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# Analysis of the Immunoglobulin Heavy Chain Variable Region of Hybridomas Producing anti-CD8 Monoclonal Antibodies.

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**ABSTRACT** **We analyzed the nucleotide sequence of immunoglobulin (Ig) heavy chain variable regions (VH) of seven hybridomas which produce anti-CD8 monoclonal antibodies. These seven antibodies were classified into four groups according to the results of a cell binding inhibition assay. The assay also indicated that CD8 antigen expressed at least four different epitopes. The VH nucleotide sequences were classified in three families. Three clones belonged to VH subgroup I(B), and two to VH subgroup V(A). Sequences in members of each family showed identical V-D-J recombinations, suggesting that the clones of each family arose from common ancestral clones. Some somatic mutations were observed in both the framework segments and the complementarity determining regions (CDR) of the I(B) clones, and only one amino acid substitution was observed. Two clones of the V(A) subgroup possessed an identical VH sequence. Although the clones of each family had originated from single ancestral clones, reactivity within families with CD8 antigen was found to vary widely. At the same time, two clones derived from different fusions, on the basis of their equal reactivities with CD8, were classified into subgroup II(A) ; subsequently, they were shown to share the same V-D-J combination. The speeds of reaction of the antibodies with CD8 antigen were investigated by flow cytometry. The observed rates of increase of brightness and final saturated brightness with these antibodies were quite different. Those results indicated that somatic mutations occurring in the VH gene of the anti-CD8 antibodies cause differences in their affinity with epitopes. Two separately originated clones that were induced by the same CD8 antigen showed identical paratopes and VH usage.** (Received January 19, 1993 and accepted February 5, 1993)

**Key Words:** Somatic mutation, Affinity maturation, Complementarity determining region (CDR), Immunoglobulin variable region gene

## 1. Introduction

The enormous diversity of antigen specific receptor of lymphocytes results from the great number of combinations of gene segments which rearranged to code for the variable domains of Ig molecules. The repertoire of Ig molecules is mediated by: (1) the diversity of the germline genome (i. e., the number of V, D, and J segments), (2) the combinatorial diversity, and (3) the junctional diversity (i. e., diversity due to the imprecise joining of the V, D, and J segments)[1-3]. The most important factor is the imprecise joining, especially on the CDR3 of Ig heavy (H) chain[1-6]. Another mechanism that affects the reactivity of antibodies is somatic mutation in Ig V region genes. This is the random process following gene rearrangement which culminates in the formation of the mature heavy (H)-and light (L)-chains. The process of replacement mutation results in progressively higher affinity, thus, this process is called

Abbreviations:
Ig, immunoglobulin, VH, heavy chain variable region, CDR, complementarity determining region, V, variable, D, diversity, J, joining, C, constant, H, heavy, L, light, FR, framework region.

affinity maturation. Many studies have addressed restricted usage of IgH gene segments[7-12], specific combinations of V-D-J gene segments[13-16], somatic mutation in the V gene of malignant lymphoma cases[17-20], and the relationship between affinity and maturation in auto reactive antibodies[21,22]. However, the reactivity of mutated antibodies with certain epitopes of antigen is still not well understood. Some papers have noted that somatic mutations alter affinity to given Ag molecule[11,12]. We analyzed the affinities of and somatic mutations in the V region genes of seven mAb clones generated against human CD8 molecules.

## 2. Materials and Methods

### 2. 1 Hybridomas

Seven hybridoma clones producing anti-CD8 monoclonal antibodies (mAbs) were used (Table 1). Those antibodies recognize the 32 kDa $\alpha$ chain of CD8 molecule in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced condition and have been shown to inhibit cytotoxic activity in autologous MLC to almost the same degree[23,24]. Six clones originated froma single fusion of the 'TD' series, and the other originated from a fusion of the 'T3' series.

**Table 1** *List of hybridoma clones used in this study*

| | subclass | l-chain | MW(RD) |
|---|---|---|---|
| TD 1B1 | G1 | k | 32 kDa |
| TD 3A2 | G2b | k | 32 |
| TD 4A2 | G2a | k | 32 |
| TD 9C6 | G1 | k | 32 |
| TD 3D6 | G1 | k | N. D. |
| T3 7D2 | G1 | k | 32 |
| TD 4C6 | G1 | k | 32 |

N. D.: not determined
MW: molecular weight
RD: reduced condition

### 2. 2 Binding inhibition assay

Determinant heterogeneity of the antigen defined by mAbs was examined by cross-blocking using unlabeled and fluorescein isothiocyanate (FITC)-labeled mAbs. Human peripheral blood lymphocytes (PBL) were incubated with unlabeled mAb at 4°C for 30 minutes (min) followed by washing with an excess volume of phosphate buffered saline (PBS). FITC-labeled mAbs were then reacted and analyzed by FACStar (Becton Dickinson, USA).

