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# Anti-mouse red blood cell monoclonal antibodies use functionally rearranged genes from the $V_H$ J558 family and are derived from the $CD5^-$ B-lymphocyte subpopulation

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## SUMMARY

The NZB mouse strain is genetically predisposed to develop, at approximately 6 months of age, a spontaneous and severe autoimmune anaemia caused by the production of pathogenic anti-mouse red blood cell (MRBC) autoantibodies. Although it is believed that the predisposition to autoimmune anaemia is multigenic in nature, the main pathogenic mechanism is attributed to anti-MRBC autoantibodies. We have generated eight anti-MRBC monoclonal antibody (mAb)-producing hybridomas derived from splenocytes of 9- and 12-month-old NZB mice with spontaneous autoimmune anaemia to dissect the molecular and cellular mechanisms resulting in the production of these pathogenic antibodies. The predominant immunoglobulin isotype was IgG2a, produced by five out of eight hybridomas (63%), while IgM, IgG1 and IgG2b were each produced by one hybridoma cell line (12%). Antigen specificity analysis of all eight hybridomas revealed that antibodies from seven out of eight hybridomas were monospecific for MRBC antigen(s). Only one hybridoma (clone 4-16-1) cross-reacted with rat RBC. None of the hybridomas produced antibodies reactive with single- or double-stranded DNA (ss- or dsDNA). Surface and cytoplasmic staining for the CD5 antigen revealed that none of the hybridomas was derived from $CD5^+$ B lymphocytes. All hybridomas cause anaemia when implanted intraperitoneally into normal BALB/c mice. Molecular studies of five of the eight anti-MRBC mAb reveal that all use functionally rearranged genes from the $V_H$ J558 gene family. Three of these five mAb used FL16.1 $D_H$ genes while one had a CDR3 that resulted from a fusion between two $D_H$ genes (SP2.3 and SP2.2) from the SP family.

## INTRODUCTION

The almost unlimited potential for V-region diversity resulting from combinatorial events during $V_H$-D-$J_H$ and $V_L$-$J_L$ gene rearrangements and subsequent somatic mutations is responsible for the existence of antibodies with specificity for self-antigen. It is of importance to determine the source of pathogenic autoantibodies and to ascertain whether they arise from natural antibodies by a process of isotype switch and somatic mutation or from the normal repertoire by antigen selection. Natural antibodies, which are present in normal germ-free animals, have the capacity to combine specifically with potential antigens including self-antigens. These antibodies are generally of the IgM isotype, may be polyreactive and have low binding affinities for antigen.[1] Further controversy surrounds the cellular origin of autoantibody-producing B lymphocytes. It is unclear whether pathogenic autoantibodies are produced by conventional B lymphocytes or the distinct B-lymphocyte subpopulation which expresses the CD5 antigen (CD5 B lymphocytes).

The anti-mouse red blood cells (MRBC) autoimmune response in NZB mice is a pathogenic autoantibody response which has been extensively studied. The response was thought to be heterogeneous as two different antigens were recognized by autoantibodies eluted from NZB RBC (AgX and Ag HB).[2] The autoantibody response is comprised of both IgM and IgG, also suggesting that the response is heterogeneous.[3] However it is now thought that the exposed antigen X is the main antigen involved in the initiation of the autoimmune response,[3] and that IgM autoantibody undergoes isotype switch resulting in IgG autoantibodies with the same Ag-binding specificity.[4]

It has previously been shown that pathogenic anti-MRBC autoantibodies are encoded by three $V_H$ gene families (J558, J606 and 3609) all distal to the D-region locus.[5,6] These studies provide some evidence that pathogenic anti-MRBC may not be derived from 'natural' autoantibodies which are, in the main, encoded by D-proximal 3′ gene families. However the molecular mechanism that results in pathogenic anti-MRBC autoantibody production remains unclear. In particular it is not known if the

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appearance of anti-MRBC-producing B lymphocytes results from the inheritance of autoantibody encoding variable region genes (IgV genes) in the germline or from antigen-driven somatic mutations in common IgV genes.

