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Variable Region Structure of Autoimmune and Anti-Viral Antibodies

by

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Abstract

Rheumatoid Arthritis (RA) is an autoimmune disease of unknown etiology that is characterized by chronic inflammation of the joints and by the presence, in high titers, of an autoantibody known as the rheumatoid factor (RF). The human cytomegalovirus, a member of the human *Herpesviridae* family, has been proposed as a potential environmental agent involved in the induction of RF production. The report of an anti-CMV antibody having structural homology to a major group of human paraproteins with RF activity, as detected by the expression of RF-associated idiotypes and by variable region sequences, provided a possible structural relationship between CMV infection and RF production.

In this thesis, the structural characterization of eight human hybridoma anti-CMV antibodies and their possible relationship to RFs is presented. All eight antibodies recognized the viral matrix phosphoprotein, known as pp65, which has previously been demonstrated to be highly immunogenic during the natural infection. Seven of these antibodies expressed a restricted number of RF-associated idiotypes. The HCV-2 anti-CMV antibody expressed the greatest number of RF-associated idiotypes and was most similar to RFs of the "PO" idiotypic family. All eight antibodies were composed of different V_H/V_L pairs, with evidence of antigen-selected somatically-induced mutations in the majority of cases. When the nucleotide sequences of these anti-CMV antibodies were compared to previously reported rearranged immunoglobulin sequences, the majority were found to share the highest degree of identity with antibodies possessing RF activity. This data provides evidence that there is extensive overlap between the autoimmune and anti-pathogen antibody repertoires.

Comparisons of the anti-CMV antibodies with homologous RF sequences revealed that most of the amino acid differences could be accounted for by putative somatic mutation events and different junctional diversity in the third complementarity determining region (CDR3) of the antibody heavy chains. This was supported by the cloning and sequencing of the variable regions of a human hybridoma RF, known as C304, which was derived from a patient with active RA. The C304 heavy and light chain sequences had high identity with one of the anti-pp65 antibodies (HCV-3) and with

a previously reported antibody directed against the human herpes simplex virus (HSV). The sequences diverged at points of suspected somatic mutations and, extensively, in the CDR3 of the heavy chains.

Although no evidence was obtained that could definitively implicate human *herpes* viruses in the induction of RFs, this work has provided an analysis of the relationship between idiotype expression and antigenic specificity. This data has enabled the identification of the sequence differences between RFs and anti-viral antibodies that potentially play a role in determining antigenic specificity and provides the basis for an experimental model to address the issue of how primary amino acid sequence determines the specificity of human antibodies.

Résumé

L'arthrite rhumatoïde est une maladie autoimmune, d'origine inconnue, characterisée par une inflammation chronique dans les articulations, et par la présence d'anticorps autoimmuns nommés facteurs rhumatoïdes (FRs). Le cytomégalovirus humain (CMV), un virus de la famille *Herpes*, a été proposé comme agent étiologique pouvant être impliqué dans la production des FRs. Une étude démontrant une ressemblance structurale, autant par l'expression d'idiotypes reliés aux FRs que par la séquence des régions variables, entre un anticorps dirigé contre le CMV et un groupe de FRs de la famille idiotypique "WA", a établi un lien possible entre l'infection par CMV et la production de FRs.

Dans cette thèse, la structure de huit anticorps humains a été étudiée et une relation avec les FRs est proposée. Ces anticorps reconaissent la phosphoprotéine de la matrice virale, nommée pp65, une protéine qui est très immunogénique lors de l'infection naturelle. L'anticorps anti-CMV, HCV-2, a exprimé le plus grand nombre d'idiotypes reliés aux FRs, et était très semblable aux FRs de la famille idiotypique "PO". Chacun des anticorps anti-pp65 était composé d'une paire de gènes V_H/V_L différente et faisait preuve de mutations somatiques sélectionnées par l'affinitée pour l'antigène. Lorsque les séquences des anticorps anti-viraux ont été comparées à toutes les séquences d'anticorps humains, elles se reapprochaient le plus des FRs. Ceci démontre l'utilisation de mêmes gènes dans les répertoires autoimmun et anti-pathogène.

La comparaison des séquences provenant des anticorps anti-CMV avec les séquences homologues provenant de FRs, démontrait que les différences entre ces anticorps pouvaient être expliquées par des événements de mutations somatiques et par la diversité dans le troisième CDR (région déterminant la complémentarité avec l'antigène) des chaînes lourdes. Cette dernière observation est appuyée par les résultats obtenu par l'analyse du FR C304 dérivé d'un patient avec arthrite rhumatoïde. Les séquences des gènes de la région variable de C304 étaient presque identiques aux séquences exprimées par un des hybridome anti-pp65 (HCV-3) et par un hybridome dirigé contre le virus herpès simplex (HSV). La différence entre ces anticorps pouvait

être attribuée à des mutations somatiques et à des différences dans les CDR3 des chaînes lourdes.

Quoique les résultats obtenus par ces études ne pouvaient démontrés un lien définitif entre les virus *Herpes* et la production de FRs, ce projet a permis l'analyse de la relation entre l'expression d'idiotype et la spécificité antigénique. De plus, l'analyse des séquences de régions variables codant les FRs et les anticorps anti-CMV a permis l'identification de différences qui sont probablement importantes dans la détermination de la spécificité de ces anticorps. Ces observations permettront l'établissement d'un modèle permettant l'étude de la relation entre la séquence des régions variables et la spécificité d'un anticorps.

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Abbreviations

APTT	activated partial thromboplastin time
bp	base pairs
BSA	bovine serum albumin
C_λ	lambda light chain constant region
C_H	heavy chain constant region
C_k	kappa light chain constant region
C_L	light chain constant region
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region (e.g. CDR1)
$CDR3_H$	third complementarity region of heavy chain
CL	cardiolipin
CMV	cytomegalovirus (human)
CRI	cross-reactive idiotype
D	diversity
dDNA	denatured deoxyribonucleic acid
DMARD	disease-modifying anti-rheumatic drug
DNA	deoxyribonucleic acid
DOPE	dioleoylphosphatidylethanolamine
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunoassay
Fc	fraction crystallizable
FR	framework region (e.g. FR1, FR2, FR3)
gly	glycine
GSDB	Genome Sequence Database
H chain	heavy chain
HPRT	hypoxanthine phosphoribosyl transferase
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1

HSV-2	herpes simplex virus type 2
HVR	hypervariable region
Id	idiotype
Ig	immunoglobulin
IgG	gamma immunoglobulin
IgG-Fc	fraction crystallizable of gamma immunoglobulin
IgM	mu immunoglobulin
J	joining
J_λ	lambda light chain joining segment
J_H	heavy chain joining segment
J_k	kappa light chain joining segment
kb	kilobase
kD	kilodalton
L chain	light chain
Mb	megabase
MC	mixed cryoglobulinemia
N nucleotide	nontemplate encoded nucleotide
O.D.	optical density
P nucleotide	palindromic nucleotide
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PL	platelet lysate
pp65	phosphoprotein of 65 kilodaltons (CMV)
Pro	proline
RA	rheumatoid arthritis
RF	rheumatoid factor
RIA	radioimmunoassay
RNA	ribonucleic acid
RSS	recombination signal sequence
SD	standard deviation

SLE	sytemic lupus erythematosus
TBE	tris borate EDTA
Tyr	tyrosine
V	variable
V_λ	lambda light chain variable region
V_H	heavy chain variable rgion
V_k	kappa light chain variable region
V_L	light chain variable region

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Preface

The Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research at McGill University reads as follows:

> "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.
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This thesis is organized in eight chapters. Chapter I is the "Introduction and Literature Review" and is divided into five major sections. The next four chapters constitute the "Experimental Results" and contain one published paper, two papers accepted for publication, and one manuscript that has been submitted for publication. Each of these four chapters possesses its own Summary, Introduction, Materials and Methods, Results, Discussion and References. Connecting texts for each of these chapters are included as Prefaces, located at the beginning of each chapter.

Chapter II is entitled, "Structural Characteristics of Four Human Hybridoma Antibodies Specific for the pp65 Protein of the Human Cytomegalovirus and Their Relationship to Human Rheumatoid Factors". The authors are J.D. Rioux, Y. Larose, B. Brodeur, D. Radzioch and M.M. Newkirk. The human anti-pp65 hybridoma cells, as well as the supernatants from these hybridomas, were provided by Dr. Y. Larose in the laboratory of Dr. B. Brodeur (National Laboratory for Immunology, Ottawa, Canada). The viral antigen specificity of these hybridoma antibodies was also determined by these collaborators. Dr. D. Radzioch was helpful in providing equipment and experimental protocols for the isolation of the cellular RNA. This manuscript has been accepted for publication in the journal *Molecular Immunology*.

Chapter III is entitled "Molecular Characterization of Human Monoclonal Antibodies Specific for the Human Cytomegalovirus: Relationship of Variable Region Sequence to Antigen Specificity and Rheumatoid Factor-Associated Idiotype Expression". The authors are J.D. Rioux, M. Ohlin, C.A.K. Borrebaeck and M.M. Newkirk. The human anti-pp65 hybridoma cells, as well as the supernatants from these hybridomas, were provided by Dr. M. Ohlin from the laboratory of Dr. C.A.K. Borrebaeck. The binding characteristics, not including the rheumatoid factor activity, of these hybridoma antibodies was determined by Dr. M. Ohlin. This manuscript has been submitted to *Clinical and Experimental Immunology*.

Chapter IV consists of a published paper entitled "Molecular Characterization of the GM 4672 Human Lymphoblastoid Cell Line and Analysis of Its Use as a Fusion Partner in the Generation of Human-Human Hybridoma Autoantibodies". This paper was published in *Human Antibodies and Hybridomas* (1993) 4:107-114. The authors are J.D. Rioux, J. Rauch, E. Zdarsky and M.M. Newkirk. Dr. E. Zdarsky was responsible for sequencing of the light chain variable region of the GM 4672 cell line. All of the hybridomas analyzed in this study were produced and characterized in the laboratory of Dr. J. Rauch. The cloning and sequencing of the GM 4672 heavy chain variable region, as well as all of the data compilation and analysis, were performed by Mr. J.D. Rioux.

Chapter V is entitled A Human Rheumatoid Factor C304 Shares V_H and V_L Gene Usage With Antibodies Specific for Ubiquitous Human Viral Pathogens. The authors are

J.D. Rioux, J. Rauch, L. Silvestri and M.M. Newkirk. The C304 hybridoma was generated in the laboratory of Dr. J. Rauch. Ms. L. Silvestri isolated the RNA and performed the amplification (PCR) of the immunoglobulin heavy chain variable region of the C304 hybridoma. All of the remaining analyses were performed by Mr. J.D. Rioux. This manuscript has been accepted for publication in the journal *Scandinavian Journal of Immunology.*

Chapter VI is the general "Discussion" for the thesis. An "Appendix" follows Chapter VI and contains information regarding antibody specificity, variable gene usage and idiotypic expression of anti-viral and control antibodies included in the Discussion. Chapter VII contains the "Bibliography", which consists of the references cited in Chapters I and VI, as well as those cited in the Appendix. Finally, Chapter VIII contains the "Claims for Original Research".

Acknowledgments

I dedicate this thesis to my mother and to my late father, to whom I am eternally grateful for everything that they have done for me over the years. My parents have always been there to provide me with love, friendship and encouragement.

I wish to thank my supervisor, Dr. Marianna M. Newkirk, for the opportunity to work on this project, as well as for her guidance and support.

I am indebted to my mentor and friend, Dr. Phil Gold, for helping me get through many times of difficulty. I wish to thank Dr. Joyce Rauch for all of her encouragement and for her thoughtful discussions. I wish to thank Dr. John Esdaile for welcoming me into his department and for his generous support. I would also like to express my appreciation to Ms. Lina Petteruti, for her friendly secretarial assistance. I wish to express my gratitude to Drs. Yolande Larose, Bernard Brodeur, Mats Ohlin and Carl Borrebaeck for their involvement in this project.

I thank all the members of my family for their patience, generosity and many words of encouragement. I thank all of my friends for their encouragement, and above all, for making life fun and interesting.

I would like to acknowledge The Arthritis Society of Canada for their financial support during my doctoral thesis.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

SECTION 1

HISTORICAL PERSPECTIVE AND RATIONALE

Although the term rheumatoid arthritis (RA) was coined near the end of the 19th century, a clear description of the clinical features of this disease was presented by Benjamin C. Brodie much earlier (Brodie, 1819; cited in Benedek, 1989). Brodie's observation that articular cartilage deterioration often followed an episode of synovitis was subsequently supported by the roentgenographic data of Nichols and Richardson (1909; cited in Hollander, 1985). It was the work of the latter investigators that characterized the atrophic and decalcified features of the these damaged joints that distinguished RA from other forms of arthritis. The concept that multiple etiologic factors, notably certain infectious agents, could be responsible for provoking this pathology was proposed by Nichols and Richardson, as well as by Frank Billings a few years later (cited in Hollander, 1985; Benedek, 1989).

During the search for a putative bacterial etiologic agent, serological studies led to the discovery of a human serum factor that was capable of agglutinating immunoglobulin-coated sheep red blood cells (Waaler, 1940). Rose and co-workers subsequently demonstrated the relationship of Waaler's "serum factor" and RA (Rose *et al.*, 1948). This discovery led not only to the renaming of the entity as "rheumatoid factor" (RF), but also to the development of the current diagnostic tests used for the detection of RFs (Pike *et al.*, 1949).

Upon discovery of the association of RFs with this disease, a specific etiological role for these autoantibodies was sought. A direct pathogenic role for RFs was placed in doubt, however, when Harris and Vaughan failed to induce any RA-associated symptoms in healthy volunteers who had high circulating RF titers maintained over a two month period by using a transfusion procedure (Harris & Vaughan, 1961). Evidence in favour of RFs playing an active role in the local inflammatory response of rheumatoid joints came when phagocytic cells isolated from synovial fluid were shown to contain

immune complexes, presumably consisting of IgM RFs bound to their gamma globulin antigens (Hollander *et al.*, 1965). The mechanism by which RF production was induced, however, remained in question.

This interest in RFs persisted into the next decade and resulted in some interesting findings. The first structural studies of human RFs (also known as anti-gamma-globulins) were performed on paraproteins isolated from patients with either mixed cryoglobulin syndrome or hypergammaglobulinemia purpura (Capra & Kunkel, 1970; Kunkel *et al.*, 1971). These and subsequent studies demonstrated that RFs from unrelated individuals were structurally similar, both by idiotypic and by primary sequence analyses. These studies were also important since they provided some of the first structural data regarding variable regions of human antibodies of defined specificity, demonstrating a relationship between variable region sequence and antigenic specificity.

These structural studies did not, however, provide further information regarding any putative etiologic agent. It was not until 1988, when a human anti-cytomegalovirus (anti-CMV) antibody was sequenced, that a structural basis could be proposed for the role of a human pathogen in the induction of RF synthesis (Newkirk *et al.*, 1988). In this study, it was reported that the EV1.15 anti-CMV antibody was structurally similar to the majority of previously characterized human RFs. These similarities were noted both with respect to idiotype expression and amino acid sequence. Since CMV was one of the many environmental agents to be proposed to putatively play a role in the etiology of RA (Hamerman *et al.*, 1982; Ford *et al.*, 1987), it was of interest to obtain a better understanding of the potential relationship between anti-CMV antibodies and human RFs. Our plan was, thus, to determine the variable region structures of a set of human antibodies that were specific for the human CMV. Since the structural knowledge of human antibodies of known specificity was almost exclusively based on antibodies with autoreactivity, this approach would also advance our goal of acquiring a better structural understanding of the humoral response to human pathogens.

The anti-viral antibodies included in this study all recognize the immunodominant viral phosphoprotein known as pp65 (Larose *et al.*, 1991; Ohlin *et al.*, 1991). Since a

pathologic relationship between primary CMV infection and the development of RA has not been established, it is most likely that any relationship found between CMV and RF synthesis would reflect the ongoing CMV/host immune system balance that predetermines viral latency and reactivation. It has indeed been demonstrated that, in post-surgery and transplantation settings, CMV reinfection or reactivation is often associated with the appearance of circulating RFs (Kantor *et al.*, 1970; Baldwin *et al.*, 1983, 1987; van der Giesen *et al.*, 1990). Disturbance of this balance may play a crucial role, whether the production of RFs is related to the humoral anti-viral response by virtue of the anti-CMV antibodies providing an antigenic stimulus to RF-producing B cells, or via a regulatory network, mediated perhaps by shared idiotypic structures. For this reason, van Zanten's observations of a heightened humoral anti-pp65 response during reinfection and reactivation of latent CMV are of importance when considering a relationship between the anti-CMV response and the induction of RFs synthesis (van Zanten *et al.*, 1991, 1993).

SECTION 2

IMMUNOGLOBULIN VARIABLE REGION

2.1 GENERAL IMMUNOGLOBULIN STRUCTURE

The general structural features of immunoglobulin (Ig) molecules have been well characterized and a thorough review of this material has been compiled by Hasemann and Capra (1989). Briefly, each Ig molecule consists of a pair of identical light (L) chains and a pair of identical heavy (H) chains (For a diagram of the basic immunoglobulin structure, please refer to figure A-1 of the Appendix). The antigenic specificity of an antibody is determined by the amino acid sequence within its variable (V) region, which is generated by the pairing of its particular V_L (L chain V region) and V_H (H chain V region). The "C" (for "constant") regions, on the other hand, are responsible for the overall structure, and for the biological functions of the Ig molecules. In humans, there are nine different H chain C region (C_H) genes. The products of these genes have different antigenic properties (see below) and effector functions. Intrachromosomal recombination within B cells is one of the mechanisms which permits the "switching" from one C_H gene to another, for example from C_μ to C_γ (ie. IgM to IgG). This permits the same V region (determining specificity) to be associated with different biological functions. (For review of the mechanism and regulation of isotype switching, see Coffman *et al.*, 1993.)

Whereas the "C" domains are relatively invariant with respect to amino acid sequence, the "V" domains are characterized by sequence variablility. This variablility is not uniformly distributed and, thus, the "V" domains are further divided into areas of high variability, known as hypervariable regions (HVRs), and conserved areas known as framework regions (FRs).

When Ig molecules are used as antigens, additional structural features can be elucidated. Certain antigenic determinants are found in all normal individuals and can

be used to separate Ig molecules into groups known as isotypes. Isotypes correspond to the different heavy chain classes and subclasses. The second category of Ig antigenic determinants represents allelic markers of Ig L and H chains and allowed for the classification of Igs into various allotypes. Both isotypic and allotypic determinants can be located in the "C" domains and are, thus, not related to antigenic specificity. The third category of antigenic determinants, called idiotypes, are located in the "V" domains and, thus, more likely to be related to specificity. Although initially defined as markers generally found on a single antibody in an individual member of a species (private idiotype), some idiotypic determinants have been detected on Igs derived from unrelated individuals and became known as cross-reactive idiotypes (CRI).

2.1.1 V REGION CONFORMATION AND ANTIGEN CONTACT RESIDUES

Statistical analysis of amino acid variability in human immunoglobulin sequences led to the delineation of three HVRs (briefly described above) in both the L and the H chains (Wu & Kabat, 1970; Kabat & Wu, 1971). The regions were postulated to contain the amino acid residues that make contact with antigen and, thus, constitute the complementarity determining regions or CDRs (Wu & Kabat, 1970; Kabat & Wu, 1971). Subsequently, X-ray analyses of crystallized antigen-antibody complexes provided formal proof of the involvement of HVR residues in antigen contact (for review see Davies *et al.*, 1989; Tulip *et al.*, 1989; Poljak, 1991). CDRs are now operationally-defined by amino acid residue position (Kabat *et al.*, 1987).

The study of a group of murine antibodies specific for lysozyme provided many important insights into the interactions between antibodies and their protein antigens. Specifically, the X-ray analyses of three different anti-lysozyme/lysozyme complexes demonstrated the involvement of an average of greater than a dozen antibody residues in the contact with antigen (Amit *et al.*, 1986; Sheriff *et al.*, 1987; Padlan *et al.*, 1989). In these three examples, amino acid residues in all six CDRs participated in antigen contact. In each case, a single FR residue, adjacent to either CDR1 or CDR2 of the H chain, was also involved in antigen/antibody interaction (Amit *et al.*, 1986; Sheriff *et al.*,

1987; Padlan *et al.*, 1989). These anti-lysozyme antibodies recognized distinct discontinuous epitopes and, not surprisingly, had different combining sites. The common denominator, however, was that the interacting surfaces were very complementary; although generally quite flat, protruding side chains of one side of the complex would lie in the depressions of the other surface (Amit *et al.*, 1986; Sheriff *et al.*, 1987; Padlan *et al.*, 1989). In this context, it can be envisioned how mutations which change the amino acid side chains participating in antigen contact, can affect binding affinity (for a review of the mutation process see section 2.3.3). It must be noted, however, that amino acid residues not directly involved in antigen contact can also alter binding affinity by affecting the positioning and orientation of the actual contact residues (Chien *et al.*, 1989; Schildbach *et al.*, 1993).

2.2 IMMUNOGLOBULIN V REGION GENES

During B lymphocyte development, Ig V regions are generated by somatic rearrangement of multiple germline genes. Specifically, H chain V regions consist of rearranged V (variable), D (diversity) and J (joining) genes, and L chains of V and J gene segments. In the H chain, CDR1 and CDR2 are encoded by the V gene, whereas the CDR3 is encoded by the D and J gene segments (and by the additional codons generated by the mechanisms described in section 2.3.2). In L chains, the V gene encodes all of CDR1 and CDR2 and the majority of CDR3, and generally the last one or two residues of CDR3 is encoded by the J gene segment (Kabat *et al.*, 1987). The location of these Ig loci, which also include their respective C (constant) genes, have been assigned to human chromosomes 14, 2 and 22, for the heavy, kappa and lambda chains, respectively. The genes within each locus have been classified into different groups or families, using various criteria that assess relatedness between the different genes. Generally it can be said that two members within a family will share a minimum of 80% nucleotide identity, and less than 70% identity between members of different families. (Pascual & Capra, 1991).

2.2.1 PHYSICAL MAP & COMPLEXITY

2.2.1.1 *THE KAPPA LOCUS*

The human kappa locus is the best defined locus of the three human Ig loci. The entire locus, which is located on the short arm of chromosome 2 at 2p11-2p12, is approximately 2 megabases (Mb) in length (Weichhold *et al.*, 1993). A detailed physical map of the locus, as well as a less detailed map of its surroundings, has recently been completed (Weichhold *et al.*, 1993). This map, in conjunction with the information obtained from the kappa gene cloning and sequencing studies, has established that the kappa locus is present in two almost complete copies (per haploid genome), which are arranged in opposite transcriptional polarity. The germline genes are clustered into regions known as B, L, A and O (reviewed in Schäble & Zachau, 1993). The B region, containing three V_k genes, is uniquely found in the copy that is proximal to the J_k-C_k regions (Lorenz *et al.*, 1988). The physical arrangement of this locus can thus be described as: telomere-C_k-J_k-B-L_p-A_p-O_p-space-O_d-A_d-L_d-centromere. The "p" and "d" subscripts refer to the copies that are proximal or distal to the J_k-C_k region, respectively. A total of 76 V_k genes have been found in the distal and proximal regions, and all have been cloned, sequenced and mapped (Lorenz *et al.*, 1988; Pargent *et al.*, 1991a; Lautner-Rieske *et al.*, 1992; Huber *et al.*, 1993a, 1993b). It is interesting to note that there are 33 gene pairs among the 76 genes sharing 95-100% identity in the coding regions, reflecting the amplification and transposition steps that are believed to have generated the particular organization of this locus (Pohlenz *et al.*, 1987).

All of the V_k genes in the 600 kilobase (kb) proximal region, except for two of the genes in the B region, are in the same transcriptional orientation as the five J_k and one C_k genes. The 36 V_k genes present in the 440 kb distal region, on the other hand, are arranged in the opposite direction (Weichhold *et al.*, 1993). Although the largely symmetrical central space (approximately 800 kb) between the distal and proximal regions has not yet been cloned and sequenced (Weichhold *et al.*, 1993), it is likely that all of the genes of this locus have been characterized (Meindl *et al.*, 1990; Schäble &

Zachau, 1993). These genes have been classified as either potentially functional (PF), pseudogenes, or as genes with minor defects (MD). The minor defects have been defined as one or two single base-pair (bp) alterations in a gene. These genes were not classified as non-functional pseudogenes since a gene, previously reported as defective, was subsequently isolated from a different individual in an unaltered form (Pech *et al.*, 1985). Nearly all of the cloning and sequencing of this locus has been carried out in the laboratory of Hans Zachau and, appropriately, they have recently written a comprehensive review of this work (Schäble & Zachau, 1993). These authors have reclassified some of the previously described genes, however since some discrepancies in classification exist, Table 2-1 is a compilation of the data from the original reports, as well as information found in the review paper by Schäble and Zachau (Lorenz *et al.*, 1988; Pargent *et al.*, 1991a; Lautner-Rieske *et al.*, 1992; Huber *et al.*, 1993a, 1993b; Schäble & Zachau, 1993). This table shows that approximately half of the 76 V genes in this locus have at least one potentially functional allele that has been described (33 PF genes and 4 PF/MD genes). Table 2-1 also highlights the fact that genes of the different V_k subgroups are interspersed in the O, A and L regions. It must also be noted that the majority of the PF and PF/MD germline genes are of the V_kI, V_kII and V_kIII subgroups (approximately 50%, 25% and 20%, respectively).

Many V_k genes reside outside of the kappa locus and are called orphons. Some were previously believed to be a part of the actual kappa locus but were since mapped to the long arm of chromosome 2, indicating that they may have derived by amplification and then been transposed to their actual position (Zimmer *et al.*, 1990). Additional kappa orphons have been located on chromosomes 1 and 22, while the specific chromosomal location of others has not yet been determined (Röschenthaler *et al.*, 1992). Even though an orphon without any sequence defects has been reported, orphons are generally considered to be pseudogenes. This is due both to their characteristic sequence defects and the fact that they are separated from the J_k-C_k region, thus unlikely to undergo functional rearrangements (Huber *et al.*, 1990).

Table 2-1. Summary of germline genes within the human kappa locus.

V GENE SUBGROUP	REGION[1]	NUMBER OF GERMLINE GENE STRUCTURES[2]			
		FUNCTIONAL	PSEUDO	MD	PF/MD
I	O	5	3	-	1
	A	-	3	2	-
	L	10	-	3	2
II	O	2	4	-	-
	A	7	5	4	-
	L	-	4	1	-
III	O	-	3	-	-
	A	2	3	-	-
	L	4	-	1	1
IV	B	1	-	-	-
V	B	1	-	-	-
VI	O	-	-	-	-
	A	1	-	2	-
	L	-	-	-	-
VII	B	-	-	1	-

[1] Genomic regions of the human kappa locus, as defined by Zachau's group (Pohlenz et al., 1987)

[2] Germline genes have been classified as "potentially functional" (functional or PF), "pseudogenes" (pseudo), genes with minor defects (MD), or genes with both PF and MD alleles (PF/MD). Data compiled from Lorenz et al., 1988; Pargent et al., 1991a; Lautner-Rieske et al., 1992; Huber et al., 1993b; Schäble & Zachau, 1993.

2.2.1.2 *THE LAMBDA LOCUS*

The sequence information, physical structure and complexity of the lambda locus, located on human chromosome 22 (band 22q11.1-q11.2), are only beginning to be elucidated. In contrast to the kappa locus, seven distinct lambda constant (C_λ) genes have been identified (Vasicek & Leder, 1990; Combriato & Klobeck, 1991). Three of these are pseudogenes, while the others encode the four serologically defined lambda isotypes (Vasicek & Leder, 1990; Combriato & Klobeck, 1991). The organization of the seven J segments is also quite distinct: each J segment is paired with a different C segment. The distance between each J_λ-C_λ pair varies from approximately 1.4 to 3.8 kb, and the intron separating the J_λ (always upstream of the C segment) and C_λ segments has an average length of 1500 bp, but may be small as 300 bp (ie. for the $J_\lambda 4$-$C_\lambda 4$ pair) (Vasicek & Leder, 1990).

The most C-proximal V_λ gene, known as V_λIII.1, was located less than 15 kb from the 5'-most $J_\lambda 1$-$C_\lambda 1$ pair (Combriato & Klobeck, 1991). Though the sequencing and localization of the remaining V_λ genes is still incomplete, the complexity of the locus is reflected by the difficulty in establishing a universal subgroup classification scheme. Many groups have modified Kabat's classification system, by adding on new subgroups to accommodate the novel structures encoded by the newly sequenced germline genes (Kabat *et al.*, 1987; Chuchana *et al.*, 1990; Daley *et al.*, 1992; Williams & Winter, 1993). In spite of this confusion, the various V_λ sequences appear to be grouped into approximately ten different sequence-related families or subgroups (Chuchana *et al.*, 1990; Williams & Winter, 1993). The total number of identified germline segments, including the most recently sequenced V_λ genes, is 34 and includes ten pseudogenes (Williams & Winter, 1993). This is probably an underestimate since, for example, only six genes of the universally accepted V_λII family have been cloned, which is approximately half the expected number (Combriato & Klobeck, 1991; Paul *et al.*, 1991; Williams & Winter, 1993). Based on the limited data and using the classification of Chuchana, the lambda families I, II, III and VII are expected to have approximately a dozen members each (Chuchana *et al.*, 1990; Williams & Winter, 1993).

Studies examining the orientation of the V_λ segments have revealed that most, if not all, are in the same transcriptional polarity as the J_λ-C_λ genes (Combriato & Klobeck, 1991).

2.2.1.3 *THE HEAVY CHAIN LOCUS*

The H chain locus has been mapped to the distal region of chromosome 14, at band 14q32.33, and has been estimated to cover a region of approximately 2.5 Mb (Berman *et al.*, 1988). An extensive review of the literature concerning this locus has recently been published (Pascual & Capra, 1991).

Some of the early mapping of this locus demonstrated that the V_H genes were physically linked to the C_H locus (Berman *et al.*, 1988; Schroeder *et al.*, 1988). The general organization of the H chain locus follows the order of telomere-V_H-D_H-J_H-C_H-centromere, with multiple genes within each region. Both the J_H and C_H complexes, containing six and nine functional genes respectively, have been physically mapped and sequenced (Ravetch *et al.*, 1981; Hofker *et al.*, 1989). A major cluster of D_H genes is located directly upstream of the J_H complex and these genes are arranged in five 9 kb-repeating units within this cluster (Siebenlist *et al.*, 1981). Each repeating unit contains a member from each of the D_{XP}, D_A, D_K, D_N, D_M and D_{LR} families (Ichihara *et al.*, 1988). There appears to exist two additional D_H families: D_{Q52} and a D_{FL16}-like family. The former is a single member "family" which is located within the J_H complex of genes (Ravetch *et al.*, 1981), and the latter appears to have multiple members which are not located in the repeating units of the major cluster (Sonntag *et al.*, 1989). In fact, D segments bearing homology to the families found within the major cluster are also found in a minor cluster, interspersed among the V_H genes (Buluwela *et al.*, 1988; Matsuda *et al.*, 1988). Also, "DIR" genes are found adjacent to some of the D_H segments, are D_H-like, and have been identified in productive rearrangements of the H chain locus (Ichihara *et al.*, 1988).

Based on sequence homologies, V_H sequences have been grouped into one single-member and six different multi-member families. Physical mapping and

sequencing strategies by a number of groups have demonstrated that the members of these different families are interspersed throughout the V_H locus (Lee *et al.*, 1987; Berman *et al.*, 1988; Walter *et al.*, 1990; van Dijk *et al.*, 1992, 1993; van der Maarel *et al.*, 1993; Matsuda *et al.*, 1993). The single V_H6 gene is the most 3' of the V_H genes, is located 77 kb upstream of the J_H complex and is immediately adjacent to the major D_H complex (Schroeder *et al.*, 1988). The recent effort of Honjo's group to structurally characterize the H chain locus resulted in the mapping and sequencing of 64 V_H genes in a 0.8 Mb DNA segment, as well as to an estimation of the total number of genes within the locus (Matsuda *et al.*, 1993). A subsequent study also characterized the newly described V_H7 family (van Dijk *et al.*, 1993). The compiled results from these two studies are found in Table 2-2 and indicate that the V_H3 family is the most complex, having the greatest number of pseudogenes and functional genes. From the quantitative hybridization results, it would appear that many genes of the V_H1 and V_H3 families have yet to be characterized (Table 2-2). Studies have also demonstrated that these families have the highest proportion of pseudogenes, nonetheless, if we assume a consistent ratio of functional/total genes for the V_H locus, the V_H1, V_H3 and V_H4 genes will still account for approximately 80% of the available repertoire of functional genes (Table 2-2, last column).

As in the case of the V_k genes, orphon V_H genes have been identified (Matsuda *et al.*, 1990). Specifically, four V_H gene segments were located on chromosome 16 and, surprisingly, two had no apparent sequence defects.

2.2.2 GENETIC POLYMORPHISM

The extent and nature of the polymorphism that exists in the human Ig loci is still not completely understood. Since the elucidation of the physical maps and germline gene sequences has only just been completed for the kappa locus and remains incomplete for the other two loci, it is difficult to establish whether new germline sequences represent new genes or simply new polymorphic alleles of previously described germline genes. In addition, the polymorphism that has been detected by restriction fragment length

Table 2-2. Summary of the V_H genes of the human heavy chain locus.

V GENE SUBGROUP	# OF GENES SEQUENCED OVER 800 kb[1]		ESTIMATED TOTAL NUMBER OF GENES IN V_H LOCUS[2]	ESTIMATED TOTAL # OF FUNCTIONAL GENES (% OF FUNCTIONAL REPERTOIRE)[3]
	TOTAL	FUNCTIONAL		
1	14	6	25	10-11 (17%)
2	3	2	7	4-5 (7%)
3	37	16	66	28-29 (47%)
4	8	7	12	10-11 (17%)
5	1	1	2	2 (3%)
6	1	1	1	1 (2%)
7[4]	6	4	6-8	4-5 (7%)
TOTAL :	70	37	119-121	59-64

[1] Results obtained from the cloning and sequencing of genes located in the 800 kilobase (kb) DNA fragment from the 3′ region of the human Ig locus (Matsuda et al., 1993).

[2] Total of number of V_H genes in the entire locus, estimated by quantitative hybridization analysis (Matsuda et al., 1993).

[3] Values represent the number of total genes that are functional, assuming that the ratio of functional to total V_H genes (functional/total) over the entire locus is the same as that determined for the 3′ 800 kilobase (kb) DNA fragment of the human Ig locus (Matsuda et al., 1993). The values in parentheses represent the proportion of the estimated total of functional genes belonging to the particular V_H subgroup.

[4] The study of the V_H7 subgroup was not limited to the 800 kb fragment. These results originated from the report of van Dijk et al., (1993).

polymorphism (RFLP) may be due to differences in noncoding regions which may not affect an individual's repertoire.

There are a few reported examples of V_k genes which exhibit minor defects in certain individuals, while appearing potentially functional in others, thus providing evidence for polymorphism at this locus. Additionally, deletion of the entire distal copy of the kappa locus has been found in three of 46 haploid genomes, from 23 unrelated individuals (Pargent *et al.*, 1991). In these same individuals, RFLP analysis detected allelic differences in eight other operationally-defined haplotypes (Pargent *et al.*, 1991).

The data regarding the lambda locus, although still preliminary, indicates that this locus displays extensive polymorphisms. When the $V_\lambda 2$ subgroup was specifically examined by RFLP analysis, approximately half of the restriction fragments were highly polymorphic (Paul *et al.*, 1991). This is supported by a more recent report describing both restriction site and sequence insertion polymorphisms (Chuchana *et al.*, 1993). In addition, Williams & Winter (1993), using the polymerase chain reaction (PCR), were unable to amplify a number of previously characterized germline genes. They were successful, however, in amplifying a number of germline sequences that were nearly identical to known gene segments, implying the existence of multiple allelic variants of these V_λ genes.

Much work is currently being done to assess the degree of polymorphism in the H chain locus. Recently, V_H3-related polymorphisms have been studied using traditional RFLP markers, sequence-specific oligonucleotides and by comparison of sequences derived from unrelated individuals (Chen *et al.*, 1989; Sasso *et al.*, 1990; Adderson *et al.*, 1993). These studies have revealed considerable polymorphism in the V_H3 genes and other regions of the H chain locus, although some genes were noted to be highly conserved. Specific examination of the V_H4 genes, by similar methods, has revealed strikingly similar results (van Dijk *et al.*, 1991; van Es *et al.*, 1992b; Weng *et al.*, 1992). Two separate groups observed that the prevalence of particular genes or markers varied greatly and concluded that the heterogeneity in the V_H3 and V_H4 families may be due predominantly to a diversity in haplotypes, rather than extensive allelic variation at

single loci (Sasso *et al.*, 1990; van Dijk *et al.*, 1992). Oligonucleotide probes to a family-specific portion of V_H framework regions demonstrated polymorphisms in the V_H1 and V_H2 families, in addition to the V_H3 and V_H4 families (Rubinstein *et al.*, 1993). These authors did not detect any polymorphism in the single V_H6 gene and did not examine either of the V_H5 or V_H7 families. An analysis of four unrelated individuals has, however, shown three of eight V_H7-specific bands to be polymorphic (van Dijk *et al.*, 1993). Although a great deal of work remains to be done, it appears that the V_H locus contains many polymorphisms but a number of highly conserved gene segments as well.

2.2.3 EXPRESSED REPERTOIRES

It has been observed that during B cell ontogeny the expressed Ig repertoire is restricted. Specifically, there is preferential expression of genes from the small V_H5 and V_H6 families, as well as particular genes from the larger families (Schroeder et al., 1987; Cuisinier et al., 1989; Schroeder & Wang, 1990; Hillson et al., 1992; van Es et al., 1993). This demonstrates that the expressed repertoire does not always reflect the genetic complexity of functional genes within the different gene families. Thus in the adult repertoire, the frequency of expression of the Ig genes from the different families may also diverge from what would be expected based on calculations of genetic complexity and therefore must be determined experimentally. Such subgroup/family V region gene analyses have been performed with respect to the expression of kappa and H chain genes in adult human B lymphocytes.

Estimates of the expressed human kappa repertoire, with respect to kappa family/subgroup, have been obtained using a number of different methods, including: 1) compilation of protein sequences of various specificities (Kabat *et al.*, 1987), 2) *in situ* hybridization with family-specific probes (Guigou *et al.*, 1990), and 3) Northern blot analysis (Mayer *et al.*, 1990). The results from these studies are summarized in Table 2-3. The actual frequencies determined for the different subgroups varied between the studies, but consistently gave a relative order of frequency of $V_\kappa I > V_\kappa III > V_\kappa II, V_\kappa IV$.

Table 2-3. V_k gene usage by human adult B lymphocytes.

FREQUENCY OF EXPRESSION (%) V_k SUBGROUP					
I	II	III	IV	SOURCE	REFERENCE
46	13	34	7	multiple[1]	Kabat et al, 1987
43	9	29	19	PBL[2]	Guigou et al., 1990
38	5	23	34	EBV clones	Guigou et al., 1990
50	19	21	10	EBV clones	Mayer et al., 1990

[1] Compilation of sequenced proteins from multiple sources.

[2] ABBREVIATIONS: PBL, peripheral blood lymphocytes; EBV clones, B cell clones derived by Epstein-Barr virus transformation of PBL.

This is different from the order predicted by genetic complexity alone: $V_\kappa I > V_\kappa II > V_\kappa III > V_\kappa IV$ (see section 2.2.1.1 and Table 2-1) and indicates the presence of selective pressures that influence the kappa repertoire (Guigou *et al.*, 1990).

The expressed repertoire of V_H genes was determined using the same methods as mentioned above and the results are summarized in Table 2-4. Again there is variation between the different studies and yet all demonstrated the highest frequency of expression of genes from the V_H3 subgroup. Approximately half of the studies found that the frequency of V_H families followed the order of $V_H3 > V_H1 > V_H4$, while the other half of the studies found $V_H3 > V_H4 > V_H1$. This finding is consistent with the high complexity of the V_H3 family, and the similar number of functional genes predicted for the V_H1 and V_H4 families (see section 2.2.1.3 and Table 2-2).

Since none of the amino acid residues of the H chain CDR3 ($CDR3_H$) are encoded by the V segment, there has been much interest in the frequency of usage of the different J_H and D_H genes in the adult repertoire. Yamada determined the J_H gene usage in 99 VDJ joinings from adult peripheral blood B cells and found a biased expression of these segments: J_H1 (1%), J_H2 (0%), J_H3 (9%), J_H4 (52%), J_H5 (15%) and J_H6 (22%) (Yamada *et al.*, 1991). The segments of the DXP (24%), DLR (18%) and DN (13%) families were found to be the most frequently expressed D_H genes in human adult peripheral B lymphocytes (Sanz, 1991).