### 2. 3 Time course flow cytometry

The reaction speed of each mAbs with cell surface antigen was analyzed. Human PBL was incubated with each mAb at 4°C for 5 min, 10 min, 20 min, and 30 min. After washing with PBS, FITC-conjugated goat anti-mouse antibody was reacted at 4°C for 30 min. Fluorescence was analyzed by FACStar.

### 2. 4 RNA preparation

RNA was extracted from each hybridoma with guanidine thiocyanate (GTC)/CsCl by the method described by Chirgwin *et al*[25]. Briefly, cells were homogenized in GTC solution containing 4M GT, 25 mM Na citrate (pH 7.0), 0.1 M 2 mercaptoethanol using Polytron homogenizer (Kinematica, Switzerland); after removing the nuclei, cell lysate was overlaid on a CsCl solution containing 5.7 M CsCl, 10 mM EDTA, and 25 mM sodium acetate. Ultra centrifugation was performed using a swing rotor

SW-41Ti and L8-M ultracentrifuge (Beckman, USA) for 18 hours at 34000 rpm.

**2. 5 cDNA synthesis**

First strand cDNAs for VH were synthesized. Five micrograms of each RNA were annealed with 25 mer oligonucleotide (#22) complementary for the sequence of the Ig joining region at 60°C for 10 min. cDNAs were synthesized with 20 units of AMV reverse transcriptase XL (Life Sciences, USA) at 42°C for 1 hour according to the recommended condition of the supplier. The sequence of the oligonucleotide is as follows[26]. #22 : 5'-GGTGACCGTGGTCCCTGCGCCCCAG-3'

**2. 6 Polymerase chain reaction (PCR)**

Sequences of Ig-VH were amplified by PCR. The primers were #22 and #29, which were designed to match with the framework (FR) of VH of mouse Ig. Both primers were phosphorylated by T4 polynucleotide kinase (Boehringer Mannheim) in advance. Amplification was performed in a 50 $\mu l$ reaction mixture containing 5 $\mu l$ of the cDNA, RNA hybrid and 50 pmole of primers using 2 units of Tth DNA polymerase (Toyobo). Thirty cycles of amplification were carried out in a programmable thermocycler (Perkin Elmer Cetus, USA). The sample was denatured at 94°C for 30 seconds (sec), annealed with a 60 sec temperate ramp from 94°C to 55°C, and incubated at 55°C for 1 sec, followed by incubation at 75°C for 2 min 30 sec. After 30 cycles, the sample was incubated at 72°C for 7 min. The sequence of the primer is as follows[27]. #29 : 5'-AGGT(CG)(AC)A(AG)CTGCAG(CG)AGTC(AT)GG-3'

**2. 7 Sequencing**

The PCR product was separated on 2% low melting point agarose gel (Sea-Plaque, FMC, USA) to get the expected DNA ranging from 329 bp to 374 bp. The purified DNA was treated with T4 DNA polymerase (Boehringer Mannheim) to blunt the ends. After the ends were polished, the DNA was ligated into a phosphatase treated SmaI site of a double strand M13 phage vector. Recombinant M13 was introduced into JM101 competent cell prepared by Hanahan's procedure[28]. Single stranded recombinant phage DNA was prepared for sequencing by Sanger's dideoxy chain termination method[29] using Sequenase kit ver. 2 (USB, USA) and $^{35}$S-dATP. Nucleotide sequences were obtained by acrylamide gel electrophoresis followed by drying and 24 hours of autoradiography. Detailed procedures for treating the insert DNA, vectors, and host cell are provided in the laboratory manual by Sambrook[30].

