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Molecular mechanisms resulting in pathogenic anti-mouse erythrocyte antibodies in New Zealand black mice

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SUMMARY

The New Zealand black (NZB) mouse strain is genetically predisposed to develop, at approximately 6 months of age, a spontaneous and severe autoimmune anaemia caused by production of pathogenic anti-mouse erythrocyte autoantibodies. In order to investigate the molecular mechanisms which lead to anti-mouse erythrocyte autoantibody production we have generated eight anti-mouse erythrocyte MoAbs producing hybridomas from splenocytes of 9- and 12-month-old NZB with spontaneous autoimmune anaemia. IgG2a was the predominant isotype, while IgM, IgG1 and IgG2b were each produced by one hybridoma cell line. All anti-mouse erythrocyte MoAbs were characterized for their antigen specificities. None of the MoAbs cross-reacted with ss- or dsDNA or with other species' erythrocytes, with the exception of one MoAb which cross-reacted with rat erythrocytes. None of the eight hybridomas was demonstrated to express surface or cytoplasmic CD5, suggesting that they derived from $CD5^-$ B lymphocytes. All hybridomas when implanted intraperitoneally into BALB/c mice caused anaemia. In order to define the genetic basis and investigate the molecular mechanisms resulting in pathogenic anti-mouse erythrocyte autoantibody production, the pattern of immunoglobulin variable region gene use has been studied. Five of the eight MoAbs whose IgV_H genes were sequenced all have functionally rearranged genes from the V_H J558 gene family. There is evidence for somatic point mutations in the complementarity-determining regions (CDR) of the IgV_H genes in all of these five MoAbs when compared with the closest known germline gene. We suggest that these nucleotide sequence changes are likely to reflect selection by an antigen-driven mechanism. Furthermore, the data indicate that pathogenic anti-mouse erythrocytes are not derived from 'natural' autoantibodies.

Keywords autoantibodies complementarity-determining region IgV_H genes B lymphocytes monoclonal antibody

INTRODUCTION

Antibodies have diverse antigen specificities resulting from the structure of the assembled V, D and J heavy chain and V and J light chain gene products. Antibodies generated during the rearrangement of germline V(D)J gene segments without the presence of foreign antigen represent the pre-immune repertoire. Due to the almost unlimited potential for V region diversity caused by the combinatorial events during V_H-D-J_H and V_L-J_L gene rearrangements and subsequent somatic mutations, antibodies with specificity for autoantigen can occur [1]. The immunoglobulin coding region is organized into largely non-overlapping V_H-gene family clusters, the most proximal to the D and J region loci being the 7183 and Q52 V_H-gene families, and J558 and 3609 V_H-gene families being most distal.

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Although it has been reported in previous molecular studies of autoantibodies that 'natural' IgM autoantibodies are encoded by genes in the germline configuration [2–6], it is now clear that somatic mutation occurs in the complementarity-determining regions (CDR) of pathogenic IgG autoantibodies [7–9]. Thus it is of importance to determine the source of these pathogenic autoantibodies, whether they arise from germline encoded 'natural' autoantibodies by a process of isotype switch and somatic mutation, or from the normal repertoire by antigen selection.

Previous molecular studies of pathogenic anti-mouse erythrocyte antibodies have shown that these autoantibodies are encoded by genes from a number of V_H gene families (J558, J606 and 3609) all distal to the D region locus [9,10]. These studies suggest that pathogenic anti-mouse erythrocytes are not derived from 'natural' autoantibodies which are preferentially encoded by genes from the most D-proximal 3′ gene families

[9,10]. Reininger *et al.* [9] demonstrated that somatic diversification in the generation of anti-mouse erythrocyte autoantibodies results from the presence of point mutations in the J_H gene segment, indicating that antigen-driven selection is the likely process by which these autoantibodies occur. Caulfield & Stanko [10] concluded from nucleotide sequence analysis of a pathogenic anti-mouse erythrocyte (G8) that it was unlike 'natural' erythrocyte-reactive antibodies which bind only to bromelain (br)-treated erythrocytes, since G8 is encoded by a gene from the V_H J558 family whereas anti-br-mouse erythrocytes are encoded by a gene from the V_H11 family [5]. These investigators suggested that pathogenic anti-mouse erythrocytes may be derived, with limited somatic mutation, from the corresponding New Zealand black (NZB) germ-line gene. However, neither group has made a direct comparison between antibody sequences and germline gene sequences in order to evaluate the frequency and distribution of somatic mutations in anti-mouse erythrocyte V_H genes [9,10].