2.2.3.1 *V REGIONS OF HUMAN HYBRIDOMA ANTI-PATHOGEN ANTIBODIES*

Human hybridoma antibodies are very useful tools in the study of expressed repertoires since, in addition to providing data with respect to overall gene usage, relationships between gene usage and antibody specificity can be examined. In this way the structural aspects of the humoral response against human pathogens can be elucidated. Such structural information is still quite limited as can be seen from a summary of the current published data on human monoclonal anti-pathogen antibodies (Table 2-5). Nevertheless, a number of observations can be made that indicate that this approach will give us relevant structural information. First, most of the hybridomas bearing lambda chains were from the I, II, III and VII families which reflects the greater genetic

Table 2-4. V_H gene usage by human adult B lymphocytes.

FREQUENCY OF EXPRESSION (%) V_H SUBGROUP								
1	2	3	4	5	6	7	SOURCE	REF[3]
15	18	65	<5	5	<5	N.D.	PBL[1]	[1]
11	3	52	19	10	5	N.D.	PBL	[2]
12	0	56	22	8	2	N.D.	EBV clones	[2]
13	1	59	20	5	2	N.D.	EBV clones	[3]
16	8	65	5	4	3	N.D.	PBL	[4]
26	<5	38	16	12	<5	<5	PBL[2]	[5]
15	1	64	12	9	1	N.D.	B.M.	[6]

[1] ABBREVIATIONS: PBL, peripheral blood lymphocytes; EBV clones, B cell clones derived by Epstein-Barr virus transformation of PBL; B.M., bone marrow.

[2] Results compiled from two separate cDNA libraries from two individuals

[3] References: [1], Cuisinier et al., 1989; [2], Guigou et al., 1990; [3], Mayer et al., 1990; [4], Zouali & Theze, 1991; [5], Stewart et al., 1992; [6], Fumoux et al.

Table 2-5. Summary of H and L Chain V Regions of Human Monoclonal Anti-Pathogen Antibodies.
(continued on following page)

MONOCLONAL ANTIBODY	PATHOGEN SPECIFICITY[1]	V_H	D_H	J_H	V_L	REFERENCE
SDZ89-104[2]	CMV (gB)	1	DLR5	6	kIIIb	Newkirk et al., 1988
H210	HAV	1	DXP1,DA1	1	L II	Lewis et al., 1993
H2	HSV-1,2	3	DK1	6	L I	Huang et al., 1992
H3	HSV-1,2	4	DXP'1	5	L III	Huang et al., 1992
V1	VZV	1	D2-C	3	L I	Huang et al., 1992
OST 577	HBV	3	?	4	L V	Andris et al., 1992
ZM1-1	HBV	3	?	4	kII	Andris et al., 1992
265-695	HBV	4	?	4	kI	Andris et al., 1992
MD3-4	HBV	5	?	3	L III	Andris et al., 1992
ED8.4	Hib (PS)	3	DXP1,4	6	N.D.	Adderson et al., 1991
SB1/D8	Hib (PS)	3	DN1	3	N.D.	Adderson et al., 1991
SB5/D6	Hib (PS)	3	?	4	L VII	Adderson et al., 1991, 1992
RAY4	Hib (PS)	3	?	4	L VII	Adderson et al., 1991, 1992
LSF2	Hib (PS)	3	?	4	L VII	Adderson et al., 1991, 1992
JB32	Hib (PS)	N.D.	N.D.	N.D.	L VII	Adderson et al., 1992
JB21	Hib (PS)	N.D.	N.D.	N.D.	L II	Adderson et al., 1992
16M3C8	Hib (PS)	N.D.	N.D.	N.D.	L II	Adderson et al., 1992
RC3	Hib (PS)	N.D.	N.D.	N.D.	kII	Adderson et al., 1992
ED6.1	Hib (PS)	N.D.	N.D.	N.D.	kI	Adderson et al., 1992
HiH2	Hib (LPS)	3	DXP5,DLR4	4	L I	Andris et al., 1993
HiH10	Hib (OMP)	2	DLS2	4	L III	Andris et al., 1993

[1] Abbreviations: CMV, cytomegalovirus; HAV, hepatitis A virus; HSV-1,2, herpes simplex virus (type 1 and 2); VZV, varicella zoster virus; HBV, hepatitis B virus; Hib, Haemophilus influenzae virus type b; HIV, human immunodeficiency virus; Rab, rabies virus; B.p. Bordella pertussis; ?, uncertain or not known.

[2] Previously known as EV1.15.

Table 2-5. Summary of H and L Chain V Regions of Human Monoclonal Anti-Pathogen Antibodies.
(continued from previous page)

MONOCLONAL ANTIBODY	PATHOGEN SPECIFICITY[1]	V_H	D_H	J_H	V_L	REFERENCE
306	HIV (gp41)	3	D21/9	3	kI	Felgenhauer et al., 1990
98-6	HIV (gp41)	4	?	4	N.D.	Andris et al., 1991
120-16	HIV (gp41)	4	?	2	N.D.	Andris et al., 1991
No.86	HIV (gp41)	1	D21/0.5	4	kI	Moran et al., 1993
188	HIV (gp41)	N.D.	N.D.	N.D.	L III	Larrick et al., 1989
257-d	HIV (gp120)	5	?	3	N.D.	Andris et al., 1991
268-d	HIV (gp120)	4	?	4	N.D.	Andris et al., 1991
No.13	HIV (gp120)	1	?	4	L II	Moran et al., 1993
S1-1	HIV (gp120)	1	D1	4	L III	Moran et al., 1993
HB4W	HIV (gp120)	2	?	5	L II	Moran et al., 1993
F105	HIV (gp120)	4	DLR4,DA1/4	5	kIIIb	Marasco et al., 1992
71-31	HIV (p24)	1	DM2-C	4	N.D.	Andris et al., 1991
mAb53	Rab (M)	3	DLR2	4	N.D.	Ikematsu et al., 1993
mAb59	Rab (RNP)	3	D21/9	4	N.D.	Ikematsu et al., 1993
mAb52	Rab (RNP)	3	DLR4	6	N.D.	Ikematsu et al., 1993
mAb105	Rab (RNP)	3	D21/10	4	N.D.	Ikematsu et al., 1993
mAb55	Rab (RNP)	3	DXP'1	6	N.D.	Ikematsu et al., 1993
mAb56	Rab (RNP)	3	D21/10	4	N.D.	Ikematsu et al., 1993
mAb58	Rab (G)	4	DXP4-C	3	N.D.	Ikematsu et al., 1993
mAb57	Rab (G)	1	DXP'1	5	N.D.	Ikematsu et al., 1993
mAb107	Rab (G)	3	DLR4	3	N.D.	Ikematsu et al., 1993
HBp2	B.p. (LOS)	6	DIR2	3	L II	Andris et al., 1993

[1] Abbreviations: CMV, cytomegalovirus; HAV, hepatitis A virus; HSV-1,2, herpes simplex virus (type 1 and 2); VZV, varicella zoster virus; HBV, hepatitis B virus; Hib, Haemophilus influenzae virus type b; HIV, human immunodeficiency virus; Rab, rabies virus; B.p. Bordella pertussis; ?, uncertain or not known.

complexity predicted for these subgroups (see section 2.2.1.2). Second, although only a few kappa-bearing hybridoma antibodies have been described, most are from the V_kI family. This is in agreement with the high genetic complexity of this family and with the observed gene usage in the peripheral blood B cell population (see above; Table 2-5). Third, the V_H gene family usage of the 37 anti-pathogen hybridoma antibodies of known H chain sequence paralleled the genetic complexity of these subgroups (Table 2-5). Specifically, the frequencies for the hybridomas were V_H1 (22%), V_H2 (5%), V_H3 (46%), V_H4 (19%), V_H5 (5%) and V_H6 (3%), compared with the predicted frequency of functional genes in these subgroups of 17%, 7%, 47%, 17%, 3% and 2%, respectively (see Table 2-2). Lastly, the frequency of expression of the J_H and D_H genes in this collection of hybridoma antibodies also reflects the repertoire expressed by normal human peripheral blood B lymphocytes.

2.3 SOMATIC GENERATION OF DIVERSITY

The distinctive characteristic of the humoral immune system is the ability to generate an antibody response to a virtually unlimited number of antigens (an estimated minimum of 10^9 to 10^{11} specific receptors), while using a limited number of germline Ig genes (Cooper, 1987; Nossal, 1987). This is thought to be achieved by three main mechanisms: 1) The random recombination of different V, D (in H chains) and J genes, resulting in combinatorial diversity; 2) The imprecise joining of the V, D and J coding ends, which can include the loss and/or addition of a variable number of nucleotides, resulting in important junctional diversity; and 3) The activation of hypermutation mechanisms that can introduce somatic mutations into the rearranged sequence.

2.3.1 REARRANGEMENT OF GERMLINE GENES

The process by which the rearrangement of germline Ig gene segments is achieved is known as V(D)J recombination. It is known that this process is site-specific, but the exact identity of the recombinase protein(s) that mediate(s) this process is still unknown. Each individual Ig gene segment is flanked by recombination signal sequences (RSS) that are specifically recognized by the recombination machinery. The RSS consist of a

palindromic heptamer and a characteristic nonamer, which are separated by a spacer of approximately 12 or 23 base pairs. It has been determined that the recombination between two genes will only occur if one segment has a RSS with a 12 bp spacer and the other has a 23 bp spacer. It has also been demonstrated that the joint formed between the two RSS (known as a signal joint) is very precise, while the joint formed between the two coding sequences (known as the coding joint) is quite variable (see section 2.3.2). In order to obtain a functional product, the individual segments must be rearranged with the same reading frame and transcriptional polarity. It is thus believed that segments of opposite transcriptional polarity must be rearranged by inversion, whereas segments of identical polarity will be rearranged by a deletion mechanism. (For review of V(D)J recombination see Tonegawa, 1983; Yancopoulos & Alt, 1986; Alt *et al.*, 1986; Max, 1989; and van Dongen & Wolvers-Tettero, 1991.)

It has been shown that the V_k genes in the distal portion of the kappa locus, as well as the B2 and B3 genes of the proximal portion, are in opposite transcriptional polarity to J_k-C_k and rearrange by an inversion mechanism, whereas the remaining proximal genes rearrange by deletion (Weichhold *et al.*, (1990). The V_λ and V_H genes, on the other hand, all appear to be in the same 5' to 3' orientation as the J_λ-C_λ and the $D_H/J_H/C_H$ genes, respectively, and rearrangement probably occurs exclusively by a deletion mechanism (Combriato & Klobeck, 1991; Shin *et al.*, 1991; Matsuda *et al.*, 1993; Matsumura *et al.*, 1994).

2.3.2 JUNCTIONAL DIVERSITY

Although the exact mechanisms by which rearrangements occur have not yet been resolved, it is clear that at some step along the pathway both strands of DNA are cleaved precisely at the border of the two heptamers. The resulting free coding ends are then susceptible to an exonuclease activity associated with the recombinase machinery that trims a variable number of nucleotides from the DNA template molecules.

Nucleotide addition to the coding ends can also occur before the coding strands are ligated together. Two non-mutually exclusive mechanisms are responsible for this acquisition of nucleotides: "N" region addition and "P" nucleotide addition. The

characteristic of the former is tha - added nucleotides are "nontemplate" additions. The additions are believed to be the product of terminal deoxynucleotidyltransferase (TdT) activity which predominantly catalyzes the addition of deoxyguanosine triphosphate (dG) residues to the 3' ends of the DNA strands. This mechanism is active in immature pre-B-cells in adult, but not fetal, lymphoid tissues and is thought to create enormous variability in the junctional regions (Engler *et al.*, 1992). "P" nucleotide addition, on the other hand, involves the insertion of nucleotides that are in the reverse complementary orientation to the 3' end of a particular DNA strand of a coding joint. This results in the formation of a palindrome of variable length. Currently, the favoured model proposes that the two DNA strands of the coding end are connected to form a hairpin structure, which is subsequently cleaved (Lafaille *et al.*, 1989; Meier & Lewis, 1993). The location of the nick which opens up the hairpin is variable, thus inserting a variable number (on average, one to three residues) of "P" nucleotides by this mechanism (Engler *et al.*, 1992; Meier & Lewis, 1993). Unlike the "N" region addition, "P" insertion does not appear to vary as a function of ontogeny (Meier & Lewis, 1993).

2.3.3 HYPERMUTATION

The progressive increase in antibody affinity, which has been noted to occur as a function of time following immunization with an antigen, is believed to be the result of somatic mutation introduced into the Ig germline genes (Griffiths *et al.*, 1984). Indeed, single point mutations, introduced by mutagenesis, have been shown to be responsible for greatly affecting affinity (several thousand fold) and for modifying fine specificity of an anti-lysozyme antibody (Lavoie *et al.*, 1992). The experimental studies designed to define the nature of the hypermutation mechanism, which is now implicit in the concept of maturation of a specific humoral immune response, have focused on various murine models. These experimental model systems have demonstrated that the fully activated mutator mechanism is dependent upon the rearrangement of the Ig gene loci (Roes *et al.*, 1989; Weber *et al.*, 1991a). Once activated, the somatic mutations introduced by the hypermutator mechanism are spatially restricted to a region

surrounding the rearranged V(D)J segments (Lebecque & Gearhart, 1990; Weber *et al.*, 1991a, 1991b). This indicated a potential involvement of cis-acting elements in the control of this mechanism. Since mutations were not observed upstream of the V gene promoter region and rearranged transgenes, containing the promoter region, were susceptible to mutation, it was proposed that the mechanism introducing the mutations relied upon specific sequences in this area and/or was linked to the transcriptional state of the gene (Lebecque & Gearhart, 1990; Weber *et al.*, 1991b). In a trangenic model system, it was also demonstrated that one or more cis elements located within or near the C_H region were requisite for hypermutation (Giusti & Manser, 1993). Furthermore, it has been shown that additional regulatory elements within the C_γ region are likely to enhance this activity; which could explain the greater frequency of mutations observed in antibodies that have undergone switching to the gamma isotype (Sohn *et al.*, 1993).

Although the mutations are found throughout the rearranged sequences, it would appear that the process is not entirely random since there is evidence of areas of higher mutability (Rogozin & Kolchanov, 1992) and of DNA strand bias (Lebeque & Gearhart, 1990; Rogozin & Kolchanov, 1992; Jacob *et al.*, 1993). The former has been explained by the influence of neighbouring base sequences (Rogozin & Kolchanov, 1992), and the latter is thought to be due to a linking of the mutator mechanism with the direction of DNA replication (Rogerson *et al.*, 1991).

The maturation of the humoral response relies heavily on the selection of clones with beneficial mutations. It is believed that four to five days after immunization with a T-dependent antigen, B lymphocytes migrate into the primary follicles of secondary lymphoid organs. The migration is followed by intense proliferation of the B lymphocytes, leading to the formation of germinal centres (Berek & Ziegner, 1993). The initiation of the hypermutation mechanism may occur during this proliferation phase, or may have even begun prior to migration (Berek *et al.*, 1991; Jacob *et al.*, 1991). Nevertheless, evidence suggests that the initial random mutations, which accumulate with time (especially in the latter part of the second week post-immunization), are subject to a selection phase within the germinal centers. Perhaps due to selective pressures imposed

by the antigen-presenting follicular dendritic cells (Berek *et al.*, 1991), clones with advantageous mutations are preferentially expanded (Berek *et al.*, 1991; Jacob *et al.*, 1991, 1993). Antibody molecules produced by B lymphocytes that have been subjected to these selective pressures, bear mutations with characteristically high replacement (ie. resulting in amino acid substitution) to silent ratio (R/S ratio) within the CDRs (Shlomchik *et al.*, 1987).

SECTION 3

HUMAN CYTOMEGALOVIRUS

3.1 GENERAL STRUCTURE AND BIOLOGY

The human cytomegalovirus (CMV), one of the seven members of the human *Herpes* family of viruses, is of great scientific interest because of its clinical importance and its worldwide prevalence. This virus is endemic in most parts of the world, with general population seropositivity ranging from 40% to 100%, depending on the country in question (Krech, 1973). A recent study of the blood donor population in the North-East region of Scotland reported that the prevalence of CMV-specific serum antibodies, of the IgM isotype, was approximately 5% (Galea & Urbaniak, 1993). Although this value was considered slightly higher than previous reports, it served as an indication of those individuals undergoing an acute infection.

The interest in this virus has been such that its entire genome has been sequenced (Chee *et al.*, 1990) and there are a number of excellent reviews covering the structure, the biology, and the virus-host immune system interactions of CMV (Sissons et al.,1986; Griffiths & Grundy, 1987; Apperley & Goldman, 1988; Landini & Michelson, 1988; Stinski, 1990).

Briefly, CMV is a large virus of approximately 180 nm in diameter composed of a nuclear core containing its genome (double-stranded DNA), a protein capsid (nucleocapsid) that surrounds the core, a poorly defined area (known as the tegument) that surrounds the nucleocapsid and a lipid bilayer that encloses the complete virion. Analysis of the CMV genome has revealed the existence of over 200 potential open reading frames (Chee *et al.*, 1990). Following infection, the expression of these genes appears to be under relatively tight temporal control. The genes transcribed within the first two hours post-infection (p.i.) are known as immediate early (IE) genes and are generally responsible for the control of the host cell's machinery for gene expression,

permitting the transcription of the early (E) genes. The early genes (2-24 hours p.i.), also known as delayed early, are responsible for controlling the production of daughter virions, primarily by enabling viral DNA replication and transcription of the late genes. The late genes are transcribed roughly 24 hours following initial infection and encode for structural components of the virions (for review see Griffiths & Grundy, 1987).

There are approximately 7-10 characterized IE gene products, of which a phosphoprotein of an estimated 72 kD is the most prominent. Among the estimated 25 E gene products, the viral DNA polymerase (p145) and a phosphorylated DNA-binding protein (pp52) are the best characterized. Approximately 55 L gene products have been reported, the majority have been post-translationally modified by glycosylation, the rest by phosphorylation. The structural glycoproteins are mainly localized in the viral envelope as three distinct complexes that have been designated as gC-I, gC-II and gC-III (Gretch *et al.*, 1988). Homologous complexes have been identified in the Herpes simplex viruses (HSV), another member of the herpes virus family, and are known as gB (gC-I) and gH (gC-III) (Cranage *et al.*, 1986, 1988). The structural phosphoproteins are mainly localized in the nucleocapsid or in the tegument. The predominant phosphoproteins have apparent molecular weights of 28, 38, 65 and 150 kD (for review see Landini & La Placa, 1991).

3.1.1 LOWER MATRIX PROTEIN (pp65)

Three types of virus particles can be isolated from *in vitro* cultures of CMV-infected cells: virions, noninfectious enveloped particles (NIEPs) and dense bodies. The dense body is a simple structure, consisting of enveloped tegument and is noninfectious since it contains no genetic material. Virions and NIEPs have more complex structures, but can be differentiated from one another since NIEPs contain no viral DNA and possess a viral assembly protein not found in mature virions (Irmiere & Gibson, 1983).

The phosphoprotein of apparent molecular weight of 65 kD (pp65), also known as the lower matrix protein, is present in all three types of virus particles (Irmiere & Gibson, 1983; Roby & Gibson, 1986). The pp65 protein is the major structural protein

of the tegument and consequently accounts for approximately 95% of the protein mass of dense bodies (Irmiere & Gibson, 1983). The sequence of the gene encoding pp65 and its location within the CMV genome have been identified (Rüger *et al.*, 1987). This gene product is non-glycosylated and is highly conserved at the level of amino acid sequence, at least between the two strains which have been characterized (Britt & Vugler, 1987; Pande *et al.*, 1990). Although it is a structural protein, pp65 is classified as a delayed early gene product. This is due to the finding that there is a low level of pp65 gene expression prior to viral replication that appears to be regulated by two IE proteins, although maximal expression occurs after the initiation of viral replication (Britt & Vugler, 1987; Depto & Stenberg, 1989). Despite the unequivocal association of protein kinase activity with pp65, the function of this protein remains undetermined (Somogyi *et al.*, 1990). Due to similarities with a tegument protein of HSV, as well as its cytoplasmic and nuclear localization in infected cells, Britt and Vugler proposed that pp65 may play a role in the control of transcription of viral genes (Britt & Vugler, 1987).

In contrast to a clear abundance in *in vitro*-derived virus particles, it has been reported that recent clinical isolates do not necessarily have pp65 as a prominent constituent (Jahn *et al.*, 1987). The importance of pp65 in natural infections has been questionned by the same group as they demonstrated that the abundant expression of pp65 was related to cell culture conditions (Klages *et al.*, 1989). More recent reports, however, have clearly shown that pp65 is the principal viral antigen in peripheral blood leukocytes during active CMV infection (Grefte *et al.*, 1992; Revello *et al.*, 1992; Ehrnst *et al.*, 1993).

3.2 HUMAN IMMUNE RESPONSE

Following primary infection of the human host, CMV is most often not eliminated from the body, but rather it establishes a persistent infection characteristic of viruses of the Herpesviridae family (Roizman, 1990). In a persistent infection, CMV survives in a latent state and possesses the potential to be reactivated. Reactivation of CMV occurs

when there is a breakdown in the host-virus balance that initially enabled the establishment of the latent infection (for review of viral persistence see Mims & White, 1984; Ahmed & Stevens, 1990). An impaired host-virus balance is observed in therapeutically (transplant recipients) and acquired (AIDS) immunosuppressed individuals, as well as in the elderly population (Musiani *et al.*, 1988; McVoy & Adler, 1989; Galea & Urbaniak, 1993). It is an important function of the human immune system to not only control primary infections but also to maintain the equilibrium that will prevent viral reactivation. The fact that this ubiquitous virus normally causes pathological disease only in individuals with either an immature or compromised immune system, clearly demonstrates the pivotal role of this system.

The immune response to CMV is quite complex, involving nonspecific cells, such as natural killer (NK) cells, as well as B and T lymphocytes. Some of the immune mechanisms, such as those involved in the lysis of CMV-infected cells or the production of virus-specific antibodies, have been elucidated and are the subject of many review articles (Sissons *et al.*, 1986; Griffiths & Grundy, 1987; Landini & La Placa, 1991).

3.2.1 HUMORAL IMMUNE RESPONSE

Although the CMV genome has approximately 200 open reading frames, which have the potential of encoding an equivalent number of proteins, the humoral response in humans has been found to be restricted to few viral proteins. The response is primarily restricted to a few nonstructural proteins, the gCI complex of structural glycoproteins and two structural phosphoproteins.

Specifically, the pp72 IE gene product and the DNA-binding pp52 E gene product are the nonstructural proteins that are immunoprecipitated with sera from acutely infected individuals. Due to its ability to efficiently induce a humoral response, the appearance of circulating antibodies to the latter viral protein has been proposed as a serological marker for primary infection in immunocompromised individuals (Landini *et al.*, 1989).

All three of the CMV glycoprotein complexes are predominantly found in the viral envelope and therefore could potentially act as targets for neutralizing antibodies. Although antibodies directed against the gCII complex have been demonstrated in human

convalescent sera, significant neutralization is not asssociated with this specificity (Kari & Gehrz, 1990; Landini & La Placa, 1991). The gCIII complex of viral glycoproteins has been shown to induce neutralizing antibodies in immunized mice, however, this complex does not appear to be immunodominant in the natural human infection (Simpson *et al.*, 1993; Landini & La Placa, 1991). In contrast to the other two complexes, gCI is responsible for inducing the majority of the virus-neutralizing activity in human sera (Britt *et al.*, 1990; Kniess *et al.*, 1991). Because of the potential therapeutic use of antibodies with similar specificity, the epitopes responsible for this neutralizing activity have been extensively examined. These studies have localized neutralizing epitopes on both the gp58 and gp116 proteins of this complex (Utz *et al.*, 1989; Banks *et al.*, 1989; Meyer *et al.*, 1990; Ohlin *et al.*, 1993).

The immunodominant structural phosphoproteins of CMV correspond to the pp150 and pp65 gene products. Despite a report that the humoral anti-pp65 response is relatively weak and not always detectable (Jahn *et al.*, 1987), subsequent reports have indicated the importance of anti-pp65 antibodies as markers of infection as well as their potential for controlling infection (van Zanten *et al.*, 1991, 1993). van Zanten's group found an increased reactivity (frequency and titer) towards pp65 in the sera of immunocompromised patients with either primary or secondary CMV infection in comparison to CMV- seropositive healthy individuals (van Zanten *et al.*, 1991, 1993). This not only demonstrated a heightened anti-pp65 response during CMV infection and reinfection/reactivation, but it also provided a possible explanation for the results of Jahn and coworkers, since the latter group used pooled seropositive sera of unspecified origin (Jahn *et al.*, 1987). Since structural phosphoproteins are primarily localized in the viral tegument and/or nucleocapsid, antibodies specific for these viral components are less likely to have any neutralizing activity. Studies with murine and human monoclonal anti-pp65 antibodies, however, have demonstrated the presence of viral epitopes on the surface of CMV-infected cells (Pereira *et al.*, 1982; Larose *et al.*, 1991). This finding could explain the neutralizing activity of some monoclonal anti-pp65 antibodies, as well as provide a mechanism by which the anti-pp65 response may play a role in defense

against CMV infection (Pereira *et al.*, 1982; Redmond *et al.*, 1986; van Zanten *et al.*, 1991).

3.2.1.1 HUMAN HYBRIDOMA ANTIBODY STUDIES

Human hybridoma antibodies have been generated in many laboratories in an attempt to: 1) identify the immunologically important epitopes present on the viral proteins, 2) characterize the human humoral response to these epitopes, and 3) produce useful diagnostic and therapeutic tools. Consequently, it is important to determine whether such monoclonal antibodies reflect the humoral response induced during infection. Table 3-1 summarizes the published information regarding human anti-CMV hybridoma antibodies. The abundance of hybridoma antibodies of the IgG_1 isotype is representative of the high prevalence and high titers of IgG_1 antibodies found in the anti-CMV immune response (Gilljam & Wahren, 1989; Mathiesen *et al.*, 1992). Hybridoma antibodies of the IgG_3 isotype, however, are under-represented. The predominance of hybridoma antibodies specific for the lower matrix protein (pp65) or the gC-I proteins is, in part, a consequence of the immunodominance of these antigens *in vivo*. In addition, the relationship between the neutralizing activity and antigen specificity that was observed in human immune sera, can also be seen in Table 3-1. The structural characterization of the variable region of one of these anti-CMV antibodies has previously been reported (Table 2-5; Newkirk *et al.*, 1988).

Table 3-1. Summary of Human Anti-CMV Hybridoma Antibodies. Part I (continued on following two pages)

MONOCLONAL ANTIBODY	ISOTYPE	SPECIFICITY: VIRAL COMPONENT	SPECIFICITY: VIRAL PROTEIN	SOURCE OF B LYMPHOCYTES[1]	NEUTRALIZATION	REF[2]
HCV-1	IgG_2k	matrix	pp65	PBL, hemoph., sero+	NO	[1]
HCV-2	IgG_2k	matrix	pp65	PBL, hemoph., sero+	NO	[1]
HCV-3	IgG_3l	matrix	pp65	PBL, hemoph., sero+	NO	[1]
HCV-4	IgG_3l	matrix	pp65	PBL, hemoph., sero+	NO	[1]
MO53	IgG_1k	matrix	pp65	PBL, normal, sero+	NO	[2]
MO58	IgG_1k	matrix	pp65	PBL, normal, sero+	NO	[2]
MO61	IgG_1l	matrix	pp65	PBL, normal, sero+	NO	[2]
MO79	IgG_1k	matrix	pp65	PBL, normal, sero+	NO	[2]
A-1	IgG	matrix	pp65 ?	PBL, normal, sero+	NO	[3]
A-2	IgG	matrix	pp65 ?	PBL, normal, sero+	NO	[3]
A-3	IgG	matrix	pp65 ?	PBL, normal, sero+	NO	[3]
A-4	IgG	matrix	pp65 ?	PBL, normal, sero+	NO	[3]
X20	IgG_1	matrix ?	pp65 ?	PBL, normal	WEAK	[4]
Z01	IgG_1	matrix ?	pp65 ?	Spleen, Hodgkin's	WEAK	[4]
MO80	IgG_1k	matrix	pp28	PBL, normal, sero+	NO	[2]
CMVHU.03	IgG	matrix	pp65	PBL, normal, sero+	N.D.	[5]
CMVHU.07	IgG	matrix	pp65	PBL, normal, sero+	N.D.	[5]

[1] Abbreviations: PBL, peripheral blood lymphocyte; hemophil., hemophiliac; sero+, seropositive; ITP, idiopathic thrombocytopenia purpura; stom. canc., stomach cancer patient; ?, uncertain or not known.

[2] References: [1], Larose et al., 1991; [2], Ohlin et al., 1991; [3], Kitamura et al., 1990; [4], Foung et al., 1989; [5], Steenbakkers et al., 1993; [6], Bron et al., 1990; [7], Masuho et al., 1987; [8], Sutherland et al., 1987; [9], Ohlin et al., 1993; [10], Erlich & Ostberg, 1990; [11], Tomiyama et al., 1990; [12], Kanoh et al., 1992; [13], Emanuel et al., 1984.

Table 3-1. Summary of Human Anti-CMV Hybridoma Antibodies. Part II (continued on following page)

MONOCLONAL ANTIBODY	ISOTYPE	SPECIFICITY: VIRAL COMPONENT	SPECIFICITY: VIRAL PROTEIN	SOURCE OF B LYMPHOCYTES[1]	NEUTRALIZATION	REF[2]
1B-8E9H5	IgG_2	matrix	pp65 ?	PBL, normal, sero+	NO	[6]
C1	IgG_1	matrix	pp65 ?	Spleen, ITP, sero+	NO	[7]
C3	IgG_1	matrix	pp65 ?	Spleen, ITP, sero+	NO	[7]
C4	IgG_1	matrix	pp65 ?	Spleen, ITP, sero+	NO	[7]
C7	IgG_1	matrix	pp65 ?	Spleen, ITP, sero+	NO	[7]
Z10	IgG_1	EA	65kD	PBL, normal	NO	[4]
C4	IgG_1	LA	48/65kD	PBL, normal, sero+	NO	[8]
ITC33	IgG_1l	gC-I	gp58	PBL, normal, sero+	NO	[9]
ITC34	IgG_1k	gC-I	gp58	PBL, normal, sero+	NO	[9]
ITC39	IgG_1l	gC-I	gp58	PBL, normal, sero+	NO	[9]
ITC48	IgG_1k	gC-I	gp58	PBL, normal, sero+	YES	[9]
ITC52	IgG_1k	gC-I	gp58	PBL, normal, sero+	YES	[9]
ITC63B	IgG_1l	gC-I	gp58	PBL, normal, sero+	YES	[9]
ITC63C	IgG_1l	gC-I	gp58	PBL, normal, sero+	YES	[9]
SDZ89-104[3]	IgG_1k	gC-I	gp58	Spleen	YES	[10]
ITC88	IgG_1k	gC-I	gp116	PBL, normal, sero+	YES	[9]
C23	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[7]

[1] Abbreviations: PBL, peripheral blood lymphocyte; hemophil., hemophiliac; sero+, seropositive; ITP, idiopathic thrombocytopenia purpura; stom. canc., stomach cancer patient; ?, uncertain or not known.

[2] References: [1], Larose et al., 1991; [2], Ohlin et al., 1991; [3], Kitamura et al., 1990; [4], Foung et al., 1989; [5], Steenbakkers et al., 1993; [6], Bron et al., 1990; [7], Masuho et al., 1987; [8], Sutherland et al., 1987; [9], Ohlin et al., 1993; [10], Erlich & Ostberg, 1990; [11], Tomiyama et al., 1990; [12], Kanoh et al., 1992; [13], Emanuel et al., 1984.

[3] Previously known as EV1.15.

Table 3-1. Summary of Human Anti-CMV Hybridoma Antibodies. Part III (continued from previous pages)

MONOCLONAL ANTIBODY	ISOTYPE	SPECIFICITY: VIRAL COMPONENT	SPECIFICITY: VIRAL PROTEIN	SOURCE OF B LYMPHOCYTES[1]	NEUTRALIZATION	REF[2]
C41	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[7]
C141	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
C176	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
C197	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
C202	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
C208	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
C242	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
C279	IgG_1	gC-I	?	Spleen, ITP, sero+	YES	[11]
I393	IgG_1k	gC-I ?	50,85,120kD	Spleen, stom. canc.	NO	[12]
K633	IgG_1l	gC-I ?	50,85,120kD	Spleen, stom. canc.	YES	[12]
E46	IgG_3l	gC-III ?	78kD	Spleen, stom. canc.	YES	[12]
K357	IgG_3l	gC-III ?	80kD	Spleen, stom. canc.	YES	[12]
K115	IgG_1k	?	50kD	Spleen, stom. canc.	NO	[12]
I351	IgG_1l	?	50kD	Spleen, stom. canc.	NO	[12]
X16	IgG_1	LA	100,65,36-38kD	PBL, normal	YES	[4]
Z02	IgG_1	?	48-50 kD	Spleen, Hodgkin's	WEAK	[4]
312.A.91.4	IgG	?	?	PBL, sero+	NO	[13]

[1] Abbreviations: PBL, peripheral blood lymphocyte; hemophil., hemophiliac; sero+, seropositive; ITP, idiopathic thrombocytopenia purpura; stom. canc., stomach cancer patient; ?, uncertain or not known.

[2] References: [1], Larose et al., 1991; [2], Ohlin et al., 1991; [3], Kitamura et al., 1990; [4], Foung et al., 1989; [5], Steenbakkers et al., 1993; [6], Bron et al., 1990; [7], Masuho et al., 1987; [8], Sutherland et al., 1987; [9], Ohlin et al., 1993; [10], Erlich & Ostberg, 1990; [11], Tomiyama et al., 1990; [12], Kanoh et al., 1992; [13], Emanuel et al., 1984.

SECTION 4

RHEUMATOID ARTHRITIS

4.1 CLINICAL ASPECTS

Rheumatoid arthritis (RA) is a chronic inflammatory disease with characteristic involvement of peripheral joints. This chronic disease has a significant socioeconomic impact and, more importantly, is associated with higher mortality rates (Gilliland, 1989; Jacobsson *et al.*, 1993). Many studies have estimated the prevalence of RA, which varies according to the population studied, and have consistently found a prevalence of approximately 1% in most Caucasian groups (reviewed in Hochberg, 1981). The prevalence of RA has been demonstrated to increase with increasing age and has also been shown to be two to three times greater in females than in males (Hochberg, 1981). The incidence of RA varies between 0.7 to 2.9 per thousand population and a secular decline in this incidence has been suggested but is not supported by the recent study of Chan and coworkers (Hochberg, 1981; Gilliland, 1989; Chan *et al.*, 1993).

The articular inflammation, observed in RA patients, usually leads to progressive joint destruction that results in permanent damage and loss of function (Zvaifler, 1988). Most of the criteria used for the classification of this disease are related to the consequences of the immunopathological events which occur in the rheumatoid joints. These include morning stiffness, joint swelling and radiographic evidence for joint erosion (Arnett *et al.*, 1987). The other criteria include the presence of rheumatoid nodules and presence of rheumatoid factor (RF).

The rheumatoid nodule is, almost without exception, pathognomonic of RA in patients with chronic synovitis (reviewed in Ziff, 1990). These nodules are believed to be the result of trauma, since they are commonly found in subcutaneous tissue overlying pressure sites. Although such nodules are only found in approximately 25% of RA patients, they are of interest because the processes which lead to their formation may be

similar to those resulting in synovitis. A key observation is that the hyperplasia of the synovial lining in rheumatoid joints is very similar to the hyperplasia involved in the formation of rheumatoid nodules. Many of the cells and mechanisms implicated in rheumatoid nodule formation are related to the articular events as well (Gay *et al.*, 1993). One of the steps believed to be common to both events is the formation of immune complexes containing RF autoantibodies which may cause the activation of monocytes and of the complement cascade (Ziff, 1990). This potential role for RF in the pathogenesis of RA is reflected in its inclusion in the classification criteria.

4.2 ETIOLOGY

The etiology and pathogenesis of RA remains unknown but are clearly multifactorial (Hochberg, 1981; Gilliland, 1989; Gay *et al.*, 1993; Sewell & Trentham, 1993). The pathogenic process, which is highly variable, appears to involve the majority of cells of the immune system, molecules (eg. cytokines) that allow for their interaction, and an unknown triggering mechanism. The multiple genetic and environmental factors, implicated in the etiology of RA, are discussed in the following sections.

4.2.1 GENETIC FACTORS

Many studies have examined the linkage of RA to the human leukocyte antigens (HLA); the gene products of the human major histocompatibility complex. Although certain HLA-DR phenotypes have been associated with a protective effect (Larsen *et al.*, 1989), many haplotypes have been associated with disease susceptibility (Gao *et al.*, 1990; Wallin *et al.*, 1991; McCusker *et al.*, 1991; Willkens *et al.*, 1991). The most notable of those believed to confer susceptibility are located in the DR cluster and share a common sequence in the ß chain (Watanbe *et al.*, 1989; Gao *et al.*, 1990; Wilkens *et al.*, 1991; Seglias *et al.*, 1992), consistent with the proposed "shared epitope" hypothesis (Gregersen *et al.*, 1987). Other analyses have proposed that RA consists of a genetically diverse group of diseases and that it is only the severe form (approximately 30% of all cases), which is linked to the HLA (Deighton et al., 1993). Indeed, a number of studies

have concluded that genetic associations with HLA are more closely related to disease severity than disease susceptibility (Calin *et al.*, 1989; Stephens *et al.*, 1989; van Zeben *et al.*, 1991).

Regardless of the actual role played by the HLA antigens or by the products of genes closely linked to this complex, other loci must be involved, since the genetic contribution of HLA to RA has been estimated to be no more than 37% (Deighton *et al.*, 1989). Much effort has been invested in evaluating the contribution of other loci, such as the immunoglobulin light and heavy chain genes and the genes of the T cell receptor. To date, however, the results indicate either no involvement or quite modest contributions associated with these loci (Moxley, 1989a, 1989b, 1992; Sanders & Grennan, 1989; Meindl *et al.*, 1990; Wallin *et al.*, 1991; Shin *et al.*, 1993).

4.2.2 ENVIRONMENTAL FACTORS

"An individual's arthritis is caused by his/her abnormal immune response to a particular infective agent." (Ford, 1991)

Although this statement has not been definitively proven, there is increasing evidence that microbiologic agents, in an appropriate genetic background, trigger the immunopathological events leading to RA. Many of the studies have used the models of bacterially-induced reactive arthritis (ReA) or virally-induced arthritides as paradigms for a connection between infection and RA. The associations between specific pathogens and the autoimmune process in RA remain tentative. The finding that synovial lymphocytes showed maximal response to different microbial agents in different patients indicates that more than one pathogen may be involved in the etiology of RA (for review see Ford, 1993). Multiple mechanisms, some involving interactions between host HLA molecules and pathogens or immunogenic peptides derived from these pathogens, have also been proposed to explain the autoimmune process in RA. It is, therefore, conceivable that more than one path leads to the development of RA. Thus, in addition to the genetic heterogeneity, the diversity of potential environmental agents and pathogenic mechanisms may account for part of the heterogeneity of RA itself (i.e.

heterogenity of disease severity, autoantibody production, HLA associations). The complexity of the interactions putatively involved may also explain why ubiquitous pathogens and relatively common genetic factors appear to be associated with a disease that occurs in only 1% of the population (for reviews see Kouri, 1985; Moreland & Koopman, 1989; Vaughan, 1989; Schwartz, 1990; Burmester, 1991; Ford, 1991, 1993).

4.2.2.1 HUMAN CYTOMEGALOVIRUS AS ETIOLOGIC AGENT

CMV, like some of the other viruses of the *Herpesviridae* family, has been postulated to be one of the potential etiologic agents in RA or to participate in the pathogenesis of this disease. Serological studies designed to examine whether there exists a greater prevalence and/or greater titer of anti-CMV antibodies in RA patients, in comparison to disease controls and/or normal controls, have revealed no statistically significant differences (Phillips *et al.*, 1976; Venables *et al.*, 1985; Ferraro & Newkirk, 1993). To determine any role of CMV in the development of RA, Walker and coworkers examined HLA identical siblings who were discordant for RA (Walker *et al.*, 1987). This group did not detect any difference in environmental exposure to CMV between the siblings who developed RA and those who did not. In contrast, studies of lymphocyte proliferation in response to specific viral antigens have provided support for a role for CMV in some patients with RA. Specifically, Ford and coworkers have shown that found that some some patients with classical seropositive RA (ie. RF-positive) repeatedly show high specific reactivity of synovial lymphocytes, but not peripheral blood lymphocytes, to CMV antigens (Ford *et al.*, 1987).