## 3. Results

**3. 1 Heterogeneity of antigenic determinant**

The binding inhibition assay revealed that the seven mAbs can be classified into four groups (Table 2). 1B1, 9C6, and OKT8 inhibited the reaction of OKT8, 1B1, and 9C6 effectively. They also showed

**Table 2** *Determinant heterogeneity of mAbs by binding inhibition assay*

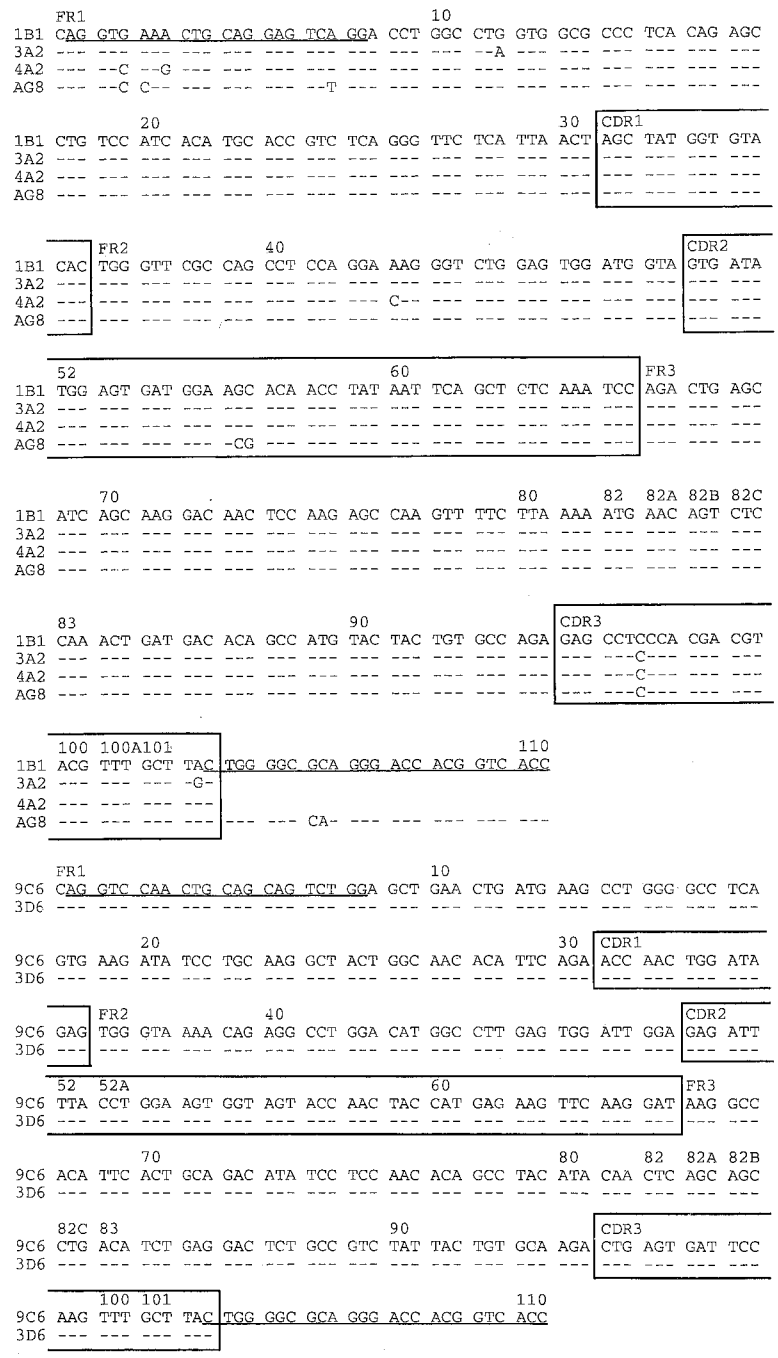
| L-2 1st. Ab | 2nd. Ab | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | OKT8 | 1B1 | 9C6 | 3A2 | 7D2 | 4C6 | 4A2 | 3D6 | Group |
| OKT8 | ++ | ++ | ++ | − | − | − | − | − | A |
| TD 1B1 | ++ | ++ | ++ | +/− | − | − | +/− | − | A |
| TD 9C6 | ++ | ++ | ++ | +/− | − | − | +/− | − | A |
| TD 3A2 | − | +/− | +/− | ++ | ++ | ++ | − | − | B |
| T3 7D2 | − | − | − | ++ | ++ | ++ | − | − | B |
| TD 4C6 | − | − | − | ++ | ++ | ++ | − | − | B |
| TD 4A2 | − | +/− | +/− | − | − | − | ++ | − | C |
| TD 3D6 | − | − | − | − | − | − | − | ++ | D |

−: <25%, +/−: 25−50%, +: 50−75%, ++: >75%

weak inhibition against 3A2 and 4A2 (Group A). 3A2, 7D2, and 4C6 were classified as another group (Group B), because of their strong inhibition over 3A2, 7D2, and 4C6. 4A2 and 3D6, which were inhibited only themselves, were classified as group C and D respectively.