We have generated eight anti-MRBC hybridomas from splenocytes of 9- and 12-month-old mice with spontaneous autoimmune anaemia in order to address these questions.

## MATERIALS AND METHODS

*Generation and characterization of monoclonal anti-mouse red blood cell antibodies*

Hybridomas in this study were derived from two fusions of splenocytes from unprimed 9- and 12-month-old NZB mice with spontaneous autoimmune anaemia, with the myeloma cell line P3X63-Ag8.653 as described previously.[7] An indirect haemagglutination assay (HA) was used to detect the anti-MRBC autoantibody activity in supernatants of established lines. Briefly, 25 $\mu$l of hybridoma culture supernatant was incubated with a 1% washed CBA MRBC suspension in the presence of goat anti-mouse Ig (1:500) (Sigma Chemical Co., St Louis, MO) for 1 hr at room temperature and haemagglutination assessed. Final titres produced by each hybridoma were determined using supernatants standardized to 5 $\mu$g Ig/ml to measure the highest dilution at which haemagglutination occurred. The immunoglobulin isotype of each hybridoma was determined by enzyme-linked immunosorbent assay (ELISA) using goat anti-mouse IgM and IgG (Sigma), and goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology, Birmingham, AL). Cross-reactivity with RBC from other species was also determined by HA, substituting MRBC with the RBC of rabbit, rat, sheep or human. Cross-reactivity with double- and single-stranded DNA (ds- or ssDNA) (Sigma) was measured by ELISA.

*Pathogenic activity of the anti-MRBC monoclonal antibodies (mAb)*

To determine the pathogenic activity of the anti-MRBC mAb haematocrits and Coombs' titres were determined in groups of three BALB/c mice (aged 3 months) at 8, 19 and 33 days after intraperitoneal (i.p.) injection of $1 \times 10^6$ hybridoma cells.

Haematocrits were determined using heparinized micro-haematocrit tubes centrifuged at 9000 ***g*** for 10 min. Coombs' titres (direct haemagglutination) were determined using a 1:100 dilution of goat anti-mouse Ig (1 mg/ml) (Sigma) incubated with 1% MRBC suspension for 1 hr at 37° in U-bottomed microtitre plates.

*Immunofluorescence studies*

Immunofluorescence experiments were based on methods previously described by Fidanza *et al.*[8] with the following modifications. Hybridoma cells ($1 \times 10^6$) were washed twice with phosphate-buffered saline (PBS) containing 0·02% sodium azide. For surface CD5 staining fluorescein isothiocyanate (FITC) anti-CD5 (FITC anti-Ly-1; Becton Dickinson, Cowley, U.K.) (1:100) v/v was added to the cell suspension and incubated for 20 min on ice. For cytoplasmic CD5 staining the cells were fixed with 1% paraformaldehyde in PBS, at room temperature for 30 min. Cells were then washed twice in PBS and suspended in saponin buffer [PBS with 0·1% saponin, 2% bovine serum albumin (BSA) and 1 mM EGTA] and incubated for a further 30 min at room temperature. FITC anti-CD5 (1:100) v/v was added to the cell suspension in saponin buffer and incubated for 20 min on ice. The cells stained either for surface or cytoplasmic CD5 were then washed and resuspended in 300 $\mu$l PBS for FACS analysis on a FACScan (Becton Dickinson). The myeloma cell line P3X63Ag8.653 was used as a negative control and the T-cell line EL4 as a positive control for CD5 staining.[8]

*Molecular studies*

RNA was extracted from hybridomas grown in tissue culture by the method of Chomzynski and Scacchi using guanidine isothiocyanate[9] and cDNA and polymerase chain reaction (PCR) amplification were based on the methods described by Orlandi *et al.*[10] Direct nucleotide sequencing of PCR-amplified DNA was performed essentially as described by Innis *et al.*,[11] with modifications as reported previously.[12] Nucleotide sequence data were analysed using the DNASTAR software package and the genebank/EMBL database.