Although there has been one report of live CMV virions isolated from the synovium of an RA patient, experience suggests this is possible only in a minority of cases (Hamerman *et al.*, 1982). These data suggest that either CMV is rarely present in the synovium or the methods used for isolation of the virions are not effective. The second possibility is likely since the tissue culture methods employed may not be appropriate to detect CMV if, at the time of biopsy, the virus is present in a latent form. For this reason, specific amplification of viral DNA from RA synovial biopsies has been

attempted. Using the polymerase chain reaction, Einsele and coworkers recently demonstrated the presence of viral genomes in eleven of 83 RA synovial biopsies (Einsele *et al.*, 1992). Although not specific to RA, detectable CMV genome was more than four times more prevalent in this population than in patients with other joint disorders. Interestingly, in the same study there also was no difference in CMV-specific serum antibody levels between the RA and non-RA patient groups. In addition, only patients with detectable viral genome within their biopsies had elevated levels of anti-CMV antibodies in their synovial fluid, relative to serum anti-CMV levels. Taken together, these data indicate that significant differences with respect to CMV can exist between RA and non-RA individuals that are not detectable in routine serological studies, perhaps due to the high prevalence of this virus in the general population.

The mechanism by which CMV may participate in the autoimmune process may be via the induction of RF synthesis. This potential relationship between RF production and the humoral immune response to CMV infection is examined in the following section (Section 5.6).

SECTION 5

RHEUMATOID FACTORS

5.1 ASSOCIATION WITH DISEASE

Rheumatoid Factors (RFs) were first described in 1940 as a human serum factor capable of mediating specific agglutination of sensitized sheep erythrocytes (Waaler, 1940). Since that time, the RF has become known as the autoantibody closely associated with RA and is currently among the criteria used for the classification of this autoimmune disease (Arnett *et al.*, 1987).

Elevated levels of serum RF have been shown to exist in 70% to 90% of patients with RA (Carson, 1989; Lipsky, 1987; Harris, 1990) and have recently been reported to be present prior to the development of clinical symptoms (del Puente *et al.*, 1988; Aho *et al.*, 1991). The report by Aho and coworkers was part of an extensive field survey in which all individuals, who were RF-positive at their inital blood sampling, developed RA within the subsequent four years. These authors therefore proposed that RA may have a long asymptomatic period characterized by the presence of circulating RFs and that an additional trigger may be required for the progression to clinical disease. Such a postulate implies that RF seropositivity may simply be a marker of disease and that these autoantibodies may not play a direct role in the pathophysiology of RA. This notion is supported by findings of RF-seropositivity in patients with autoimmune diseases other than RA, such as systemic lupus erythematosus (SLE) and scleroderma (Carson, 1989; Fong *et al.*, 1985). In addition, IgM paraproteins with RF activity can be found in individuals with non-autoimmune diseases, such as mixed cryoglobulinemia (MC), Waldenström's macroglobulinemia and chronic lymphocytic leukemia (CLL), (Brouet *et al.*, 1974; Preud'homme & Seligmann, 1972). Indeed, RFs are often detectable in normal individuals undergoing chronic bacterial or viral infections and in apparently healthy individuals, as well (Vaughan, 1972; Wernick *et al.*, 1981; Zvaifler, 1988). The

actual levels in the circulation, however, may be of clinical relevance. For example, RF titers in healthy individuals are usually significantly lower than in RA patients (Wernick *et al.*, 1981). In addition, the use of methotrexate, an important disease-modifying anti-rheumatic drug (DMARD), has been shown to suppress RF levels in RA patients and these lower levels were found to be related to clinical improvement (Alarcón *et al.*, 1990). Similar declines in RF titers have also been observed during therapy with many of the other DMARDs (Bluestone & Goldberg, 1973; Wernick *et al.*, 1983; Hanly *et al.*, 1986). Moreover, IgM-RF seropositivity, at entry into an eight year longitudinal study was predictive of a more aggressive disease outcome for a group of RA patients with recently diagnosed RA (Tuomi *et al.*, 1988). Despite these and other reports correlating IgM RF levels with clinical status (Cats & Hazevoet, 1970; Westedt et al., 1985; Robbins et al., 1986a), it would appear that RFs of the IgG and IgA isotypes may be more important (Scott *et al.*, 1981; Teitsson *et al.*, 1984; Hannonen *et al.*, 1988).

5.2 ANTIGENIC SPECIFICITY

The autoantigen recognized by RFs is believed to be the Fc portion of immunoglobulins, predominantly the Fc of immunoglobulins of the gamma isotype. This is also the operational definition for this autoantibody/autoantigen interaction, as clinical diagnostic tests and experimental assays detect antibody binding to either intact IgG or purified IgG-Fc fragments (Singer & Plotz, 1956; Roberts-Thomson *et al.*, 1985; Gioud-Paquet *et al.*, 1987; Newkirk, 1992a). It is perhaps more accurate to state that the term "RF" encompasses a group of autoantibodies with a range of fine antigenic specificities, all capable of binding epitopes located in the junction of the C_H2 and C_H3 domains. This area has been shown to overlap with the binding sites of the bacterial proteins A and G (Jefferis *et al.*, 1984; Sasso *et al.*, 1988; Stone *et al.*, 1989). Using either purified IgG-Fc or recombinant preparations of chimeric IgG, it has clearly been shown that individual RFs have specific binding patterns with respect to the different gamma chain subclasses and allotypes (Newkirk *et al.*, 1990; Artandi *et al.*, 1991; Robbins *et al.*, 1993). Since RFs can be of any of the circulating Ig H chain isotypes

(Torrigiani & Roitt, 1967; Carson *et al.*, 1981; Otten *et al.*, 1991), some will possess the property of self-association. More specifically, any IgG RF that has the specificity for its own IgG subclass and allotype has the potential to self-associate. Self-association of IgG RFs has been demonstrated to form dimers in vitro or dimers and multimers in vivo (Lu *et al.*, 1992; Otten *et al.*, 1992).

5.3 MECHANISMS OF INDUCTION

Although the potential binding sites located on IgG-Fc are currently under investigation, it remains unclear how and why IgG molecules trigger the production of RFs. Furthermore, it is still unknown whether IgG is the actual triggering antigen. This uncertainty is reflected in the diversity of mechanisms proposed for the induction of RF synthesis, which include:

1) *Polyclonal B cell activation.* This hypothesis is related to the observation that a number of B cell mitogens are capable of inducing RF production (Slaughter *et al.*, 1978; Koopman & Schrohenloher, 1980; Tsoukas *et al.*, 1980) as well as the observation that a relatively large proportion of B cell precursors (which varies depending upon the report) are committed to RF production (Vischer *et al.*, 1988; Burastero *et al.*, 1990; Hirohata *et al.*, 1990; He *et al.*, 1993).

2) *Anti-idiotypic response to anti-bacterial or anti-viral antibodies.* Plotz postulated that autoantibodies are immunoregulatory "internal image" anti-idiotypic antibodies directed against anti-viral antibodies (Plotz, 1983). The discovery of Fc-binding proteins produced by a number of different human pathogens (Furukawa *et al.*, 1975; Langone, 1982; Yee *et al.*, 1982; Xu-Bin *et al.*, 1989; Bell *et al.*, 1990), has led to the suggestion that anti-idiotypic antibodies produced during the humoral response to such bacterial or viral proteins could lead to the production of RFs (Mouritsen, 1986; Oppliger *et al.*, 1987; McCormick *et al.*, 1988; Nardella *et al.*, 1988; Schröder & Christensen, 1982; Tsuchiya *et al.*, 1990; Williams & Malone, 1992).

3) *RF-producing B cell clones are triggered by immune complexes located in the synovium.* It has been postulated that RFs are synthesized in a traditional humoral

response to antigen (ie. IgG) and in turn could participate in immune complex formation, in the synovia, along with IgG antibodies directed against either exogenous or endogenous antigens present in the joints (Winchester *et al.*, 1970; Munthe & Natvig, 1971; Rowley *et al.*, 1987; Lafyatis *et al.*, 1992). It has been of some interest to try and understand why self IgG molecules should elicit an immune response resulting in high and sustained RF titers in patients with RA. Although acquisition of antigenic markers characteristic of isologous but not autologous IgG was reported to occur as a consequence of immune aggregation (Williams *et al.*, 1992a), it is unclear how such a mechanism could be restricted to individuals who develop RA. Consequently, structural changes which were specific to RA and that increased immunogenicity of the C_H2/C_H3 region, were sought. Although no primary sequence defects, due to genetic mutations in the Fc region, have been identified (Naiki *et al.*, 1992), it is possible that defects in glycosylation may increase the avidity of some RFs towards IgG-Fc (Artandi *et al.*, 1991). The latter finding, however, remains controversial since changes in the oligosaccharide, located between the C_H2 domains, have been shown either to have no effect on RF binding or even to decrease binding, depending on the isotype of the RF (Newkirk *et al.*, 1990; Newkirk & Rauch, 1993).

4) *RF-bearing B cells are activated by antigens other than autologous IgG.* It was postulated that at least some RFs, due to their relatively low affinity, may actually be specific for an antigen other than IgG-Fc. An example of such an interaction was decribed by Williams and co-workers when they detected RA-derived RFs that were cross-reactive with native $ß_2$-microglobulin molecules (Williams *et al.*, 1992b; Williams *et al.*, 1993a; Williams *et al.*, 1993b). The epitopes responsible for this cross-reactivity were reported to be homologous to amino acid sequences corresponding to a number of procaryotic and eucaryotic proteins, suggesting that there may be an exogenous trigger for the production of these autoantibodies (Williams *et al.*, 1993a).

Regardless of the mechanism involved in the production of RFs, it is important to note the differences that are thought to exist between RFs from healthy and RA populations. Most important are the observations that RFs derived from RA patients may

have different antigenic specificities than those derived from healthy individuals, appear to be predominantly monospecific rather than polyreactive, and may be derived from a pool of B cells which is selectively and differentially expanded in patients with RA but not in healthy individuals (Dalal *et al.*, 1990; Rudolphi *et al.*, 1993; He *et al.*, 1993). Even within individual RA patients, there is evidence for differential antigenic specificities when comparing RFs from the serum to those from the synovium (Robbins *et al.*, 1986). The fact that there may be preferential homing of RF-producing B cells to the synovium may provide a selective mechanism, although this specific question has not been thoroughly addressed (Moynier *et al.*, 1992).

5.4 IDIOTYPE EXPRESSION

In 1974 Jerne proposed a formal network theory and highlighted one of the immune system's properties; that of dualism (Jerne, 1974). The dualism that is most important with respect to antibody structure is *"antibody molecules can recognize as well as be recognized"*. The practical implication was that the structure of an antibody's antigen binding site, or paratope, could be probed with immunological reagents. Xenogeneic and isologous antisera could be generated to examine the antigenic determinants (also known as epitopes) present on a set of antibodies. These included the set of epitopes, collectively known as an idiotype (or set of idiotopes), expressed by the variable regions of an antibody. Thus it was recognized early on that the structural features of an antibody's polypeptide chains that defined antigenic specificity would also determine the pattern of idiotopes displayed by that antibody molecule (Jerne, 1974). The experimental evidence for the existence of such idiotypic determinants originated from studies on human and rabbit antibodies in 1963 (Kunkel, 1963; Oudin, 1963). It was later demonstrated that antibodies of similar specificity could express certain idiotopes not found on antibodies of a different specificity (Williams, 1968). This demonstration of cross-reactive idiotypes (CRI) present on human cold agglutinins (antibodies which bound to the I antigens of human erythrocytes) provided clear evidence for a structure-function relationship within the antibody combining site. This was further

supported by the finding that the L chain V regions of the cold agglutinins were very similar in primary amino acid sequence, originating from a restricted number of genes (Capra *et al.*, 1972).

Studies on human antibodies with RF activity (at the time known as anti-γ-globulin activity) were initiated on serum immunoglobulins isolated from patients with either a mixed cryoglobulin (MC) syndrome or hypergammaglobulinemic purpura (Capra & Kunkel, 1970; Kunkel *et al.*, 1973). Many of these patients were shown to have relatively high concentrations of paraproteins with RF activity which could be isolated in sufficient quantity to enable structural studies. Using absorbed rabbit antisera, Kunkel's group defined the CRI system for the MC-derived prototypical RFs (Kunkel *et al.*, 1973). These investigators were able to demonstrate that the RFs studied could be separated into two main groups with respect to CRI expression. The majority of RFs (60%) from unrelated individuals were shown to express a CRI found on one particular RF paraprotein (known as "WA") but not on another RF paraprotein (known as "PO") or on proteins that did not have RF activity. Conversely, RFs of the "PO" group expressed a CRI present on this paraprotein but not of those from the "WA" group or on proteins without RF activity. Two proteins of the newly defined "PO" group were found to have very similar H chain V region sequence but expressed L chains from two different families (Capra & Kunkel, 1970). Subsequent work, however, accumulated evidence for restricted L and H chain V gene usage for these prototypical RFs which could be predicted by expression of the respective CRI (reviewed in Newkirk & Capra, 1987; and section 5.5.1, found below). The subsequent description of another CRI (known as "BLA"), associated with a subset of polyreactive RFs that bind DNA-histone, was also associated with a distinct V region gene usage (Agnello *et al.*, 1980; Agnello & Barnes, 1986; Barnes *et al.*, 1990). Taken together, this work supported the previous finding that antibodies with similar specificities, even when isolated from unrelated individuals, display important structural similarities in their V regions.

In order to further define the idiotypic structures present on RFs, murine monoclonal reagents were generated using the MC-derived, as well as some RA-derived,

RFs as immunogens. These monoclonal anti-idiotypic reagents can be grouped into three major categories: 1) those that recognize determinants believed to be located on the RF L chains, 2) those that recognize determinants believed to be located on RF H chains, and 3) those that recognize conformational determinants, which depend on the association of both the heavy and light chains.

Table 5-1 summarizes the pertinent information regarding the better characterized murine monoclonal anti-idiotypic reagents (used in the current study). The vast majority of these anti-RF reagents were generated using WA-like (V_kIIIb/V_H1) and PO-like (V_kIIIa/V_H3) MC-derived RFs as immunogens. Most were shown to recognize idiotopes that were preferentially associated with antibodies with RF activity, although numerous studies demonstrated that expression of these idiotopes did not confer RF activity (Mageed *et al.*, 1986a; Lydyard *et al.*, 1990; Mageed *et al.*, 1990; Abderrazik *et al.*, 1992). Compilation of available data on RFs of known primary sequence and idiotype (Id) expression allowed for the identification of consensus amino acid sequences representing Id-positive RFs. This approach resulted in the characterization of the genetic loci, and corresponding alleles, encoding the 6B6.6, 17.109 and G6 (see Table 5-1). All of the studies that evaluated the presence of Id-positive Igs or of Id-associated genes have found that normal individuals, as well as autoimmune patients, are able to express these RF-associated Ids. Most appear to be expressed in small quantities within the circulation, but their prevalence in RA patients, with respect to either RF-precursor B cells or to circulating levels, has been found to be either greater, equivalent or even lower than in normal individuals ({Mageed *et al.*, 1990; Schrohenloher *et al.*, 1990; Shokri *et al.*, 1991}, {Croweley *et al.*, 1990; Shokri *et al.*, 1991; Abderrazik *et al.*, 1992}, {Shokri *et al.*, 1991}, respectively). There are also a number of reports providing evidence that these Ids are more commonly detected on RFs with variable regions that have little or no somatic mutations (Radoux *et al.*, 1986; Liu *et al.*, 1989; Lydyard *et al.*, 1990; Crowley *et al.*, 1990; Yang *et al.*, 1993; Newkirk *et al.*, 1993). Consequently, putative mutational events have been related to modulation of idiotype

Table 5-1. Summary of Murine Monoclonal Anti-RF Antibodies. (continued on following page)

NAME	TYPE[1]	IMMUNOGEN (V_L, V_H)[2]	REPORTED SPECIFICITY & RELEVANT INFORMATION	REF[3]
B12	L	KO (kIIIb, VH1)	Predominantly kIII, but also other k subgroups	[1]
C7	L	KO (kIIIb, VH1)	Similar, but not identical to B12	[1]
			Similar frequency on RF from RA & normal (N)	[2]
C6	L	KO (kIIIb, VH1)	Recognizes a FR sub-subgroup determinant on kIIIb	[3]
JG-B1	L	GLO (kIIIb, VH3)	Specific for kIIIb sub-subgroup	[4]
686.6	L	COR (kIIIa, VH2)	Specific for kIIIa sub-subgroup	[5]
			Expressed in 50% sero+ RA but in small quantities	[5]
			Encoded by the kv328 & kv328h5 loci	[6]
			Similar RFLP patterns for the two loci in RA patients and N individuals	[6,7]
			Preferential association with a subset of VH4	[8]
17.109	L	SIE (kIIIb, VH1)	Subset of the kIIIb sub-subgroup	[9]
			Encoded by the kv325 germline gene	[10]
			Preferential association with subset of VH1	[11]
108.12	L	GOL (kIIIb, ?)	Specific for kIIIb sub-subgroup	[12]

[1] L, L chain related Ids; H, H chain related Ids; C, conformational Ids

[2] L chain/ H chain V regions expressed by the RF immunogen; "?", not known.

[3] References: [1], Mageed et al., 1986b; [2], Abderrazik et al., 1992; [3], Lydyard et al., 1990 [4], Greenstein et al., 1984; [5], Schrohenloher et al., 1990; [6], Liu et al., 1989; [7], Yang et al., 1991; [8], Silverman et al., 1990; [9], Carson & Fong, 1986; [10], Radoux et al., 1986; [11], Silverman et al., 1988; [12], Posnett et al., 1986.

Table 5-1. Summary of Murine Monoclonal Anti-RF Antibodies. (continued from previous page)

NAME	TYPE[1]	IMMUNOGEN (V_L, V_H)[2]	REPORTED SPECIFICITY & RELEVANT INFORMATION	REF[3]
H1	H	KOK (kIIIb, VH1)	Present on a subset of VH1 subgroup	[13]
G6	H	KOK (kIIIb, VH1)	Present on a subset of VH1 subgroup	[13]
			Encoded by hv1051 germline gene	[14]
G8	H	KO (kIIIb, VH1)	Present on VH1 subgroup; higher in RA than N	[15]
B6	H	HER (kIII, VH3)	Present on a subset of VH3 subgroup	[16]
D12	H	HER (kIII, VH3)	Present on some VH3 H chains	[3]
			Relatively high level common to RA, SLE and N	[17]
			Predominantly associated with polyreactive RFs	[18]
G7	H	AN (lambda, VH3)	Expressed on some VH3	[18]
102.2	C	GOL (kIIIb, ?)	kIII + mu chain	[12]
128.4	C	GLO (kIIIb, VH3)	Predominantly IgMk with RF activity	[12]
86.3	C	GLO (kIIIb, VH3)	Present on a subgroup of WA RFs	[19]
B4	C	GLO (kIIIb, VH3)	Private Id found on GLO-RF	[4]

[1] L, L chain related Ids; H, H chain related Ids; C, conformational Ids

[2] L chain/ H chain V regions expressed by the RF immunogen; "?", not known.

[3] References: [3], Lydyard et al., 1990 [4], Greenstein et al., 1984; [12], Posnett et al., 1986; [13], Mageed et al., 1986a; [14], Yang et al., 1993; [15], Mageed et al., 1990; [16], Crowley et al., 1990; [17], Shokri et al., 1991; [18], Newkirk et al., 1993; [19], Posnett et al., 1992.

expression observed during normal development and during the progression of RA (Lydyard *et al.*, 1990; Kouri *et al.*, 1990).

5.5 VARIABLE REGION STRUCTURE

5.5.1 HUMAN PARAPROTEINS WITH RF ACTIVITY

The first two completely sequenced V regions of human RFs, were the POM and LAY MC-derived RFs (Capra *et al.*, 1971; Capra & Kehoe, 1974; Capra & Klapper, 1976). The H chains of these two "PO" RFs were quite similar and both belonged to the V_H3 family. Their light chains were, however, from different Vk subgroups: POM from the V_kIIIa sub-subgroup and LAY from the V_kI subgroup. This led these investigators to conclude that the "PO" CRI was most likely located on the RF H chain.

Using specific antisera, Kunkel and co-workers were able to establish that all of the WA-positive RFs studied were part of the V_kIIIb sub-subgroup (Kunkel *et al.*, 1974). The complete amino acid sequences of the variable regions of two "WA" RFs (SIE and WOL) were then elucidated (Andrews & Capra, 1981a, 1981b, 1981c). Sequence similarities between these two antibodies were found in both the H and L chains. The light chains were of the V_kIIIb sub-subgroup and the H chain V regions from the V_H1 family. Further sequence analyses demonstrated that the V_kIIIb/V_H1 V region pair was associated with the "WA" CRI (Ledford *et al.*, 1983; Pons-Estel *et al.*, 1984; Newkirk *et al.*, 1987).

The BLA serum RF, the MC-derived prototype for the "BLA" CRI, has also been sequenced. Barnes and co-workers demonstrated its use of V_kIIIa and V_H4 V region genes (Barnes *et al.*, 1990). This specific pairing would appear to be important since Agnello *et al.* (Agnello & Barnes, 1986) have demonstrated that the "BLA" CRI is a heat-labile conformational antigen (Agnello & Barnes, 1986).

These associations between CRI expression and the use of certain V_L/V_H pairs suggested structural features that were important for the binding of RFs to IgG-Fc. As summarized in Table 5-2, the accumulated sequence data for the human paraproteins with

Table 5-2. V_L and V_H Gene Usage in Human Paraproteins with RF activity.[1]

RF	ORIGIN	V_L	V_H	RF	ORIGIN	V_L	V_H
BOR	MC[2]	kIIIb	1	KES	SLE	kI	3
KAS	MC	kIIIb	1	CHA	UN	kI	3
SIE	MC	kIIIb	1	LAY	MC	kI	3
WOL	MC	kIIIb	1	POM	MC	kIIIa	3
ARL	UN	kIIIb	1	SFL	HP	kIIIa	3
BLO	UN	kIIIb	1	MIL	UN	kIIIa	3
DRI	UN	kIIIb	1	RIV	MC	kIIIa	3
FRA	UN	kIIIb	1	GLO	MC	kIIIb	3
KOK	MC	kIIIb	1	PAY	MC	kIIIb	3
NEU	MC	kIIIb	1	KOH	UN	lambda	3
GAR	MC	kIIIb	1	DIN	UN	kIII	4
PAL	UN	kIII	1	STI	UN	kIIIa	4
BEL	UN	kIII	1	BLA	MC	kIIIa	4
McD	UN	kIII	1	51	UN	kIIIa	4
SOU	UN	kIII	1	108	UN	kIIIa	4
COR	UN	kIIIa	2	ORI	UN	kIIIa	4
CUR	MC	kIIIb	2	LES	UN	kIIIa	4
GOT	MC	kIIIb	2	LEW	UN	kIIIa	4

[1] Adapted from Randen et al., 1992b.

[2] Abbreviations: MC, mixed cryoglobulinemia; HP, hypergammaglobulinemia purpura SLE, systemic lupus erythematosus; CLL, chronic lymphocytic leukemia; UN, origin is unclear but majority are from MC or Waldenströms macroglobulinemia.

RF activity implicated the V_kIII chain in this antigenic specificity since nearly 90% of these RFs were kIII-positive. There appeared to be a restricted use of V_H gene segments, as well, since the majority expressed H chains from either the V_H1 or V_H3 families.

5.5.2 HUMAN HYBRIDOMA RFs FROM NORMAL INDIVIDUALS AND AUTOIMMUNE PATIENTS

Although nearly 75% of individuals suffering from MC have arthralgias, they lack the synovitis and other clinical features that are characteristic of RA. For this reason, it was unclear whether the results obtained for the MC-derived RFs could be generalized to those involved in autoimmunity. It was not until the advent of human hybridoma technology that the study of RFs from autoimmune patients and normal individuals was possible. Summaries of the structural data obtained from RF-producing B cells, cloned almost exclusively from RA patients, are found in Tables 5-3, 5-4 and 5-5. In addition to their derivation, these RFs may be more relevant since many are monospecific and have relatively high affinity values, as indicated by their dissociation constants (Table 5-5).

In contrast to the MC-derived RFs, there is a striking diversity of L chain V region genes expressed by human hybridoma RFs (Table 5-3). Nearly all the V gene families are represented, with no more than 30% from the V_kIII subgroup. In addition, lambda L chain genes account for approximately half of all the sequences, as opposed to the 3% found for MC-derived RFs. The majority of H chains are encoded by the V_H1 and V_H3 families, as previously shown for the paraproteins. Although V_H3-encoded H chains predominate in the RA population (Table 5-4), this V_H distribution closely matches the frequency of V gene expression observed in unselected peripheral blood lymphocytes from a healthy donor (Table 2-4).

An additional advantage of these human hybridoma RF studies is that the primary sequence data was obtained at the nucleotide level, rather than by amino acid sequencing. This, along with the information regarding the Ig loci, has revealed that the V regions of these RFs also derive from a diverse set of germline V_L and V_H genes, as seen in Tables 5-3 and 5-4. This assignment of putative germline genes allows for the evaluation

Table 5-3. V_L Gene Usage of Human Hybridoma Rheumatoid Factors. (continued on following page)

RF	IgH	ORIGIN	GENE FAMILY	PUTATIVE GERMLINE GENE (% IDENTITY)	REFERENCES
D1	G3	RA, synov.	kIIIa	Vg (99.6%)	Olee et al., 1992
L1	G1	RA, synov.	L I	1v1L1 (98.6%)	Olee et al., 1992
C6	M	RA, synov.	kI	HK102 (98.2%)	Ermel et al., 1993
G9	M	RA, synov.	L III	Humlv318 (99.3%)	Ermel et al., 1993
D5	M	RA, synov.	kIIIa	Vg (96.2%)	Ermel et al., 1993
RF-TS7	G2	RA, synov.	L II	HuL2-4A (98.6%)	Randen et al., 1993
RF-SJ5	G1	RA, synov.	kI	kvi4 (94.7%)	Randen et al., 1993
RF-KL1	M	RA, synov.	kI	Vd (97.5%)	Victor et al., 1991
RF-TS3	M	RA, synov.	kII	A23 (100%)	Victor et al., 1991
RF-TS1	M	RA, synov.	kIIIb	kv325 (99.3%)	Victor et al., 1991
RF-TS2	M	RA, synov.	kIIIa	kv328 (97.0%)	Victor et al., 1991
RF-SJ1	M	RA, synov.	L I	?	Victor et al., 1991
RF-SJ2	M	RA, synov.	L I	?	Victor et al., 1991
RF-SJ3	M	RA, synov.	kIIIb	kv325 (98.7%)	Victor et al., 1991
C1	G2	RA, synov.	L IX (?)	?	Lu et al., 1993
C2	G1	RA, synov.	L I	1v1L1 (90.0%)	Lu et al., 1993
HAF10	M	RA, synov.	L (?)	?	Robbins et al., 1990
YES8c	M	RA, B.M.	kIIIb	kv325 (98.3%)	Ezaki et al., 1991

ABBREVIATIONS: IgH, Ig H chain isotype; RA, RA patient; synov., synovium; PBL, peripheral blood lymphocyte; serum, RF protein was isolated from serum (not a hybridoma Ab); JRA, juvenile rheumatoid arthritis patient; polyA, polyarthritis patient; SLE, SLE patient; N, normal individual; N.D., not determined; ?, germline origin is uncertain or not reported.

Table 5-3. V_L Gene Usage of Human Hybridoma Rheumatoid Factors. (continued from previous page)

RF	IgH	ORIGIN	GENE FAMILY	PUTATIVE GERMLINE GENE (% IDENTITY)	REFERENCES
mAB111	M	RA, PBL	L I	1v117 (96.9%)	Mantovani et al., 1993
mAB112	M	RA, PBL	kIIIb	kv325 (97.2%)	Mantovani et al., 1993
mAB113	M	RA, PBL	kIIIb	kv325 (97.6%)	Mantovani et al., 1993
mAB114	M	RA, PBL	kIIIa	kv328h5 (96.5%)	Mantovani et al., 1993
mAB63	M	RA, PBL	L IV	?	Harindranath et al., 1991
mAB67	M	RA, PBL	L I	?	Harindranath et al., 1991
mAB65	A1	RA, PBL	L IV	?	Harindranath et al., 1991
mAB61	M	RA, PBL	L I	?	Harindranath et al., 1991
mAB60	A1	RA, PBL	L III	?	Harindranath et al., 1991
C304	M	RA, PBL	L I	DPL8 (99.7%)	Rioux (unpublished)
AN	M	RA, serum	L III	N.D.	Natvig et al., 1988
RF-KL5	G1	JRA, synov.	kIV/kVI ?	? (<74%)	Randen et al., 1993
hRF1	G4	polyA, synov.	kII	A3 (99.7%)	Weisbart et al., 1991
RF-KES1	M	SLE, PBL	kI	V52 (96.0%)	Victor et al., 1991
Ab47	M	N, PBL	N.D.	N.D.	Sanz et al., 1989
SSH23	M	N, PBL	kII	A23 (90.0)	Stüber et al., 1992
RF-TMC1	M	N, PBL	kIII	38K (93.8%)	Victor et al., 1991

ABBREVIATIONS: IgH, Ig H chain isotype; RA, RA patient; synov., synovium; PBL, peripheral blood lymphocyte; serum, RF protein was isolated from serum (not a hybridoma Ab); JRA, juvenile rheumatoid arthritis patient; polyA, polyarthritis patient; SLE, SLE patient; N, normal individual; N.D., not determined; ?, germline origin is uncertain or not reported.

Table 5-4. V_H Gene Usage of Human Hybridoma Rheumatoid Factors. (continued on following page)

RF	IgH	ORIGIN	GENE FAMILY	PUTATIVE GERMLINE GENE (% IDENTITY)		REFERENCES
D1	G3	RA, synov.	3	VH26	(91.8%)	Olee et al., 1992
L1	G1	RA, synov.	1	hv1L1	(96.1%)	Olee et al., 1992
C6	M	RA, synov.	3	VH26	(87.6%)	Ermel et al., 1993
G9	M	RA, synov.	4	VH4.18	(98.6%)	Ermel et al., 1993
D5	M	RA, synov.	3	GL-SJ2	(92.7%)	Ermel et al., 1993
RF-TS7	G2	RA, synov.	1	hv1L1	(99.3%)	Randen et al., 1993
RF-SJ5	G1	RA, synov.	3	1.9III	(93.3%)	Randen et al., 1993
RF-KL1	M	RA, synov.	3	VH26	(95.1%)	Pascual et al., 1990
RF-TS3	M	RA, synov.	1	4.16	(99.2%)	Pascual et al., 1992
RF-TS1	M	RA, synov.	1	hv1263	(94.5%)	Pascual et al., 1990
RF-TS2	M	RA, synov.	3	1.9III	(97.5%)	Pascual et al., 1990
RF-SJ1	M	RA, synov.	3	1.9III	(95.7%)	Pascual et al., 1990
RF-SJ2	M	RA, synov.	3	hv3005	(98.5%)	Pascual et al., 1990
RF-SJ3	M	RA, synov.	3	1.9III	(99.4%)	Pascual et al., 1992
C1	G2	RA, synov.	1	hv1f10	(92.0%)	Lu et al., 1993
C2	G1	RA, synov.	4	71-4	(95.0%)	Lu et al., 1993
HAF10	M	RA, synov.	1	hv1f10	(98.0%)	Soto-Gil et al., 1990
YES8c	M	RA, B.M.	1	hv1263	(93.2%)	Ezaki et al., 1991

ABBREVIATIONS: IgH, Ig H chain isotype; RA, RA patient; synov., synovium; PBL, peripheral blood lymphocyte; serum, RF protein was isolated from serum (not a hybridoma Ab); JRA, juvenile rheumatoid arthritis patient; polyA, polyarthritis patient; SLE, SLE patient; N, normal individual; N.D., not determined; ?, germline origin is uncertain or not reported.

Table 5-4. V_H Gene Usage of Human Hybridoma Rheumatoid Factors. (continued from previous page)

RF	IgH	ORIGIN	GENE FAMILY	PUTATIVE GERMLINE GENE (% IDENTITY)	REFERENCES
mAB111	M	RA, PBL	1	V1-2 (96.9%)	Mantovani et al., 1993
mAB112	M	RA, PBL	1	hv1263 (92.8%)	Mantovani et al., 1993
mAB113	M	RA, PBL	1	hv1263 (93.5%)	Mantovani et al., 1993
mAB114	M	RA, PBL	3	HHg19 (91.4%)	Mantovani et al., 1993
mAB63	M	RA, PBL	4	VH4.21 (100%)	Harindranath et al., 1991
mAB67	M	RA, PBL	4	V79 (99.3%)	Harindranath et al., 1991
mAB65	A1	RA, PBL	4	V71-2 (87.3%)	Harindranath et al., 1991
mAB61	M	RA, PBL	4	VH4.18 (97.6%)	Harindranath et al., 1991
mAB60	A1	RA, PBL	3	VH11 (92.1%)	Harindranath et al., 1991
C304	M	RA, PBL	3	VH26 (99.6%)	Rioux (unpublished)
AN	M	RA, serum	3	N.D.	Natvig et al., 1988
RF-KL5	G1	JRA, synov.	3	DP-31 (96.9%)	Randen et al., 1993
hRF1	G4	polyA, synov	N.D.	N.D.	Weisbart et al., 1991
RF-KES1	M	SLE, PBL	3	12-2 (87.0%)	Pascual et al., 1992
Ab47	M	N, PBL	1	?	Sanz et al., 1989
SSH23	M	N, PBL	2	VII-b (99.0%)	Stüber et al., 1992
RF-TMC1	M	N, PBL	4	4-21 (99.6%)	Pascual et al., 1992

ABBREVIATIONS: IgH, Ig H chain isotype; RA, RA patient; synov., synovium; PBL, peripheral blood lymphocyte; serum, RF protein was isolated from serum (not a hybridoma Ab); JRA, juvenile rheumatoid arthritis patient; polyA, polyarthritis patient; SLE, SLE patient; N, normal individual; N.D., not determined; ?, germline origin is uncertain or not reported.

Table 5-5. Human Hybridoma Rheumatoid Factors. Dissociation constants and sequences of heavy chain complementarity determining region 3 ($CDR3_H$). (continued on following page)

RF	K_D [1]	$CDR3_H$ SEQUENCE	D_H	J_H	REF [2]
D1	5.2×10^{-7}	SGYRGGDY	DK4	4	[1]
L1	4.1×10^{-7}	EYFYDGSDLKPSDVFDI	D21/9, DXP'1	3	[1]
C6	N.D.	DRELLWFGELLN	DXP1	4	[2]
G9	N.D.	RVVGVDHTFDY	DXP4, D3-C	4	[2]
D5	N.D.	GLSRVTTHTFDI	DFL16, D3	3	[2]
RF-TS7	N.D.	N.D.	N.D.	N.D.	[3]
RF-SJ5	N.D.	N.D.	N.D.	N.D.	[3]
RF-KL1	6.8×10^{-7}	LRSGLVPYYFDS	?	4	[4]
RF-TS1	7.7×10^{-8}	EDPYGDYVANPFDI	?	3	[4]
RF-TS2	7.1×10^{-8}	DRVAVYASVFFIDSFDI	?	3	[4]
RF-TS3	7.4×10^{-8}	EDSNGYKIFDY	?	4	[4]
RF-SJ1	2.4×10^{-8}	GVYCSSSSCYSYYYYHYMDV	DLR2	6	[4]
RF-SJ2	2.7×10^{-6}	GRFCSGGSCYSYYYYYYMDV	DLR2	6	[4]
RF-SJ3	4.6×10^{-8}	WGGYCTNGVCYRGGYGMDV	DLR1	6	[4]
C1	N.D.	EGPSITQKIVASFDF	DLR1-C, D3	4	[5]
C2	N.D.	QRVSGRDLDY	D21/7 or DXP'1, DN1	4	[5]
HAF10	N.D.	DSRGGDLLTGHHCIDY	?	4	[6]
YES8c	N.D.	GIASAGTLNYFFY	?	4	[7]

ABBREVIATIONS: K_D, dissociation constant; V_H, H chain V gene family; ?, no germline origin reported; N.D., not determined.

[1] K_D values for native human IgG or IgG-Fc were obtained from the following references: Harindranath et al., 1991; Lu et al., 1992; Olee et al., 1992; Randen et al., 1992; Mantovani et al., 1993.

[2] References: [1], Olee et al., 1992; [2], Ermel et al., 1993; [3], Randen et al., 1993; [4], Pascual et al., 1990; [5], Lu et al., 1993; Robbins et al., 1990; [7], Ezaki et al., 1991.

Table 5-5. Human Hybridoma Rheumatoid Factors. Dissociation constants and sequences of heavy chain complementarity determining region 3 ($CDR3_H$). (continued from previous page)

RF	K_D [1]	$CDR3_H$ SEQUENCE	D_H	J_H	REF[2]
mAB111	6.3×10^{-7}	GGYCTTSNCNLKDTFVI	DLR4	3	[8]
mAB112	4.6×10^{-7}	EGRSSDYSNPFDY	DN4-C, DA1 or DA4	4	[8]
mAB113	3.1×10^{-7}	EGRSSDYSNPFDY	DN4-C, DA1 or DA4	4	[8]
mAB114	6.8×10^{-7}	GDYYDYSGNYIDAFDA	D21/9	3	[8]
mAB63	4.0×10^{-5}	GGSVLRFLEWLLYPAFDY	DXP4	4	[9]
mAB67	4.2×10^{-5}	VTGSTFWSGYYTRGYYFDY	DXP4	4	[9]
mAB65	1.2×10^{-4}	WGYYNSNGSPLVYYFYGQNV	DXP1	6	[9]
mAB61	6.0×10^{-7}	LGPDDYTLDGMDV	DLR1	6	[9]
mAB60	2.0×10^{-7}	IGGGTNSPDS	DLR1	5	[9]
C304	N.D.	YQYYYDSSGYYYNWFDP	D21/9	5	[10]
AN	N.D.	N.D.	N.D.	N.D.	[11]
RF-KL5	N.D.	N.D.	N.D.	N.D.	[3]
hRF1	N.D.	N.D.	N.D.	N.D.	[12]
RF-KES1	N.D.	LLEVSYPGLDV	Dpseudo	6	[4]
Ab47	N.D.	N.D.	N.D.	4	[13]
SSH23	N.D.	WTVDSSGYYLGFDY	D21/9	4	[14]
RF-TMC1	N.D.	GRMRG...	N.D.	N.D.	[4]

ABBREVIATIONS: K_D, dissociation constant; V_H, H chain V gene family; ?, no germline origin reported; N.D., not determined.

[1] K_D values for native human IgG or IgG-Fc were obtained from the following references: Harindranath et al., 1991; Lu et al., 1992; Olee et al., 1992; Randen et al., 1992; Mantovani et al., 1993.

[2] References: [3], Randen et al., 1993; [4], Pascual et al., 1990; [8], Mantovani et al., 1993; [9], Harindranath et al., 1991; [10], Rioux et al., (unpublished); [11], Natvig et al., 1988; [12], Weisbart et al., 1991; [13], Sanz et al., 1989; [14], Stüber et al., 1992.

of somatic mutation events. Evidence for both near-germline encoded V regions (Pascual *et al.*, 1990; Ezaki *et al.*, 1991; Pascual *et al.*, 1992) and somatically mutated sequences exists (Harindranath *et al.*, 1990; Olee *et al.*, 1992; Randen *et al.*, 1992; Mantovani *et al.*, 1993; Ermel *et al.*, 1993), indicating that while some RF-producing B cells have probably undergone antigen-driven selection, others have not.

The third CDR of the RF H chains displayed a great variety of lengths and sequences (Table 5-5). The average length of these $CDR3_H$ domains (approximately 14 amino acid residues) is comparable to the values reported for normal human peripheral B cells (Sanz, 1991; Wu *et al.*, 1993). Despite the rearrangement of a J_H4 segment in over half of the RFs (Table 5-5), this predominant expression of J_H4 genes is not likely to be related to antigenic specificity since this pattern is almost identical to what is seen in adult peripheral blood lymphocytes (Yamada *et al.*, 1991). A great diversity of D_H germline gene usage for these RFs has also been reported (summarized in Table 5-5). In summary, although there is some Ig V region gene usage similar to the MC-derived RFs, most RFs originating from patients with autoimmune disorders are derived from germline genes distinct from those encoding the "WA", "PO", and "BLA" CRI (Victor *et al.*, 1991).