### 3. 2 Genetic heterogeneity of VH sequences

Nucleotide sequences of the VH region are shown in Figure 1. The three clones 1B1, 3A2, and 4A2 showed the same CDR3 sequence except for one nucleotide substitution at amino acid #102 (Figure 1a).



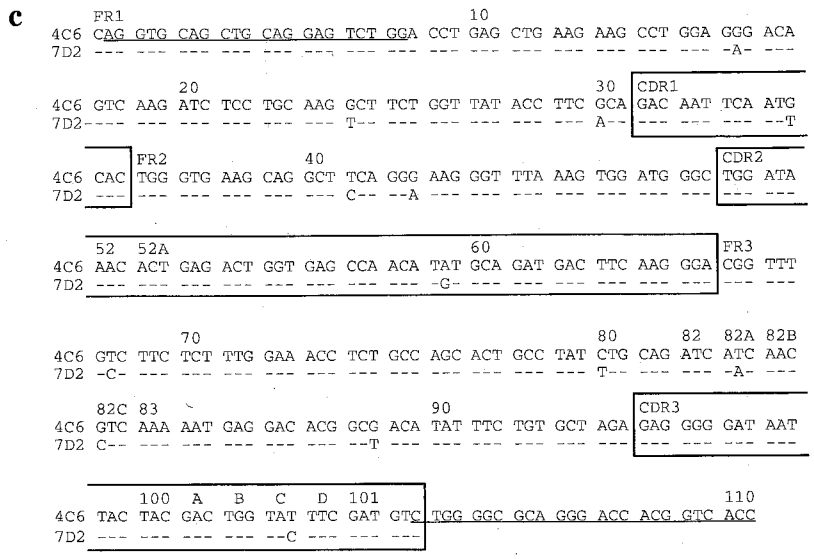



**Fig. 1** Sequences of VH regions of anti-CD8 mAbs. Sites of primers are underlined. The number of amino acids is quoted from Kabat[26]. AG8, found in the GENETYX data base, is almost identical with 1B1 (see text).

Two clones, 9C6 and 3D6, possessed the same CDR3 (Figure 1b). These results suggested that those two groups arose from two common ancestral clones, respectively. One extra codon "C" was observed in CDR3 of the first group in the subsequent chain of 96 amino acids (see discussion). The frequencies of nucleotide substitutions are summarized in Table 3. Two nucleotide substitutions were observed between 1B1 and 3A2, and one nucleotide susbstituion was observed between 1B1 and 4A2. No substitution was observed between 9C6 and 3D6 (Figure 1b). Two VH sequences were obtained from both 4C6

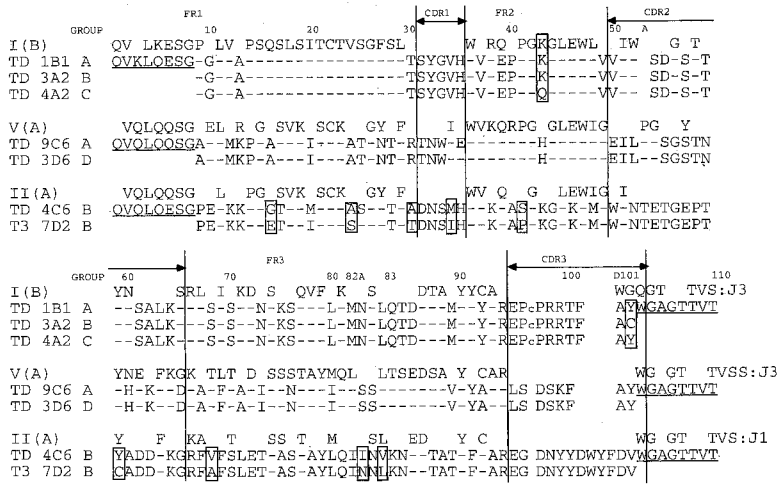



**Fig. 2** Deduced amino acid sequences of VH are shown. The amino acid translation is given in standard one-letter symbols. Numbering and invariant residues are indicated according to Kabat[26]. An amino acid substitution was indicated by a box. The extra "C" at next to #96 was observed in CDR3 of the I(B) group.