We now report the characterization and nucleotide sequence analysis of five anti-mouse erythrocyte MoAbs. All eight hybridomas studied were pathogenic, and the five which have been sequenced were encoded by genes from the V_H J558 gene family. These molecular studies show unequal distribution of numerous somatic mutations within the V_H region when compared with the closest known germline gene, suggesting that selection by antigen is important in the generation of these antibodies.

MATERIALS AND METHODS

Generation and characterization of anti-mouse erythrocyte MoAbs

The hybridomas described in this study were the product of two fusions of splenocytes from unprimed 9- and 12-month-old NZB mice with spontaneous autoimmune anaemia and fusion partner P3X63Ag8653 carried out essentially according to established procedures [11]. An indirect haemagglutination assay (HA) was used to detect the anti-mouse erythrocyte autoantibody activity in the culture supernatants of established lines. Final titres produced by each hybridoma were determined using supernatants standardized to 5 μg immunoglobulin/ml to measure the highest dilution at which haemagglutination occurred. The immunoglobulin isotype of each MoAb was determined by ELISA using goat anti-mouse IgM and IgG (Sigma Chemical Co., St Louis, MO), and goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology, Birmingham, AL). Cross-reactivity with erythrocytes from other species (rabbit, rat, sheep and human) was also determined by HA. Cross-reactivity with double stranded (ds) and single stranded (ss) DNA (Sigma) was measured by ELISA.

Pathogenic activity of the anti-mouse erythrocyte MoAbs was determined by haematocrit and Coombs titre measurement in groups of three BALB/c mice (aged 3 months) over 33 days after intraperitoneal (i.p.) injection of 1×10^6 hybridoma cells.

Immunofluorescence staining for surface CD5 expression was performed using 1×10^6 hybridoma cells. The cells were washed twice with PBS containing 0·02% sodium azide before addition of FITC anti-CD5 (1:100) (Becton Dickinson, Cowley, UK) to the cell suspension. The cells were then 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) was added to the cell suspension in saponin buffer and incubated for 20 min on ice. Cells stained either for surface or cytoplasmic CD5 expression were then washed and resuspended in 300 μl PBS for FACS analysis on a FACScan (Becton Dickinson). The myeloma cell line P3X63Ag8653 was used as a negative control, and the T cell line EL4 as a positive control for CD5 staining.

RNA extraction and cDNA synthesis

RNA was extracted from hybridomas grown in tissue culture by the method of Chomzynski & Scacchi using guanidine isothiocyanate [12]. cDNA synthesis and polymerase chain reaction (PCR) amplification were based on the methods described by Orlandi *et al.* [13]. Briefly, cDNA synthesis was as follows: a 50-μl reaction mixture containing 1 μg mRNA, 0·5 μg Oligo (dT), 250 μM of each dNTP, 10 mM dithiothreitol, 100 mM Tris HCl (pH 8·3), 10 mM $MgCl_2$, 140 mM KCl and 100 U M-MLV reverse transcriptase (BRL, Bethesda, MD) was incubated for 1 h at 37°C. Products of transcription were used in PCR without any processing. PCR amplification protocol used the following oligonucleotide primers: V_H1FOR [5′d(TGAGGAGACG-GTGACCGTGGTCCCTTGGCCCCAG)] and V_H1BACK degenerate primer [5′d(AGGT(C/G)A(G/A)CTGCAG(G/C)AGTC(T/A)GG)]. For PCR amplification a 100-μl reaction mixture containing 5 μl of template cDNA, 1 μg each of V_H1FOR and V_H1BACK primers, 1·25 mM of each dNTP, 67 mM Tris HCl (pH 8·8), 17 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 5 U of *Thermus aquaticus* (Taq) polymerase (Perkin Elmer Cetus, Norwalk, CT) was overlaid with mineral oil. The PCR cycles were as follows: cycle 1 for 1 round, 93°C for 7·5 min, 42°C for 1 min, and 72°C for 1·5 min, followed by cycle 2, 93°C for 1·5 min, 42°C for 1 min, 72°C for 2 min for 30 rounds, then 72°C for 7 min. PCR amplification products were separated on 1·5% low melting point (LMP) agarose gels and products of the appropriate size ~350 bp were cut from the gel. DNA was recovered by a modification of the freeze-thaw method using a 0·45-μm filter unit (Ultrafree-MC Millipore, Bedford, MA) [14].