5.6 RELATIONSHIP TO THE ANTI-CMV IMMUNE RESPONSE

The difficulty in correlating RF synthesis with an anti-viral immune response in RA patients is likely two-fold: prevalence and time. As previously discussed, the prevalence of CMV in the general population is high and thus makes it difficult to establish an association between CMV-seropositivity and any disease variable. The second point relates to the difficulty in establishing a relationship between two events which do not have well-defined origins with respect to time. Specifically, at the time of diagnosis, the majority of patients already have high RF titers. As well, most of these individuals are CMV-seropositive (as in the general population) and, almost without exception, the time of infection, reinfection or reactivation is not defined. For these reasons, many of the documented associations between CMV infection and RF production

originate from a more controlled clinical setting unrelated to RA: post-operative infections. In these instances, serological CMV status prior to surgery is defined, the time of infection can be established, and other variables (ie. RF titers) can be monitored.

One of the first studies to use this approach was in the context of open heart surgery, where three of ten postperfusion infections had a coincident appearance of circulating RF in high titers (Kantor *et al.*, 1970). Moreover, the serum RFs became undetectable when the mononucleosis-like syndrome, caused by the CMV infection, had resolved. This CMV-related disturbance was not restricted to the induction of RF production since six of the remaining patients developed immunologic abnormalities unrelated to RF synthesis (Kantor, 1970). Studies of transplant recipients have provided supporting evidence for the connection between CMV and RF synthesis. Baldwin's group demonstrated that the majority of kidney recipients who developed severe CMV infection, had simultaneously become RF-positive (Baldwin *et al.*, 1983). These RFs were predominantly of the IgM isotype and were believed responsible for the immune complexes that were detectable in the circulation, as well as in the glomeruli of the kidneys (Baldwin *et al.*, 1983). It had been suggested that the appearance of RFs in this population was related to graft rejection; however, in a large study of nearly 150 patients there was a strong correlation between circulating RFs and CMV infection, and not between RFs and transplant rejection (Baldwin *et al.*, 1987). Another group of investigators also observed that the majority of kidney recipients, who developed a secondary CMV infection (patients were seropositive prior to transplantation), also developed detectable levels of circulating RFs (Van Der Giessen *et al.*, 1990).

In RA, although it has not been possible to establish the time of infection in relation to the appearance of RFs, a correlation between the co-existence of circulating RFs and anti-CMV antibodies has been reported both in RA patients as well as in normal controls (Ferraro & Newkirk, 1993). It has also been reported that the occurence of reactivation of latent CMV is greater in RA patients than in normal controls, in part as a consequence of immunosuppressive therapies (Dowling *et al.*, 1976). Periodic reactivation of latent CMV in RA patients that would trigger anti-viral antibody synthesis

could therefore result in immune complexes consisting of virus, anti-viral antibody and RFs, for which there is currently some evidence (McCormick *et al.*, 1992).

A potential problem is that much of the information in this area has been obtained from cases of systemic CMV infections, while the devastating effects in RA are focussed in the joints. This dilemma can be resolved by proposing that similar events occur in the joints (see section 4.2.2.1 regarding the presence of live particles and/or latent CMV within this anatomical location), or that synovial lymphocytes participate in the systemic anti-viral response, as it has been demonstrated for the case of *Haemophilus influenzae* (Pelton *et al.*, 1985).

Furthermore, there is evidence for a structural relationship between the anti-CMV humoral response and human RFs. This work, based on the expression of RF-associated CRI as well as L and H chain V region primary sequence data, demonstrated similarities between an anti-CMV antibody and MC-derived RFs of the "WA" idiotypic family (Newkirk *et al.*, 1988).

EXPERIMENTAL RESULTS

CHAPTER II

Structural Characteristics of Four Human Hybridoma Antibodies Specific for the pp65 Protein of the Human Cytomegalovirus (CMV) and Their Relationship to Human Rheumatoid Factors.

PREFACE

It had previously been reported that one anti-CMV antibody, directed against the viral glycoprotein gp58, expressed RF-associated idiotypes and had sequence similarities characteristic of RFs within the "WA" idiotypic family. Since little was known about the V regions expressed by human anti-viral antibodies, this chapter examines the RF-associated idiotype expression and complete sequences of the variable regions of four human hybridoma antibodies specific for the pp65 antigen of the human CMV.

SUMMARY

Four human hybridoma antibodies directed against the human CMV were characterized with respect to their immunoglobulin gene usage and expression of rheumatoid factor (RF) associated idiotypes and variable region epitopes. The aims of these experiments were: 1) to characterize the immunoglobulin gene usage of four antibodies directed against a single protein of a human pathogen and 2) to examine how this humoral response may be linked to the production of RFs, autoantibodies found in the majority of patients with rheumatoid arthritis (RA). All four anti-CMV antibodies were of the gamma heavy chain isotype and were specific for the immunodominant 65 kilodalton viral matrix phosphoprotein (pp65). The four anti-pp65 antibodies expressed different light (L) and heavy (H) chain variable region gene combinations. These were: $V_k III/V_H 3$, $V_\lambda 1/V_H 3$, $V_\lambda 1/V_H 4$ and $V_\lambda 3/V_H 3$, respectively for the HCV-2, HCV-3, HCV-63 and HCV-65 hybridoma cell lines. Although none had RF activity, each of these antibodies expressed a unique set of RF-associated determinants, implying different

three-dimensional configurations of the variable regions of these antibodies. The HCV-2 antibody, however, had the most extensive similarities to human RFs since it not only expressed the greatest number of RF-associated determinants but also had a protein sequence that was very homologous to RFs of the "Po" idiotypic family. Furthermore, predicted germline gene usage by anti-CMV antibodies and RFs suggest that some are encoded by identical or similar genes and that the different specificities are achieved by somatic mutations in the L and H chain complementarity determining regions (CDRS) and genetic diversity in the H chain CDR3.

INTRODUCTION

The human cytomegalovirus (CMV) is the largest of the human herpesviridae and is highly prevalent in both industrialized and non-industrialized nations, where approximately 60% to nearly 100% of all adult individuals are infected. Like the other members of the herpes family, CMV causes a persistent infection and can remain in the host in a latent, non-replicative, state with the potential to be reactivated (Apperley and Goldman, 1988).

Although the CMV genome has the potential of encoding over 200 different proteins, the immunocompetent host appears to generate a strong antibody response to only a limited number of different polypeptides. Specifically, the immunodominant polypeptides are the immediate early phosphoprotein of 72 kDa, the early phosphoprotein of 52 kDa, the structural glycoprotein complex (gCI) of 55-58 kDa and 93-130 kDa and the structural phosphoproteins of 65 kDa and 150 kDa (Landini and La Placa, 1991). The importance of the host's immune response to this virus is evident by the fact that CMV is a major cause of morbidity and mortality in individuals with either immature or compromised immune systems (as reviewed in Griffiths and Grundy, 1987; Apperley and Goldman, 1988). Even in an immunocompetent host, primary CMV infection can disrupt the host's immune system and lead to clinical abnormalities. These abnormalities commonly include polyclonal hypergammaglobulinemia and elevated titers of a number of different autoantibodies (Wager *et al.*, 1968; Horwitz *et al.*, 1979; Berlin *et al.*, 1977).

Because of these interactions, CMV has the potential to deregulate the normal immune status and play a role in autoimmune diseases such as rheumatoid arthritis (RA). RA is as a chronic inflammatory disorder of unknown etiology and is characterized by an inflammatory process involving multiple joints (Short *et al.*, 1957). Autoantibodies known as rheumatoid factors (RFs) are found circulating in up to 75% of RA patients. RFs appear to bind to a number of different, but possibly overlapping, epitopes present on the Fc portion of IgG molecules. These epitopes are generally believed to be located in the cleft between the C_H2 and C_H3 domains of the gamma chain (Stone *et al.*, 1989; Jefferis *et al.*, 1984). Structural relationships between RFs from unrelated individuals were first demonstrated by Kunkel and coworkers who used rabbit antisera to classify human IgM paraproteins with RF activity into two major cross-reactive idiotypic families known as Wa and Po (Kunkel *et al.*, 1973). Subsequent work demonstrated that the sharing of an idiotype was related to similarities in the protein sequence of the variable regions of the L and H chains of the antibody molecules (reviewed in Newkirk and Capra, 1987).

Interestingly, CMV virions have been isolated from the synovial cells from joints of an RA patient (Hamerman *et al.*, 1982). There is also evidence that CMV may remain latent in the synovial membrane (Einsele *et al.*, 1992) which is important since it has been shown that the rheumatoid synovial membrane has the ability to participate in anti-viral immune responses (Pelton *et al.*, 1985). A correlation between the presence of anti-CMV antibodies and RFs, both in renal transplant patients (Baldwin *et al.*, 1987) and in patients with RA (Ferraro and Newkirk, 1993), has also been documented. A possible relationship between the immune response to CMV and RFs was further suggested by the identification of an IgG anti-CMV antibody showing a striking structural similarity to IgM RFs expressing the Wa idiotype (Newkirk *et al.*, 1988).

In this report, four human hybridoma anti-CMV antibodies, specific for the immunodominant lower matrix phosphoprotein (pp65), were studied to examine whether they were structurally related to human RFs. The variable (V) regions of the light (L) and heavy (H) chains of the four hybridomas were cloned and sequenced. Additionally,

the anti-CMV antibodies were analyzed for their expression of RF-associated idiotypes and V region epitopes. We found that each of the four anti-pp65 kDa hybridoma antibodies used a unique set of V region genes. However, one of the four antibodies was found to be similar to RFs of the Po idiotypic family, both in the expression of specific idiotypes and the use of related L and H chain genes.

MATERIALS AND METHODS

Cell lines

The human hybridoma cell lines HCV-2, HCV-3, HCV-63 and HCV-65 were obtained from the National Laboratory for Immunology (Laboratory Centre for Disease Control, Ottawa, Ontario). They were derived by somatic fusion of lymphocytes from CMV-seropositive individuals with the heteromyeloma Sp2/HPT, as previously described (Brodeur *et al.*, 1987; Martin *et al.*, 1988; Larose *et al.*, 1991). The peripheral blood lymphocytes (PBLs) of a hemophiliac patient were used in the generation of the HCV-2 and HCV-3 hybridoma cell lines, as previously described (Larose *et al.*, 1991). Axillary lymph nodes of a breast cancer patient were used as a source of B-lymphocytes for the production of the HCV-63 and HCV-65 hybridoma cell lines.

Hybridoma antibody characterization

Immunoglobulin class and light chain specificities were determined by ELISA using commercially available reagents (ICN Biomedicals, Costa Mesa, CA; Fisher, Orangeburg, NY). The specificity of the hybridoma antibodies was tested for CMV reactivity by indirect immunofluorescence (IFA), radioimmunoprecipitation and neutralization of virus infectivity as previously described (Larose *et al.*, 1991). All antibodies were tested, by IFA, for virus reactivity using the Towne and Davis prototype strains of CMV (ATCC, Rockville, MD), laboratory CMV strain AD-169 (from the culture collection of the Laboratory Centre for Disease Control, Ottawa, Ontario), as well as with the four other human herpesviruses using commercial slides (Electro-nucleonics Inc., Columbia, MD). The characterization of the HCV-2 and

HCV-3 antibodies was previously reported (Larose *et al.*, 1991) whereas that of the HCV-63 and HCV-65 is new and is described herein.

RF assays

RF activity was tested both by ELISA and by an immunoblot assay. For the ELISA, wells were coated overnight with purified human Fc fragments (IgG1) at a concentration of 0.01 mg/ml. The plates were washed, purified "test" antibodies were then added at a concentration of 0.01 mg/ml and incubated at 37°C for 2 hours. Bound antibodies were either detected by the use of affinity-purified horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fragments of kappa chain-specific antisera (Protos Immunoresearch, San Francisco, CA) or of affinity-purified biotinylated $F(ab')_2$ fragments of lambda chain-specific antisera (EY Laboratories, San Mateo, CA) followed by HRP-conjugated avidin-D. All assays included positive- and negative-control antibodies in addition to wells incubated with PBS/Tween alone. Optical densities (O.D.) at 492 nm were determined using an ELISA plate reader (SLT Labinstruments, model EAR 400RT, Fisher Scientific, Montreal, Quebec).

The immunoblot assay involved the separation of human IgG Fc fragments by sodium dodecyl (SDS) polyacrylamide gel electrophoresis (PAGE) with transfer to nitrocellulose followed by incubation and then detection of bound, RFs as previously described (Newkirk, 1992b).

Idiotype and V-region subgroup epitope expression

The anti-RF mouse monoclonal antibody reagents used in this study were generated against several different human RFs and have been shown to recognize V region determinants on these RFs. Specific information regarding their production, specificity and prevalence of expression on human RFs, has previously been described (References in Table 1 and Crowley *et al.*, 1988; Posnett *et al.*, 1986; Newkirk *et al.*, 1993; Shokri *et al.*, 1991). In the current study, the expression of RF-associated idiotypes or V-region subgroup epitopes was determined for each anti-CMV antibody by a previously described ELISA (Newkirk *et al.*, 1987).

Positive controls for idiotype/ V region epitope expression included the EV1.15 anti-CMV antibody and the BOR (Newkirk *et al.*, 1987), GLO (Capra and Kehoe, 1975) and SCZ (clinical sample from a patient with mixed essential cryoglobulinemia was kindly provided by Dr. D. Danoff, Montreal General Hospital) human RFs. The results, with the background (PBS/Tween blank) subtracted, are represented as follows: -, <0.10; +, 0.10-0.50; ++, >0.50-1.00; +++, >1.00-1.50; ++++, >1.50.

Protein sequencing

The HCV-2 hybridoma antibody was purified by affinity chromatography using Protein-A Sepharose (Bio-Rad Laboratories, Mississauga, Ontario). H and L chains were separated by SDS-PAGE in the presence of mercaptoethanol (0.72 M) and transferred to polyvinyl paper (Immobilon, Millipore Laboratories, St-Laurent, Quebec) according to the method of Matsudaira (Matsudaira, 1987). Amino-terminal sequence of the HCV-2 L chain was obtained by automated Edman degradation using a gas phase sequencer (model 470 Applied Biosystems, Foster City, CA) with an on-line HPLC phenylthiohydantoin amino acid identification system.

PCR amplification of immunoglobulin V regions

Total cellular RNA was extracted from 10^6 hybridoma cells with guanidinium thiocyanate followed by centrifugation through a 5.7 M cushion of cesium chloride (Chirgwin *et al.*, 1979). First strand cDNA was directly synthesized from 1 μg of total RNA using oligonucleotide primers specific for the appropriate constant region domain (gamma, kappa or lambda). The primers used were:

a) gamma chains: 5'-AAGCTTCTGCAGAAGGGTTGGGCCCTTGGTGGAGGC-3' or
5'-CCAAGCTT(CT)GCCAGGGGGAAGACCGA-3'

b) kappa chains: 5'-CAGAATTCAACTGCTCATCAGAT-3'

c) lambda chains: 5'-TGGCTTGGAGCTCCTCAGAGGA-3' or
5'-CCAAGCTTGAAGCTCCTCAGAGGAGGG-3' (Larrick *et al.*, 1989)

Each cDNA preparation was subsequently amplified by the polymerase chain reaction (Saiki *et al.*, 1988) using a Perkin-Elmer thermal cycler (model 480, Cetus

Perkin Elmer, Emeryville, CA). The 3' oligomers were the same as those used for the cDNA synthesis, whereas the 5' primers used were:

a) gamma chains: 5'-CAGGTGCAGCTGGTGGAGTCTGG-3' (Hillson *et al.*, 1992) or an equimolar mixture of

5'-GGGAATTCATGGACTGGACCTGGAGG(AG)TC(CT)TCT(GT)C-3',
5'-GGGAATTCATGGAG(CT)TTGGGCTGA(CG)CTGG(CG)TTT(CT)T-3'and
5'-GGGAATTCATG(AG)A(AC)(AC)(AT)ACT(GT)TG(GT)(AT)(CGT)C(AT)(CT)(CG)CT(CT)CTG-3' (Larrick *et al.*, 1989)

b) kappa chains: 5'-GGTACCCAGTCTCCATCCTCCCTGTCT-3'

c) lambda chains: 5'-ATGGCCTGGACTCCTCTCCTTCT-3' or

5'-GGGAATTCATG(AG)CCTG(CG)(AT)C(CT)CCTCTC(CT)T(CT)CT(CG)(AT)(CT)C-3' (Larrick *et al.*, 1989)

Subcloning and sequencing

Each PCR product was electrophoresed on a preparative 1.5% agarose/Tris borate EDTA (TBE) gel (Sambrook *et al.*, 1989) and the appropriate band was excised and purified using Prep-A-Gene (Biorad, Mississauga, Ontario). The purified PCR products were prepared for blunt-end cloning by simultaneous incubation with 10 units of T4 polynucleotide kinase (Pharmacia Inc., Baie d'Urfe, QUE.) and 10 units of DNA polymerase-I (New England Biolabs LTD., Mississauga, ONT.) for one hour at 37°C. The PCR products were purified as before and subcloned into the Sma I site of the pGEM-3zf cloning vector (Promega, Madison, WI) (Sambrook *et al.*, 1989).

Both sense and anti-sense strands were sequenced with the SP6 and T7 sequencing primers using the T7 DNA sequencing kit (Pharmacia, Baie d'Urfe, Quebec). The sequences obtained were compared with all of the immunoglobulin sequences in the GenBank and EMBL databases using the computer programs of the Genetic Computer Group (Devereux *et al.*, 1984).

RESULTS

Viral antigen specificity and RF assay of the hybridoma anti-CMV antibodies.

All four hybridoma antibodies were of the gamma isotype and were specific for CMV since all were shown to react with CMV-infected cells but not with cells infected with herpes simplex type 1 and 2, varicella-zoster, or Epstein-Barr virus (data not shown). Radioimmunoprecipitation was carried out to identify the specific viral proteins recognized by the HCV-63 and HCV-65 hybridoma antibodies (Figure 1). These antibodies recognized a viral protein with an apparent molecular weight of 68 kDa (lanes 2 and 4), corresponding to the viral pp65 lower matrix protein. This viral protein was also recognized by the HCV-2 and HCV-3 hybridoma antibodies (Larose *et al.*, 1991) and by human convalescent serum (lane 6). It was not, however, immunoprecipitated by the four hybridoma antibodies or by the convalescent serum when using mock-infected cells (lanes 3, 5 and 7 and Larose *et al.*, 1991). When the hybridoma antibodies were tested for neutralizing activity against the CMV strain AD-169, none showed significant neutralizing titers (Larose *et al.*, 1991; and data not shown).

None of the anti-CMV antibodies were found to possess any RF activity either by ELISA or by immunoblot assays.

Idiotype and V region epitope expression.

As summarized in Table 1, the four anti-CMV hybridoma antibodies were found to express a restricted number of RF-associated idiotypes and V region epitopes. These reactivities were specific since all four anti-CMV antibodies were negative when tested with at least 13 other anti-idiotype/ V region epitope reagents (data not shown). The B12, C7 and 17-109 epitopes, most commonly expressed by V_KIII sub-group polypeptides, were identified on the HCV-2 antibody whereas only the B12 and/or C7 epitopes were detected on the other three anti-CMV antibodies. The HCV-2, HCV-3 and HCV-65 hybridoma antibodies reacted with at least one of the anti-idiotypic antibodies (B6, D12 and G7) that had been generated against RFs expressing V_H3 H chains. These results demonstrated that the HCV-2 antibody appeared to be the most structurally similar

FIGURE 1. Autoradiogram of CMV proteins recognized by the HCV-63 and HCV-65 hybridoma antibodies. Human foreskin fibroblast cells were infected with human CMV (lanes 2, 4 and 6) or mock-infected (lanes 3, 5 and 7). Foreskin cells were labeled with [^{35}S] methionine, lysed, immunoprecipitated with the hybridoma antibodies and electrophoresed on a 10% SDS polyacrylamide gel under reducing conditions. Lanes 1 and 8: [^{14}C] standard proteins; lanes 2 and 3: HCV-63; lanes 4 and 5: HCV-65; lanes 6 and 7: human convalescent serum.

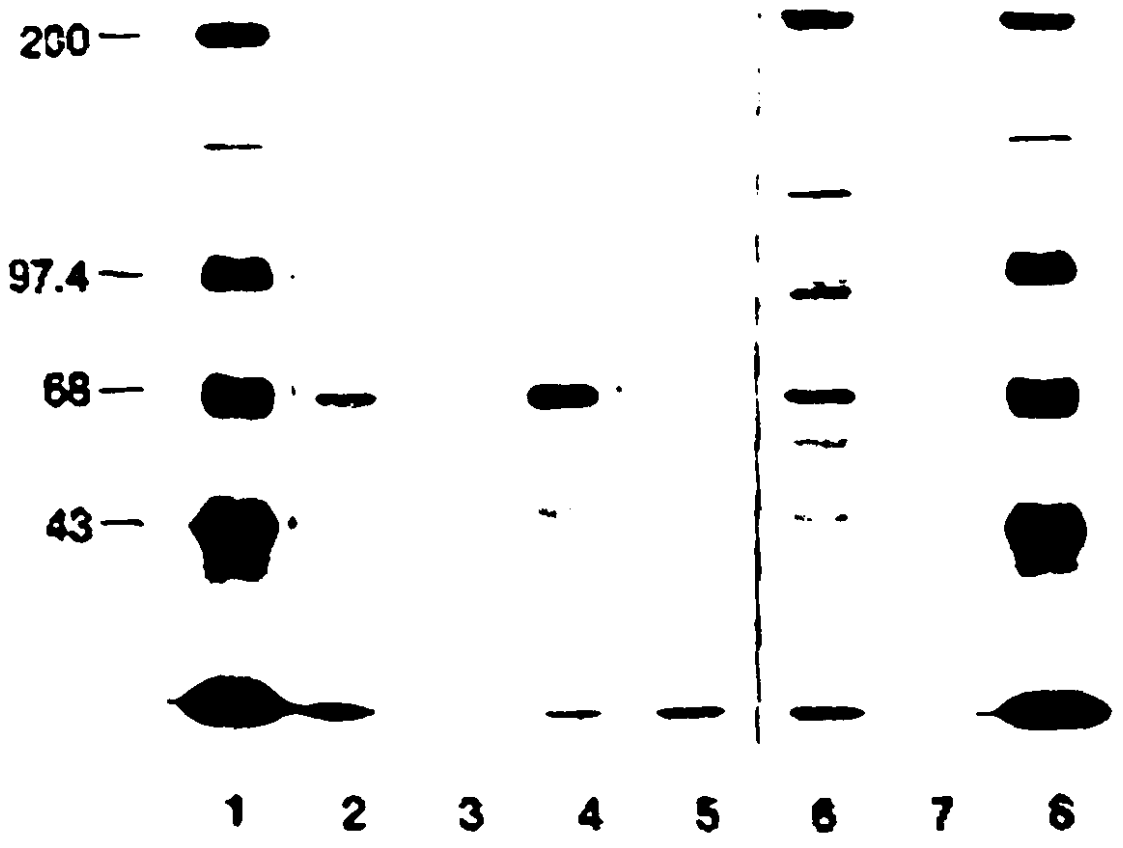

200
97.4
68
43
1
2
3
4
5
6
7
8

Table 1. Expression of rheumatoid factor-associated idiotypes and/or V region epitopes by four hybridoma antibodies directed against the human CMV.

Anti-Id name	Reference	ELISA Score of four anti-CMV Abs			
		HCV-2	HCV-3	HCV-63	HCV-65
a) Light chain determinants					
	Mageed et al. (1986)	+++	+	+	++
C7	Mageed et al. (1986)	++++	+	-	+
17-109	Carson and Fong (1983)	+	-	-	-
b) Heavy chain determinants					
B6	Crowley et al. (1990)	+	+	-	-
D12	Lydyard et al. (1990)	+	-	-	-
G7		+	+	-	+
c) Conformational determinants					
86.3	Gorevic and Frangione (1991)	++	-	-	-
102.2	Gorevic and Frangione (1991)	+	-	+	+

to the previously studied RFs, in particular those of the "Po" idiotypic family (Newkirk, 1992a; Klapper and Capra, 1976; Capra and Kehoe, 1974).

H chain V regions

The nucleotide and predicted amino acid sequences of the V_H genes expressed by the four anti-CMV hybridomas are shown in Figure 2 and are aligned with their putative germline genes. Three of the four hybridomas have rearranged gene segments from the V_H3 family and the fourth, HCV-63, from the V_H4 family. The V_H3 segment expressed by the HCV-2, HCV-3 and HCV-65 hybridomas had the greatest identity to the previously described germline genes V_H26 (92.0%), WHG26 (93.7%) and WHG16 (92.8%), respectively (Matthysens and Rabbits, 1980; Chen *et al.*, 1988; Kuppers *et al.*, 1992). The V_H gene segment expressed by the HCV-63 hybridoma was found to be 96.0% identical to the V71-4 germline gene, a member of the small V_H4 family (Kodaira *et al.*, 1986). When the three V_H3 heavy chains were compared, the amino acid identities ranged from 69% to 79%. The HCV-63 amino acid sequence on the other hand, derived from a V_H4 gene, was no more than 48% identical to the three V_H3-encoded anti-pp65 antibody H chains.

The assignment of the putative germline genes allowed an estimation of the somatic mutation events which likely occurred during the maturation of the respective B-lymphocytes. This analysis predicted a greater frequency of somatic mutations in the CDRs than in the FRs, and that the majority of the mutations in the CDRs resulted in amino acid changes, whereas most in the FRs were silent (Figure 2 and data not shown).

The greatest evidence for selection of replacement mutations was in the CDR2 of the HCV-65 and HCV-2 antibodies where there were, respectively, 8 and 9 times more replacement mutations than silent mutations.

H chain CDR3 regions.

When comparing the sequences from the four hybridoma antibodies, the greatest divergence was found in the CDR3 of the heavy chains (Figure 3). The four CDR3 regions differed not only in sequence, but also in length. The length of the CDR3s varied

FIGURE 2. The nucleotide and predicted amino acid sequences of the V_H segments expressed by the four hybridoma anti-CMV antibodies. These sequences are available under Genome Sequence Database (GSDB) accession numbers L26906 (HCV-2), L26903 (HCV-3), L26904 (HCV-63) and L26905 (HCV-65). The V_H26 (Chen *et al.*, 1988), WHG26 (Kuppers *et al.*, 1992), WHG16 (Kuppers *et al.*, 1992) and V71-4 (Kodaira *et al.*, 1986) germline genes are shown aligned to the hybridoma sequences to which they are most homologous. In this and subsequent figures, dashed lines indicate sequence identity between the germline genes and their respective hybridoma sequences whereas dots represent gaps between the different sequences. In addition, the complementarity determining regions (CDRs) are indicated and the amino acid positions are numbered according to Kabat *et al.* (1991).

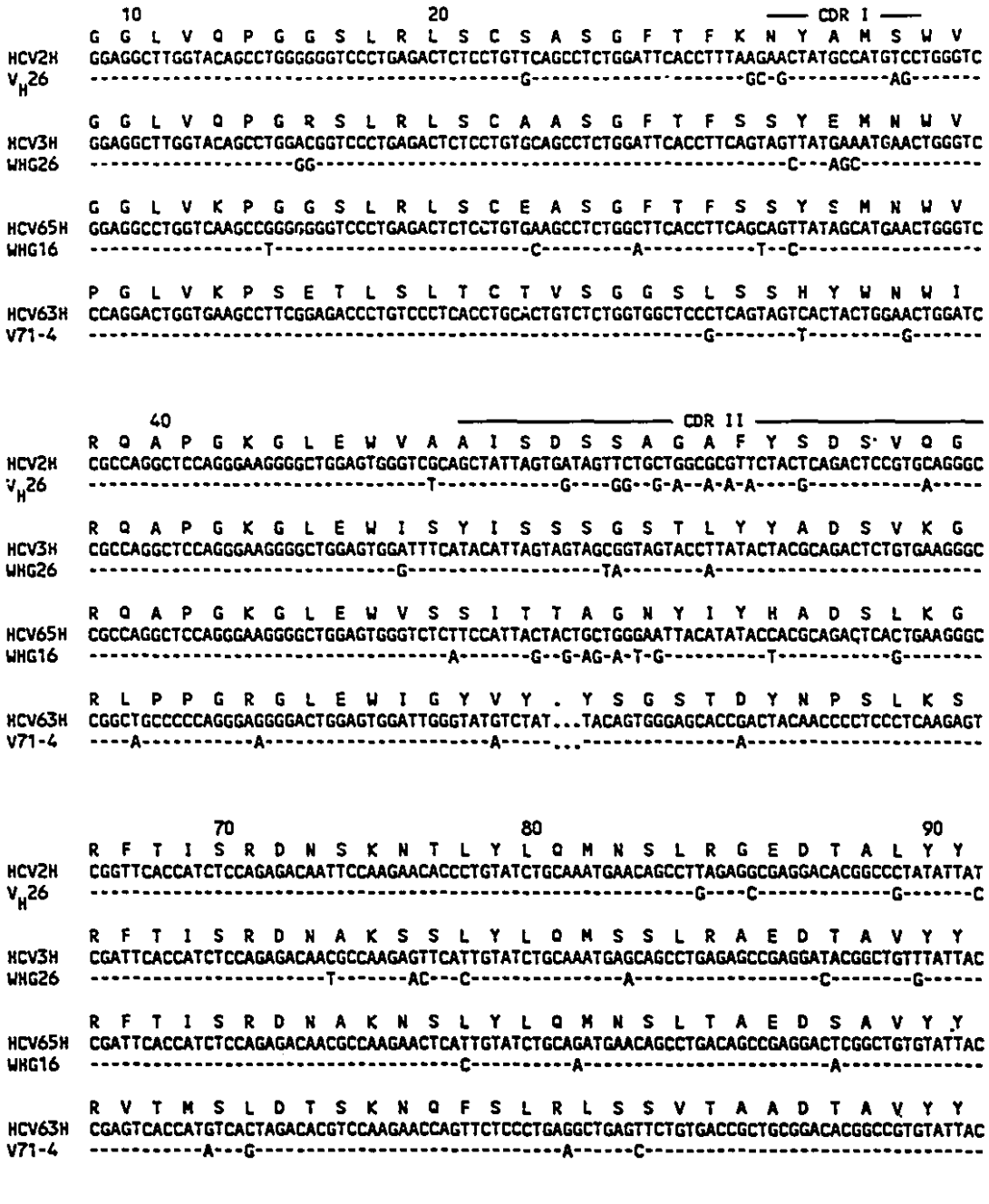
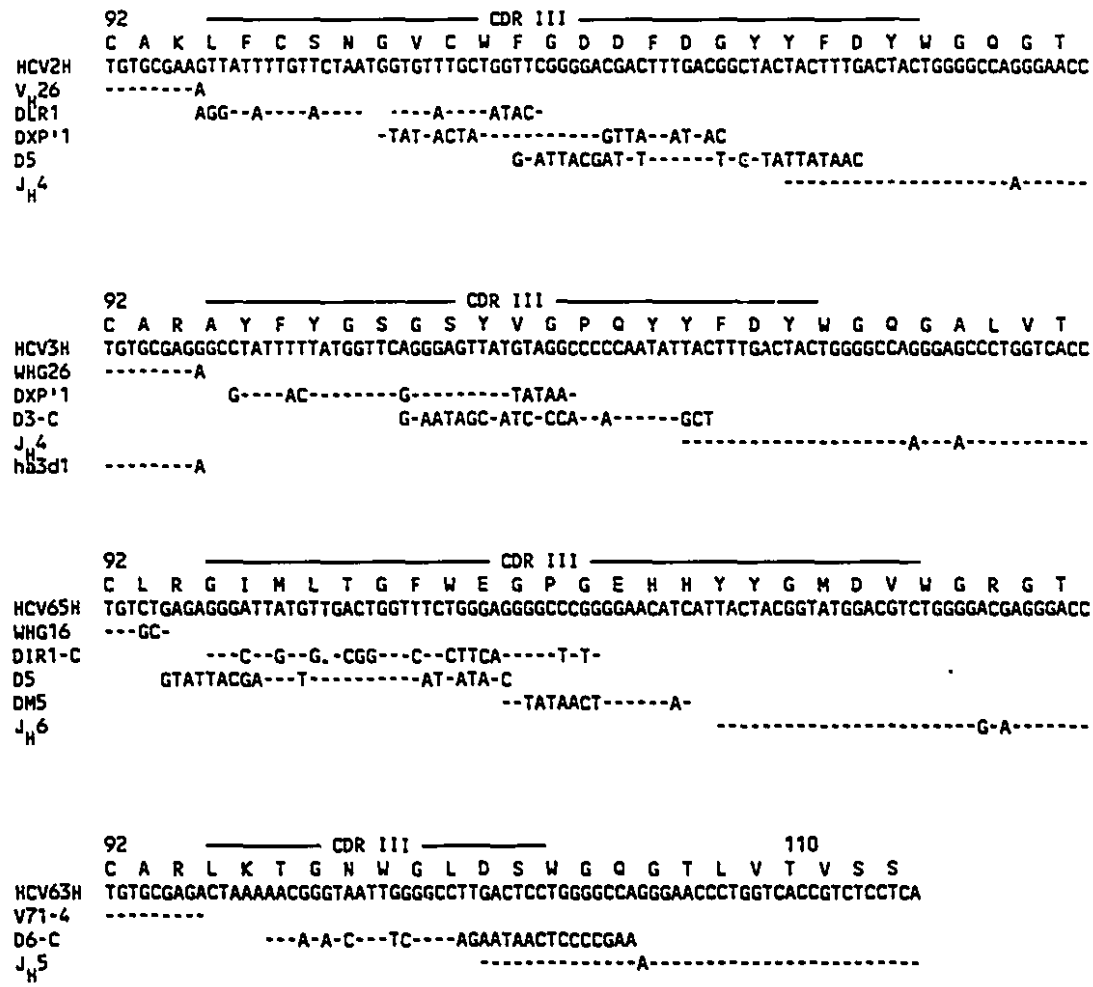

10
20
CDR I
G G L V Q P G G S L R L S C S A S G F T F K N Y A M S W V
HCV2H GGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTTCAGCCTCTGGATTCACCTTTAAGAACTATGCCATGTCCTGGGTC
V_H26 ----------G-------GC-G----------AG-------
G G L V Q P G R S L R L S C A A S G F T F S S Y E M N W V
HCV3H GGAGGCTTGGTACAGCCTGGACGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGTTATGAAATGAACTGGGTC
WHG26 ----------GG----------C---AGC----------
G G L V K P G G S L R L S C E A S G F T F S S Y S M N W V
HCV65H GGAGGCCTGGTCAAGCCGGGGGGGTCCCTGAGACTCTCCTGTGAAGCCTCTGGCTTCACCTTCAGCAGTTATAGCATGAACTGGGTC
WHG16 ----------T----------C---------A----------T--C----------
P G L V K P S E T L S L T C T V S G G S L S S H Y W N W I
HCV63H CCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCCTCAGTAGTCACTACTGGAACTGGATC
V71-4 ----------G--------T---------G-------
40
CDR II
R Q A P G K G L E W V A A I S D S S A G A F Y S D S V Q G
HCV2H CGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCGCAGCTATTAGTGATAGTTCTGCTGGCGCGTTCTACTCAGACTCCGTGCAGGGC
V_H26 ----------T----------G----GG--G-A--A-A-A----G----------A-----
R Q A P G K G L E W I S Y I S S S G S T L Y Y A D S V K G
HCV3H CGCCAGGCTCCAGGGAAGGGGCTGGAGTGGATTTCATACATTAGTAGTAGCGGTAGTACCTTATACTACGCAGACTCTGTGAAGGGC
WHG26 ----------G----------TA--------A----------
R Q A P G K G L E W V S S I T T A G N Y I Y H A D S L K G
HCV65H CGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCTTCCATTACTACTGCTGGGAATTACATATACCACGCAGACTCACTGAAGGGC
WHG16 ----------A-------G--G-AG-A-T-G----------T----------G--------
R L P P G R G L E W I G Y V Y . Y S G S T D Y N P S L K S
HCV63H CGGCTGCCCCCAGGGAGGGGACTGGAGTGGATTGGGTATGTCTAT...TACAGTGGGAGCACCGACTACAACCCCTCCCTCAAGAGT
V71-4 ----A----------A----------A-----...----------A----------
70
80
90
R F T I S R D N S K N T L Y L Q M N S L R G E D T A L Y Y
HCV2H CGGTTCACCATCTCCAGAGACAATTCCAAGAACACCCTGTATCTGCAAATGAACAGCCTTAGAGGCGAGGACACGGCCCTATATTAT
V_H26 ----------G----C----------G-------C
R F T I S R D N A K S S L Y L Q M S S L R A E D T A V Y Y
HCV3H CGATTCACCATCTCCAGAGACAACGCCAAGAGTTCATTGTATCTGCAAATGAGCAGCCTGAGAGCCGAGGATACGGCTGTTTATTAC
WHG26 ----------T-------AC---C----------A----------C--------G------
R F T I S R D N A K N S L Y L Q M N S L T A E D S A V Y Y
HCV65H CGATTCACCATCTCCAGAGACAACGCCAAGAACTCATTGTATCTGCAGATGAACAGCCTGACAGCCGAGGACTCGGCTGTGTATTAC
WHG16 ----------C----------A----------A----------
R V T M S L D T S K N Q F S L R L S S V T A A D T A V Y Y
HCV63H CGAGTCACCATGTCACTAGACACGTCCAAGAACCAGTTCTCCCTGAGGCTGAGTTCTGTGACCGCTGCGGACACGGCCGTGTATTAC
V71-4 ----------A---G----------A------C----------

FIGURE 3. The nucleotide and predicted amino acid sequences of the H chain CDR3s of the four hybridoma anti-CMV antibodies. The most homologous D_H (Ichihara *et al.*, 1988; Bakhshi *et al.*, 1987; Siebenlist *et al.*, 1981; Matsuda *et al.*, 1990) and J_H (Ravetch *et al.*, 1981) genes are shown aligned to each of the hybridoma sequences. The "-C" suffix denotes that the reverse complementarity strand of the particular D_H gene is shown.


92 CDR III
C A K L F C S N G V C W F G D D F D G Y Y F D Y W G Q G T
HCV2H TGTGCGAAGTTATTTTGTTCTAATGGTGTTTGCTGGTTCGGGGACGACTTTGACGGCTACTACTTTGACTACTGGGGCCAGGGAACC
V_H26 --------A
DLR1 AGG--A----A---- ----A----ATAC-
DXP'1 -TAT-ACTA----------GTTA--AT-AC
D5 G-ATTACGAT-T------T-G-TATTATAAC
J_H4 ----------A------
92 CDR III
C A R A Y F Y G S G S Y V G P Q Y Y F D Y W G Q G A L V T
HCV3H TGTGCGAGGGCCTATTTTTATGGTTCAGGGAGTTATGTAGGCCCCCAATATTACTTTGACTACTGGGGCCAGGGAGCCCTGGTCACC
WHG26 --------A
DXP'1 G----AC--------G---------TATAA-
D3-C G-AATAGC-ATC-CCA--A------GCT
J_H4 ----------A---A----------
hd3d1 --------A
92 CDR III
C L R G I M L T G F W E G P G E H H Y Y G M D V W G R G T
HCV6SH TGTCTGAGAGGGATTATGTTGACTGGTTTCTGGGAGGGGCCCGGGGAACATCATTACTACGGTATGGACGTCTGGGGACGAGGGACC
WHG16 ---GC-
DIR1-C ---C--G--G.-CGG---C--CTTCA-----T-T-
D5 GTATTACGA---T----------AT-ATA-C
DM5 --TATAACT------A-
J_H6 ----------G-A-------
92 CDR III 110
C A R L K T G N W G L D S W G Q G T L V T V S S
HCV63H TGTGCGAGACTAAAAACGGGTAATTGGGGCCTTGACTCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
V71-4 ---------
D6-C ---A-A-C---TC----AGAATAACTCCCCGAA
J_H5 ----------A----------

from 10 to 21 amino acids and the contribution of the J_H segment was either 2, 4 or 6 amino acids. The remaining residues (8 to 17) were either encoded by D_H or DIR genes, or contributed by N segment addition.