and 7D2. One of each showed an in-frame productive sequence that was highly homologous each other, including almost same CDR3 (Figure 1c). Another one of VH sequence was the out-of-frame and no similarity was observed (data not shown). Thus, a total of only 13 discrepant nucleotides were seen between 4C6 and 7D2. This result suggested that VH sequences of both alleles were obtained from different lineage, and same VH usage occurred by chance.

### 3. 3 Analysis of amino acid sequences

Deduced amino acid sequences are shown in Figure 2. Three groups were classified as mouse Ig VH subgroup I(B), V(A), and II(A), according to the alignment of the invariant residues[26]. The codon

**Table 3** *Homology of anti-CD8 VH genes and candidate germ-line genes*

| VH genes | Nucleotide Differences | | | %Homology | | |
|---|---|---|---|---|---|---|
| | FR | CDR | Total | FR | CDR | Total |
| TD 1B1 | 0 | 0 | 0 | 100 | 100 | 100 |
| TD 3A2 | 1 | 1 | 2 | 99.5 | 99 | 99.3 |
| TD 4A2 | 1 | 0 | 1 | 99.5 | 100 | 99.7 |
| AG8 | 0 | 2 | 2 | 100 | 98 | 99.3 |
| TD 9C6 | 0 | 0 | 0 | 100 | 100 | 100 |
| TD 3D6 | 0 | 0 | 0 | 100 | 100 | 100 |
| TD 4C6 | 0 | 0 | 0 | 100 | 100 | 100 |
| T3 7D2 | 10 | 3 | 13 | 95 | 97 | 96 |
| | Amino Acids Differences | | | %Homology | | |
| | FR | CDR | Total | FR | CDR | Total |
| TD 1B1 | 0 | 0 | 0 | 100 | 100 | 100 |
| TD 3A2 | 0 | 1 | 1 | 100 | 97 | 99 |
| TD 4A2 | 1 | 0 | 1 | 98.5 | 100 | 99 |
| AG8 | 0 | 1 | 1 | 100 | 97 | 99 |
| TD 9C6 | 0 | 0 | 0 | 100 | 100 | 100 |
| TD 3D6 | 0 | 0 | 0 | 100 | 100 | 100 |
| TD 4C6 | 0 | 0 | 0 | 100 | 100 | 100 |
| T3 7D2 | 7 | 2 | 9 | 90 | 94 | 91 |

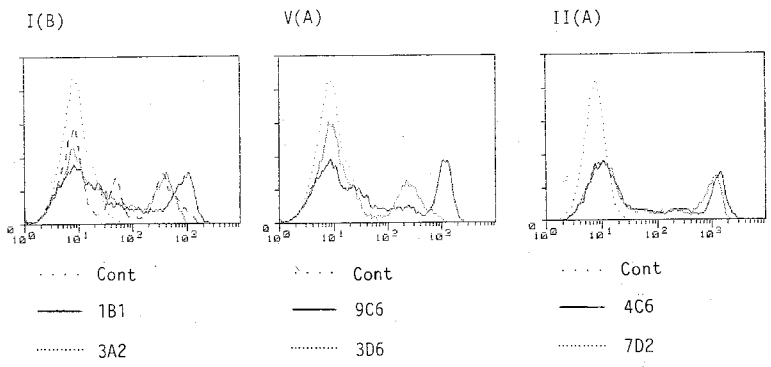



**Fig. 3** Brightness was compared by FACStar overlay histogram. 1B1 showed the brightest fluorescence of the I(B) subgroup. 4A2 showed two positive peaks.
9C6 stained brighter than 3D6 among V(A) family. Two members of II(A) subgroup, 4C6 and 7D2, showed almost the same brightness.
cont ; negative control

"C", which would be expected to destroy the viability of this protein, turned out to be an integral part of this consensus sequence (see discussion). A single amino acid substitution was observed in CDR3 of 3A2 compared with 1B1. Also in 4A2, a single amino acid replacement was observed in FR2. No productive mutation was observed between 9C6 and 3D6. Seven and two differing amino acids were found in FRs and CDRs, respectively, between 4C6 and 7D2. However, the sequence of CDR3 was identical.