Nucleotide sequencing

Direct nucleotide sequencing of PCR-amplified DNA was performed essentially as described by Innis *et al.* [15], with modifications as reported [16]. Reaction products were analysed on 5% urea polyacrylamide gels. Nucleotide sequence data were analysed and comparisons made using the DNAstar software package and the genebank/EMBL data base.

RESULTS

Characterization of anti-mouse erythrocyte MoAbs

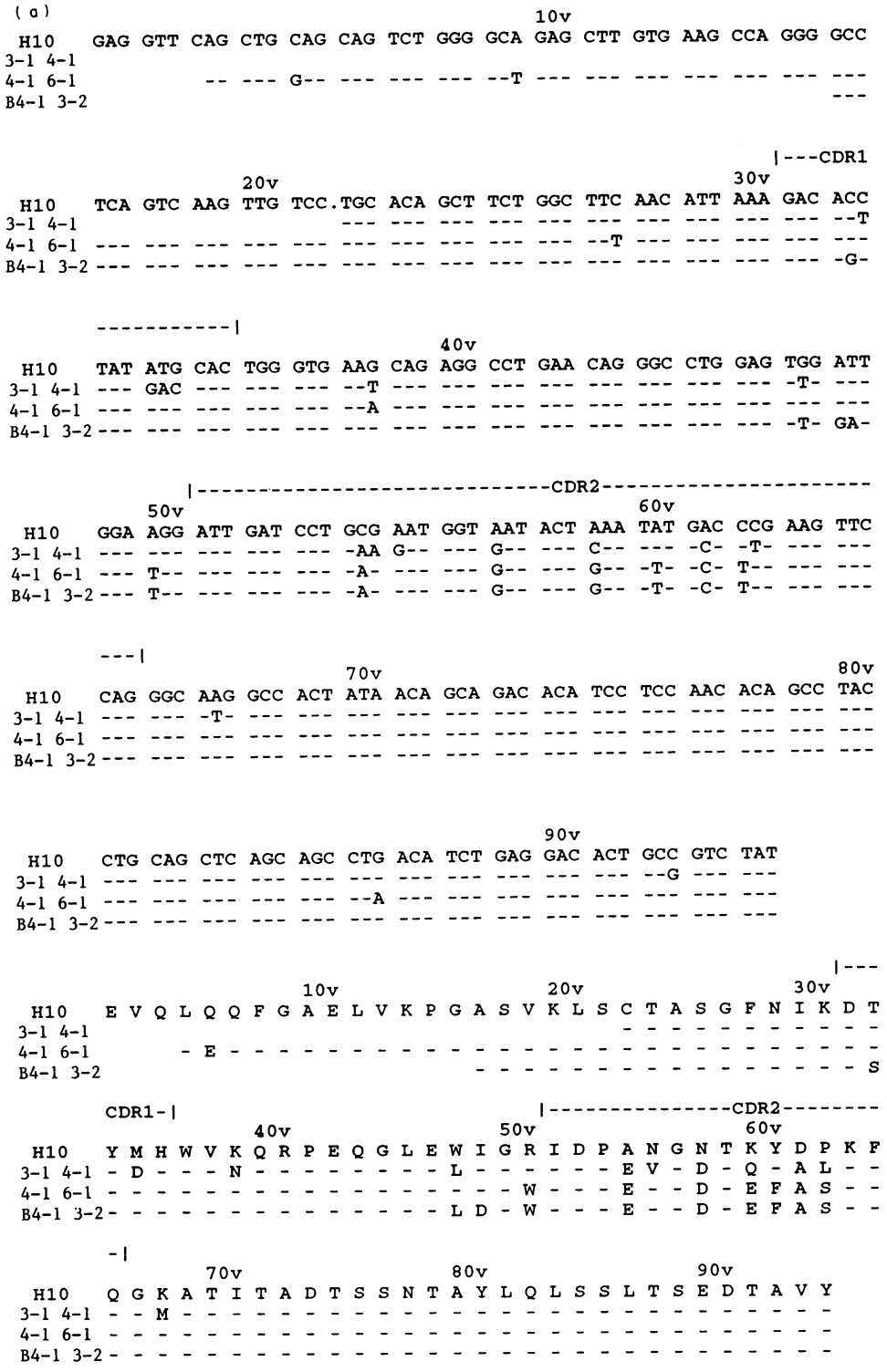
Antibody isotypes, HA titres, surface and cytoplasmic CD5 expression, anti-ds and ssDNA reactivity and cross-reactivity with other species' erythrocytes of the eight anti-mouse erythrocyte MoAbs are summarized in Table 1.