The HCV-63 antibody had the smallest CDR3, with eight amino acids encoded by a single D segment, with some possible N segment addition, and two amino acids encoded by the J_H segment. The reverse complementarity strand of the D6 gene (Bakhshi *et al.*, 1987) had the highest homology to this CDR3 sequence and appeared to be joined to a J_H5 gene segment that had a single silent mutation (Ravetch *et al.*, 1981). The HCV-3 antibody had a longer CDR3 region of 18 amino acids. The majority of this sequence probably originated from a double D joining of the DXP'1 and D3 segments, with the second D being inverted (Ichihara *et al.*, 1988; Siebenlist *et al.*, 1981). The HCV-2 antibody had a very long CDR3 of 21 amino acids, which suggests that multiple D_H segments were fused together. Indeed, it would appear that as many as three D_H genes may have been rearranged. The sequence comparisons indicated that the DLR1 gene (Ichihara *et al.*, 1988) may have encoded the beginning of the CDR3 and that the rest could be encoded by the DXP'1 and D5 genes (Ichihara *et al.*, 1988; Bakhshi *et al.*, 1987). The HCV-65 clone also had a CDR3 of 21 amino acids where six of these were encoded by a J_H6 segment (Ravetch *et al.*, 1981). The 5' end of this CDR3 had high homology to both the D5 gene (Bakhshi *et al.*, 1987) and to the reverse complementarity strand of the DIR1 gene (Ichihara *et al.*, 1988) and thus either the D5 or the DIR1 could have been fused to the DM5 gene (Matsuda *et al.*, 1990) to generate this region. It is also possible that the D5 and DIR1 genes were fused together, considering the significant partial homology between these two sequences.

The average length of these four anti-pp65 antibody H chain CDR3s (52.5 nucleotides) was significantly longer than what has been reported for adult peripheral B-lymphocytes (average 31 nucleotides) (Sanz, 1991), and may be an important structural feature of these H chain variable regions.

L chain V regions

The nucleotide sequences of the L chain V regions expressed by the four hybridoma anti-CMV antibodies are shown aligned with their putative germline genes in figures 4 and 5. The HCV-2 antibody, the only hybridoma to express a kappa L chain, had rearranged a V_k gene segment from the V_kIIIa family that was 96.5% identical to the previously described V(g) germline gene (Pech and Zachau, 1984). The majority (7/8) of the nucleotide differences between this expressed sequence and the V(g) germline gene were located in the CDRs and most (6/7) resulted in amino acid changes (Figure 4 and data not shown).

The complete nucleotide sequences of the variable regions of the three lambda-bearing hybridomas HCV-3, HCV-63 and HCV-65 are shown in Figure 5. The HCV-3 and HCV-63 antibodies both express light chain genes from the lambda-1 family and are respectively 94% to 95% identical to the V_λ1.2 germline gene nucleotide sequence (Bernard *et al.*, 1990). The third lambda chain, expressed by the HCV-65 hybridoma, was found to be from the lambda-3 family. Even though this L chain sequence was only 79% identical to the V_λ3.1 germline gene (Combriato and Klobeck, 1991), it was 93% identical to the cML13 clone which has been classified as part of the V_λIIIb subgroup (Combriato and Klobeck, 1991). All three hybridomas were found to have rearranged the J_λ2 gene (Udey and Blomberg, 1987). When the entire V_λ and J_λ segments of the three lambda chains were compared, amino acid sequence identities were 54%, 56% and 90%, respectively, for the following pairs: HCV-3 vs HCV-65, HCV-63 vs HCV-65 and HCV-3 vs HCV-63.

Although we were unable to assign a germline gene sufficiently identical to the HCV-65 lambda sequence, the putative germline genes identified for the HCV-3 and HCV-63 enabled us to estimate the somatic mutations which may have occurred in these lambda chains. It would appear that both the HCV-3 and HCV-63 L chains have a pattern of mutations in their CDRs that is compatible with positive antigen selection (Schlomchik *et al.*, 1987).

FIGURE 4. The nucleotide and predicted amino acid sequences of the kappa L chain expressed by the HCV-2 antibody. The putative germline V_K gene, V(g) (Pech and Zachau, 1984), and the reported sequence for the J_K4 (Hieter *et al.*, 1982) gene are shown aligned to the HCV-2 sequence (GSDB accession number L26899). Also shown, for comparison, is the L chain sequence Humka3d1 (Olee *et al.*, 1992) expressed by the D1 hybridoma which produces a self-associating IgG RF.

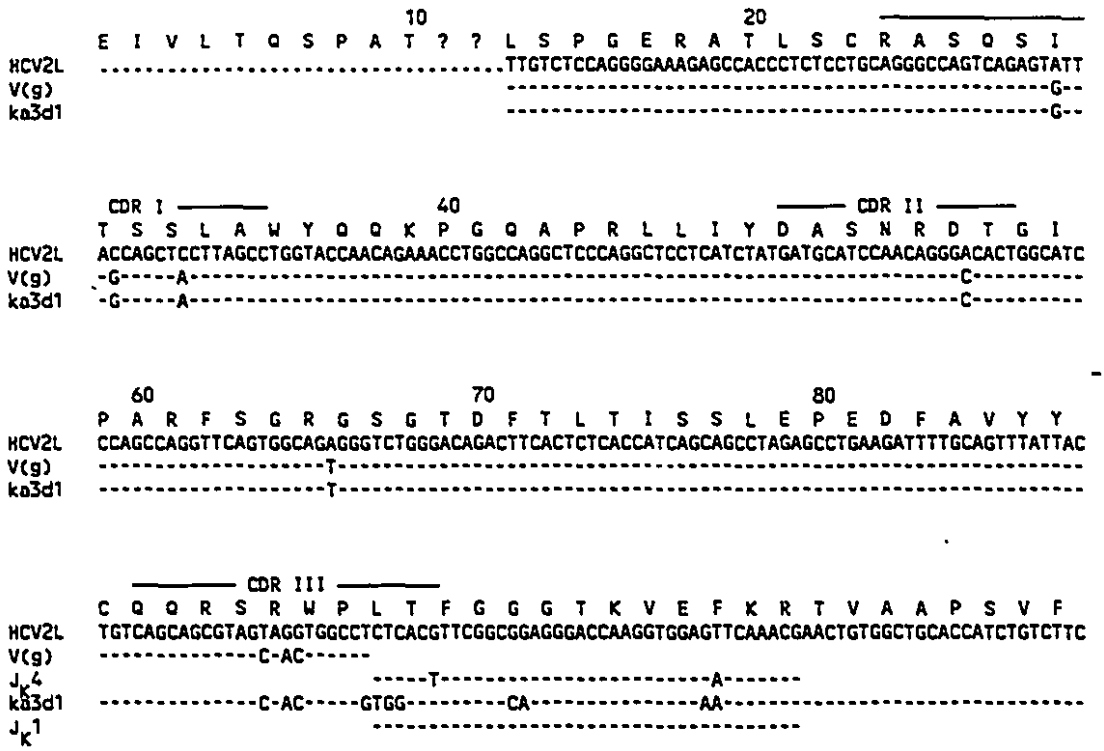
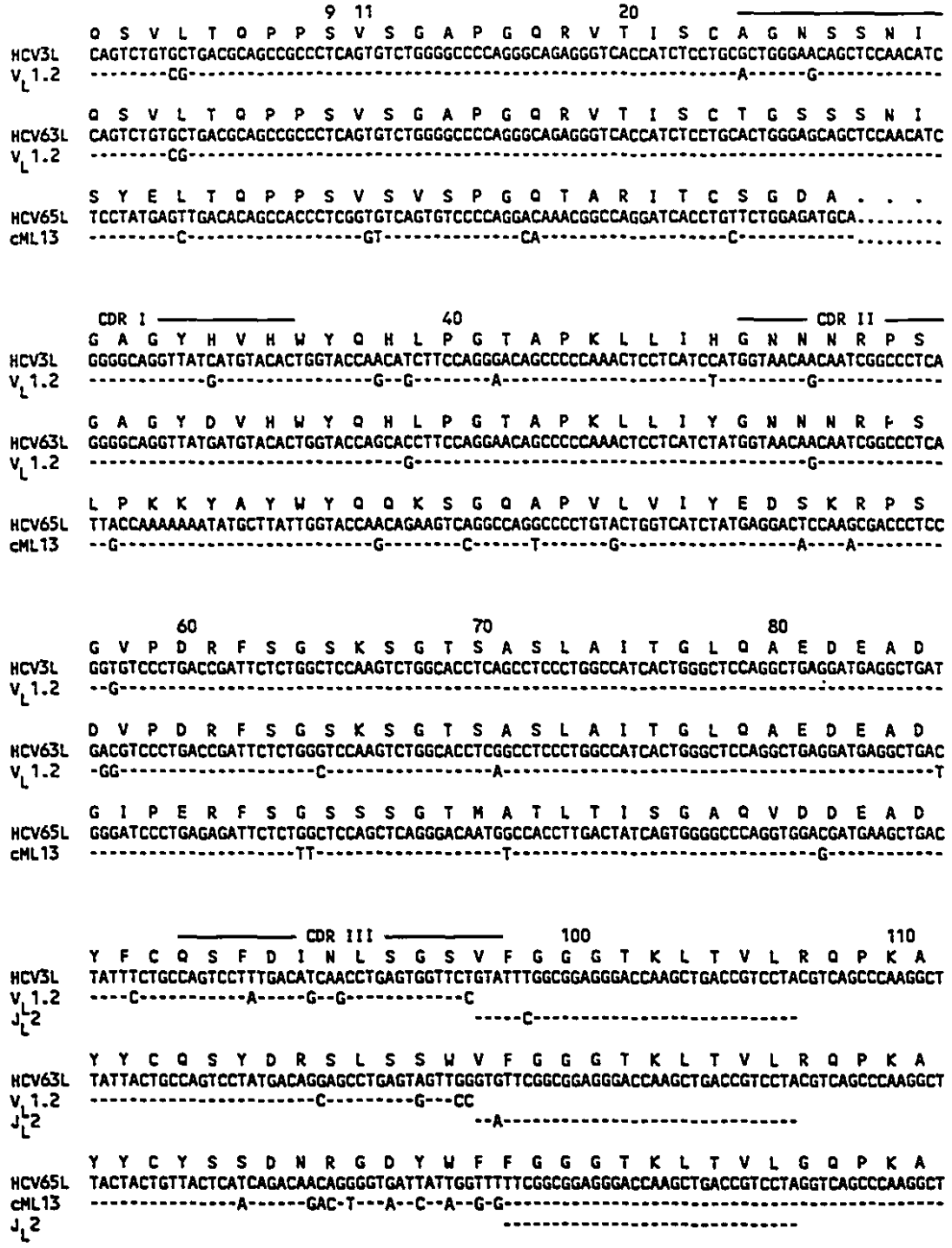

10
20
E I V L T Q S P A T ? ? L S P G E R A T L S C R A S Q S I
HCV2L .....TTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATT
Vκ(g) ----------G--
κa3d1 ----------G--
CDR I
40
CDR II
T S S L A W Y Q Q K P G Q A P R L L I Y D A S N R D T G I
HCV2L ACCAGCTCCTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGACACTGGCATC
Vκ(g) -G-----A----------C----------
κa3d1 -G-----A----------C----------
60
70
80
P A R F S G R G S G T D F T L T I S S L E P E D F A V Y Y
HCV2L CCAGCCAGGTTCAGTGGCAGAGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTAC
Vκ(g) ----------T----------
κa3d1 ----------T----------
CDR III
C Q Q R S R W P L T F G G G T K V E F K R T V A A P S V F
HCV2L TGTCAGCAGCGTAGTAGGTGGCCTCTCACGTTCGGCGGAGGGACCAAGGTGGAGTTCAAACGAACTGTGGCTGCACCATCTGTCTTC
Vκ(g) ----------C-AC------
Jκ4 -----T----------A-------
κa3d1 ----------C-AC-----GTGG---------CA----------AA----------
Jκ1 ----------

FIGURE 5. The nucleotide and predicted amino acid sequences of the lambda L chains expressed by the HCV-3, HCV-63 and HCV-65 hybridoma antibodies. These sequences are available under GSDB accession numbers L26900 (HCV-3), L26901 (HCV-63) and L26902 (HCV-65). The $V_l1.2$ germline gene (Bernard *et al.*, 1990) is the putative germline gene encoding the HCV-3 and HCV-63 antibodies and is shown aligned to these sequences. The HCV-65 sequence is shown aligned with the cML13 clone (Combriato and Klobeck, 1991) which is the most homologous sequence in the GenBank and EMBO databases. The cML13 is a rearranged sequence from a human spleen and is included for comparison because no appropriate germline gene could be found.


9 11 20
Q S V L T Q P P S V S G A P G Q R V T I S C A G N S S N I
HCV3L CAGTCTGTGCTGACGCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGAGGGTCACCATCTCCTGCGCTGGGAACAGCTCCAACATC
V_L1.2 --------CG----------A------G----------
Q S V L T Q P P S V S G A P G Q R V T I S C T G S S S N I
HCV63L CAGTCTGTGCTGACGCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGAGGGTCACCATCTCCTGCACTGGGAGCAGCTCCAACATC
V_L1.2 --------CG----------
S Y E L T Q P P S V S V S P G Q T A R I T C S G D A . . .
HCV65L TCCTATGAGTTGACACAGCCACCCTCGGTGTCAGTGTCCCCAGGACAAACGGCCAGGATCACCTGTTCTGGAGATGCA.....
cML13 ---------C----------GT----------CA----------C----------.....
CDR I 40 CDR II
G A G Y H V H W Y Q H L P G T A P K L L I H G N N N R P S
HCV3L GGGGCAGGTTATCATGTACACTGGTACCAACATCTTCCAGGGACAGCCCCCAAACTCCTCATCCATGGTAACAACAATCGGCCCTCA
V_L1.2 ----------G----------G--G--------A----------T---------G----------
G A G Y D V H W Y Q H L P G T A P K L L I Y G N N N R P S
HCV63L GGGGCAGGTTATGATGTACACTGGTACCAGCACCTTCCAGGAACAGCCCCCAAACTCCTCATCTATGGTAACAACAATCGGCCCTCA
V_L1.2 ----------G----------G----------
L P K K Y A Y W Y Q Q K S G Q A P V L V I Y E D S K R P S
HCV65L TTACCAAAAAAAATATGCTTATTGGTACCAACAGAAGTCAGGCCAGGCCCCTGTACTGGTCATCTATGAGGACTCCAAGCGACCCTCC
cML13 --G----------G---------C------T-------G----------A----A---------
60 70 80
G V P D R F S G S K S G T S A S L A I T G L Q A E D E A D
HCV3L GGTGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCACTGGGCTCCAGGCTGAGGATGAGGCTGAT
V_L1.2 --G----------
D V P D R F S G S K S G T S A S L A I T G L Q A E D E A D
HCV63L GACGTCCCTGACCGATTCTCTGGGTCCAAGTCTGGCACCTCGGCCTCCCTGGCCATCACTGGGCTCCAGGCTGAGGATGAGGCTGAC
V_L1.2 -GG----------C----------A----------T
G I P E R F S G S S S G T M A T L T I S G A Q V D D E A D
HCV65L GGGATCCCTGAGAGATTCTCTGGCTCCAGCTCAGGGACAATGGCCACCTTGACTATCAGTGGGGCCCAGGTGGACGATGAAGCTGAC
cML13 ----------TT----------T----------G----------
CDR III 100 110
Y F C Q S F D I N L S G S V F G G G T K L T V L R Q P K A
HCV3L TATTTCTGCCAGTCCTTTGACATCAACCTGAGTGGTTCTGTATTTGGCGGAGGGACCAAGCTGACCGTCCTACGTCAGCCCAAGGCT
V_L1.2 ----C----------A-----G--G----------C
J_L2 -----C----------
Y Y C Q S Y D R S L S S W V F G G G T K L T V L R Q P K A
HCV63L TATTACTGCCAGTCCTATGACAGGAGCCTGAGTAGTTGGGTGTTCGGCGGAGGGACCAAGCTGACCGTCCTACGTCAGCCCAAGGCT
V_L1.2 ----------C---------G---CC
J_L2 --A----------
Y Y C Y S S D N R G D Y W F F G G G T K L T V L G Q P K A
HCV65L TACTACTGTTACTCATCAGACAACAGGGGTGATTATTGGTTTTTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTCAGCCCAAGGCT
cML13 ----------A------GAC-T---A--C--A--G-G----------
J_L2 ----------

Structural comparisons with human RFs.

The HCV-2 antibody, which we found to express idiotypic and epitopic determinants characteristic of the "Po" family of RFs, also had H and L chain sequence similarities to this group of autoantibodies. Table 2 summarizes the sequence similarities between the HCV-2 anti-CMV antibody and the prototype paraprotein of the "Po" idiotypic family (POM), as well as to two related paraproteins with RF activity; for which the amino acid sequences are available (Klapper and Capra, 1976; Capra and Kehoe, 1974; Newkirk, 1992a). The degree of identity found between HCV-2 and the "Po" RFs was equivalent to that found within the group of RFs.

Subsequent comparison of the four anti-CMV antibodies, to all rearranged Ig sequences found in the GenBank and EMBL databases, revealed that the majority had the greatest identity to either RFs or fetal-derived antibodies (Table 3). The most significant similarity was found between two anti-CMV antibody sequences and the D1 IgG self-associating RF (Olee *et al.*, 1992). Specifically, the HCV-2 L chain and the HCV-3 H chain had greater than 93% identity to the D1 RF sequences. Both the anti-viral antibody and the RF L chains appear to be encoded by the Vg gene, although predicted mutations led to nine nucleotide differences between these two sequences. The majority of these differences (8/9), however, were located in the CDRs and resulted in seven amino acid differences (Figure 2).

Both the HCV-3 and the D1 H chains appear to be encoded by the same germline gene, having 93.7% and 99.6% identity, respectively, to the WHG26 gene. The majority (6/9) of amino acid differences between the V_H segments expressed by the RF and anti-CMV antibody are located in the CDR1 and CDR2. Both of these antibodies have rearranged a J_H4 gene but differ in the length of their CDR3s. The D1 H chain has a CDR3 of eight amino acids, whereas the CDR3 of the HCV-3 antibody is 18 amino acids long. The presence of an Asp and multiple Gly residues was common to these two CDR3 regions; however, the presence of a Pro and a considerably greater number of Tyr residues in the HCV-3 antibody distinguished these two variable regions (data not shown). No information was available regarding RF-asociated idiotype or V-region epitope expression of the D1 RF.

Table 2. Comparison of the L and H chain sequences of the HCV-2 anti-CMV hybridoma antibody to human paraproteins of the "Po" idiotypic family.

		% AMINO ACID IDENTITY							
		L chain V gene				H chain V gene			
	(M/G)[1]	POM	RIV	SFL	HCV-2	POM	RIV	SFL	HCV-2
POM	(M)	100				100			
RIV	(M)	82	100			79	100		
SFL	(G)	82	98	100		79	87	100	
HCV-2	(G)	81	90	89	100	76	79	82	100

[1] The heavy chain isotype of each antibody is indicated as either mu (M) or gamma (G).

Table 3. Summary of published antibodies with the greatest homology to the anti-CMV antibodies.

Anti-pp65 Antibody		Most homologous human antibody sequences			
		Antibody	Specificity	Identity	Reference
HCV-2	Kappa	D1	RF	94.6%	Olee et al. (1992)
	Gamma	30P1	fetal	91.9%	Schroeder et al. (1987).
HCV-3	Lambda	cML18	unknown	92.2%	Combriato & Klobeck (1991)
		L1	RF	87.6%	Olee et al. (1992)
	Gamma	D1	RF	93.3%	Olee et al. (1992)
HCV-63	Lambda	L1	RF	85.0%	Olee et al. (1992)
	Gamma	58P2	fetal	95.9%	Schroeder et al. (1987)
		mAB63	RF	90.0%	Harindranath et al. (1991)
HCV-65	Lambda	cML13	unknown	93.0%	Combriato & Klobeck (1991)
	Gamma	D1	RF	88.5%	Olee et al. (1992)

In these two examples, and in others not shown, it was apparent that similar or identical germline gene usage could be found for RFs and anti-CMV antibodies. In addition, amino acid sequence differences were evident but were found to be restricted primarily to the CDRs, with the greatest differences being located in the H chain CDR3.

DISCUSSION

Four human hybridoma antibodies directed against the pp65 phosphoprotein of the human CMV have been characterized with respect to their Ig gene usage and their expression of determinants previously associated with human RFs. Surprisingly little information is currently available about the human antibody repertoire to viral pathogens and, although a number of recent reports have provided information on antibodies of different viral specificities (Newkirk *et al.*, 1988; Andris *et al.*, 1992; Andris *et al.*, 1991; Felgenhauer *et al.*, 1990; Scott *et al.*, 1991; Ikematsu *et al.*, 1993; Huang *et al.*, 1992), none have focussed on antibodies that all react with the same viral protein.

Our structural analysis of the four anti-pp65 antibodies revealed that each expressed a unique set of RF-associated idiotypes and V region epitopes. Although the precise structures recognized by the monoclonal anti-idiotypic/epitopic reagents are not known, some of these antibodies are believed to recognize determinants within the antigen-binding cleft. These results suggest that the three-dimensional structure of the variable regions of each antibody is also unique and, therefore, we would predict that the four antibodies recognize different epitopes on the viral pp65 protein.

The observation that three out of four anti-CMV antibodies co-expressed the light chain determinants recognized by the C7 and B12 antibodies is consistent with the previous finding that these reagents had similar, but not identical, patterns of binding to a panel human RFs (Mageed *et al.*, 1986). Since the expression of these two epitopes was previously found to be associated with V_KIII-bearing RFs, it was not surprising to detect the greatest expression on HCV-2, the only V_KIII L chain among the four anti-CMV antibodies. Although the exact determinants recognized by the C7 and B12 reagents are not known, it appears that related structures are found on antibodies not expressing kappa L chains, as attested to by the reactivity with the HCV-3, HCV-63 and

HCV-65 antibodies and concurs with the findings of a previous report (Mageed *et al.*, 1986). It is possible that the lower reactivity on non-V_KIII L chains may be the result of somatic mutation (Lydyard *et al.*, 1990) creating a structure that is cross-reactive with the original epitope recognized by the mouse mAb.

The HCV-2, HCV-3 and HCV-65, all V_H3-bearing antibodies, were shown to express at least one determinant previously associated with RFs that utilize V_H3 H chains. Since the immunogens used to generate the B6, D12 and G7 monoclonal reagents were all from the V_H3 family, the most likely explanation for the lack of detection of any RF-associated H chain idiotypes on the HCV-63 antibody is that its H chain is derived from a gene belonging to the V_H4 family. It is interesting that the conformational determinant recognized by the 102.2 antibody is present on three of the four anti-CMV antibodies, since this determinant was thought to be restricted to IgM RFs.

It is important to note that the majority of the reports characterizing the monoclonal anti-idiotypic and anti-epitopic reagents used in this study have indicated that the expression of these determinants does not necessarily confer RF activity (Mageed *et al.*, 1986; Lydyard *et al.*, 1990; Abderrazik *et al.*, 1992). Indeed, none of the four anti-CMV antibodies possessed detectable RF activity. In addition, the presence of these determinants has been reported not only in polyclonal sera from RA patients but from healthy individuals as well (Shokri *et al.*, 1991). Taken together, these results suggest that several of the anti-idiotypic/epitopic monoclonal reagents are more specific for V region amino acid sequences than for actual antigen-binding specificity. For this reason, these reagents have proven to be valuable tools for demonstrating restricted gene usage (notably, certain V_KIII, V_H1 and V_H3 genes) of paraproteins and of early B-lymphocytes (Newkirk and Capra, 1987; Lydyard *et al.*, 1990). As demonstrated by this study, these monoclonal reagents are also very useful in identifying structurally similar antibodies from apparently distinct antibody populations. This was particularly evident when HCV-2, the anti-CMV antibody expressing the greatest number of RF-associated determinants, was shown to be structurally homologous to one of the major idiotypic groups of RFs.

The sequencing of the variable regions of both the L and H chains of these four human antibodies, specific for a single protein of CMV, has also allowed us to further examine the Ig gene usage in response to a specific exogenous antigen. It was observed that each anti-pp65 antibody expressed a unique V_L/V_H pair and, in agreement with the idiotype results, likely indicates different epitopic specificities for these antibodies. Although the variable region sequences for the anti-pp65 antibodies were diverse, the majority were most closely related to either RFs or fetally-derived rearranged clones.

In the case where the anti-CMV antibody sequences were found to derive from identical or similar germline genes to those used by an IgG RF, the predicted amino acid sequences were not identical. The majority of the amino acid differences between the sequences were attributable to somatic mutations in the CDRs of both the L and H chains of the anti-CMV antibodies and to genetic diversity in the H chain CDR3. The clinical correlations between the presence of CMV, or of antibodies directed against this virus, and circulating RFs (Einsele *et al.*, 1992; Baldwin *et al.*, 1987; Ferraro and Newkirk, 1993) may therefore be related to the structural similarities between the RF and anti-CMV antibody populations. It is of interest that polyreactive fetal clones have also been shown to express some of the cross-reactive epitopes examined in this study and, through mutation and selection, may potentially give rise to specific high-affinity clones (Lydyard *et al.*, 1990). One possibility, may be that the immune response to an immunodominant CMV protein is idiotypically and/or structurally connected to autoreactive clones, providing a link between the humoral anti-viral response and the production of RFs in autoimmunity.

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CHAPTER III

Molecular Characterization of Human Monoclonal Antibodies Specific for the Human Cytomegalovirus: Relationship of Variable Region Sequence to Antigen Specificity and Rheumatoid Factor-Associated Idiotype Expression.

PREFACE

The discovery of V region similarities between human anti-pp65 antibodies and human RFs, described in the previous chapter, warranted further examination of the relationship between these two antibody populations. Antibodies with known fine specificity for the pp65 antigen of CMV had been reported by Dr. Borrebaeck's group at The Lund University in Sweden. This chapter examines the V region sequence and idiotypic expression of these antibodies of defined epitopic specificity. Together with the results from the previous chapter, these results form the largest group of complete V region sequences of human hybridoma antibodies directed against a single antigen of a human pathogen.

SUMMARY

Human hybridoma antibodies, specific for the viral matrix protein pp65 of the human cytomegalovirus (CMV), were studied in order to gain insight into the relationship between variable region primary structure and antigen specificity of human immunoglobulins directed against exogeneous antigens. The four anti-pp65 antibodies MO53, MO58, MO61 and MO81 expressed different heavy and light chain variable region gene combinations, respectively: $V_\kappa I/V_H 3$, $V_\kappa I/V_H 1$, $V_\lambda I/V_H 3$ and $V_\kappa I/V_H 4$. Three of the four antibodies expressed a limited number of rheumatoid factor (RF) -associated idiotypes. Five of the heavy and light chain variable regions also had sequence similarities to these autoantibodies, but none had any detectable RF activity.

Comparisons of the anti-pp65 sequences to known germline and rearranged immunoglobulin sequences revealed important mechanisms responsible for the generation of the anti-viral specificity, as well as possible explanations for the epitopic fine specificities of these antibodies. Although putative somatic mutations were sometimes located in the heavy chain CDR2, the domain with the greatest potential for encoding epitopic specificity was the heavy chain CDR3. It was also observed that sequence differences in the heavy chain FR3 could potentially contribute to the antigen specificity of these anti-viral antibodies. It appears that structural similarities between anti-CMV antibodies and human RFs reflects the use of identical or highly homologous variable region germline gene usage.

INTRODUCTION

In humans, the humoral immune response leads to the production of antibodies capable of recognizing specific molecular structures. The specific recognition function of antibodies is a result of areas of hypervariability in the primary sequence of the light and heavy chain variable regions. From crystallographic studies, these complementarity determining regions (CDRs) have been shown to contain the majority of the amino acid residues which are in direct contact with antigen [1]. Much information is currently available regarding the somatic mechanisms involved in the generation of antibody variable regions. These mechanisms include rearrangement of multiple gene segments as well as hypermutation of these rearranged sequences. B lymphocytes expressing high affinity receptors, as a result of these mechanisms, are then selected by antigen and stimulated to proliferate within germinal centers [2].

It is clear that the first two CDRs of light and heavy chain variable regions are encoded, respectively, by either V_k or V_λ and V_H genetic elements. These genetic elements are found in distinct loci on chromosomes 2, 22 and 14 of the human genome [3-6]. The third CDR of light chains is almost exclusively encoded by the V segment in addition to one or two residues which are encoded by the J segment. In contrast, there exists multiple mechanisms responsible for the generation of the heavy chain CDR3, none of which include residues encoded by V_H segments [7]. As a result of these

mechanisms, this region has the greatest variability in length and sequence which has important consequences regarding antibody specificity [reviewed in ref. 8].

In contrast, what is still poorly understood is how this sequence variability translates into specific structure of the antibody combining site. The majority of information concerning variable region sequence and antigen specificity is derived from studies of mouse monoclonal antibodies. Due to the increasing amount of evidence that monoclonal antibodies of human origin are superior immunotherapeutic agents, as compared to murine antibodies [9], it is important to understand the antibody sequence/antigen specificity relationship for human immunoglobulins. This structural information, however, has largely been limited to human antibodies with autoreactivities. For this reason, we have been interested in the human humoral response to exogeneous antigens. Specifically we have studied four human monoclonal antibodies specific for the pp65 viral matrix protein of a ubiquitous human pathogen, the human cytomegalovirus (CMV). The precise antigenic specificities of the hybridoma antibodies, analyzed in the present study, have previously been reported [10]. It was therefore of interest to determine the primary sequence of the variable regions of these well characterized human antibodies, and then correlate with fine antigenic specificity. In addition, the variable region sequences obtained for the anti-pp65 antibodies were analyzed in relation to all available human immunoglobulin sequences including other human anti-viral antibodies, as well as human antibodies with autoreactivity. We were particularly interested in the relationship of the anti-CMV antibodies to rheumatoid factors (RFs), autoantibodies found in the majority patients with rheumatoid arthritis (RA) [11]. This interest stems from reports of the potential pathogenic role of CMV in RA [12], the correlation of anti-CMV antibodies with RFs in RA patients [13] and our prior findings of structural similarity of anti-CMV antibodies to human RFs [14,15]. The results obtained have increased our understanding of the relationship which exists between variable region gene sequence and the specificity of antigenic binding sites of human antibodies.

MATERIALS AND METHODS

Cell lines

The MO53, MO58, MO61 hybridoma cell lines were established as previously described [10]. Briefly, peripheral human B cells obtained from a CMV-seropositive donor were transformed with Epstein-Barr virus. Antigen-specific lymphoblastoid cells were subsequently fused to the human x mouse heterohybridoma K6H6/B5, and the hybrids were cloned at least twice prior to use. Similarly, the hybridoma MO81 was established by an identical procedure. This cell line produces an antibody identical to the previously described antibody MO79 [10].

Determination of antigen-specific reactivity

All four IgG1 hybridoma antibodies were demonstrated to specifically recognize the viral pp65 matrix protein [10]. All four hybridoma antibodies were subsequently tested for specific binding to tetanus toxoid, calf thymus histones, single- and double-stranded DNA, RNA, acid soluble collagen, porcine thyroglobulin, KLH and bovine insulin, as previously described [16].

The anti-pp65 antibodies were also tested for RF activity, both by an ELISA and by an immunoblot assay. In the ELISA assay [17], Immunolon-1 ELISA plates (Fisher Scientific, Montreal, Quebec) were coated overnight with purified human Fc fragments from an IgG myeloma protein at a concentration of 0.01 mg/ml. The plates were washed, purified "test" antibodies (0.010 mg/ml) were added and incubated at 37°C for 2 hours. Bound antibodies were either detected by the use of affinity-purified horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fragments of kappa chain-specific antisera (Protos Immunoresearch, San Fracisco, CA) or by affinity-purified biotinylated $F(ab')_2$ fragments of lambda chain-specific antisera (E.Y. Laboratories, San Mateo, CA) followed by HRP-conjugated avidin-D (Vector Laboratories, Burlingame, CA). All assays included positive and negative control antibodies, in addition to wells with PBS/Tween alone. Optical densities (O.D.) at 492 nm were determined using an ELISA plate reader (SLT Labinstruments, model EAR 400RT, Fisher Scientific, Montreal, Quebec).

The immunoblot assay involved the separation of human IgG Fc fragments by sodium dodecyl (SDS) polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose, followed by incubation with the anti-pp65 or control antibodies and then detection of bound antibodies as previously described [18].

RF-associated idiotype expression

The anti-RF mouse monoclonal antibody reagents used in this study were generated against several different human RFs and have been shown to recognize V region determinants on these RFs. Specific information regarding their production, specificity and prevalence of expression on human RFs has previously been described [17, 19-22]. In the current study, the expression of RF-associated idiotypes or V-region subgroup epitopes was determined for each anti-CMV antibody by a previously described ELISA [23].

The background staining was determined for each anti-idiotype/anti-V region monoclonal reagent using PBS/Tween, instead of hybridoma antibody, and was subtracted from the respective O.D. values for the test antibodies. Positive controls for idiotype/ V region epitope expression included the EV1.15 anti-CMV antibody [14], and the BOR [23] and SCZ (clinical sample from a patient with mixed essential cryoglobulinemia was kindly provided by Dr. D. Danoff, Montreal General Hospital) human RFs. The results, with the background subtracted, are represented by the following scores:

-, $O.D._{492}$ <0.10; 1, $O.D._{492}$ >0.10-0.50; 2, $O.D._{492}$ >0.50-1.00; 3, $O.D._{492}$ >1.00-1.50.

PCR amplification of immunoglobulin V regions

Total cellular RNA was extracted from 10^5 hybridoma cells with guanidinium thiocyanate followed by centrifugation through a 5.7 M cushion of cesium chloride cushion. First strand cDNA was directly synthesized from total RNA using an oligo-dT primer as described by Larrick et al. [24]. Each cDNA preparation was subsequently amplified by the polymerase chain reaction [25] using a Perkin-Elmer thermal cycler

(model 480, Cetus Perkin Elmer, Emeryville, CA). The conditions for the amplifications are described by Larrick *et al.*, [24]. For the heavy chain of clone MO61, it was necessary to perform a second round of amplification using 0.001 ml from the first amplification reaction and 20 pmoles of each of the following primers:

Zu43b: 5'-TTCCTTGTTGC(AT)(AC)TTTTAAAAGGTGTC-3'
C_H1: 5'-AAGCTTCTGCAGAAGGGTTGGGCCCTTGGTGGAGGC-3'

The conditions used for the second round of amplification (30 cycles) were: Melt 94°C, 1 minute; primer anneal 60°C, 50 seconds; primer extension 72°C, 1.5 minutes.

Subcloning and sequencing

Each PCR product was electrophoresed on a preparative 1.5% agarose/Tris borate EDTA (TBE) gel [26] and the appropriate band was excised and purified using Prep-A-Gene (Biorad, Mississauga, Ontario). The purified PCR products were prepared for blunt-end cloning by simultaneous incubation with 10 units of T4 polynucleotide kinase (Pharmacia Inc., Baie d'Urfe, QUE.) and 10 units of DNA polymerase-I (New England Biolabs LTD., Mississauga, ONT.) for one hour at 37°C. The PCR products were purified as described above and subcloned into the Sma I site of the pGEM-3zf cloning vector (Promega, Madison, WI) [26].

Both sense and anti-sense strands were sequenced with the SP6 and T7 sequencing primers, using the T7 DNA sequencing kit (Pharmacia, Baie d'Urfe, Quebec). The sequences obtained were compared to all of the available immunoglobulin sequences in the GenBank and EMBO databases using the computer programs of the Genetic Computer Group [27].

RESULTS

Binding characteristics of the anti-pp65 antibodies

The ability of the four hybridoma antibodies to react with the viral 65-kD protein was previously demonstrated by radioimmunoprecipitation [10]. These antibodies were

subsequently tested for RF activity by ELISA (Figure 1A) and by immunoblotting (data not shown) and were negative. In addition, these antibodies were also tested for other autoreactivities, such as anti-DNA, but were shown to be monospecific for pp65 since all were negative against this panel of autoantigens (Figure 1B).

Expression of RF-associated idiotypes

A comparison of the RF-associated idiotypes expressed by the four anti-pp65 hybridoma antibodies, to those found on an anti-gp58 antibody (ITC63B) and two IgM paraproteins with RF activity (BOR and SCZ), revealed that a restricted number of idiotypes were present on three of the four anti-pp65 antibodies (Table 1). Interestingly, the C7 and B12 light chain idiotypes, which have previously been shown to be expressed by antibodies bearing V_kIII light chains [20], were present on both V_kI- and V_λI-bearing anti-pp65 antibodies (Tables 1 & 2). The B6 monoclonal antibody, believed to recognize the product of the V_HIII gene GL-SJ2 [28], bound to the two V_HIII-positive anti-pp65 antibodies (MO53 and MO61). The B6-positive antibodies showed nearly three-fold greater amino acid identity to the GL-SJ2 gene product than did the B6-negative antibodies (data not shown).

Light chain V-gene usage

Three of anti-pp65 -producing hybridomas expressed V_kI genes, while the fourth expressed a gene from the V_λI family (Table 2). Comparison of the nucleotide sequences of these light chains, to previously published germline genes, revealed that the putative germline gene for all of these light chains had a minimum of 94% identity with the hybridoma antibody sequence (Table 2). It appeared that the three V_kI light chains were probably encoded by different germline genes, as can be seen from the alignment of the predicted amino acid sequences of the light chains (Figure 2a). The maximum similarity between the three V_kI chains was 80% identity, that which was observed for the MO53/MO58 light chain pair. Although the absolute number of amino acid differences between these sequences was highest in the framework regions, the greatest variability (relative to sequence length) was seen in the CDRs. The extent and nature of the somatic

FIGURE 1a. Rheumatoid factor activity. Rheumatoid factor activity of the human monoclonal RF BOR (0.001 mg/ml) and pp65-specific human monoclonal antibodies (at the 10-fold higher concentration of 0.010 mg/ml), as determined by ELISA.

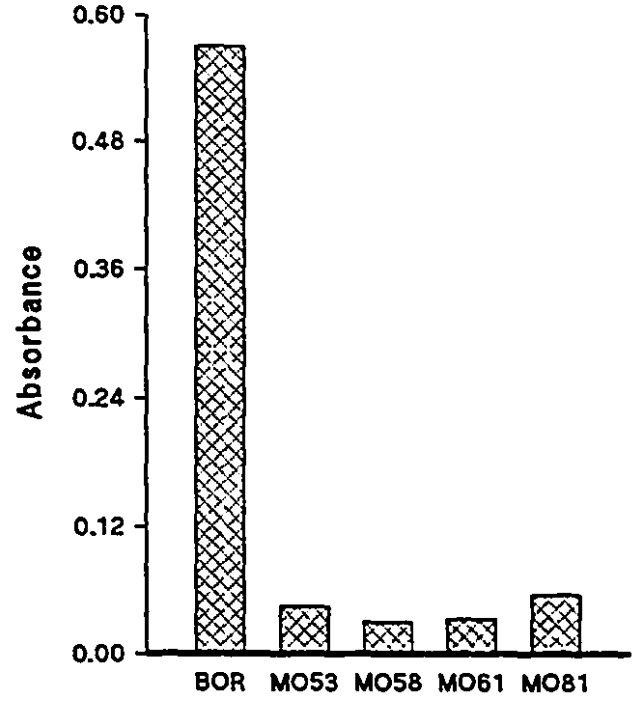

0.60
0.48
0.36
0.24
0.12
0.00
Absorbance
BOR
MO53
MO58
MO61
MO81

FIGURE 1b. Binding characteristics of the anti-pp65 antibodies. Reactivity pattern of human monoclonal antibody B7 15A2 (IgG1 control, specific for tetanus toxoid) as well as of MO53, MO58, MO61 and MO81 against a variety of antigens. The antigens are (from left to right): nuclear extract of CMV-infected fibroblasts (CMV), bovine insulin, calf thymus histones, single-stranded DNA, double-stranded DNA, RNA, acid-soluble collagen, porcine thyroglobulin (Tg), keyhole limpet hemocyanin (KLH) and tetanus toxoid (TT). None of the pp65-specific antibodies recognize nuclear extracts obtained from CMV-noninfected fibroblasts (not shown).