### 3. 4 Affinity and epitope analysis by flow cytometry

In the overlay histogram, 1B1, 9C6, and 4C6 showed stronger luminosity than the other mAbs (Figure 3), which indicates these three mAbs had higher affinity with the antigen than the other ones. Only 4A2 showed two positive peaks. In the time course flow cytometry assay, 1B1, 9C6, and 4C6 showed a time dependent increase of strong brightness (Figure 4), while the other mAbs reached the plateau in a short time except for the dull positive peak of 4A2. Two color analyses by flow cytometry indicated that these dull positive cells co-expressed CD11b very strongly (data not shown). This suggested a difference in the population resulting from showing high expression of CD8.

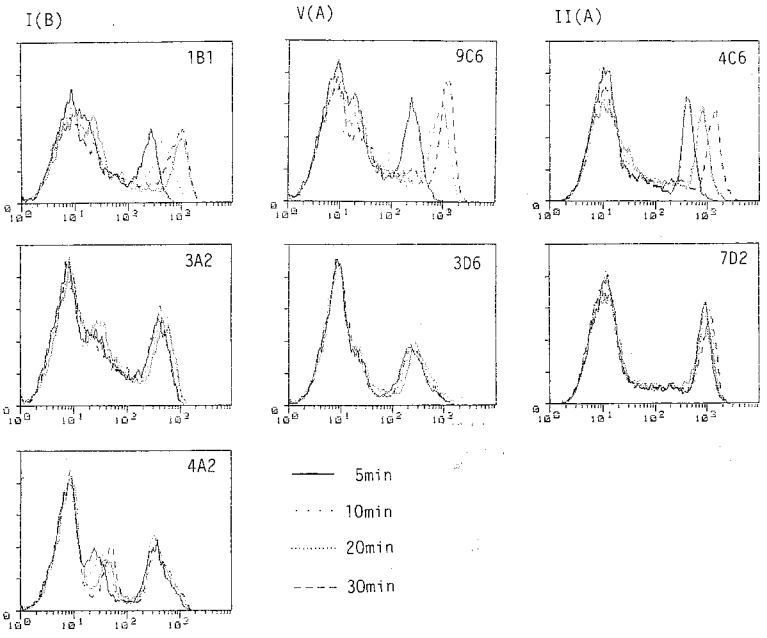



**Fig. 4** Time course flow cytometry analysis was performed by reacting antibodies for 5 min, 10 min, 20 min, and 30 min. Time dependent increase of fluorescence intensity was observed in the histogram of 1B1, 9C6, 4C6, and in the dull positive peak of 4A2.

## 4. Discussion

The enormous diversity of the whole repertoire of Ig stems from (1) the number of V, D, and J segments, (2) the number of possible V-(D)-J combinations, and (3) junctional diversity. Within the variable domain of each Ig chain, the antigen binding site is composed of three hypervariable regions, or complementarity determining regions (CDR). Two of these CDRs, CDR1 and CDR2, are encoded in germline sequences of the V gene segment, while the third (CDR3) is encoded by the junctional segment that has the highest degree of junctional diversity. This junctional region (CDR3) is known to determine the specific reactivity of Ig to antigen.

We studied seven mAbs that recognize the epitopes on a CD8 molecule. A cell binding inhibition assay classified those mAbs into four groups. That suggested there are at least four different major antigenic determinants on the CD8 molecule. The VH sequences of those mAbs were then determined to analyze the relationship between epitopes and affinity. Seven VH sequences were obtained, and were classified into three families according to the identity of CDR3 construction. The VH sequences of 9C6 and 3D6 were classified as group V(A); they had identical sequences in every FR and CDR3. Although these mAbs clearly arose from a common ancestral clone, they are here classified into different groups because they show distinctly differing activities in the binding inhibition assay. The reason for this appears to be the structural change in the light chain variable region (VL) which takes place during somatic mutations. It is generally supposed that the H-chain plays the major role in determining the properties of the idiotope of an Ig species, but our results suggested that the change of VL altered the reactivity of mAbs from group A to group D in spite of possessing identical VH.