Final HA titres of between 1:512 and 1:4096 were observed. No cross-reactivity with either ssDNA or dsDNA or with sheep, rabbit, rat, or human erythrocytes was observed for seven of the eight MoAbs. All hybridomas produced only one heavy chain

isotype, implying clonality. The clonality was further confirmed using the PCR protocol (described later) to demonstrate a single band for functional V_H gene rearrangement. One MoAb (4–16–1) cross-reacted with rat erythrocytes. The predominant isotype was IgG2a (5/8 (62·5%)), while IgM, IgG1 and IgG2b were each produced by one hybridoma. All hybridomas were negative for surface and cytoplasmic CD5 expression (Table 1).

Pathogenicity of anti-mouse erythrocyte MoAbs

The pathogenicity of the eight anti-mouse erythrocyte MoAbs was determined and is summarized in Table 2. All hybridomas when injected intraperitoneally into BALB/c mice caused reduction in haematocrit (by 17–26·5%) and raised Coombs titres (in excess of 1:8192) (Table 2) over the course of the experiment (33 days). Intraperitoneal injection of



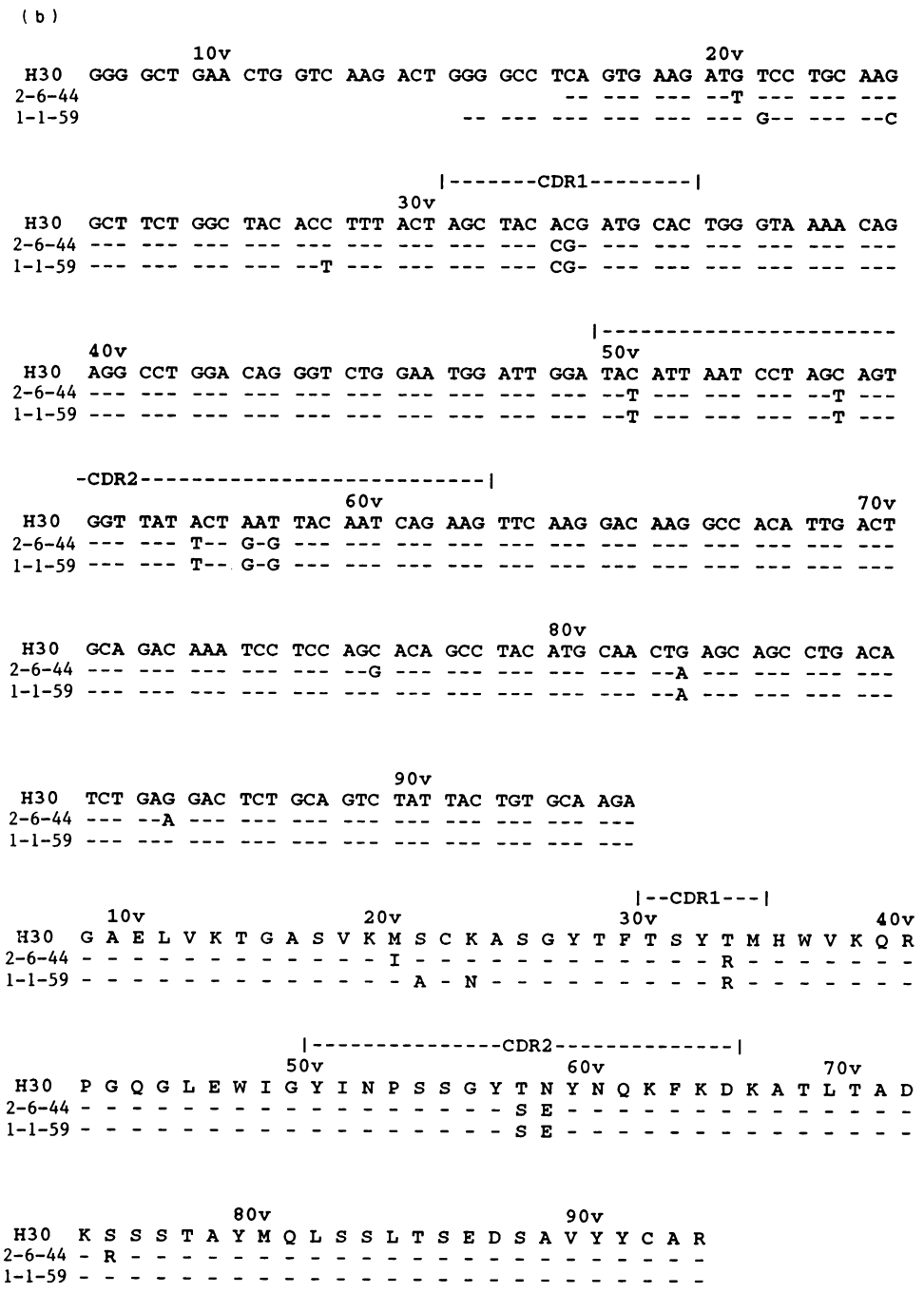


Fig. 1. Nucleotide sequences of DNA encoding the V_H regions of five anti-mouse erythrocyte MoAbs and predicted amino acid sequence. Numbering of amino acids and complementarity-determining regions (CDRs) is according to Kabat *et al.* [35]. (-) indicates nucleotide or amino acid is identical to that in the germline gene. (a) DNA sequences which had identity with the germline gene H10. (b) DNA sequences which had identity with the germline gene H30.

P3X63Ag8653 myeloma cells [8] or medium alone caused neither a decrease in haematocrit nor raised Coombs titre in the BALB/c mice (Table 2).

Nucleotide sequence analysis of the V_H regions of anti-mouse erythrocytes

Nucleotide sequences of the portions of cDNA encoding V_H regions and the corresponding deduced amino acid sequences of five of the eight anti-mouse erythrocyte MoAbs are presented in Fig. 1. Comparison of the V_H sequences with members of the 14 known V_H families [17] indicated that all five derived from the V_H J558 family. Each anti-mouse erythrocyte V_H gene exhibited at best 86–94% nucleotide homology with the closest germline gene. The five sequenced MoAbs could be divided into two subgroups based on homology to two different germline genes. The first subgroup of two MoAbs showed homology with the

Table 1. Summary of the characteristics of the eight anti-mouse erythrocyte MoAbs

Anti-mouse erythrocyte MoAbs, fusion (1), fusion (2)	Antibody isotype	Antibody HA titre, 5 μg immunoglobulin/ml	Surface and cytoplasmic CD5 expression +/−	Autoantigen cross-reactivity (dsDNA, ssDNA)	Cross-reactivity with other species' erythrocytes (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	−	−	−

HA, Haemagglutination assay.