## RESULTS

### Characterization of anti-MRBC mAb

HA titres were determined for all eight anti-MRBC mAb, standardized to 5 $\mu$g Ig/ml. All eight mAb gave titres of at least 1:512 and two (clones 2-6-44 and 2-7-6) had titres of 1:4096. None of the eight mAb reacted with either ss-DNA or ds-DNA

**Table 1.** Summary of the characteristics of the eight anti-MRBC hybridomas

| Anti-MRBC mAb Fusion (1) Fusion (2) | Antibody isotype | Antibody HA titre (5 $\mu$g Ig/ml) | Surface and cytoplasmic CD5 expression (+/−) | Autoantigen cross-reactivity (dsDNA, ssDNA) | Cross-reactivity with other species RBC (sheep, rabbit, rat, human) |
|---|---|---|---|---|---|
| 2-6-44 (1) | IgG2a | 1:4096 | − | − | − |
| 2-4-A1 (1) | IgG2b | 1:1024 | − | − | − |
| 2-7-6 (1) | IgM | 1:4096 | − | − | − |
| 1-1-59 (1) | IgG2a | 1:1024 | − | − | − |
| 4-16-1 (2) | IgG1 | 1:512 | − | − | −/+ (rat) |
| 3-1-3 (2) | IgG2a | 1:2048 | − | − | − |
| B4-13-2 (2) | IgG2a | 1:1024 | − | − | − |
| 3-14-1 (2) | IgG2a | 1:1024 | − | − | − |

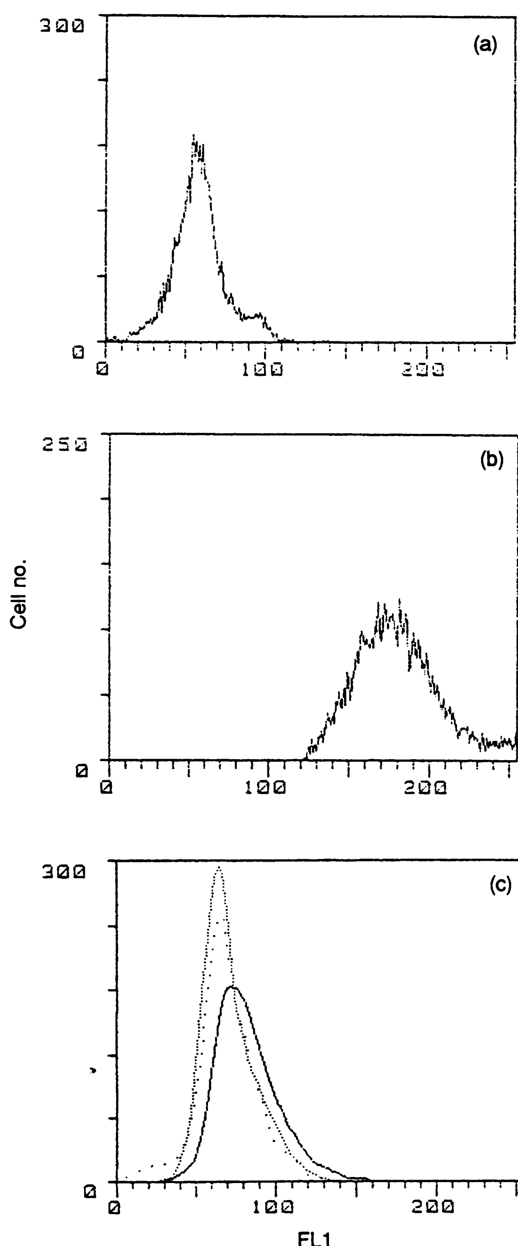


**Figure 1.** FACScans of three hybridoma cell lines [1-1-59 (——), 2-7-6 (· · ·) and B4-13-2 (·····)] stained with FITC anti-CD5 for cytoplasmic CD5 (c). EL4 (b) and P3X63Ag8.653 (a) are positive and negative controls respectively.