The three clones in I(B) all contained one extra codon "C" just after the #96 amino acid. This indicated that those sequences were not the ones originally encoded on the VH of those clones, but had been copied from the other allele that, but for the random nature of somatic mutation, would not participate in making the products. Nevertheless, the sequences clearly indicated that those three were subclones derived from a common ancestral clone. Furthermore, the sequences in the constant region suggested that 3A2 (Ig-G2b) and 4A2 (Ig-G2a) had derived from 1B1 (Ig-G1). These results led us to conclude that somatic mutations occurring in these clones were responsible for the changes in the reactivity from group A (1B1) to group B (3A2) or C (4A2). A homology search in GENETYX data base revealed a previously registered mouse IgVH with extremely high homology with 1B1. The sequence, MUSVHAG8 (accession number X58634) is given in Figure la as AG8. The only difference between 1B1 and AG8 is a single component at 56. We suspect that both 1B1 and AG8, with their unusual extra nucleotide, are nonsense recombinations. In a homology analysis of VH sequences using a vast data base, Kabat reported finding some clones that had almost identical VH sequences[31].

The relationships between clones are summarized in Figure 5. The affinities of 3A2 and 4A2 were weaker than 1B1; however, a clone with higher affinity (square) did, presumably, exist at the time of

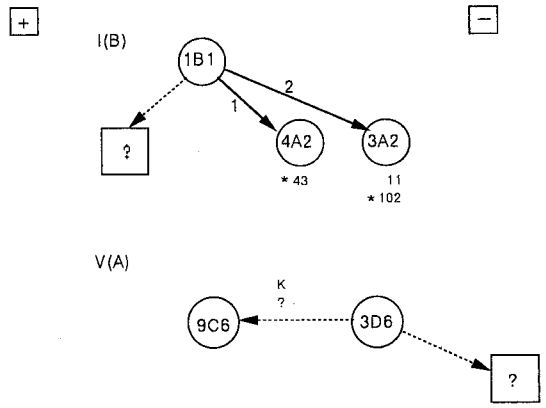



**Fig. 5** Clonal relationships of the five VH sequences (circles) and hypothetical subclones (square) are shown. Distance between the clones are proportional to the number of base mutations as indicated along the lines. The mutations are listed by location according to the amino acid number. Productive mutations are listed by asterisk.

cell fusion. As well as in the I(B) family, a clone with lower affinity (square) should also have existed in the V(A) family.

It is well accepted that junctional diversity gives rise to the tremendous flexibility of Ig. The results presented here clearly demonstrate, however, that somatic mutation also plays a key role in constructing antigen-specific Ig molecules. Furthermore, somatic mutation alone is sufficiently powerful a process to forge differing idiotypes even from originally identical daughter cells of a single ancestral clone. In addition, two clones arising from separate fusions expressed almost identical VH sequences. This suggested that the epitope on CD8 recognized by 4C6 and 7D2 may direct the V region selection in immunized mice that resulted in the formation of antibodies expressing a closely related VH gene and a highly homologous CDR3 sequence. In the process of Ig formation using a limited number of VH gene segments against the vast variety of antigens existing in nature, common VH conformation may happen more frequently than we suppose.

## 5. Conclusion

We analyzed seven mAbs' VH sequences that recognize the same CD8 Ag. Although a binding inhibition assay classified the mAbs into four groups, it proved more useful to consider these as three VH families. Somatic mutation was shown to charge not only the affinity of resulting Ig, but also the idiotope, even among monoclonal products. Two products of differing monoclonal origins were found to have the same paratopes and the same VH usage.

The nucleotide sequence data reported in this paper will appear in the DDJB, EMBL, and GenBank Nucleotide Sequence Databases with the following accession number D14170, D14171, D14172, D14173, D14174, D14175, and D14176.

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# 抗CD8モノクローナル抗体VH領域の解析

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私達は，同一抗原のCD8を認識する7つのモノクローナル抗体のVH領域を解析した．その結果，3つのVHファミリーが使われており，I（B）ファミリーの3クローンはそれぞれ1つのアミノ酸の違いであり，V（A）ファミリーの2クローンは全くの同一のVHを有しており，それぞれ1つのクローンから派生したと考えられた．また，同一ファミリー内のクローンは別のエピトープグループに属しており，affinityの違いも見られ，これはsomatic mutationによるものと考えられた．また，別々のfusionから得た2つのクローンはII（A）ファミリーに属し，同じエピトープグループに分類された．これらはCDR3が全く同じで同じVHを使用していた．