Table 2. Autoimmune anaemia induced by eight anti-mouse erythrocyte hybridomas. Groups of three BALB/c mice were injected intraperitoneally with either 1×10^6 hybridoma cells, 0·5 ml culture medium (control) or 1×10^6 P3X63Ag8653 myeloma cells (control)

Anti-mouse erythrocyte MoAbs, fusion (1), fusion (2)	Coombs titre at 33 days	Per cent reduction in haematocrit at 33 days
Medium alone	—	—
P3X63Ag8.653 cells	—	—
2-6-44 (1)	>1:8192	17
2-4-A1 (1)	>1:8192	25·5
2-7-6 (1)	>1:8192	26·5
1-1-59 (1)	>1:8192	20
4-16-1 (2)	>1:8192	19
3-1-3 (2)	>1:8192	25·5
B4-13-2 (2)	>1:8192	22·5
3-14-1 (2)	>1:8192	20

germline gene H30 [18], the second subgroup to the germline gene H10 [19].

MoAbs 2-6-44 and 1-1-59, both from fusion 1, had 92% and 86% sequence homology with the closest germline gene H30, and shared a number of identical mutations in the CDRs that resulted in amino acid replacements. In CDR1 at position 33, threonine was interchanged for arginine in both MoAbs. In the CDR2 at position 58, threonine was replaced by serine, a conservative interchange, in both MoAbs, and asparagine at position 59 was replaced in both MoAbs by glutamic acid, which is a relatively conservative interchange. MoAbs 2-6-44 and 1-1-59 shared identical amino acids at the start of the CDR3. The MoAb 1-1-59 sequence was available for the whole of the CDR3 to be analysed (Fig. 2).

MoAbs 3-14-1, 4-16-1 and B4-13-2, all from fusion 2, had 88%, 94% and 93% homology with the closest germline gene H10, and some similar mutations in the CDR2 were observed for all MoAbs. In the CDR2 at position 54 alanine, at position 57 asparagine, and at position 61 aspartic acid were replaced with glutamic acid, aspartic acid and alanine, respectively, in all three MoAbs. Only the replacement at position 57 was a relatively conservative interchange. In the CDR3 of all three MoAbs at position 98 a threonine was present. There were a number of other unique replacements in the CDRs of all three MoAbs which were not shared by all the members of the group.

We were able to assign the CDR3 from four of the sequenced MoAbs to known D_H genes, but not to J_H genes. The D_H gene usage of MoAbs 1-1-59, 2-14-1, 4-16-1 and B4-13-2 are shown in Fig. 2. MoAb 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) with nucleotide additions at the junctions of the two D_H genes. The three other MoAbs appear to use D_H gene FL16.1.

The ratios of replacement to silent nucleotide changes in the

```
                SP2.3 (4)                N        SP2.2

          TCT ACT ATG GTT ACG AC                TCT ACT ATG ATT ACG AC
1-1-59    --G G-- TC- -A-              GGA G    -- --- T-- -C- --T
```