and no cross-reactivity was observed with sheep, rabbit, rat, or human RBC in seven of the eight mAb. One mAb (clone 4-16-1) cross-reacted with rat RBC. IgG2a was the predominant isotype [5/8 (63%)], while IgM, IgG1 and IgG2b were each produced by one hybridoma cell line. All hybridomas were negative for both surface and cytoplasmic CD5 expression (Table 1). Figure 1 demonstrates the FACScan of P3X63Ag8.653 myeloma cell line (negative control) (Fig. 1a), EL4 T cell line (positive control) (Fig. 1b) and three of the eight hybridomas (clones 1-1-59, 2-7-6, B4-13-2) (Fig. 1c) for cytoplasmic CD5 expression. FACScan for surface CD5 expression are not shown as these were essentially identical to those for cytoplasmic expression. EL4 was positive for both surface (data not shown) and cytoplasmic CD5 (Fig. 1b) and P3X63Ag8.653 was negative for both surface (data not shown) and cytoplasmic CD5 (Fig. 1a), all hybridomas were negative for both surface (data not shown) and cytoplasmic CD5 (Fig. 1c).

### Pathogenicity of anti-MRBC mAb

All hybridomas caused a reduction in haematocrit (by 17–26·5%) when injected i.p. into BALB/c mice and a raised Coombs' titre (in excess of 1:8192) at 33 days (Table 2). Haematocrits and Coombs' titres were observed to increase over the course of the experiment, measurements were taken at day 8 where the per cent reduction in haematocrit ranged from 2 to 18% and the Coombs' titre ranged from 0 to 1:277 (Table 2) and at 19 days where the per cent reduction in haematocrit ranged from 12 to 23% and the Coombs' titre ranged from 1:1024 to 1:8192 (Table 2). The i.p. injection of P3X63Ag8.653 myeloma cells or medium alone caused no decrease in haematocrit or raised Coombs' titre in the BALB/c mice (Table 2).

### Molecular studies of anti-MRBC mAb

cDNA encoding $V_H$ regions from five of the eight anti-MRBC mAb were sequenced and compared with members of the 14[13] known $V_H$ families in the genebank/EMBL database indicated that all five derived from the $V_H$ J558 family and could be assigned to one of two germline genes H10[14] or H30[15] (Table 3). We were also able to determine the $D_H$ gene usage of four of the five sequenced mAb. Three mAb (clones 2-14-1, 4-16-1 and B4-13-2) were found to use the $D_H$ gene FL16.1 whereas the D region in clone 1-1-59 appears to result from the fusion between two $D_H$ genes from the SP family (SP2.3[4] and SP2.2) (Table 3).

## DISCUSSION

The first demonstration of the transfer of autoimmune haemolytic anaemia was by Cooke *et al.* in which hybridomas derived from CBA and NZB mice immunized with rat RBC were implanted into syngeneic or allogeneic nude mice. These hybridomas secreted monoclonal anti-erythrocyte autoantibodies cross-reactive with mouse and rat RBC.[16] Two other groups have previously reported the production of anti-MRBC from unprimed NZB mice[17,18] and demonstrated that these antibodies are pathogenic when either hybridomas[16] or the purified mAb[18] are transferred into BALB/c mice. These groups[17,18] have described hybridomas which produced a range of immunoglobulin isotypes and demonstrated that pathogenic anti-MRBC reacted exclusively with MRBC and had no cross-reactivity with other species' RBC. All anti-MRBC mAb reported here reacted with MRBC in HA and had high end-point HA titres measured at a fixed immunoglobulin concentration. IgG2a was the predominant isotype produced by these hybridomas (5/8, 63%); the other isotypes produced were IgM, IgG1 and IgG2b. Shibata *et al.*[18] reported the production of eight anti-MRBC hybridomas predominantly of the IgM isotype. Caulfield *et al.*[17] described the production of one IgM anti-MRBC hybridoma. In agreement with both groups, seven of the eight pathogenic hybridomas described here produced anti-MRBC which reacted exclusively with MRBC, however one hybridoma (clone 4-16-1) produced IgG1 autoantibodies which cross-reacted with rat RBC.

