BioTechnology* **11** (1993), 1145–1149.

[18] M. Borretzen, C. Chapman and F.K. Stevenson et al., Structural analysis of VH4-21 encoded human IgM allo- and autoantibodies against red blood cells, *Scand J Immunol* **42** (1995), 90–97.

[19] L. Edelman, C. Margaritte and H. Chaabihi et al., Obtaining a functional recombinant anti-rhesus (D) antibody using the baculovirus-insect cell expression system, *Immunology* **91** (1997), 13–19.

[20] T. Paterson, J. Innes and L. McMillan et al., Variation in IgG1 heavy chain allotype does not contribute to differences in biological activity of two human anti-Rhesus (D) monoclonal antibodies, *Immunotechnology* **4** (1998), 37–47.

[21] T.Y. Chang and D.L. Siegel, Genetic and immunological properties of phage-displayed human anti-Rh(D) antibodies: implications for Rh(D) epitope topology, *Blood* **91** (1998), 3066–3078.

[22] S. Miescher, M. Vogel and C. Biaggi et al., Sequence and specificity analysis of recombinant human Fab Anti-Rh D isolated by phage display, *Vox Sang* **75** (1998), 278–287.

[23] H.P. Brezinschek, R.I. Brezinschek and P.E. Lipsky, Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction, *J Immunol* **155** (1995), 190–202.

[24] H.P. Brezinschek, S.J. Foster and R.I. Brezinschek et al., Analysis of the human V_{H} gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral CD5+AgM+ and CD5-AgM+ B cells, *J Clin Invest* **99** (1997), 2488–2501.

[25] G.J. Silverman and D.A. Carson, Structural characterization of human monoclonal cold agglutinins: evidence for a distinct primary sequence-defined VH4 idiotype, *Eur J Immunol* **20** (1990), 351–356.

[26] K.M. Thompson, J. Sutherland and G. Barden et al., Human monoclonal antibodies against blood antigens preferentially express a VH4.21 variable region gene-associated epitope, *Scand J Immunol* **34** (1991), 509–518.

[27] L.E. Silverstein, L.C. Jefferies and J. Goldman et al., Variable region gene analysis of pathologic human autoantibodies to the related i and I red cell antigens, *Blood* **78** (1991), 2372–2386.

[28] S.J. Thorpe, C.E. Turner and F.K. Stevenson et al., Human monoclonal antibodies encoded by the V4-34 gene segment show cold agglutinin activity and variable multireactivity which correlates with the predicted charge of the heavy-chain variable region, *Immunology* **93** (1998), 129–136.

[29] M. Zouali, B-cell superantigens: implication for selection of the human antibody repertoire, *Immunology Today* **16** (1995), 399–405.

[30] E.E. Adderson, P.G. Shackelford and A. Quinn et al., Restricted immunoglobulin V_{H} usage and VDJ combinations in the human response to Haemophilus influenzae type b capsular polysaccharide, *J Clin Invest* **91** (1993), 2734–2743.

[31] R.M.T. de Wildt, R.M.A. Hoet and W.J. van Venrooij et al., Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire, *J Mol Biol* **285** (1999), 895–901.

[32] N.C. Hughes-Jones, J.M. Bye and B.D. Gorick et al., Synthesis of Rh Fv phage-antibodies using V_{H} and V_{L} germline genes, *Br J Haematol* **105** (1999), 811–816.

[33] I.M. Tomlinson, S.C. Williams and S.J. Corbett et al., V Base Directory of Human V Gene Sequences. http://www.mrc-cpe.cam.ac.uk/imt_doc/vbase_home_page.html (internet), 1996.