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                FL16.1

          TTT ATT ACT TAC GGT AGT AGC TAC
3-1 4-1                --- TTC -C- -*- *-
4-1 6-1                --- T-- -C- -*- *-
B4-1 3-2               --- T-- --- -*- *-
```

Fig. 2. Analysis of CDR3 regions from four of five sequenced hybridomas. *, Gap introduced; N, nucleotide addition.

Table 3. Ratio of replacement to silent nucleotide changes in the V_H genes of the five anti-mouse erythrocyte hybridomas, both in the framework and complementarity-determining regions (CDR). Also summarized is the per cent identity with the closest germline gene for each hybridoma

Hybridoma, fusion (1), fusion (2)	Framework	CDR region	Per cent identity with J558
2-6-44 (1)	1·0	3·33	92 (i)
1-1-59 (1)	1·0	14·5	86 (i)
3-14-1 (2)	3·0	14·0	88 (ii)
4-16-1 (2)	0·5	8·0	94 (ii)
B4-13-2 (2)	4·0	5·0	93 (ii)

framework and CDRs compared with the germline genes are summarized in Table 3. The ratio of replacement to silent nucleotide changes for all five sequences was consistently greater in the CDRs than in the framework regions.

DISCUSSION

Several groups have reported the production of anti-mouse erythrocytes from unprimed NZB mice [10,20] and demonstrated that these antibodies are pathogenic when either hybridomas [10] or purified MoAbs [20] are transferred into BALB/c mice. These groups described hybridomas that produced a range of antibody isotypes, and demonstrated that pathogenic anti-mouse erythrocytes reacted exclusively with mouse erythrocytes and had no cross-reactivity with other species' erythrocytes. In the present study we report the production, from unprimed NZB mice, of anti-mouse erythrocyte MoAbs with similar properties to those reported [10,19]. All anti-mouse erythrocyte MoAbs reported here reacted with mouse erythrocytes in HA and had high endpoint HA titres measured at a fixed immunoglobulin concentration. IgG2a was the predominant isotype produced by these hybridomas (5/8, 62·5%), the other isotypes being IgM, IgG1 and IgG2b. Shibata *et al.* [20] reported the production of eight anti-mouse erythrocyte hybridomas with the same range of isotypes, but with IgM predominant. Caulfield & Stanko [10] described the production of one IgM anti-mouse erythrocyte hybridoma. In agreement with both groups, seven of the eight hybridomas described here produced anti-mouse erythrocytes that reacted exclusively with mouse erythrocytes, but one hybridoma (4-16-1), produced IgG1 autoantibodies cross-reactive with rat erythrocytes.

Caulfield & Stanko reported that the sequence of their IgM anti-mouse erythrocytes was similar to that of an anti-DNA autoantibody sequence [10], but no cross-reactivity was observed with ss- or dsDNA within our panel of anti-mouse erythrocyte hybridomas, nor sequence identity with any reported anti-DNA autoantibodies. In addition, we have shown that all eight anti-mouse erythrocytes are most likely derived from $CD5^-$ B lymphocytes. This was demonstrated by immunofluorescence staining of the hybridomas for cytoplasmic CD5. Fidanza *et al.* [21] have recently reported that hybridomas derived from CD5 B lymphocytes, which normally lose expression of surface CD5 during the fusion with the myeloma cell line, retain cytoplasmic expression of the CD5 antigen. By detecting the expression or non-expression of cytoplasmic CD5 in our eight anti-mouse erythrocyte MoAbs we have been able to identify the cellular origin of these hybridomas (whether derived from $CD5^+$ or $CD5^-$ B lymphocytes) (Sadigh *et al.*, manuscript submitted). Although many autoantibodies have been reported to be produced by CD5 B lymphocytes [22], it would appear that anti-mouse erythrocytes are not.

All our anti-mouse erythrocyte reactive hybridomas caused anaemia when implanted into BALB/c mice. The pathogenicity of the panel of anti-mouse erythrocytes was similar, and no significant differences could be observed between hybridomas of different isotype or endpoint titre in the HA. The results for the panel of anti-mouse erythrocytes were most similar to those of Caulfield & Stanko [10], with equivalent endpoint haematocrits and Coombs titre results. The pathogenic activities of the anti-mouse erythrocytes generated by Shibata *et al.* [20] were more marked than those obtained here or by Caulfield & Stanko [10], and caused greatly reduced haematocrits and death of recipient mice within a short period of time. However, this is probably due, in part, to the fact that in the study of Shibata *et al.* [20] purified MoAbs were injected at high concentration. These high concentrations would not be achieved as rapidly when implanting hybridoma cell lines, as time is required for the hybridoma tumours to grow and produce antibody.