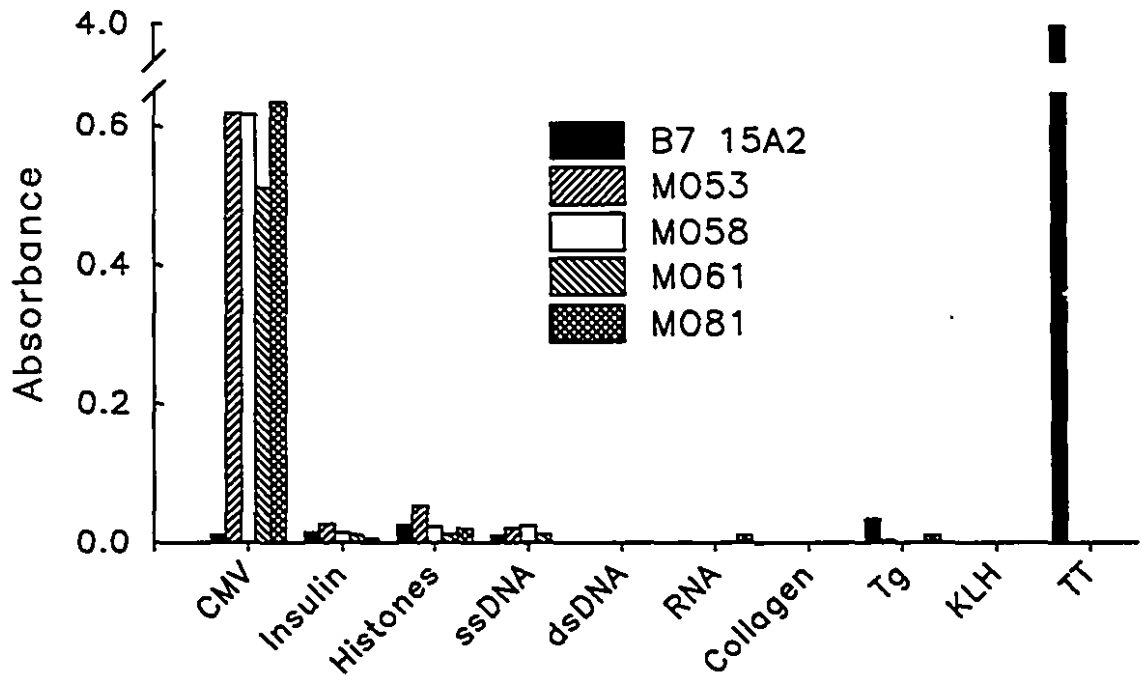

4.0
0.6
0.4
0.2
0.0
Absorbance
B7 15A2
M053
M058
M061
M081
CMV
Insulin
Histones
ssDNA
dsDNA
RNA
Collagen
Tg
KLH
TT

Table 1. Expression of rheumatoid factor (RF) -associated idiotypes.

			ELISA Score					
			"LC" Ids[1]			"HC" Ids[2]		
Antibody	Isotype	Specificity	C7	C6	B12	G6	B6	D12
MO53	IgG1, k	CMV - pp65	1	-	1	1	1	-
MO58	IgG1, k	CMV - pp65	1	-	-	-	-	-
MO61	IgG1, l	CMV - pp65	1	-	2	-	1	-
MO81	IgG1, k	CMV - pp65	-	-	-	-	-	-
ITC63B	IgG1, l	CMV - gp58	-	-	-	-	-	-
BOR	IgM, k	hIgG-Fc	3	3	3	3	-	-
SCZ	IgM, k	hIgG-Fc	2	2	2	-	2	2

[1] LC Ids: Light chain associated idiotypes; C7 & B12 [20]; C6 [22].

[2] HC Ids: Heavy chain associated idiotypes; G6 [19]; B6 [21]; D12 [22].

Table 2. Summary of V_L and V_H gene usage by four IgG anti-CMV antibodies.

Cell line	VL gene family	Germline gene[1]	Percent identity	JL	VH gene family	Germline gene[1]	Percent identity	JH
MO53	k I	KLVA20	95.1%	Jk3	VH3	hv3005	87.7%	JH4
MO58	k I	KL11A	96.7%	Jk1	VH1	HG3	92.6%	JH4
MO61	l I	LVE	98.4%	Jl2	VH3	WHG16	97.6%	JH4
MO81	k I	HK102	94.0%	Jk1	VH4	V71-2	88.1%	JH4

[1] Refers to germline gene, from the GenBank and EMBO databases, with the closest homology.

FIGURE 2a. The predicted amino acid sequences of the variable regions of the light chains of four anti-pp65 antibodies. The MO58, MO61 and MO81 sequences were aligned with the MO53 sequences to facilitate comparisions between the antibodies. Dashes indicate amino acid identity with the MO53 sequences and dots represent gaps between the different sequences. The complementarity determining regions (CDRs) are indicated and the amino acid positions are numbered according to Kabat [29].

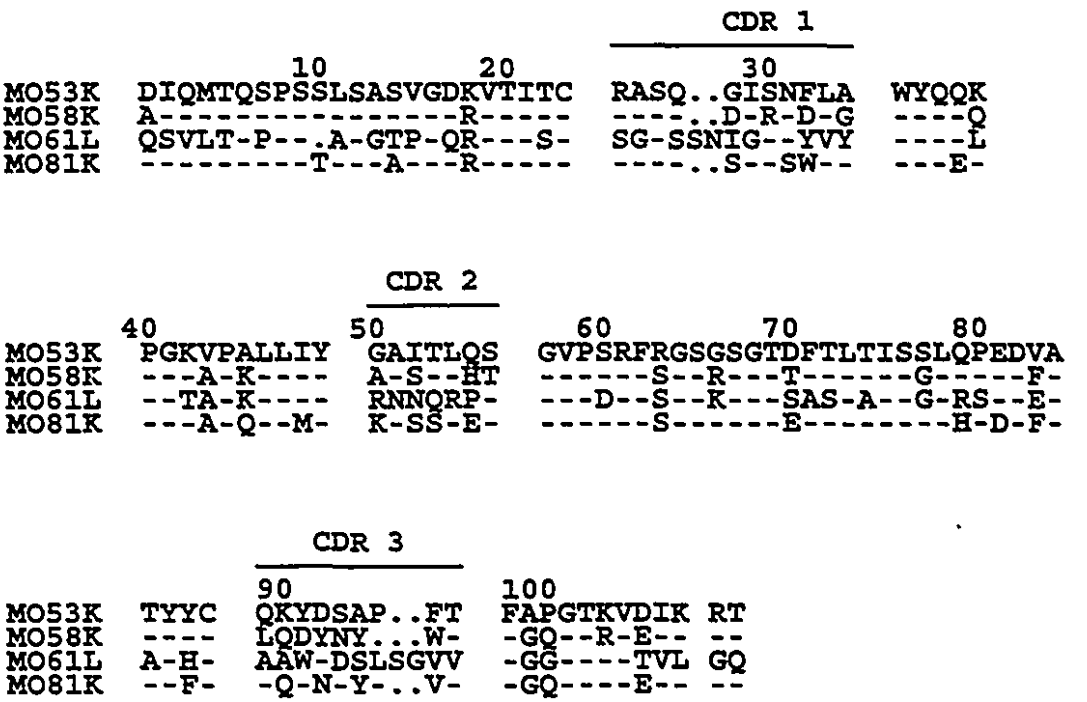
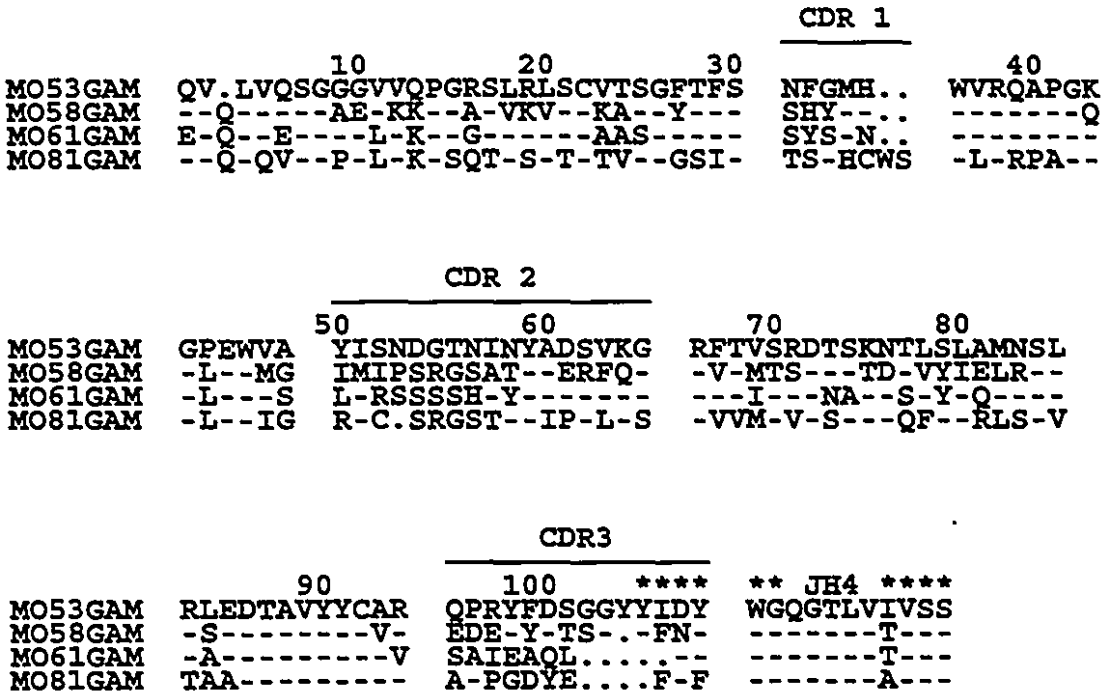

CDR 1
CDR 2
CDR 3
10
20
30
40
50
60
70
80
90
100
MO53K DIQMTQSPSSLSASVGDKVTITC RASQ..GISNFLA WYQQK PGKVPALLIY GAITLQS GVPSRFRGSGSGTDFTLTISSLQPEDVA TYYC QKYDSAP..FT FAPGTKVDIK RT
MO58K A----------R----- -----..D-R-D-G ----Q ---A-K---- A-S--HT ------S--R---T------G-----F- ----- LQDYNY...W- -GQ--R-E-- --
MO61L QSVLT-P--.A-GTP-QR---S- SG-SSNIG--YVY ----L --TA-K---- RNNQRP- ---D--S--K---SAS-A--G-RS--E- A-H- AAW-DSLSGVV -GG----TVL GQ
MO81K ----------T---A---R----- -----..S--SW-- ---E- ---A-Q--M- K-SS-E- ------S------E--------H-D-F- --F- -Q-N-Y-..V- -GQ----E-- --

FIGURE 2b. The predicted amino acid sequences of the variable regions of the heavy chains of four anti-pp65 antibodies. The MO58, MO61 and MO81 sequences were aligned with the MO53 sequences to facilitate comparisions between the antibodies. Dashes indicate amino acid identity with the MO53 sequences and dots represent gaps between the different sequences. The complementarity determining regions (CDRs) are indicated and the amino acid positions are numbered according to Kabat [29].


CDR 1
10
20
30
40
MO53GAM QV.LVQSGGGVVQPGRSLRLSCVTSGFTFS NFGMH.. WVRQAPGK
MO58GAM --Q-----AE-KK--A-VKV--KA--Y--- SHY--.. -------Q
MO61GAM E-Q--E----L-K--G------AAS----- SYS-N.. --------
MO81GAM --Q-QV--P-L-K-SQT-S-T-TV--GSI- TS-HCWS -L-RPA--
CDR 2
50
60
70
80
MO53GAM GPEWVA YISNDGTNINYADSVKG RFTVSRDTSKNTLSLAMNSL
MO58GAM -L--MG IMIPSRGSAT--ERFQ- -V-MTS---TD-VYIELR--
MO61GAM -L---S L-RSSSSH-Y------- ---I---NA--S-Y-Q----
MO81GAM -L--IG R-C.SRGST--IP-L-S -VVM-V-S---QF--RLS-V
CDR3
90
100
**** ** JH4 ****
MO53GAM RLEDTAVYYCAR QPRYFDSGGYYIDY WGQGTLVIVSS
MO58GAM -S--------V- EDE-Y-TS-.-FN- -------T---
MO61GAM -A---------V SAIEAQL.....-- -------T---
MO81GAM TAA--------- A-PGDYE....F-F -------A---

mutation events which occurred in these variable regions, as determined from the alignment of the nucleotide sequences of the hybridoma antibodies with their respective germline genes (Figure 3), are summarized in Table 3. This comparison demonstrated that approximately the same number of mutations had arisen in both the framework and complementarity determining regions. Moreover, the ratio between mutations which resulted in amino acid changes and those that did not (R:S ratio) demonstrated that the mutations in these light chains may not be necessary for the specificity of these antibodies [36].

Heavy chain V-gene usage

The MO53, MO58, MO61 and MO81 hybridomas expressed heavy chain genes of the V_H3, V_H1, V_H3 and V_H4 families, respectively (Table 2). The most homologous germline genes currently available in the databases were 88% to 98% identical to the anti-pp65 antibody sequences. Not only do the expressed sequences derive from different gene families, but the putative germline genes that encode them are dispersed along a 300 kilobase segment of the V_H locus on human chromosome 14 [5]. The comparison of the anti-CMV antibody sequences to their respective germline genes (Figure 4 and Table 3) indicated that CDR2 of the MO58 and MO81 heavy chains had a R:S ratio of seven and the framework regions of the same antibodies had ratios below 2.9, the ratio predicted by random mutation. The other two antibodies had ratios consistent with random mutation events. When the predicted amino acid sequences of the four heavy chains were compared (Figure 2b), many differences were seen between the four anti-pp65 antibodies. This not only included differences in primary sequence, but also in the length of the two CDRs encoded by the V_H genes.

Heavy chain CDR3 regions

The great diversity in amino acid sequence seen in the heavy chain CDR3 regions (Figure 2b) can be explained by N segment addition and by differential D segment utilization. Although all four hybridomas have rearranged J_H4 segments, the different somatic mutation and splicing events contributed to the distinct CDR3 sequences (Figure

FIGURE 3. The nucleotide sequences of the light chain variable regions of the four anti-pp65 antibodies. These sequences are available under Genome Sequence Database (GSDB) accession numbers L26890 (MO53), L26891 (MO58), L26896 (MO61) and L26893 (MO81). Each sequence is aligned with its putative germline gene. Dashes indicate nucleotide identity between the hybridoma sequence and the repective germline gene. Dots represent gaps between the different sequences. References for the germline "V" and "J" genes are: KLVA20 [30]; KL11A [31]; LVE [32]; HK102 [33]; J_k1 and J_k3 [34] and J_L2 [35].

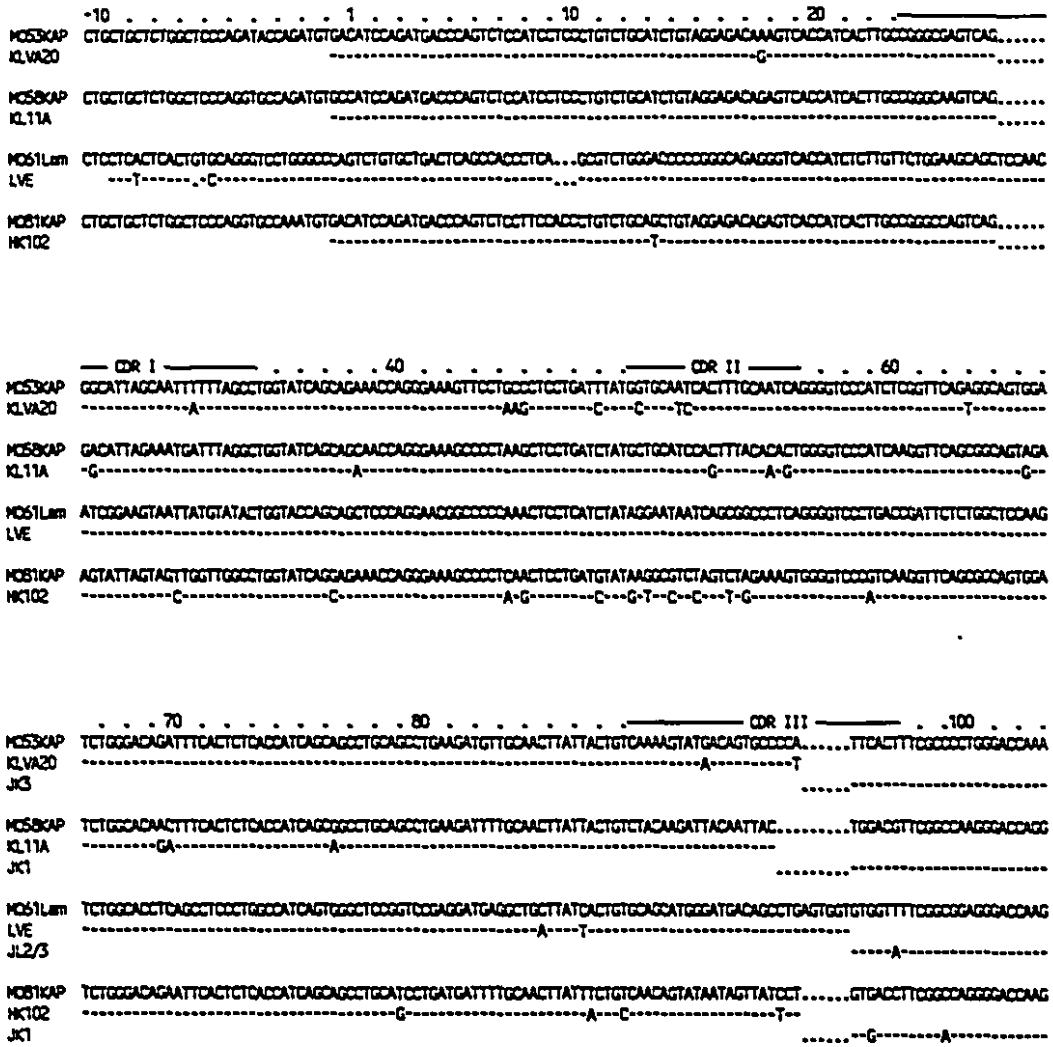
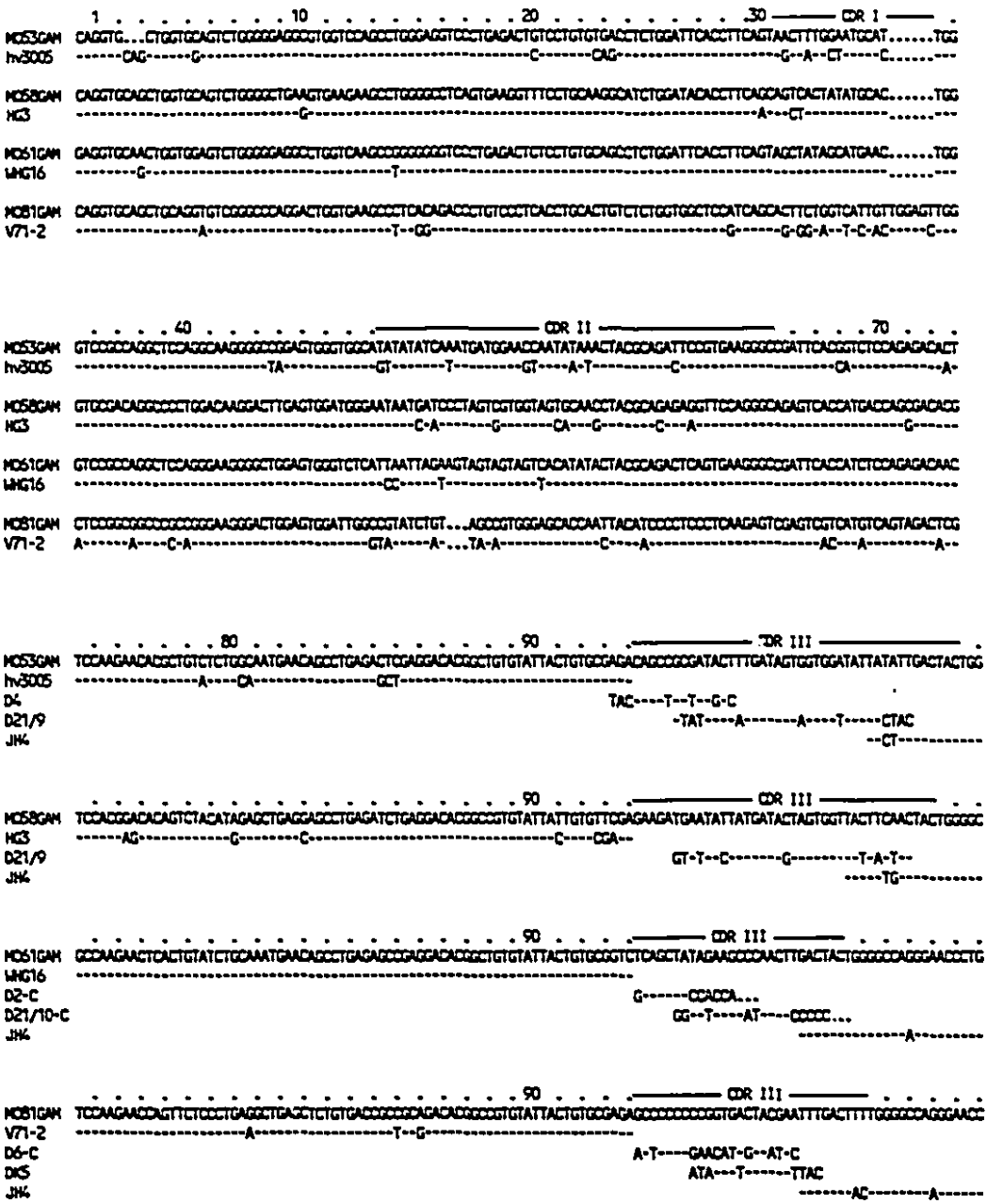

-10 . . . . . 1 . . . . . 10 . . . . . 20 . . .
MOS3KAP CTGCTGCTCTGGCTCCCAGATACCAGATGTGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAAAGTCACCATCACTTGCCGGGCGAGTCAG.....
KLVA20 ----------G----------.....
MOS8KAP CTGCTGCTCTGGCTCCCAGGTGCCAGATGTGCCATCCAGATGACCCAGTCTCCATCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAG.....
KL11A ----------.....
MO61Lam CTCCTCACTCACTGTCACAGGTCCTGGGCCCAGTCTGTGCTGACTCAGCCACCCTCA...GCGTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAAGCAGCTCCAAC
LVE ---T-------.-C----------...----------
MOB1KAP CTGCTGCTCTGGCTCCCAGGTGCCAAATGTGACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCAGCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCCAGTCAG.....
HK102 ----------T----------.....
— CDR I ——— . . . . 40 . . . . . .—— CDR II —— . . . 60 . . . . . .
MOS3KAP GGCATTAGCAATTTTTTAGCCTGGTATCAGCAGAAACCAGGGAAAGTTCCTGCCCTCCTGATTTATGGTGCAATCACTTTGCAATCAGGGGTCCCATCTCCGTTCAGAGGCAGTGGA
KLVA20 ----------A----------AAG---------C----C----TC----------T---------
MOS8KAP GACATTAGAAATGATTTAGGCTGGTATCAGCAGCAACCAGGGAAACCCCCTAAGCTCCTGATCTATGCTGCATCCACTTTACACACTGGGGTCCCATCAAGGTTCAGCGGCAGTAGA
KL11A -G----------A----------G------A-G----------G--
MO61Lam ATCGGAAGTAATTATGTATACTGGTACCAGCAGCTCCCAGGAACGGCCCCCAAACTCCTCATCTATAGGAATAATCAGCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAG
LVE ----------
MOB1KAP AGTATTAGTAGTTGGTTGGCCTGGTATCAGCAGAAACCAGGGAAAGCCCCTAACTCCTGATGTATAAGCGGTCTAGTCTAGAAAGTGGGGTCCCGTCAAGGTTCAGCGGCAGTGGA
HK102 ----------C----------C----------A-G---------C---G-T--C--C---T-G----------A----------
. . . 70 . . . . . 80 . . . . . .———— CDR III ———— . .100 . . .
MOS3KAP TCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATGTTGCAACTTATTACTGTCAAAGTATGACAGTGCCCCA.....TTCACTTTCGGCCCCTGGGACCAAA
KLVA20 ----------A----------T
JK3 .....----------
MOS8KAP TCTGGCACAACTTTCACTCTCACCATCAGCGGCCTGCAGCCTGAAGATTTTGCAACTTATTACTGTCTACAAGATTACAATTAC.....TGGACGTTCGGCCAAGGGACCAGG
KL11A ---------GA----------A----------
JK1 .....----------
MO61Lam TCTGGCACCTCAGCCTCCCTGGCCATCAGTGGGCTCCGGTCCGAGGATGAGGCTGCTTATCACTGTGCAGCATGGGATGACAGCCTGAGTGGTGTGGTTTTCGGCGGAGGGACCAAG
LVE ----------A----T----------
JL2/3 -----A----------
MOB1KAP TCTGGGACAGAATTCACTCTCACCATCAGCAGCCTGCATCCTGATGATTTTGCAACTTATTTCTGTCAACAGTATAATAGTTATCCT.....GTGACGTTCGGCCAGGGGACCAAG
HK102 ----------G----------A---C----------T--
JK1 .....--G---------A----------

FIGURE 4. The nucleotide sequences of the heavy chain variable regions of the four anti-pp65 antibodies. These sequences are available under Genome Sequence Database (GSDB) accession numbers L26894 (MO53), L26892 (MO58), L26898 (MO61) and L26895 (MO81). Each sequence is aligned with its putative germline gene. Dashes and dots are as in figure 3. References for the germline genes are: hv3005 [36]; HG3 [37]; WHG16 [38]; V71-2 [39]; D2 & D4 [40]; D21/9 & D21/10 [41]; D6 [42] and DK5 [43].


1 . . . . . 10 . . . . . 20 . . . . . 30 CDR I
MOS3GAM CAGGTG...CTGGTGCAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTGTCCTGTGTGACCTCTGGATTCACCTTCAGTAACTTTGGAATGCAT.....TGG
hv3005 ------CAG------G----------C--------CAG----------G--A--CT-----C.....---
MOS8GAM CAGGTGCAGCTGGTGCAGTCTGGGGCTGAAGTGAAGAAGCCTGGGGCCTCAGTGAAGGTTTCCTGCAAGGCATCTGGATACACCTTCAGCAGTCACTATATGCAC.....TGG
HG3 ----------G----------A---CT----------.....---
MO61GAM GAGGTGCAACTGGTGGAGTCTGGGGGAGGCCTGGTCAAGCCGGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTATAGCATGAAC.....TGG
WHG16 --------G----------T----------.....---
MOB1GAM CAGGTGCAGCTGCAGGTGTCGGGGCCAGGACTGGTGAAGCCCTCACAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCACTTCTGGTCATTGTTGGAGTTGG
V71-2 ----------A----------T--GG----------G------G-GG-A--T-C-AC-----C---

40 . . . . . CDR II . . . . . 70 . . .
MOS3GAM GTCCGCCAGGCTCCAGGCAAGGGGCCTGGAGTGGGTGGCATATATATCAAATGATGGAACCAATATAAACTACGCAGATTCCGTGAAGGGCCGATTCACCGTCTCCAGAGACAACT
hv3005 ----------TA----------GT--------T---------GT----A-T----------C----------CA----------A-
MOS8GAM GTGCCACAGGCCCCTGGACAAGGACTTGAGTGGATGGGAATAATGATCCCTAGTCGTGGTAGTGCAACCTACGCAGAGAGGTTCCAGGGCAGAGTCACCATGACCAGGGACACC
HG3 ----------C-A-------G-------CA---G-------C---A----------G------
MO61GAM GTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCATTAATTAGAAGTAGTAGTAGTCACATATACTACGCAGACTCAGTGAAGGGCCGATTCACCATCTCCAGAGACAAC
WHG16 ----------CC-----T----------T----------
MOB1GAM CTCCGGCGGCCCCCGGGAAGGACTGGAGTGGATTGGCCGTATCTGT...AGCGGTGGGAGCACCAATTACATCCCCTCCCTCAAGAGTCGAGTCGTCATGTCAGTAGACTCG
V71-2 A------A----C-A----------GTA-----A-...TA-A----------C----A----------AC---A---------A--

80 . . . . . 90 . . . . CDR III . . .
MOS3GAM TCCAAGAACACGCTGTCTCTGCAATGAACAGCCTGAGACTGGAGGACACGGCTGTGTATTACTGTGCGAGACAGCCGGCGATACTTTGATAGTGGTGGATATTATATTGACTACTGG
hv3005 ----------A----CA----------GCT----------
D4 TAC----T--T--G-C
D21/9 -TAT----A-------A----T-----CTAC
JH4 --CT----------
. . . . . 90 . . . . CDR III . . .
MOS8GAM TCCACGGACACAGTCTACATAGAGCTGAGGAGCCTGAGATCTGAGGACACGGCCGTGTATTATTGTGTTCGAGAAGATGAATATTATGATACTAGTGGTTACTTCAACTACTGGGGC
HG3 ------AG----------G---------C----------C----CGA--
D21/9 GT-T--C-------G----------T-A-T--
JH4 -----TG----------
. . . . . 90 . . . . CDR III . . . . . .
MO61GAM GCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGGTCTCAGCTATAGAAGCCCCAACTTGACTACTGGGGCCAGGGAACCCTG
WHG16 ----------
D2-C G------CCACCA...
D21/10-C GG--T----AT----CCCCC...
JH4 ----------A---------
. . . . . 90 . . . . CDR III . . . . . .
MOB1GAM TCCAAGAACCAGTTCTCCCTGAGGCTGAGCTCTGTGACCGCCGCAGACACGGCCGTGTATTACTGTGCGAGAGCCCCCCCCGGTGACTACGGAATTTGACTTTTGGGGCCAGGGAACC
V71-2 ----------A----------T--G----------
D6-C A-T----GAACAT-G--AT-C
DK5 ATA---T------TTAC
JH4 -------AC---------A------

Table 3. Putative somatic mutations in the V genes of four anti-CMV antibodies.

ANTIBODY	L chain/ H chain	% Mutations[1]		Replacement : Silent					
		FRs	CDRs	FR1	FR2	FR3	CDR1	CDR2	CDR3
MO53	LC	2.8	8.0	1:0	3:1	1:0	1:0	3:0	1:1[2]
	HC	7.0	19.7	3:2	1:1	7:2	3:2	6:2	N/A[3]
MO58	LC	2.4	5.6	0	1:0	4:0	1:0	3:0	0
	HC	5.3	15.2	1:1	0:1	6:3	1:1	7:1	N/A
MO61	LC	1.0	0	0	0	2:0	0	0	0
	HC	1.0	6.0	0:2	0	0	0	3:1	N/A
MO81	LC	4.3	10.6	1:0	3:1	2:2	0:1	2:4	1:0
	HC	7.4	24.6	3:2	3:2	5:2	6:3	7:1	N/A

[1] Calculated as the number of predicted mutations/100 nucleotides of sequence.

[2] The "J" segment is excluded.

[3] Not applicable (N/A) since this region is not encoded by the V_H gene.

4). In all four cases, it was possible to assign germline D segments that were likely to encode for the majority of the amino acids in this region (Figure 4). The MO53 CDR3 had a 23 nucleotide stretch that was 87% identical to the D21/9 segment and which may have been fused to a portion of the D4 gene. The D21/9 segment also appears to have been rearranged in the MO58 hybridoma, where there was 86% identity over a length of 22 nucleotides. The fusion of two inverted D segments could account for the entire portion of the MO61 heavy chain CDR3, which was not encoded by the J_H4 segment. Specifically, the fusion of the D2 and D21/10 segments had 76% identity with the MO61 sequence. Fusion of the DK5 gene to an inverted D6 segment resulted in a sequence that was 67% identical to the CDR3 of MO81. This fusion of two D segments, in addition to 9 nucleotides from the J_H4 gene, could encode for the entire 10 amino acids of the MO81 CDR3.

The similarity in mechanisms for the generation of the MO53 and MO58 heavy chain CDR3s is reflected in their primary amino acid sequences (Figure 2b). These two CDR3 sequences differ in length by one amino acid residue, both have four tyrosine residues and have a similar number of acidic and basic residues. The MO61 and MO81 CDR3 regions, on the other hand, are shorter and have only a single tyrosine residue.

Structural similarity to human autoantibodies

All light and heavy chain variable regions expressed by the anti-pp65 antibodies had a minimum of 87% identity with previously published rearranged immunoglobulin sequences. The majority of these sequences corresponded to antibodies with RF activity, but also included sequences of other autoimmune or fetal antibodies (Table 4).

Although the MO53 anti-CMV antibody did not have any anti-DNA activity, its sequence was highly related to an IgM anti-DNA antibody. Specifically, the MO53 light chain had the highest identity with the III-2R anti-DNA antibody [45]. There was 93% nucleotide identity over the entire variable region, resulting in a total of four amino acid differences in the CDRs. Both the MO53 and III-2R antibodies had heavy chains which were most identical (greater than 86%) to to two different rearranged clones derived from a single fetal liver [46].

Table 4. Summary of published antibodies with the greatest homology to the anti-CMV antibodies.

Anti-pp65 Antibody		Most homologous human antibody sequences			
		Antibody	Specificity	Identity	Reference
MO53	Kappa	III-2R	anti-DNA	93%	[44]
	Gamma	56P1	unknown (fetal)	87%	[45]
MO58	Kappa	2E7	anti-platelet	92%	[46]
	Gamma	HAF10	RF	92%	[47]
MO61	Lambda	L1	RF	96%	[49]
		mAb61	RF	97%	[48]
		mAb67	RF	98%	[48]
	Gamma	D1	RF	95%	[49]
MO81	Kappa	C6	RF	95%	[50]
	Gamma	mAb67	RF	87%	[48]

The MO58 light chain was most homologous to the anti-platelet autoantibody 2E7 [47]. The anti-gpIIb-IIIa antibody 2E7 and MO58 were 92% identical over the entire light chain variable region. The rearranged immunoglobulin sequence with the highest homology to the MO58 heavy chain was expressed by a hybridoma secreting an IgM RF [48]. These two rearranged V_H nucleotide sequences were 92% identical and were derived from the same germline gene. The heavy chain CDR3s of these two antibodies were approximately the same length (13 vs 16 amino acids) but of completely divergent sequence (data not shown).

Both the light and heavy chain variable regions of the MO61 antibody were most homologous to rearranged sequences corresponding to antibodies with RF activity. Specifically, the MO61 light chain variable region was 96%, 97% and 98% identical, respectively, to the light chains of the L1, mAb61 and mAb67 RFs [49,50]. The resulting amino acid differences between MO61 and the mAb61 and mAb67 RFs were located either in the CDR3 or just flanking this region. The L1 light chain, however, also differed in the other CDRs. The V_H sequence of MO61 was most homologous to the D1 IgG RF [50], having 95% identity at the nucleotide level. All but one of the resulting amino acid differences were due to putative somatic mutation events. The two antibodies rearranged a J_H4 segment, differed in CDR3 length by one amino acid (8 vs 9), but had different amino acid sequences encoded by their D segments (data not shown).

The light chain variable region with the highest degree of homology to the V_k expressed by the MO81 anti-pp65 antibody also belonged to an antibody with RF activity. The nucleotide sequence of the variable region of MO81 was 95% identical to the kappa chain expressed by the C6 RF [51] and there were only three amino acid differences in the CDR regions. The MO81 kappa chain was the only sequence of the four anti-pp65 antibodies to be significantly homologous to any previously reported rearranged sequence corresponding to an anti-viral antibody. Specifically, the MO81 sequence was 93% identical to the light chain expressed by a monoclonal antibody specific to the gp41 of the HIV-1 [52]. The MO81 V_H segment was most identical to the C6B2 anti-DNA antibody and to the mAb67 RF [53] with 89% and 87% identity,

respectively. There was substantial difference in the length of the CDR3 between the two autoantibodies and the anti-pp65 antibody. The MO81 CDR3 was 10 amino acids long whereas the C6 and mAb67 CDR3 regions consisted of 18 and 19 amino acids, respectively, and there was no apparent sequence homology between the three CDR3 domains (data not shown).

DISCUSSION

The structural characteristics of four human monoclonal antibodies, with known specificity for the pp65 protein of human CMV, were studied in order to gain insight into the human humoral response to foreign antigens. Although three of four antibodies expressed RF-associated idiotypes, none had any detectable RF activity. This is consistent with previous findings that the presence of these idiotypic structures does not confer RF activity upon an antibody [15,20,21]. Antibodies which express the same idiotypic determinants usually derive from identical or similar germline genes, as we saw for the B6 idiotypic determinant in confirmation of a previous report [54]. It appeared, however, that the C7 and B12 determinants, V_KIII-associated idiotypes, were present on antibodies bearing light chains other than V_KIII, as has been previously reported [20]. It is possible that co-expression of some idiotypic determinants may have arisen as a result of somatic mutation [22], which is a probable event in these IgG anti-pp65 antibodies.

Regarding the relationship between variable region sequence and antigenic specificity, the present study demonstrated that four different light/heavy chain V gene combinations were capable of generating specific anti-pp65 antibodies. The two most identical light chains were found on the MO53 and MO58 antibodies. These two antibodies expressed V_H genes belonging to two different families but had similar heavy chain CDR3. These similarities in light and heavy chain sequences appear to be sufficient to enable these antibodies to recognize partially overlapping epitopes as determined by binding to linear synthetic peptides [10]. In competition experiments, previous studies also demonstrated that the MO81 antibody was able to compete with the MO53 and MO61 antibodies for binding to the intact antigen. MO81 was unable,

however, to bind any of the linear peptides indicating a specificity for a highly conformational epitope [10]. Such specificity is reflected in the MO81 variable region which was found to be significantly different from the other antibodies studied.

The role of somatic mutation in the generation of these specificities is predominantly restricted to the heavy chains. Although mutations were detected in all light chains, the R:S ratios that were observed did not indicate any clear evidence of antigen selection. On the other hand, two of the heavy chains had R:S ratios indicative of selection of mutations residing in the CDR2s. The conclusions drawn from this evaluation, however, must be accompanied with the caveat that the germline genes attributed to these sequences may be incorrect, since it is possible that the appropriate genes have yet to be isolated.

Although it is difficult to assess the role of somatic mutation events in the heavy chain CDR3, it appears that such events may have at least been important in the 3' end of this region. All four hybridomas had rearranged a J_H4 gene, a preferential usage previously noted in both pre-B cells and peripheral blood B cells [55,56]. Despite this, the last two residues of CDR3 were different in three of these antibodies, potentially having an important structural role in this region.

A significant number of amino acid changes, putatively resulting from somatic mutation events, were located in the interval of the heavy chain FR3 that is important for defining V_H clans [57]. When the hybridoma antibody sequences were compared to the appropriate clan consensus sequences, the majority of amino acid differences had resulted from somatic mutation (data not shown). These amino acid changes potentially function to modify the specificity provided by the basic clan structure. The location of this FR3 ß loop, located next to the CDR1 and CDR2 domains, may extend into the actual antigen binding site. These changes may therefore affect the structure of the combining site or may even participate in the direct contact with antigen [57].

Comparisons of the anti-pp65 sequences to all previously reported rearranged immunoglobulin sequences revealed that most resembled RFs, other autoantibodies and fetal antibodies of unknown specificities. Although there is an apparent bias in the number of autoantibody sequences in the current databases, this result clearly indicates

that anti-viral and autoantibodies can derive from identical or similar germline genes. The comparison of antibodies with similar germline gene usage, but different specificities, has enabled further examination of the relationship between variable region primary sequence and specificity of antibody combining sites. From our studies it was apparent that the greatest sequence differences resided in the CDR domains, including the heavy chain CDR3 which was seen to differ both in length and sequence. This was most evident in the MO61 anti-pp65 antibody which had light and heavy chain variable regions 98% identical to germline sequences and were also highly homologous to RF sequences. The two mutations in the MO61 light chain, resulted in amino acid changes, and were located adjacent to the CDR3 domain and could potentially mediate binding to antigen. In the heavy chain, there were three replacement mutations in the CDR2 which resulted in differences with the RF sequence. Although similar in length, the anti-viral antibody MO61 and the D1 RF had different heavy chain CDR3 sequences.

These results demonstrate that antibodies specific for a single exogenous protein can result from different recombination events involving multiple genes from distinct gene families. This report also provides evidence that the expression of identical or similar germline genes can result in the production of antibodies with specificity for either self or exogenous antigens. The greatest difference between the anti-pp65 antibodies and the autoantibodies resided in the heavy chain CDR3, indicating that this domain is most likely critical for the generation of antibodies with distinct epitopic specificities for a single exogenous antigen and of antibodies capable of distinguishing between foreign and self antigens.

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CHAPTER IV

Molecular Characterization of the GM 4672 Human Lymphoblastoid Cell Line and Analysis of Its Use as a Fusion Partner in the Generation of Human-Human Hybridoma Autoantibodies.