**Table 2.** Autoimmune anaemia induced by eight anti-MRBC hybridomas. Groups of three BALB/c mice were injected i.p. with either $1 \times 10^6$ hybridoma cells, 0·5 ml culture medium (control) or $1 \times 10^6$ P3X63Ag8.653 myeloma cells (control). Haematocrit values and Coombs' titres are given at days 8, 19 and 33. The data are compiled from the three mice in each group injected with either one of the eight hybridoma cell lines, the control cell line or culture medium

| Anti-MRBC mAb Fusion (1) Fusion (2) | Coombs' titre | | | % reduction in haematocrit | | |
|---|---|---|---|---|---|---|
| | Day 8 | Day 19 | Day 33 | Day 8 | Day 19 | Day 33 |
| Medium alone | — | — | — | — | — | — |
| P3X63Ag8.653 cells | — | — | — | — | — | — |
| 2-6-44 (1) | — | 1:2901 | >1:8192 | 4 | 12 | 17 |
| 2-4-A1 (1) | 1:261 | 1:3413 | >1:8192 | 18 | 23 | 25·5 |
| 2-7-6 (1) | 1:277 | 1:2901 | >1:8192 | 15 | 19 | 26·5 |
| 1-1-59 (1) | 1:149 | 1:2730 | >1:8192 | 9 | 15 | 20 |
| 4-16-1 (2) | 1:5 | 1:1194 | >1:8192 | 2 | 12 | 19 |
| 3-1-3 (2) | 1:21 | >1:8192 | >1:8192 | 14 | 20 | 25·5 |
| B4-13-2 (2) | 1:266 | 1:4096 | >1:8192 | 7·5 | 22·5 | 22·5 |
| 3-14-1 (2) | 1:8 | 1:1024 | >1:8192 | 4 | 13 | 20 |

**Table 3.** Molecular study of five anti-MRBC mAb

| Anti-MRBC mAb Fusion (1) Fusion (2) | $V_H$ family usage | Closest known germline gene | % similarity of rearranged gene with closest germline gene | $D_H$ gene expressed |
|---|---|---|---|---|
| 2-6-44 (1) | $V_H$ J558 | H30 | 92 | NA |
| 1-1-59 (1) | $V_H$ J558 | H30 | 86 | Fusion of SP2.3 [4] and SP2.2 |
| 4-16-1 (2) | $V_H$ J558 | H10 | 94 | FL16.1 |
| B4-13-2 (2) | $V_H$ J558 | H10 | 93 | FL16.1 |
| 3-14-1 (2) | $V_H$ J558 | H10 | 88 | FL16.1 |

NA, not available.

Shibata *et al.*[18] reported that only anti-MRBC reactive exclusively with MRBC were pathogenic while non-pathogenic anti-MRBC cross-reacted with rat, human, sheep, rabbit and chicken RBC. The one IgM anti-MRBC mAb generated by Caulfield *et al.*[17] also reacted exclusively with MRBC. However, we have demonstrated one pathogenic hybridoma (clone 4-16-1) which produces Ig isotype IgG1 which cross-reacts with rat RBC. Perhaps as a consequence of this cross-reactivity and correspondingly reduced affinity for the cross-reactive determinant this clone had the lowest HA titre of the eight mAb.

Although both groups reported pathogenic anti-MRBC reactive solely with MRBC, Shibata *et al.*[18] stressed that the anti-MRBC mAb studied may not represent the full spectrum of pathogenic anti-MRBC autoantibodies occurring in the NZB mouse and this has been demonstrated with clone 4-16-1.