The present molecular analysis of anti-mouse erythrocytes provides the first demonstration that autoantibodies whose pathogenicity has been demonstrated *in vivo* are likely to be derived by antigenic selection. The analysis of cDNA encoding the V_H regions of five pathogenic anti-mouse erythrocyte MoAbs from NZB mice reveals several important points. First, genes from the V_H J558 family encode all five pathogenic MoAbs, indicating that these pathogenic autoantibodies may be restricted in V_H family usage. However, in contrast to our findings that anti-mouse erythrocyte MoAbs may be restricted in V_H family usage, Reininger *et al.* reported that pathogenic anti-mouse erythrocyte autoantibodies can be encoded by genes from the V_H 3609, J606 and J588 gene families [9].

Second, the fact that all functionally rearranged V_H gene segments from the anti-mouse erythrocyte autoantibodies could be assigned to 5′ proximal V region gene families supports the suggestion by Kofler *et al.* [23] that the spontaneously arising autoantibodies originate from the same repertoire as antibodies elicited against foreign antigens.

Third, no utilization of genes from the V_H gene families most proximal to the D region locus was observed in the anti-mouse erythrocyte autoantibodies. It has been previously demonstrated that the V_H gene families most proximal to the D region locus rearrange frequently in B lymphocytes from fetal or neonatal mice as well as in pre-B lymphocyte lines [24,25], in contrast to adult mature B cells which utilize V_H gene families at frequencies that correlate with family size [26]. In natural autoantibodies a similar biased utilization of V_H gene families proximal to the D region locus has been reported by several investigators [27–29], but has not been confirmed by others [2,7,30]. Our observation that all five anti-mouse erythrocyte MoAbs are encoded by members of the 5′ most proximal V_H gene families further argues against the preferential rearrangement of V_H families proximal to the D regions for the generation of autoantibodies, and is in agreement with previous studies of anti-mouse erythrocyte autoantibodies [9,10].

We were able to assign the CDR3 from four of the sequenced

MoAbs to known D_H genes. The D_H genes in MoAbs 2-14-1, 4-16-1 and B4-13-2 all appear to be similar to the FL16.1 germline gene. However, in all three gaps had to be introduced to maximize the degree of homology. All three MoAbs use the FL16.1 in reading frame I, which is consistent with the predominance of this reading frame in the available nucleotide sequences. The CDR3 region in hybridoma 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. It also appears that there are four nucleotide additions at the junction between both D_H genes. None of the MoAbs appears to be using D_HQ52 gene which is over-represented in natural autoantibodies produced during fetal life [31]. Furthermore, the reading frames did not yield tyrosine-rich sequences as has been suggested [32].

We observed a high incidence of somatic mutation within the CDRs of these autoantibodies, suggesting that antigen selection may be important in the generation of anti-mouse erythrocyte antibodies. In studies of antibodies to exogenous antigens an increased frequency of replacement mutations is associated with high affinity antibodies [33,34]. Similarly, it is likely that some of the somatic mutations observed here contribute to a more efficient binding to mouse erythrocytes, thereby enhancing the pathogenicity of the anti-mouse erythrocyte autoantibodies. All of the five V_H genes investigated were found to have between 86% and 94% identity with the closest known germline gene, and therefore it was possible to make a direct estimation of the frequency of somatic mutations in the anti-mouse erythrocyte V_H gene. A greater frequency of mutations was consistently found in the CDRs when compared with the framework regions. A number of identical amino acid replacements in the CDRs were shared within each of the two groups (from first and second fusion) of anti-mouse erythrocyte autoantibodies, and it is possible that these replacements may account for increased affinity of the antibody for mouse erythrocytes. It is likely that the somatic mutations observed in the CDRs reflect the selection of these autoantibodies by an antigen-driven mechanism.

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