PREFACE

A very large number of human hybridoma autoantibodies has been generated using the GM 4672 lymphoblastoid cell line as a fusion partner. The structural information regarding these well characterized antibodies is of great interest. It had previously been determined that this cell line, prior to fusion with B lymphocytes, was capable of secreting Ig molecules. However, the sequences corresponding to the rearranged V region genes of this fusion partner were not known. This chapter reports the V region sequences expressed by this cell line and provides evidence that the GM 4672 genes are not used in the hybridoma lines produced using this fusion partner.

SUMMARY

The GM 4672 lymphoblastoid cell line has been used in cell hybridization experiments with peripheral blood lymphocytes (PBLs) in order to generate human-human hybridomas that secrete immunoglobulins directed against a number of different autoantigens. The GM 4672 cells were fused with PBLs isolated from patients with either rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) or from normal individuals and the resulting hybridomas were screened for reactivity to platelets, erythrocytes, DNA, cardiolipin (CL), human IgG-Fc, phosphatidylethanolamine (PE), and for lupus anticoagulant activity. This report analyzes the results from 149 fusion

experiments completed over a period of 9 years. Fifty to sixty-six percent of the fusion experiments resulted in immunoglobulin-secreting clones, with an average of 15 clones/fusion. The hybridoma antibodies were predominantly of the IgM heavy chain isotype and 67% expressed kappa light chains. Although most hybridoma antibodies (78%) recognized a single autoantigen, 22% recognized more than one autoantigen and were considered polyreactive. In addition, the light and heavy chain varible regions of the antibody secreted by the GM 4672 cell line were amplified by the polymerase chain reaction (PCR) technique and sequenced. The GM 4672 light chain was encoded by a V_kI gene and used a J_k4 minigene. The GM 4672 heavy chain was derived from the rearrangement of a gene from the V_H4 subgroup and used a J_H4 minigene. The 8 amino acid long diversity region was generated by the fusion of the DK1 and DLR2 genes. The hybridomas generated in fusion experiments, when examined, did not appear to secrete antibodies using the immunoglobulin variable regions derived from the GM 4672 cells. This report demonstrates that the GM 4672 cell line can efficiently immortalize B-lymphocytes from autoimmune patients and normal individuals for the purpose of functional and structural studies of human autoantibodies.

INTRODUCTION

The generation of human monoclonal antibody-producing cell lines by somatic cell hybridization involves the fusion of human B-lymphocytes with an immortal fusion partner cell line. One such fusion partner is the GM 4672 lymphoblastoid cell line, a hypoxanthine phosphoribosyl transferase (HPRT)-deficient cell line that secretes immunoglobulin molecules of the IgG2-kappa isotype. GM 4672 has been used to generate specific monoclonal hybridoma antibodies reactive with parasitic antigens (1), cytoskeletal proteins (2), cardiolipin (3), DNA (4,5,6,7) and the Fc-portion of IgG molecules (5).

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are two autoimmune disorders that are characterized by the presence of circulating antibodies directed against self components. Human hybridoma technology provides the potential to immortalize peripheral blood lymphocytes responsible for the production of

autoantibodies from RA and SLE patients. This same methodology can be used to generate hybridoma antibodies from control patients and healthy individuals. The hybridoma antibodies can then be studied at both the protein and nucleic acid levels for functional and structural characteristics that may identify important similarities or differences between the antibodies.

Human hybridoma antibodies have been the subject of many structural studies. Most have utilized methods involving the polymerase chain reaction (PCR) to rapidly clone antibody variable regions, using RNA isolated from the individual hybridoma cell lines (8,9,10,11). It is important to know the sequence of the fusion partner's rearranged genes in order to ascertain that none of the sequences obtained are attributable to the fusion partner.

In the present study, the GM 4672 lymphoblastoid cell line has been studied for its ability to generate human hybridoma autoantibodies from peripheral blood lymphocytes derived from healthy individuals and from patients with rheumatic diseases. In addition, the complete sequences of the light and heavy chain variable regions of the immunoglobulin synthesized by the GM 4672 cell line are reported. A total of 58 light and heavy chain variable regions of the human hybridoma antibody molecules have been shown to derive from the fused peripheral blood lymphocytes and not from the fusion partner itself.

MATERIALS AND METHODS

Cell Lines

The GM 4672 human lymphoblastoid cell line was obtained from the Cell Repository Institute of Medical Research, Camden NJ. All other cell lines were human hybridomas derived by fusion of peripheral blood lymphocytes from patients and normal individuals with the GM 4672 parental cell line, as previously described (12,13). The patient group included individuals with SLE, RA, peripheral neuropathy and women having undergone multiple spontaneous abortions without any other evident clinical features. On average, 2×10^7 lymphocytes were fused with 2×10^7 GM 4672 cells. Although the total number of cells fused (lymphocytes + GM 4672 cells) did vary

between 2 x 10^7 and 6 x 10^7, the 1:1 cell ratio was maintained and the cells were always seeded in 2.0 ml wells at a concentration of 4 X 10^5 cells/well. This study includes cell hybridization experiments performed between November 1981 and October 1990. Multiple fusions were done on lymphocytes obtained from some individuals over the period of the study and each was analyzed independently.

Antibody Isotype and Specificity Screening

Hybridoma supernatants were tested for immunoglobulin secretion, immunoglobulin isotype and reactivity to a number of different autoantigens including platelet lysate (PL), cardiolipin, phosphatidylethanolamine and human IgG-Fc fragments (rheumatoid factors) by enzyme-linked immunoassays (ELISA), as previously described (14,15). Samples were considered positive if the $OD_{410} > 0.08$ in the anti-platelet assay, $OD_{410} > 0.15$ in the anti-CL assay, $OD_{410} > 0.12$ in the anti-PE assay or $OD_{492} > 0.25$ in the rheumatoid factor (RF) assay.

Binding to Fc-fragments of human IgG was also tested by radioimmunoasay (RIA), as was the binding to denatured DNA (dDNA) (5,13). Binding to Fc fragments was considered significant if cpm values were > 1000 and if the binding to Fab fragments and/or to bovine serum albumin (BSA) < 1000 cpm. Antibodies were defined as having anti-dDNA reactivity if their binding to dDNA exceeded 1200 cpm and their binding to BSA was less than 1000 cpm.

Detection of lupus anticoagulant activity in hybridoma supernatants was performed using a modified activated partial thromboplastin time (APTT) assay (16). A hybridoma antibody was defined as having lupus anticoagulant activity if its APTT value exceeded that of the GM 4672 control by greater than 6 seconds.

Coombs positivity was tested by a hemagglutination assay on papain-treated red blood cells (17).

All of the above-mentioned cut-off values were greater than the mean plus two standard deviations of 5 known negative samples in repeated assays.

The data on antigen specificity derive from hybridoma clones that were obtained from 81 consecutive fusion experiments done over a period of four years (September

1986 to October 1990) and not all were tested for every specificity. Antibodies which recognized only a single antigen were considered as monoreactive whereas those which recognized two or more antigens were termed polyreactive. Polyreactive antibodies in general were not broadly reactive and were unreactive to some of the antigens tested.

Amplification of Immunoglobulin Variable Regions

Total cellular RNA was extracted with guanidinium thiocyanate followed by centrifugation through a dense cushion of cesium chloride (18). First strand cDNA was then directly synthesized from 1 μg of unselected RNA using oligonucleotide primers specific for the appropriate constant region domains. Each cDNA preparation was subsequently amplified by the polymerase chain reaction (PCR) using the Perkin-Elmer thermal cycler (Cetus Perkin Elmer, Emeryville, CA, USA). All oligonucleotide primers, as well as the reaction conditions for the cDNA synthesis and PCR reactions, have been previously described (8). The one exception was for the GM 4672 kappa chain variable region that was amplified for 30 cycles using the following conditions: denaturing 94°C, 1 minute; annealing 42°C, 30 seconds; extension 72°C, 2 minutes. The sequences of the oligonucleotide primers (J.L. Hillson, personal communication) used for the GM 4672 kappa amplification were:

C_k: 5'-CAGAATTCAACTGCTCATCAGAT-3'

V_k: 5'-GGTACCCAGTCTCCATCCTCCCTGTCT-3'

DNA Sequencing

Each PCR product was electrophoresed on a preparative 1.5% agarose/Tris borate EDTA (TBE) gel and the appropriate band was excised and purified using Prep-A-Gene (Biorad, Mississauga, Ontario). The purified bands were either directly sequenced or subcloned into the pGEM-3zf cloning vector (Promega, Madison, WI) and then sequenced using the T7 DNA Sequencing kit (Pharmacia, Baie d'Urfe, Quebec). Sequence data obtained was analyzed using computer programs of the Genetic Computer Group (19).

Identification of Variable Gene Family Utilization

The light and heavy variable gene families for each of the hybridoma antibodies were determined either by nucleic acid sequencing (as described above) or by anti-peptide analysis of purified hybridoma proteins (as described in reference 20). Briefly, the anti-peptide analysis was performed using antibodies that had been raised against peptides representing the framework regions that distinguish the different heavy and light chain gene families. These imunoglobulin variable region gene-specific antisera were used in a Western blot of purified hybridoma antibodies and detected using ^{125}I-protein A (ICN Radiochemicals Inc., Irvine, CA) by autoradiography.

RESULTS

Evaluation of the cell hybridization experiments with GM 4672

The GM 4672 lymphoblastoid cell line has been used to produce human hybridoma antibodies from peripheral blood lymphocytes in order to generate autoantibodies representative of those found in the circulation of healthy and autoimmune individuals. Over a period of nine years, 180 separate fusion experiments were performed. Thirty-one of these experiments were lost due to contamination. Seventy-five of the remaining experiments, or 50% (see Table 1), were considered successful since they gave rise to antibody-producing clones. During those nine years, there were three periods where 10-21 successive fusion experiments resulted in failure to produce clones. Fusion efficiency was restored either by thawing an earlier aliquot of cells or by ordering a new batch of GM 4672 cells from the Cell Repository. Thus, with the exclusion of the experiments that failed to produce clones due to poor GM 4672 cells, the fusion success rate was 66%.

The 75 successful hybridization experiments generated 1127 antibody-producing clones. Between 1 and 146 distinct clones were produced per fusion, with an average of 15 clones/fusion (SD = 22.0). Although there was a slightly higher success rate when using lymphocytes derived from RA and SLE than from normal individuals, the average number of clones generated per successful fusion experiment was not significantly different (Table 1).

Table 1. Analysis of the success rate and average number of clones per successful fusion for cell hybridization experiments using peripheral blood lymphocytes (PBLs) from different sources.

PBL SOURCE	NUMBER OF FUSION EXPERIMENTS	NUMBER OF FUSIONS PRODUCING ANTIBODY-SECRETING CLONES (% SUCCESS)	AVERAGE NUMBER OF CLONES/ SUCCESSFUL FUSION (SD)
RA	54	29 (53.7)	16.6 (28.7)
SLE	52	34 (65.4)	15.6 (17.2)
SA	13	3 (23.1)	1.0 (0.0)
PN	1	0	NA
NL	22	9 (40.9)	12.7 (17.6)
UNKOWN	7	0	NA
TOTAL	149	75	

* Abbreviations: rheumatoid arthritis (RA), standard deviation (SD), systemic lupus erythematosus (SLE), spontaneous abortion (SA), peripheral neuropathy (PN), not applicable (NA), normal (NL).

Hybridoma Antibody Isotypes and Antigenic Specificities

Although not an exhaustive characterization, Tables 2a and 2b summarize the results obtained from the isotype and specificity testing of up to 534 different antibody-producing hybridoma clones. Ninety-six percent of the clones produced antibodies of the IgM isotype, while only 4% secreted IgG molecules. The light chain isotype distribution (67% kappa, 33% lambda) was found to be similar to the kappa/lambda ratio found in the human circulation.

The hybridoma supernatants were also tested for anti-dDNA, rheumatoid factor, lupus anticoagulant, anti-CL, anti-PE, anti-platelet and anti-erythrocyte (Coombs) activities and the results are shown in Table 2. Between 205 and 522 clones were tested for the individual reactivities and the results show that hybridomas could be generated for each of these specificities. The incidence of clones with the desired specificities varied between 3.0 and 22.4% of the number of clones tested; the least frequent was Coombs positivity and the most frequent was lupus anticoagulant activity.

Seventy-eight percent of the clones tested were found to be monoreactive, that is, hybridoma antibodies that recognized a single autoantigen, while 22% recognized two or more autoantigens (i.e. were polyreactive).

GM 4672 Light and Heavy Variable Region Sequences

The nucleotide sequences, along with the deduced amino acid sequences, of the immunoglobulin variable regions expressed by the GM 4672 fusion partner are shown in Figures 1a and 1b.

The GM 4672 light chain variable region derived from the rearrangement of a gene from the V_kI subroup to a J_k4 minigene. The expressed V_kI sequence most closely resembled the 08/018 germline gene (21), being 94% identical at the nucleotide level. A computer search of the GenBank and EMBL databanks for the closest related expressed sequence to the GM 4672 gamma chain revealed that an anti-turkey egg white lysozyme antibody (22) was 86% identical over a 268 nucleotide stretch that included the J segment.

Table 2a. Characteristics of the human hybridoma antibodies derived by fusion of human peripheral blood lymphocytes (PBLs) with the GM 4672 lymphoblastoid cell line.

Summary of light and heavy chain isotypes

ISOTYPE REAGENT	NUMBER OF CLONES TESTED*	% POSITIVE CLONES
anti-Mu	534	95.7
anti-Gamma	534	4.3
anti-Kappa	325	67.0
anti-Lambda	325	33.0

* Results were compiled from 81 fusions, including fusions B28 to C10 (September 1986 to October 1990).

Table 2b. Characteristics of the human hybridoma antibodies derived by fusion of human peripheral blood lymphocytes (PBLs) with the GM 4672 lymphoblastoid cell line.

Summary of reactivities against a panel of autoantigens.

SPECIFICITY	NUMBER OF CLONES TESTED*	% POSITIVE CLONES
anti-platelet	240	6.7
lupus anticoagulant	205	22.4
anti-erythrocyte (Coombs)	233	3.0
anti-dDNA	522	10.5
anti-cardiolipin	483	4.1
anti-IgG (RF)	414	10.6
anti-PE	386	6.2

* Results were compiled from 81 fusions, including fusions B28 to C10 (September 1986 to October 1990).

FIGURE 1a. The nucleotide and corresponding translated amino acid sequence of the GM 4672 light chain variable region. The complementarity determining regions (CDR) are indicated and the amino acid positions are numbered according to Kabat *et al.* (24). The 08/018 germline gene (21) has been aligned with the GM 4672 kappa sequence and the predicted amino acid differences are indicated by asterisks (dashed lines indicate identity at the nucleotide level).

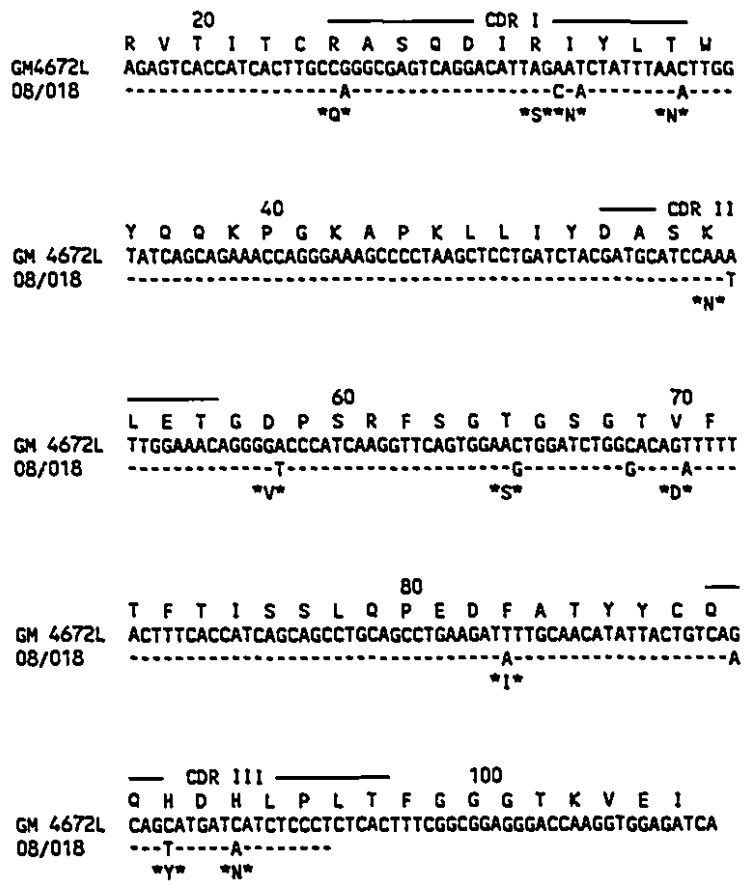
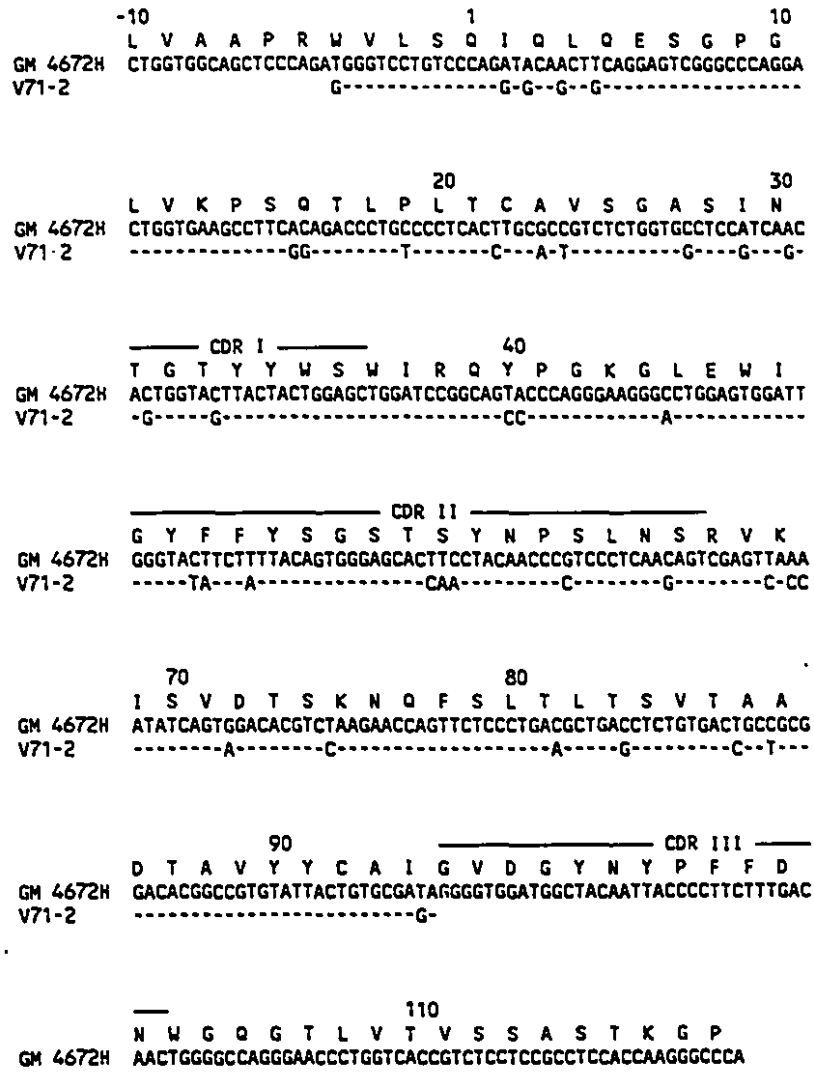

20
CDR I
R V T I T C R A S Q D I R I Y L T W
GM4672L AGAGTCACCATCACTTGCCGGGCGAGTCAGGACATTAGAATCTATTTAACTTGG
08/018 ----------A----------C-A--------A----
Q *S**N* *N*
40
CDR II
Y Q Q K P G K A P K L L I Y D A S K
GM 4672L TATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTACGATGCATCCAAA
08/018 ----------T
N
60
70
L E T G D P S R F S G T G S G T V F
GM 4672L TTGGAAACAGGGGACCCATCAAGGTTCAGTGGAACTGGATCTGGCACAGTTTTT
08/018 ----------T----------G---------G----A----
V *S* *D*
80
T F T I S S L Q P E D F A T Y Y C Q
GM 4672L ACTTTCACCATCAGCAGCCTGCAGCCTGAAGATTTTGCAACATATTACTGTCAG
08/018 ----------A----------A
I
CDR III
100
Q H D H L P L T F G G G T K V E I
GM 4672L CAGCATGATCATCTCCCTCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCA
08/018 ---T-----A--------
Y *N*

FIGURE 1b. The nucleotide and corresponding translated amino acid sequence of the GM 4672 heavy chain variable region. The complementarity determining regions (CDR) are indicated and the amino acid positions are numbered according to Kabat *et al.* (24). The V71-2 germline gene (23) has been aligned with the GM·4672 gamma sequence and the predicted amino acid differences are indicated by asterisks (dashed lines indicate identity at the nucleotide level).


-10 1 10
L V A A P R W V L S Q I Q L Q E S G P G
GM 4672H CTGGTGGCAGCTCCCAGATGGGTCCTGTCCCAGATACAACTTCAGGAGTCGGGCCCAGGA
V71-2 G----------G-G--G--G----------
20 30
L V K P S Q T L P L T C A V S G A S I N
GM 4672H CTGGTGAAGCCTTCACAGACCCTGCCCCTCACTTGCGCCGTCTCTGGTGCCTCCATCAAC
V71-2 ----------GG--------T-------C---A-T----------G----G---G-
CDR I
40
T G T Y Y W S W I R Q Y P G K G L E W I
GM 4672H ACTGGTACTTACTACTGGAGCTGGATCCGGCAGTACCCAGGGAAGGGCCTGGAGTGGATT
V71-2 -G-----G----------CC----------A----------
CDR II
G Y F F Y S G S T S Y N P S L N S R V K
GM 4672H GGGTACTTCTTTTACAGTGGGAGCACTTCCTACAACCCGTCCCTCAACAGTCGAGTTAAA
V71-2 -----TA---A----------CAA---------C--------G--------C-CC
70 80
I S V D T S K N Q F S L T L T S V T A A
GM 4672H ATATCAGTGGACACGTCTAAGAACCAGTTCTCCCTGACGCTGACCTCTGTGACTGCCGCG
V71-2 --------A--------C----------A-----G---------C--T---
90
CDR III
D T A V Y Y C A I G V D G Y N Y P F F D
GM 4672H GACACGGCCGTGTATTACTGTGCGATAGGGGTGGATGGCTACAATTACCCCTTCTTTGAC
V71-2 ----------G-
110
N W G Q G T L V T V S S A S T K G P
GM 4672H AACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCCGCCTCCACCAAGGGCCCA

The heavy chain variable region was encoded by a member of the V_H4 family and was 88% identical, at the nucleotide level, to the V71-2 germline gene (23). It was joined to a J_H4 minigene by a 24 base pair-long diversity region generated by the fusion of the DK1 and DLR2 genes (25,26). The expressed gene that most closely resembled the GM 4672 gamma chain was the HUMVHP30 sequence (27). There is 90% identity between the two expressed V_H segments; both have rearranged J_H4 minigenes but their diversity segments differ in length by 21 nucleotides.

Hybridoma Variable Gene Family Utilization

Fourteen hybridoma light chain variable regions have been sequenced (data not shown). Although six of these were encoded by genes from the V_kI subgroup and none of them were identical to the GM 4672 kappa chain. In addition, the light chain gene family usage of another 24 hybridomas was determined by anti-peptide analysis. Nine of the twenty-four were predicted to be encoded by V_kI genes but were not sequenced to definitively prove that they did not originate from the GM 4672 cell line. In total, the light chain variable region gene expression was determined for 38 different hybridoma clones and was as follows: 15 V_kI, 4 V_kII, 14 V_kIII, 1 V_k5, 2 $V_\lambda I$ and 2 $V_\lambda III$.

Nine hybridoma heavy chain variable regions were also sequenced (data not shown). None were encoded by members of the V_H4 family. An additional 22 hybridoma heavy chain variable regions were assigned to gene families by anti-peptide analysis. Only three of these had rearranged genes from the V_H4 subgroup. Since the immunoglobulin "class switch" mechanism involves the loss of the intervening genomic region encoding previously expressed heavy chain constant regions genes, any hybridoma of the IgG1 or IgG3 subclasses would not be derived from the GM 4672 fusion partner except in the very rare instance of sister chromatid exchange. Two of these V_H4-bearing hybridomas were tested for IgG subclass expression; one was an IgG1 and the other an IgG3. An additional 18 IgG-secreting hybridomas were classified with respect to their gamma subclass and 16 of these were determined to be either of the IgG1 or IgG3 subclasses (data not shown). To summarize, of the hybridoma antibodies for which the heavy chain variable region gene utilization was determined, the majority were derived

from genes of the V_H3 family (22/31) with the remainder being derived from the V_H1 (6/31) and V_H4 (3/31) families.

DISCUSSION

Human-human hybridomas can be generated by fusion of B-lymphocytes to an immortalized partner cell line to produce monoclonal antibodies of defined specificity. One of the few human B-lymphoblastoid fusion partners described to date is the GM 4672 cell line which we have used for cell hybridization experiments over the past decade. In this communication we document the functional and structural characteristics of human monoclonal autoantibodies derived from fusion with the GM 4672 cell line, as well as the variable region gene sequences of the light and heavy chains expressed by GM 4672 cells.

We have reported in the past that, in fusion experiments using human PBLs and the GM 4672 cell line, approximately 11% of the wells plated were positive for immunoglobulin secretion (13). Similar mean yields have been observed in a number of other reports (1,12,6), although one report noted that the yield varied between 12%-87%, depending on the fusion conditions (6). In the present study, we examined a much larger number of fusion experiments and were interested in evaluating the percentage of fusions that successfully produced immunoglobulin-secreting clones. We found that at least half of our fusion experiments were successful and that slightly greater success was obtained with PBLs derived from autoimmune patients than from healthy individuals, although the two groups yielded approximately equivalent numbers of clones per fusion.

The use of the GM 4672 fusion partner in our experiments appeared to favour the production of immunoglobulins of the IgM isotype (96%). This characteristic is most likely due to the fusion partner's ability to selectively fuse with a subset of B-lymphocytes. Experience in our laboratory with the use of a different fusion partner, the SP2/HPT heteromyeloma, in contrast resulted in the production of hybridoma antibodies primarily of the IgG isotype (80-85%) under the same experimental conditions (data not shown).

The ability to produce human hybridoma autoantibodies varied depending on the specificity sought and the source of the PBLs used in the fusion experiments. The results obtained for the anti-DNA specificity, however, were similar to previous reports (6,13). The relatively high frequency of hybridoma antibodies with lupus anticoagulant activity can be attributed to a testing bias, since approximately half of all the clones tested for this specificity were derived from patients who were lupus anticoagulant-positive (data not shown). The high percentage (78%) of autoantibody-producing hybridomas that were monoreactive is most likely due to the high stringency of the cutoff values used in this study, resulting in the exclusion of polyreactive antibodies with low avidity. Taken together, these results clearly demonstrate that the GM 4672 lymphoblastoid cell line is a useful fusion partner to generate human-human hybridoma antibodies specific for a variety of autoantigens, by fusion with B-lymphocytes derived from autoimmune patients or normal individuals. Our sequence data of the immunoglobulin genes rearranged and expressed by the GM 4672 cell line indicated that the latter produces antibody molecules with a V_kI light chain and a V_H4 heavy chain. This is in contrast to the V_kI light chain and V_H1 heavy chain usage predicted by Atkinson et al (28). These authors predicted the usage of a gene from the V_H1 subgroup based on their findings of [^{3}H] leucine incorporation at amino acid (a.a.) position 4 but not at positions 5, 11, 18 and 20. Although not commonly found at position 5 in any of the heavy chain subgroups, leucine is found at positions 11, 18 and 20 for subgroups V_H2, V_H3 and V_H4 but not V_H1 (24). Our nucleotide sequence of the GM 4672 heavy chain predicts the existence of a leucine residue at positions 4, 11, 18 and 20. It is possible that Atkinson et al. (28) did not detect the leucine residues at positions 11, 18 and 20 due to low efficiency at the labeling or Edman degradation steps. Since three independent bacterial clones containing the GM 4672 heavy chain PCR insert had identical sequences, we are confident that this sensitive assay gave reliable results. Furthermore the V_kI/V_H4 usage of GM 4672 has also been independently found by another group (29).

No known specificity has yet been attributed to the antibody secreted by the GM 4672 cells. The 08/018 germline gene, which may encode the V_kI light chain variable region expressed by the GM 4672 cells, has been reported to be functionally rearranged

and expressed during the human anti-Haemophilus influenzae type b polysaccharide response (21). We found that the GM 4672 heavy chain variable region most closely resembled a V_H-J_H rearrangement previously found in malignant cells from a patient with chronic lymphocytic leukemia (27). This suggests that the heavy and/or light chains produced by the GM 4672 cell scan, as would be expected, bind antigen. It is, thus, possible that a hybridoma derived by fusion with the GM 4672 cell line could secrete a hybrid antibody molecule generated by the pairing of immunoglobulin chains derived from both the B-lymphocyte and the GM 4672 cell. Moreover, since the PCR technique is sensitive enough to amplify the variable regions from single cells (8), artifacts could arise when cloning and sequencing hybridomas. For example, some hybridomas generated by fusion with GM 4672 cells could produce low levels of GM 4672-derived transcripts, which might be cloned and sequenced in addition to the more abundant lymphocyte-derived transcripts.

To address these possibilities, we have sequenced 14 light chain and 9 heavy chain variable regions of GM 4672-derived hybridomas that had been amplified using the PCR technique. None of the sequences obtained were identical to the GM 4672 sequences. The anti-peptide analysis also demonstrated that the majority of the hybridomas express immunoglobulin genes from families other than the V_kI and V_H4 sugroups found to be rearranged in the GM 4672 cell line. In addition, the great majority of the hybridoma antibodies were of a heavy chain isotype or subclass that was incompatible with the expression of the GM 4672 heavy chain. This strongly suggests that the majority, if not all, of the hybridomas produced by fusion with the GM 4672 lymphoblastoid cell line express the immunoglobulin genes rearranged in the donor B-lymphocyte. The most likely explanation of this would be that the immunoglobulin genes endogeneous to the GM 4672 fusion partner are either lost, since genetic material is often shed by hybridoma clones (3), or are nonfunctional in the immunoglobulin-secreting clones. These findings should facilitate future structural studies of human hybridoma antibodies generated using the GM 4672 lymphoblastoid cell line.

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CHAPTER V

A Human Rheumatoid Factor C304 Shares V_H and V_L Gene Usage With Antibodies Specific for Ubiquitous Human Viral Pathogens.

PREFACE

As the majority of structural information regarding human RFs has been generated by amino acid sequence analysis of IgM paraproteins isolated from patients with a lymphoproliferative disorder known as mixed cryoglobulinemia, it was of great interest to analyze sequences of human hybridoma RFs derived from patients with rheumatoid arthritis (RA). This chapter examines the nucleotide sequences of the V region genes expressed by a hybridoma RF, derived from a RA patient with active disease, and relates them to sequences of known anti-viral antibodies.

SUMMARY

Analysis of the variable region gene sequences of a human hybridoma rheumatoid factor (RF), derived from a patient with RA, revealed the expression of genes from the $V_\lambda I$ and $V_H 3$ families. Specifically, the C304 RF had rearranged the DPL8/Humlv1042 and VH26 germline V_λ and V_H genes, respectively. This gene usage has also been observed in the rearrangement of human anti-viral antibodies specific for the herpes group of viruses. This overlap between the autoimmune and anti-viral antibody gene repertoires suggests a possible structural relationship between the immune response directed against ubiquitous pathogens and the induction of RF production.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized, in the majority of patients, by the presence of circulating autoantibodies known as rheumatoid factors (RFs) [1]. Although RA is an autoimmune disease of unknown

etiology, studies of RA synovial fluid lymphocytes have revealed potential microbiologic causative agents including viruses of the Herpesviridae family [reviewed in reference 2]. It has also been suggested that viruses from this group may be involved in the production of RFs, which are antibodies specific for the Fc portion of IgG molecules. Specifically, due to their ability to induce IgG Fc receptors on the surface of infected cells, the herpes simplex virus (HSV) and the cytomegalovirus (CMV) may potentially elicit anti-idiotypic antibodies with RF activity [3,4]. In addition, we have found significant structural similarities between RFs and anti-CMV antibodies [5-7], providing further support for the hypothesis that the human immune response against herpes viruses may be linked to RF production. In the present study, we report the sequence of a human RF derived from a patient with active RA and demonstrate the usage of light and heavy chain immunoglobulin variable region genes that are expressed by human anti-viral antibodies specific for herpes viruses.

MATERIALS AND METHODS

Generation of the RF-secreting hybridoma

50 ml of venous blood was obtained from a 68 year old female patient who fulfilled the American College of Rheumatology criteria for RA [1]. At the time of fusion, this patient had active RA with multiple swollen joints and a RF titer of 1/1663. The peripheral blood lymphocytes (PBL) were isolated on a Ficoll-Hypaque gradient and immediately fused with the GM 4672 human lymphoblastoid cell line (Cell Repository Institute of Medical Research, Camden, NJ) at a cell ratio of 1:1 using 44.4% polyethylene glycol 1540 (J.T. Baker Chemical Co., Phillipsburg, NJ) [8].

Antibody specificity

The hybridoma supernatant was tested for RF activity by ELISA as previously described [9]. Binding to denatured DNA (dDNA) was tested by radioimmunoassay [8]. Reactivity to a number of other autoantigens including lysed platelets (PL), cardiolipin (CL) and dioleoylphosphatidylethanoloamine (DOPE) was tested by ELISA, as previously described [10]. Coombs positivity was tested by hemagglutination assay on

papain-treated red blood cells [11]. Reactivity against herpes simplex virus types 1 and 2 (HSV-1, -2) was tested by indirect immunofluorescence and by competitive inhibition of specific anti-HSV-1 and anti-HSV-2 antibodies, using commercially available slides (Diagnostic Products Corp., Los Angeles, CA). Reactivity against the human cytomegalovirus (CMV) was tested by indirect immunofluorescence using MRC-5 cells (American Type Culture Collection, Rockville, MD) infected with the Davis prototype strain of CMV, as previously described [12].

Amplification of Immunoglobulin Variable Regions

Total cellular RNA was extracted from 10^6 hybridoma cells with guanidinium thiocyanate followed by centrifugation through a 5.7 M cushion of cesium chloride [13]. First strand cDNA was directly synthesized from 1 μg of total RNA using oligonucleotide primers specific for the appropriate constant region domain (lambda or mu). The primers (20 pmoles/0.020 ml reaction volume) used were:

C_λ: 5'-TGGCTTGGAGCTCCTCAGAGGA-3' (J.L. Hillson, personal communication)

C_{mu}: 5'-CCAAGCTTAGACGAGGGGGAAAAGGGTT-3'[14]

Each cDNA preparation was subsequently amplified by the polymerase chain reaction [15] using a Perkin-Elmer thermal cycler (model 480, Cetus Perkin Elmer, Emeryville, CA). The 50 μl reaction mixture contained 10 μl of the cDNA, 1.5 mM $MgCl_2$, 20 pmoles 3' primer, 40 pmoles 5' primer and 2 units of Thermus aquaticus (Taq) thermostable DNA polymerase (Promega, Madison, WI) in the magnesium-free buffer supplied by the manufacturer. The 3' primers were the same as above. The 5' primers, corresponding to the relatively conserved lambda chain (amino acid position -20 to -13) and heavy chain (position -20 to -12) leader sequences, were:

a) lambda chain: 5'-ATGGCCTGGACTCCTCTCCTTCT-3'

b) mu chain: an equimolar mixture of

5'-GGGAATTCATGGACTGGACCTGGAGG(AG)TC(CT)TCT(GT)C-3',

5'-GGGAATTCATGGAG(CT)TTGGGCTGA(CG)CTGG(CG)TTT(CT)T-3' and

5'-GGGAATTCATG(AG)A(AC)(AC)(AT)ACT(GT)TG(GT)(AT)(CGT)C(AT)(CT)(CG)CT(CT)CTG-3' [14]

Subcloning and sequencing

Each PCR product was electrophoresed on a preparative 1.5% agarose/Tris borate EDTA (TBE) gel [16] and the appropriate band was excised and purified using Prep-A-Gene (Biorad, Mississauga, Ontario). The purified PCR products were prepared for blunt-end cloning by simultaneous incubation with 10 units of T4 polynucleotide kinase (Pharmacia Inc., Baie d'Urfe, QUE.) and 10 units of DNA polymerase-I (New England Biolabs LTD., Mississauga, ONT.) for one hour at 37°C. The PCR products were purified as above and subcloned into the Sma I site of the pGEM-3zf cloning vector (Promega, Madison, WI) [16]. Both sense and anti-sense strands were sequenced with the SP6 and T7 sequencing primers using the T7 DNA sequencing kit (Pharmacia, Baie d'Urfe, Quebec). The sequences obtained were compared to all of the immunoglobulin sequences in the GenBank and EMBL databases using the computer programs of the Genetic Computer Group [17].

RESULTS

Binding characteristics of the C304 hybridoma antibody

Initial screening of the C304 hybridoma supernatant revealed that the IgM antibody secreted by this clone was a monospecific RF. This was demonstrated when the C304 antibody was shown to have anti-human IgGFc (RF) activity but no anti-dDNA, anti-PL, anti-CL, anti-DOPE or Coombs reactivities (data not shown). Following sequence analysis of the C304 antibody, tests for reactivity against HSV-1, HSV-2 and CMV were performed and found to be negative (data not shown).

C304 RF variable region sequences

Figures 1 and 2 show the sequences obtained for the C304 light and heavy chain variable regions, respectively. The C304 light chain was found to belong to the $V_\lambda I$ family and was 99.7% identical to the recently described DPL8/Humlv1042 germline gene [18,19]. The single nucleotide difference was located at the V/J junction and had no effect on the predicted amino acid sequence (Fig. 1). The rearranged J segment was most identical to the $J_\lambda 2$ germline gene, but diverged from the latter sequence by a total

FIGURE 1. The nucleotide and predicted amino acid sequences of the lambda light chain variable region of the C304 RF. The germline DPL8 [18] and J_L2 [27] sequences are shown aligned with the C304 sequence (available under GSDB accession number L26908). In this figure, dashes represent sequence identity with the RF sequence. The complementarity determining regions (CDRs) and amino acid positions are indicated according to Kabat *et al.* [28].