Caulfield and Stanko[6] reported that the sequence of the heavy chain variable region of their IgM anti-MRBC was similar to that of an anti-DNA autoantibody, however no cross-reactivity was observed with ss- or ds-DNA within our panel of anti-MRBC hybridomas and there was no sequence identity with any reported anti-DNA autoantibodies. In addition, we have shown that all eight anti-MRBC, in agreement with Fidanza *et al.*,[8] are most likely to be derived from CD5 negative B lymphocytes. This was demonstrated by immunofluorescence staining of the hybridomas for surface and cytoplasmic CD5. CD5 B lymphocytes when fused with myeloma cells during the generation of hybridomas lose surface CD5 expression and so it is not possible to determine the B-lymphocyte cellular origin of a hybridoma. However, we have been able to identify the cellular origin of hybridomas (whether derived from CD5 positive or negative B lymphocytes) by cytoplasmic staining for CD5 (S. Sadigh, B. Scott, R. A. Mageed, A. Malcolm, E. Andrew and R. N. Main, manuscript submitted). This is in contrast to reports that many naturally occurring autoantibodies are produced by a discrete subset of B lymphocytes bearing the CD5 antigen.[19] It would appear that anti-MRBC may be produced by $Lyb5^+$ B lymphocytes as introduction of $X^{id}$ into NZB mice, which deletes $Lyb5^+$ B lymphocytes, ameliorates autoimmune disease including autoimmune haemolytic anaemia.[20] This finding supports our evidence that anti-MRBC are not produced by $CD5^+$ B lymphocytes as $Lyb5^+$ B lymphocytes appear to be a distinct subpopulation of B lymphocytes, not present

until late in ontogeny,[21] whereas the $CD5^+$ B lymphocyte subpopulation is present very early in ontogeny.[22]

Recent studies of the mechanism responsible for pathogenic anti-MRBC antibody production using mice transgenic for functionally rearranged anti-MRBC antibody genes suggest that B lymphocytes responsible for the anti-MRBC response express the CD5 antigen and are 'hidden' within the peritoneal cavity. These cells escape antigen-induced apoptosis or clonal anergy mechanisms, which prevent the self-reactive B lymphocytes mounting a response, due to the lack of contact with the red blood cell antigen.[23] The self-generating property of CD5 B lymphocytes allows these cells to proliferate in the absence of T cells and antigen stimulation in contrast to the proliferation of conventional B lymphocytes in germinal centres.

This shielding of self-reactive peritoneal B lymphocytes has been proposed to explain the propensity of NZB mice to autoimmune disease.[24] However, our work would suggest that this is not the complete picture as we have demonstrated that our anti-MRBC mAb are likely to be derived from CD5 negative B lymphocytes. It must also be remembered that the shielding theory of self-reactive B lymphocytes is based on findings in a transgenic mouse which may not be a representative model of the naturally occurring murine disease.

All eight anti-MRBC-reactive hybridomas reported in this study caused anaemia following implantation into BALB/c mice. The pathogenicity of the panel of anti-MRBC was similar and no significant differences could be observed between hybridomas of different isotypes or end-point titres in the HA. The results for the panel of anti-MRBC were most similar to those of Caulfield *et al.*[17] with equivalent haematocrit end-point and Coombs' titres. The pathogenic activities of the anti-MRBC generated by Shibata *et al.*[18] were more marked than those obtained by us or by Caulfield *et al.*,[17] resulting in greatly reduced haematocrits and death of the mice within a short period of time. However, this was probably due, in part, to the fact that in the study reported by Shibata *et al.*[18] purified mAb were used at high concentration. Comparable concentrations are not achieved following implantation of hybridoma cell lines, as in the current study, since time is required for the hybridoma tumours to grow and produce high levels of antibody.

The analysis of cDNA encoding the $V_H$ regions of five of the eight pathogenic anti-MRBC mAb revealed that all were encoded by genes from the $V_H$ J558 gene family. The use of this single $V_H$ family for all five anti-MRBC mAb may indicate a restricted use of $V_H$ family by these autoantibodies. The observation that all five anti-MRBC mAb were encoded by a 5′ proximal $V_H$ family is in contrast to the reported biased utilization of $V_H$ gene families proximal to the D region in the generation of natural autoantibodies.[25] Unlike previous molecular studies of anti-MRBC we were able to assign five of the eight mAb to known germline genes H10 and H30 with identity of between 86 and 94%. We were able to assign four of the sequenced mAb to known germline $D_H$ genes. The $D_H$ genes in mAb 2-14-1, 4-16-1 and B4-13-2 appear to be the FL16.1 germline gene in reading frame I and the $D_H$ gene usage in mAb 1-1-59 appears to be the result of a fusion between two $D_H$ genes from the SP family, SP2.3 [4] and SP2.2 both in reading frame II.

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