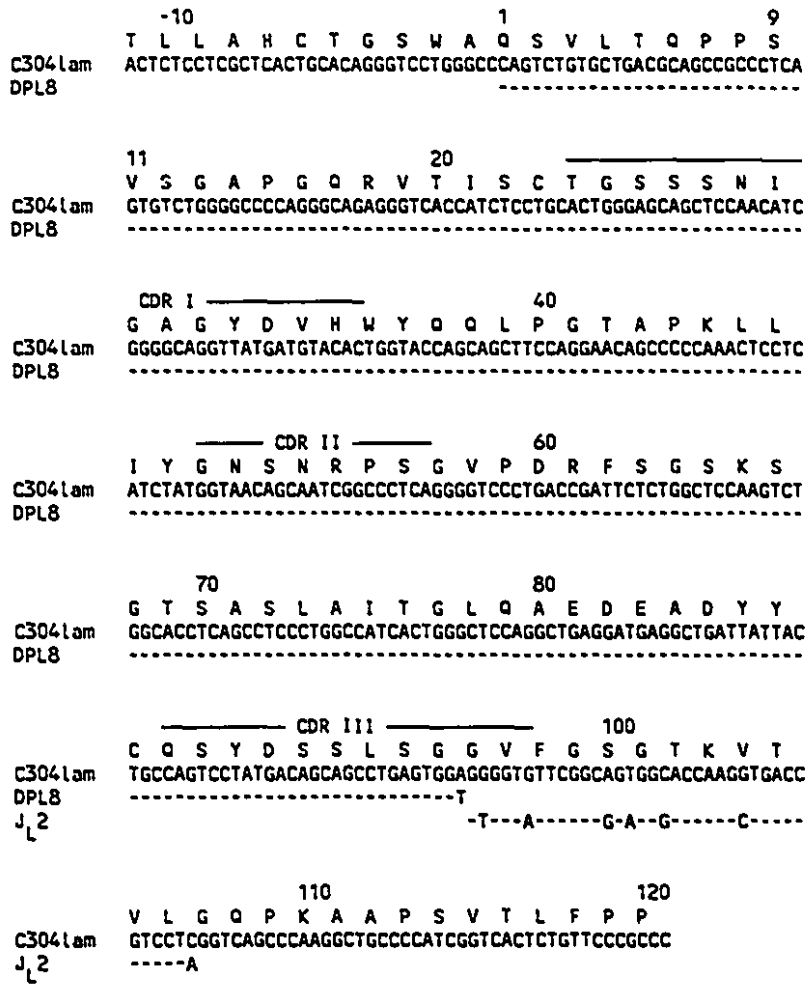
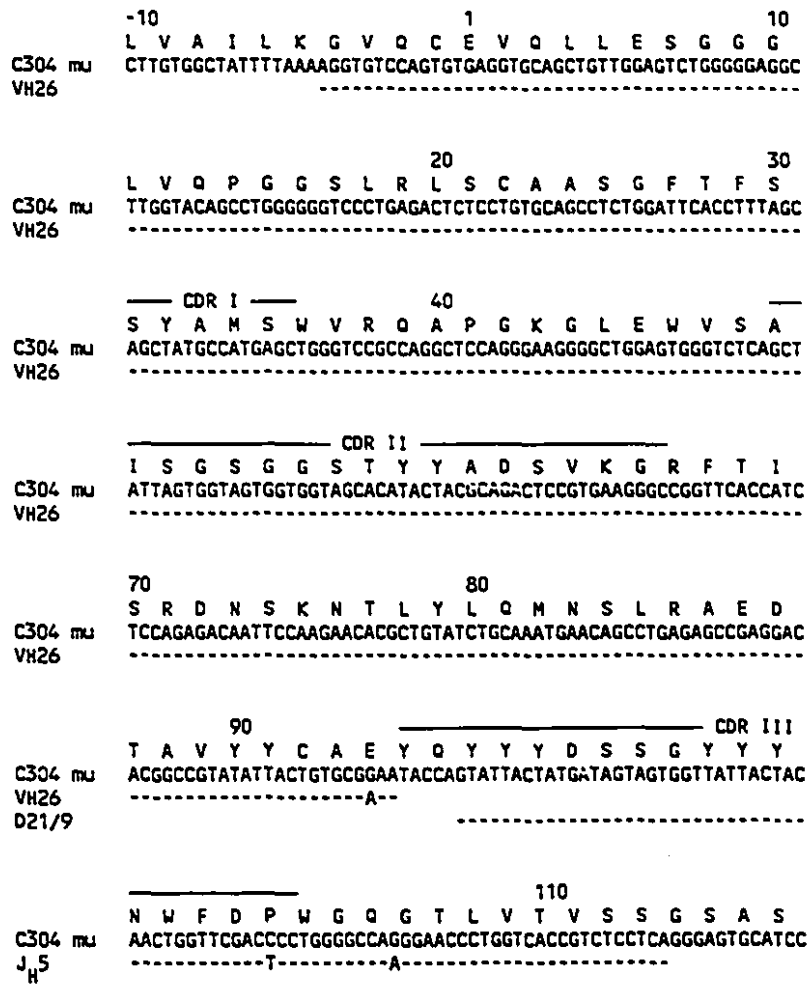

-10 1 9
T L L A H C T G S W A Q S V L T Q P P S
C304lam ACTCTCCTCGCTCACTGCACAGGGTCCTGGGCCCAGTCTGTGCTGACGCAGCCGCCCTCA
DPL8 ----------
11 20
V S G A P G Q R V T I S C T G S S S N I
C304lam GTGTCTGGGGCCCCAGGGCAGAGGGTCACCATCTCCTGCACTGGGAGCAGCTCCAACATC
DPL8 ----------
CDR I
40
G A G Y D V H W Y Q Q L P G T A P K L L
C304lam GGGGCAGGTTATGATGTACACTGGTACCAGCAGCTTCCAGGAACAGCCCCCAAACTCCTC
DPL8 ----------
CDR II
60
I Y G N S N R P S G V P D R F S G S K S
C304lam ATCTATGGTAACAGCAATCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCT
DPL8 ----------
70 80
G T S A S L A I T G L Q A E D E A D Y Y
C304lam GGCACCTCAGCCTCCCTGGCCATCACTGGGCTCCAGGCTGAGGATGAGGCTGATTATTAC
DPL8 ----------
CDR III
100
C Q S Y D S S L S G G V F G S G T K V T
C304lam TGCCAGTCCTATGACAGCAGCCTGAGTGGAGGGGTGTTCGGCAGTGGCACCAAGGTGACC
DPL8 ----------T
J_L2 -T---A------G-A--G------C-----
110 120
V L G Q P K A A P S V T L F P P
C304lam GTCCTCGGTCAGCCCAAGGCTGCCCCATCGGTCACTCTGTTCCCGCCC
J_L2 -----A

FIGURE 2. The nucleotide and predicted amino acid sequences of the heavy chain variable region of the C304 RF. The germline V_H26 [20,21], D21/9 [29] and J_H5 [30] sequences are shown aligned with the C304 sequence (available under GSDB accession number L26907). In this figure, dashes represent sequence identity with the RF sequence. The complementarity determining regions (CDRs) and amino acid positions are indicated according to Kabat *et al.* [28].


-10 1 10
L V A I L K G V Q C E V Q L L E S G G G
C304 mu CTTGTGGCTATTTTAAAAGGTGTCCAGTGTGAGGTGCAGCTGTTGGAGTCTGGGGGAGGC
VH26 ----------
20 30
L V Q P G G S L R L S C A A S G F T F S
C304 mu TTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGC
VH26 ----------
CDR I 40
S Y A M S W V R Q A P G K G L E W V S A
C304 mu AGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCT
VH26 ----------
CDR II
I S G S G G S T Y Y A D S V K G R F T I
C304 mu ATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATC
VH26 ----------
70 80
S R D N S K N T L Y L Q M N S L R A E D
C304 mu TCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGAC
VH26 ----------
90 CDR III
T A V Y Y C A E Y Q Y Y Y D S S G Y Y Y
C304 mu ACGGCCGTATATTACTGTGCGGAATACCAGTATTACTATGATAGTAGTGGTTATTACTAC
VH26 ----------A--
D21/9 ----------
110
N W F D P W G Q G T L V T V S S G S A S
C304 mu AACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGGAGTGCATCC
J_H5 ----------T----------A----------

of 7 nucleotides. These nucleotide differences in J_λ resulted in three amino acid changes (Fig. 1).

The C304 hybridoma had rearranged a V_H segment from the V_H3 family. Specifically, the C304 V_H segment was 99.6% identical to the VH26 gene [20,21] (Fig. 2). The single nucleotide difference resulted in a lysine to glutamate amino acid change directly adjacent to the CDR3 (Fig. 2). The CDR3 was almost exclusively encoded by an unmutated D21/9 gene that had been rearranged to a J_H5 segment. The single replacement mutation in the rearranged J_H segment resulted in a change from serine to proline at the last position of the CDR3 (Fig. 2).

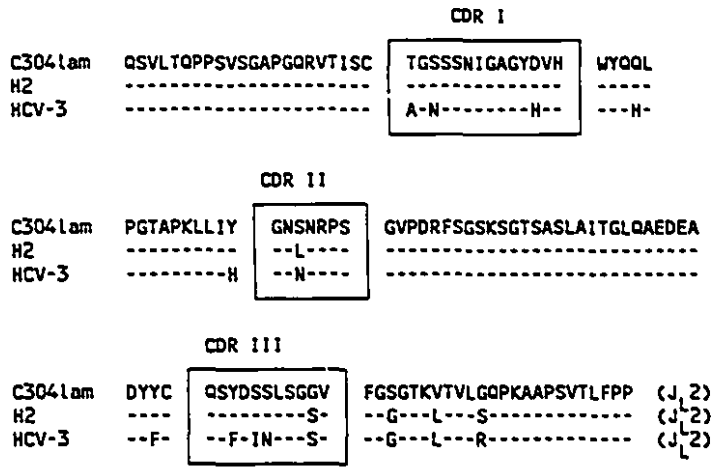
Comparison of the C304 variable region to other antibody variable regions

The variable region gene sequences obtained for the C304 antibody were found to be distinct from the sequences reported for the rearranged immunoglobulin genes endogenous to the GM 4672 lymphoblastoid cell line [22]. Since this indicated that the sequences obtained for the C304 clone were derived from the donor B lymphocyte, rather than from the fusion partner, they were compared to all currently available sequences of human antibodies. The published antibody, of known specificity, with the greatest identity with the C304 RF was the H2 anti-viral antibody [23]. The specificity of the H2 antibody is for the human herpes simplex viruses types 1 and 2. The nucleotide identity between the V region segments of the C304 and H2 antibodies was 98.6% and 95.4% for the light and heavy chains, respectively (data not shown). Analysis of the H2 sequences revealed that the C304 and H2 antibodies apparently derived from the same germline V_λ and V_H genes (data not shown) [23]. Consequently, all of the amino acid differences between the C304 RF and the anti-HSV antibody (Fig. 3) potentially arose as a result of somatic mutation events within these variable regions.

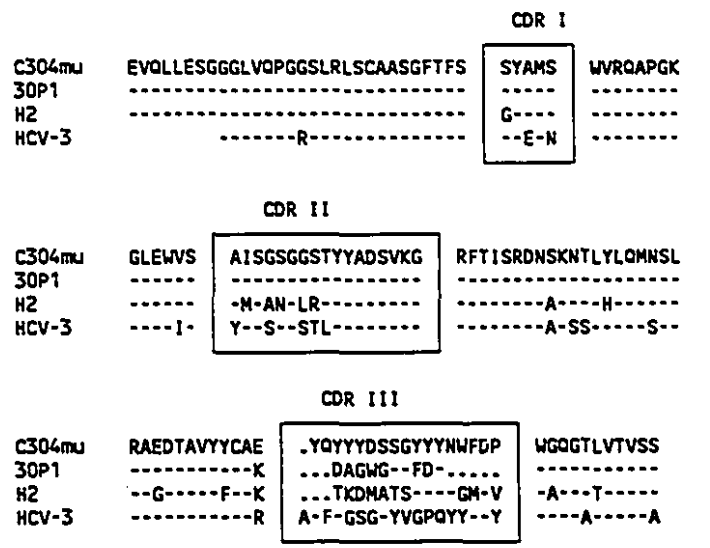
Another anti-viral antibody, HCV-3 [6], showed significant homology to C304 and may also be derived from the same $V_\lambda I$ germline gene and a related V_H3 germline gene. The HCV-3 antibody, specific for the human cytomegalovirus (CMV), was 95.6% identical to DPL8 gene and 93.9% identical to the VH26 gene (data not shown). The amino acid sequence alignment, shown in Figure 3, indicated that the putative somatic

FIGURE 3. Comparison of the C304 RF variable region amino acid sequences to two anti-viral antibody sequences. The A) light and B) heavy chain sequences of an anti-HSV antibody (H2) [23] and an anti-CMV antibody (HCV-3) [6] are shown aligned with the C304 sequence. Also shown is the rearranged heavy chain sequence of the 30P1 human fetal liver clone [24]. The dots represent junctional gaps between the different sequences.

a)


CDR I
C304lam QSVLTQPPSVSGAPGQRVTISC TGSSSNIGAGYDVH WYQQL
H2 ---------- ---------- -----
HCV-3 ---------- A-N--------H-- ---H-
CDR II
C304lam PGTAPKLLIY GNSNRPS GVPDRFSGSKSGTSASLAITGLQAEDEA
H2 ---------- --L---- ----------
HCV-3 ---------H --N---- ----------
CDR III
C304lam DYYC QSYDSSLSGGV FGSGTKVTVLGQPKAAPSVTLFPP (J L 2)
H2 ---- ---------S- --G---L---S---------- (J L 2)
HCV-3 --F- --F-IN---S- --G---L---R---------- (J L 2)

b)


CDR I
C304mu EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGK
30P1 ---------- ----- --------
H2 ---------- G---- --------
HCV-3 -------R---------- --E-N --------
CDR II
C304mu GLEWVS AISGSGGSTYYADSVKG RFTISRDNSKNTLYLQMNSL
30P1 ------ ---------- ----------
H2 ------ -M-AN-LR--------- --------A----H------
HCV-3 ----I- Y--S--STL-------- --------A-SS-----S--
CDR III
C304mu RAEDTAVYYCAE .YQYYYDSSGYYYNWFDP WGQGTLVTVSS
30P1 ----------K ...DAGWG--FD-..... ----------
H2 --G-----F--K ...TKDMATS----GM-V -A---T-----
HCV-3 ----------R A-F-GSG-YVGPQYY--Y ----A-----A

mutations that occurred in this anti-CMV antibody were significantly different from those found in the anti-HSV antibody and C304 RF.

The C304 V_H segment was nearly identical (minimum 99% identity) to a group of rearranged fetal liver clones with unknown specificities [24-26]. Despite the near identity in the V_H segments, there was very little sequence similarity between the CDR3s of these antibodies and the C304 heavy chain. Of this group of fetal clones, the 30P1 fetal liver clone had the greatest homology to the C304 CDR3 and is shown in figure 3. There was also little sequence similarity between the heavy chain CDR3s of the anti-viral antibodies and the C304 RF, apart from the "YYYN" sequence shared between the RF and the H2 anti-HSV antibody.

DISCUSSION

In order to gain a better understanding of the relationship between the normal humoral immune response to exogenous antigens and the production of autoantibodies, we have been interested in structural comparisons of anti-viral antibodies and human RFs. In the present study, we determined the sequence of the light and heavy chain variable regions of a RF derived from a RA patient with active disease. This RF was encoded by known V, D and J germline genes and represents a germline-encoded autoantibody.

The light chain expressed by the C304 RF was most homologous to previously reported anti-HSV and anti-CMV antibodies [6,23]. Although the lambda chain of the anti-HSV antibody was previously reported to be encoded by the lv1s2 germline gene, we have found it to have greater identity with the DPL8/Humlv1042 gene (data not shown) [23]. This recently described gene was also the putative germline gene for the C304 RF. This result contradicts the hypothesis that the light chain variable region genes expressed by anti-pathogen antibodies are distinct from autoantibody-related genes [23].

The V_H segment expressed by the C304 RF has previously been shown to encode both autoantibodies and anti-viral antibodies [20,23]. The VH26 gene has also been reported to be expressed by a number of clones isolated from fetal liver libraries [24-26]. The preferential utilization of this gene segment during fetal life [24] may account for

its apparent over-representation in the adult human B cell repertoire [31], although this may potentially be influenced by genetic variation at the V_H locus [32]. The C304 heavy chain was found to diverge significantly from the fetal and anti-viral antibodies in the CDR3, consistent with the view that this region is important for distinct antigenic specificities [6,7,33].

In conclusion, our light chain data indicate the potential limitations of the current sequence databases with respect to human antibodies of known specificity. Thus, it may be difficult to claim with certainty cases of germline gene usage restricted to either the normal or autoimmune repertoires. This will become less of a problem as more sequences of antibodies of different specificities, as well as the entire set of germline genes, are elucidated. Shared gene usage between anti-viral and autoimmune antibodies, as observed in this study, may thus become more evident and ultimately provide a better understanding of the structural relationship between human viral pathogens and autoantibody synthesis.

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CHAPTER VI

DISCUSSION

One of the aims of this thesis was to study the possible link between RF synthesis and the immune response directed against the human CMV. The experimental data presented here demonstates that many anti-CMV antibodies possess structural features, revealed by idiotype analysis and V region sequencing, that are common to previously described human RFs. As one might expect, this relationship was not observed for all of the anti-CMV antibodies studied. Nor was the structural relationship exclusive to this particular pathogen, since similar observations were made for an RA-derived RF and a previously characterized antibody specific for the human HSV types 1 and 2.

The study of eight monoclonal anti-CMV antibodies, derived from three different individuals, also enabled the structural characterization of the humoral response to a single antigen of a ubiquitous human pathogen. The structural complexity of this humoral response is demonstrated by the diversity in V region structure of eight hybridoma anti-pp65 antibodies and likely reflects the complexity in the structure of the protein antigen itself, which is evidenced by the different epitopic specificities of these anti-viral antibodies.

This work has also demonstrated shared usage of V genes between the autoimmune and anti-pathogen repertoires and highlights the importance of the diversity-generating mechanisms in $CDR3_H$ and of the hypermutation process in defining antigen specificity.

IDIOTYPE EXPRESSION

In the 1960's, using rabbit anti-idiotypic sera, RFs isolated from unrelated individuals were demonstrated to have similar V region structures. A decade later, amino acid sequence analyses of V regions provided further evidence that RFs within these cross-reactive idiotypic groups were structurally similar (reviewed in Newkirk & Capra, 1987). Monoclonal anti-idiotypic antibodies were subsequently generated to define more precisely the structures shared between RFs. Since the expression of the idiotypes defined by the polyclonal and monoclonal antibody preparations was believed to be related to the RF specificity, it was intriguing to find the RF-related idiotypic structures present on an anti-CMV antibody known as EV1.15 (Newkirk *et al.*, 1988).

The data presented in Chapters II and III show that these RF-associated idiotypes are expressed by many anti-CMV antibodies (seven of the eight anti-pp65 antibodies studied). The HCV-2 anti-CMV antibody, in particular, expressed a considerable number of idiotypes previously demonstrated to be found on human RFs. The fact that the EV1.15 and HCV-2 antibodies bear V regions characteristic of the two major idiotypic families, known as "WA" and "PO", respectively, warrants a closer analysis.

The murine anti-idiotypic antibodies used in the current and previous studies were generated by using human RFs as immunogens, and were believed to be specific for idiotypic determinants only. The actual structures recognized by these different reagents, however, have not yet been identified. It has been reported, however, that the idiotypes recognized by the 17.109, 6B6.6 and G6 antibodies are encoded by the kv325, kv328/kv328h5 and hv1051 germline genes, respectively (Radoux *et al.*, 1986; Liu *et al.*, 1989; Yang *et al.*, 1993). Therefore, unless these particular genes are exclusively rearranged in B cells producing RFs, it must be expected that these idiotypes will be present on antibodies of different specificities. This indeed appears to be the case for the LH1:114 anti-digoxin antibody. This antibody expresses a light chain which is identical to the predicted kv325 gene product (Danielsson *et al.*, 1991) and expresses the 17.109 idiotype (Appendix, Table A-1). The expression of the RF-associated idiotypes on the anti-CMV antibodies reported in the Chapters II and III, as well as on a number of control non-RF, non-anti-CMV antibodies (summarized in Tables A-1 and A-2), therefore, is more related to V gene usage than to antigenic specificity.

Although related to V gene usage, the correlation between the expression of the V region epitopes, recognized by the anti-idiotypic antibodies, and the expression of particular germline genes is not absolute. For example, the ITC52 antibody expresses a light chain that diverges from the kv325 gene product by only two amino acid residues (Ohlin *et al.*, 1994) yet is negative for the 17.109 idiotype (Table A-2). In addition, many of the anti-CMV antibodies that express the "V_κIII-specific" idiotypes, B12 and C7, are actually encoded by lambda germline genes. It would therefore appear that the potential of a gene to encode a particular idiotypic determinant can be modulated, perhaps as a result of somatic mutation. Formal proof of this necessitates the precise

identification of the idiotypic determinants in order to demonstrate either the loss or acquisition of an idiotype as a result of processes such as somatic mutation. In collaboration with Drs Borrebaeck and Ohlin (Lund University, Sweden), preliminary data concerning the B12 idiotype indicates that somatic mutation events, and perhaps even allelic differences, in either of two positions of a nine amino acid stretch can effectively modulate the recognition of the determinant by the monoclonal B12 antibody (unpublished observations). Further characterization of this and other determinants will clarify the structural basis for the shared idiotypic expression on antibodies of different specificity.

SEQUENCE SIMILARITIES BETWEEN RFs AND ANTI-VIRAL ANTIBODIES

Many examples of sequence similarities between anti-viral antibodies and human RFs were described in the preceding chapters. Seven of the eight anti-pp65 antibodies had either one or both Ig chains with greatest sequence identity to rearranged sequences corresponding to rheumatoid factors derived from patients with RA. This can partially be explained by the apparent bias of the current databases. This bias has resulted from the interest in elucidating the structural mechanisms involved in the development of autoimmunity. Consequently, many autoantibodies have been sequenced and have contributed significantly to our structural knowledge of human immunoglobulins. In contrast to the limited heterogeneity originally observed for the human paraprotein RFs (see Table 5-2), human hybridoma RFs derived from patients with RA show a diversity in V_H and V_L gene usage (see Tables 5-3 and 5-4). In addition, the majority of the RFs are poorly mutated (greater than 97% identity with their putative germline genes; see Tables 5-3 & 5-4). Thus, any anti-pp65 antibody with substantial sequence homology to a germline gene previously found to encode a RF can be expected to demonstrate high homology to this RF as well.

Another difficulty in the interpretation of the sequence similarities is that when both the L and H chains from the anti-pp65 antibodies were similar to RF sequences, most often they were derived from different RFs (see Table 3, Chapter II; Table 4, Chapter III). Since many crystallographic studies of antigen/antibody complexes have

demonstrated the participation of amino acid residues from all six antibody CDRs in the specific physical interaction with antigen (Amit *et al*, 1986; Sheriff *et al.*, 1987; Padlan *et al.*, 1989), the relevance of comparing the anti-viral antibodies to Ig chains from distinct RFs needs to be established. This could be accomplished by demonstrating that the H and L chains from these particular RFs could be interchanged without any loss of activity. At the present time, there is no such experimental data regarding these RFs in the literature.

In contrast to the sequence similarities described above, the data presented in Chapter V are more clearly interpretable since the anti-viral antibodies and the C304 RF are all derived from the same, or at least very closely related, V_H and V_λ germline genes. Consequently, the majority, if not all, of the amino acid differences observed between the V segments expressed by these antibodies (see Figure 3, Chapter V) can be attributed to somatic mutation events (see following section). In addition to providing further evidence for overlap between the autoimmune and anti-pathogen Ig repertoires, this data provides the opportunity to elucidate the exact structural features/requirements of the different Ig V regions that determine the anti-HSV, anti-CMV and anti-IgG-Fc specificities of these antibodies. The amino acid substitutions with significant potential for affecting the binding properties are the nonconservative changes in the CDRs, as well as in the last position of the H chain FR3. The latter has arisen from the only putative somatic mutation in the C304 RF V_H segment (Figure 2, Chapter V) and can potentially influence the structure of the antigen binding site of this antibody or even directly participate in the contact with antigen. The diversity in length and sequence of the $CDR3_H$ of these three antibodies is also an important structural feature that must be considered as having a key role in differentiating between the different specificities. Further study of these antibodies could provide direct evidence for the critical role of this region in determining the antigenic specificity that has been postulated in the previous experimental chapters as well as in a number of other studies (Sanz, 1991; Wu *et al.*, 1993; Rock *et al.*, 1994).

EVIDENCE FOR SOMATIC MUTATIONS

In the chapters describing the experimental data, the role of somatic mutation events in determining antigen specificity was evaluated by computer-aided comparison of the hybridoma sequences to known germline genes. Since all of the functional genes, including allelic variants, within the Ig loci have not yet been identified, it is important to be cautious when drawing conclusions based solely on this comparative analysis. In addition, it is important to evaluate the possibility that some apparent mutations are actually a result of errors introduced by the Taq DNA polymerase employed in the polymerase chain reaction (PCR). To address the latter issue, a minimum of three independent clones were isolated for each hybridoma sequence. Single nucleotide differences between the clones are likely to represent polymerase induced errors rather than different RNA transcripts being produced by the hybridoma cell line. Comparison of all of the clones reported herein revealed 18 differences in over 21,000 bases sequenced (data not shown), which corresponds to an observed error rate of 1/1184 (approximately 2.4×10^{-5}/bp per cycle) and is in the range previously reported (Dunning *et al.*, 1988; Loh *et al.*, 1989). The PCR conditions outlined in the methods sections in the previous chapters have been established as the conditions required for high fidelity DNA synthesis by the Taq DNA polymerase (Eckert & Kunkel, 1990). As the error rate determined above is in excellent agreement with the expected error rate, this indicates that the majority of errors due to the amplification step have been detected and excluded from the somatic mutation analysis. This conclusion is also supported by the very low number of putative mutations observed in two of the ten hybridoma antibodies sequenced (C304 and MO61).

The qualitative features of the sequence differences between the hybridoma sequences and their putative germline genes also support the conclusion that they have resulted from somatic hypermutation. Firstly, the majority of the antibodies had putative mutations with replacement/silent substitution ratios that provided evidence of antigen selection in the CDRs but not in the FRs. Secondly, when the nucleotide substitutions were categorized with respect to the four possible transitions and eight possible transversions, the pattern of exchanges was characteristic of the hypermutator

mechanism. One of the important differences observed provided evidence for a strand bias in the mutations observed (data not shown). Specifically the frequency of "A to N" exchange was double that of the reciprocal "T to N" exchange, a pattern that has previously been linked to the asymmetric hypermutator mechanism (Jacob *et al.*, 1993).

The eight anti-pp65 antibodies described herein can therefore provide some important information regarding the level of somatic mutation that occurs during the normal immune response to exogeneous antigens. In these anti-CMV antibodies, there appeared to be a two-fold greater mutation rate in the H chains when compared to the group of L chains, with an average of 21 (range 6-34) and 11 (range 4-17) mutations per V segment, respectively. These levels of somatic mutation were almost identical to those calculated for the previously published anti-pathogen antibody H chains, but significantly lower than the L chains of this same group. Specifically, by compiling the available results from previously sequenced anti-pathogen antibodies (summarized in Table 2-5), both the L and H chains were found to have an average of 19 mutations per V segment (range 5-38). These results, however, are subject to the same caveats that were expressed for the anti-pp65 antibodies regarding the incompleteness of the characterization of the Ig loci. This problem can be avoided by studying the somatic mutation level in human B lymphocytes that have rearranged the nonpolymorphic V_H6 gene (the only member of this family). Such a study revealed an average of 10 mutations (range 2-19) per V segment in IgM molecules of adult peripheral blood mononuclear cells (van Es *et al.*, 1992a). It is reasonable to expect a greater number of mutations in the anti-pp65 antibodies since they are all IgG molecules, a situation where there is expected to be an enhanced activity of the hypermutator mechanism (Sohn *et al.*, 1993). A definitive measure of somatic mutation levels in the normal human anti-pathogen response can only be achieved once the entire set of immunoglobulin genes (including allelic variants) have been characterized and only when, in the case of polymorphic genes, the expressed sequence is compared with the germline sequence from the same individual.

GENERAL IMPLICATIONS

A) REGULATION OF RF PRODUCTION

In light of the rationale for the present thesis, as stated at the beginning of the Introduction, one important question that should be addressed is whether the experimental data presented here clearly establish a relationship between the anti-CMV humoral response and RF production.

The answer to this question is that the mechanism by which RF production is triggered remains unresolved. As stated above, the exact relationship between the RFs and the anti-CMV antibodies, as examined by idiotype expression and primary sequence, is not clear. There are two possible explanations for this: first, no relationship exists, and second, a relationship exists but is not detectable by the methodology employed. In view of the body of information previously reported implicating CMV as one of the environmental agents in RA, as well as the reported associations between CMV infection/reinfection and the appearance of circulating RFs, the latter possibility must be examined.

Although the initial studies, based on human paraproteins, suggested that the majority of RFs could be grouped into three main idiotypic families, this no longer appears to be the case. In fact, the diversity of RF V regions, which is now becoming quite evident, would indicate that it is unlikely that a single structural feature (idiotype or other marker) will be common to all RFs. Thus when speaking of "RFs", it should be emphasized that this is a group of autoantibodies with diverse epitopic specificities and subject to diverse regulatory forces. It is, therefore, more likely that multiple idiotypic structures are responsible for the regulation of this group of autoantibodies. Although the relationship between the natural anti-idiotypic antibodies and the current monoclonal anti-idiotypic reagents is debatable (Jefferis, 1993a, 1993b; Kazatchkine & Coutinho, 1993), the fact that the RF-associated anti-idiotypic antibodies recognize determinants present on antibodies of different specificities is quite relevant. Current immune network theories, which are based on observations of high connectivity between B cells, predict that antibodies of different specificities are involved in multiple, dynamic interconnections (Holmberg *et al.*, 1989; Avrameas, 1991; Varela & Coutinho, 1991;

Avrameas & Ternynck, 1993). Therefore, the observation of shared idiotypes on an anti-digoxin antibody, as well as on anti-viral antibodies and RFs, does not exclude the possibility of a regulatory relationship between the latter two antibody populations. Furthermore, these theories have predicted that antigenic stimulation by exogenous antigens, leading to significant clonal expansion, is likely to modulate the immune network, favouring an alteration of the dynamic interactions of autoantibodies within the network. This disturbance of the regulatory interactions, normally maintained by the network, is predicted to result in the expansion of the autoantibody-producing clones due to the availability of autoantigens (Varela & Coutinho, 1991; Avrameas & Ternynck, 1993). This theoretical framework can easily be applied to the situation of repeated antigenic stimulation provided by CMV infections/reinfections, resulting in RF production.

Admittedly speculative, this hypothesis does provide a possible mechanism to explain how various microbiologic agents might produce similar immunologic disturbances, as well as explaining how a single agent could produce different manifestations in different individuals (observations that are reviewed in Ford, 1991, 1993). Moreover, this hypothesis highlights the probable complexity in the interactions between environmental agents and the immune system that may be involved in RA. Finally, this cautions against drawing conclusions regarding causal relationships based solely on correlative studies of serologic markers.

B) STRUCTURAL BASIS FOR ANTIGENIC SPECIFICITY

Until three years ago, structural information regarding antigen/antibody interactions was based solely on data provided by studies of the murine immune system or of human autoantibodies. Since 1991, approximately a dozen reports have emerged from studies of human hybridoma antibodies directed against human pathogens (see Table 2-5). The earliest of these provided evidence of restricted gene usage in the humoral response to *Haemophilus influenzae* type b (Adderson *et al.*, 1991; 1992). This, however, was likely a reflection of the repetitive nature of the polysaccharide antigen. The data on the eight anti-pp65 antibodies, presented in the earlier chapters, constitutes

the largest group of hybridoma antibodies directed against a single exogenous antigen to have been fully characterized with respect to H and L chain V region sequences. All eight antibodies were found to express different V_H/V_L pairs, using V genes from the largest families. In contrast to the anti-*H. influenzae* data, this demonstrated that a single exogenous antigen could elicit a humoral response with extensive Ig gene heterogeneity.

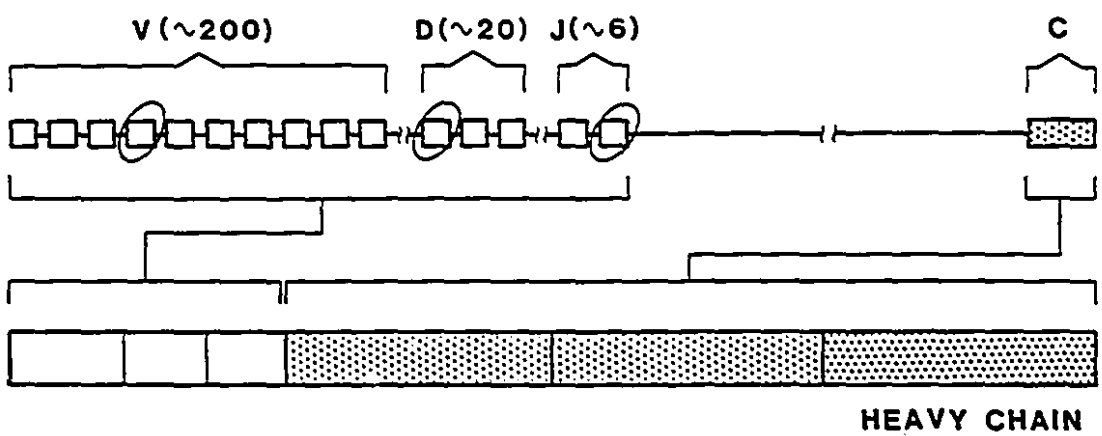
Although the majority of the anti-pp65 antibodies had evidence for antigen-selected somatically-induced mutations, the sequence of the MO61 antibody demonstrated that high affinity IgG antibodies need not be highly mutated (affinity values from Ohlin *et al.*, 1991). This suggests that the particular V_H/V_λ gene pair rearranged in the MO61 hybridoma can encode for this specificity in a near germline configuration or that the junctional diversity plays a predominant role in determining this specificity (or a combination of both).

The cloned variable regions of these anti-CMV antibodies and of the C304 RF can now be used as useful tools for determining the structural basis for the different fine specificities of these antibodies. Direct proof of the importance of the putative mutations, of particular V_H/V_L pairings, and of the $CDR3_H$ length and sequence, with respect to determining pp65 epitopic specificity and distinguishing between the anti-viral and RF specificities, can be determined experimentally. This can be achieved by V_H/V_L exchange, CDR3H swapping and site-directed mutagenesis experiments, followed by testing of the expressed proteins for binding characteristics. This approach has the potential to provide the necessary information to expand our understanding of the basic structural requirements that determine certain antigenic specificities. This basic knowledge, which is required to address the issues of whether limited point mutations can separate the anti-pathogen and autoimmune repertoires and whether junctional diversity is essential, should ultimately lead to the improved design of recombinant antibody reagents specific enough to be useful in the clinical setting.

APPENDIX

FIGURE A-1. Basic immunoglobulin structure. Schematic diagram representing human immunoglobulins. A representative heavy chain variable region is shown to be derived from the rearrangement of particular germline V, D and J gene segments. The light and heavy chain variable (V) and constant (C) regions are indicated.

Antibody Structure



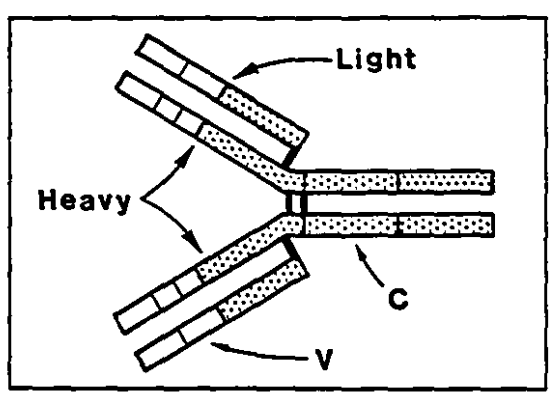


Table A-1. Summary of the hybridoma antibodies studied for the expression of RF-associated idiotypes.

ANTIBODY	SOURCE[1]	ISOTYPE	SPECIFICITY[2]	GENE FAMILY		REF.[3]
				VH	VL	
HCV-2	A	IgG_k	CMV, pp65	3	k IIIa	[1]
HCV-3	A	IgG_l	CMV, pp65	3	L I	[1]
HCV-63	A	IgG_l	CMV, pp65	4	L I	[1]
HCV-65	A	IgG_l	CMV, pp65	3	L III	[1]
MO53	B	IgG_k	CMV, pp65	3	k I	[1]
MO58	B	IgG_k	CMV, pp65	1	k I	[1]
MO61	B	IgG_l	CMV, pp65	3	L I	[1]
MO81	B	IgG_k	CMV, pp65	4	k I	[1]
MO80	B	IgG_k	CMV, pp28	N.D.	N.D.	
MO188	B	IgG_k	CMV, gp116	N.D.	k IIIa	[2]
ITC33	B	IgG_l	CMV, gp58	5	L II	[3]
ITC39	B	IgG_l	CMV, gp58	3	L III	[3]
ITC48	B	IgG_k	CMV, gp58	4	k IV	[3]
ITC52	B	IgG_k	CMV, gp58	5	k IIIb	[3]
ITC63B	B	IgG_l	CMV, gp58	5	L III	[3]
B7	B	IgG_l	TT	N.D.	N.D.	
MO6	B	IgM_k	Digoxin	3	k II	[4]
LH1:92	B	IgM_l	Digoxin	3	L I	[4]
LH1:114	B	IgM_k	Digoxin	4	k IIIb	[4]
BOR[4]		IgM_k	IgG-Fc	1	k IIIb	[5]

[1] Source of the hybridoma supernatants: A, The National Laboratory for Immunology Ottawa, Canada (kindly provided by Drs. B. Brodeur and Y. Larose); B, Department of Immunotechnology, Lund University, Sweden (kindly provided by Drs. C.A.K. Borrebaeck and M. Ohlin).

[2] Abbreviations: CMV, human cytomegalovirus; TT, tetanus toxoid.

[3] References regarding variable gene usage: [1], the present thesis; [2], Dr. Mats Ohlin (personal communication); [3], Ohlin et al., 1994; [4], Danielsson et al., 1991. [5], Newkirk et al., 1987.

[4] BOR is a human paraprotein with RF activity and was one of the positive controls included in this study (source Dr. M.M. Newkirk).

Table A-2. Expression of RF-associated idiotypes by human hybridoma antibodies.

ANTIBODY	RF-ASSOCIATED IDIOTYPES[1]									
	B12	C7	17.109	C6	G6	B6	D12	G7	86.3	102.2
HCV-2	3[2]	4	1	0	1	1	1	1	2	1
HCV-3	1	1	0	0	0	1	0	1	0	0
HCV-63	1	0	0	0	0	0	0	0	0	1
HCV-65	2	1	0	0	0	0	0	1	0	1
MO53	1	1	0	0	1	1	0	0	0	1
MO58	0	1	0	0	0	0	0	0	0	0
MO61	2	1	0	0	0	1	0	0	0	0
MO81	0	0	0	0	0	0	0	0	0	0
MO80	1	1	0	0	0	0	0	1	0	1
MO188	2	2	0	0	0	0	0	0	0	0
ITC33	1	0	0	0	0	0	0	0	0	0
ITC39	0	0	0	0	0	0	0	0	1	0
ITC48	0	0	0	0	0	0	0	0	0	0
ITC52	1	1	0	0	0	0	0	0	0	0
ITC63B	0	0	0	0	0	0	0	0	0	0
B7	1	1	0	0	0	1	0	0	0	1
MO6	0	1	0	1	0	0	0	0	0	1
LH1:92	2	1	0	0	0	1	0	0	0	0
LH1:114	4	4	2	3	1	0	0	0	1	3
BOR	4	4	3	3	4	0	0	0	1	2

[1] All of these antibodies were negative for the following idiotypes: B9, E5, 16.65, EV1.15, C10, 16.72, 16.84, A5 and C8.

[2] The idiotype expression results are expressed as ELISA scores (determined by optical density measurements at 492 nm): 0, $O.D._{492}$ <0.10; 1, $O.D._{492}$ >0.10 - 0.50; 2, $O.D._{492}$ >0.50 - 1.00; 3, $O.D._{492}$ >1.00 - 1.50; 4, $O.D._{492}$ >1.50.

CHAPTER VII

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CHAPTER VIII

CLAIMS FOR ORIGINAL RESEARCH

1. The immunoglobulin heavy and light chain variable regions that are endogenously rearranged in the GM 4672 lymphoblastoid cell line, were cloned and sequenced. Analysis of the human hybridoma cell lines derived from this fusion partner provided evidence that the heavy and light chain variable regions expressed by the hybridomas originate from the fused peripheral blood lymphocytes and not from the GM 4672 cell.

2. The immunoglobulin heavy and light chain variable regions of eight human hybridoma anti-CMV antibodies, specific for the viral phosphoprotein pp65 (also known as the lower matrix protein), were cloned and sequenced. Each antibody was found to express a different V_H/V_L pair.

3. The expression of RF-associated idiotypes were documented in a group of anti-cytomegalovirus antibodies, as well as on some antibodies without RF or anti-viral activity. A comparative analysis of the variable region sequences and idiotype expression of these antibodies indicated that although it is related to V gene usage, the expression of these idiotypes can be modulated. This was associated both with a lack of idiotype expression on antibodies encoded by idiotype-related genes and significant idiotype expression on antibodies expressing genes not previously associated with these idiotypes.

4. Analysis of the hybridoma sequences provided evidence for somatic mutation events that are potentially important for the fine specificity of these antibodies. It was demonstrated, however, that it is possible for an an IgG anti-CMV antibody (MO61) to have high affinity for its antigen and, yet, have very few mutations.

5. The cloning and sequencing of the Ig V region genes of a human hybridoma RF (C304) derived from a patient with RA provided further evidence that these autoantibodies can be germline encoded.

6. Sequence comparisons, in particular between the C304 RF and anti-viral antibodies (including the HCV-3 anti-CMV antibody also characterized in this study), indicate a critical role for the $CDR3_H$ in determining antigenic specificity.

7. Comparisons between the sequences obtained in this study (RF and anti-CMV) and known rearranged sequences, demonstrated significant overlap in the anti-pathogen and autoimmune Ig gene repertoires.