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Epitope Promiscuity of Human Monoclonal Autoantibodies to T-Cell Receptor-Combining Site Determinants

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Abstract

To characterize the binding specificity and light- and heavy-chain variable region usage in monoclonal human autoantibodies (mAAbs) to T-cell receptors, we constructed heterohybridomas from peripheral blood B cells of three rheumatoid arthritis (RA) patients. From a panel of more than 200 heterohybridomas secreting IgM autoantibodies binding to T-cell receptor Vβ chain first complementarity determining segments (CDR1), we characterized two IgM/λ molecules from a single patient in detail. These bound to both CDR1 peptide epitopes and intact TCR of recombinant single-chain T-cell receptor constructs, and to T-cell surface TCR. Spectratype analysis using epitopes mimicking a set of 24 Vβ genes indicated that one molecule bound only a few members of the set, whereas the second showed considerable epitope promiscuity by binding to more than half of the tested CDR1 peptides. Both mAAbs used variants of a Vλ3 gene that were very similar to one another and to the germline gene. The epitope-promiscuous autoantibody used a V_H4 gene identical to a germline prototype, while the other incorporated a V_H3 sequence differing in only a single residue from its germline prototype. The CDR3s of both were large and distinct from each other as well as from the corresponding segments of rheumatoid factors and "cold agglutinins" using the same or related V_H germline genes. These mAAbs offer models for deciphering the basis of epitope promiscuity, and serve as candidates for direct use in immunomodulation because they are of intrinsic human origin and do not require molecular engineering to adapt them for use in therapy.

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Introduction

Although antibodies have historically been noted for their exquisite specificity in recognition of antigens *(1)*, numerous examples of polyspecific antibodies have been documented *(2–11)*. These are usually IgM immunoglobulins that bind with relatively low affinity to a number of distinct proteins or nucleic-acid determinants. Most recently, murine MAbs induced to retroviral glycoproteins *(12,13)* and monoclonal antibodies specific for T-cell receptors that were generated using spleens from retrovirally infected mice *(14)* have been shown to exhibit promiscuity in their capacity to bind sets of peptides having little sequence identity to one another. In addition, some human monoclonal IgG myeloma proteins can bind both recombinant TCR structures and synthetic peptide epitopes corresponding to public CDRs, most notably CDR1 *(15)*. Examples were found of IgG myelomas that were relatively specific (they predominantly bound a single peptide-defined epitope), whereas others were promiscuous—they bound to the majority of members of a set of 24 synthetic peptides duplicating the CDR1 epitopes of distinct human Vβ families. The present study was designed to gain information on the binding properties and V_H and V_L gene usage of human monoclonal autoantibodies (mAAbs) specific for human T-cell receptor epitopes as determined using recombinant Vα/Vβ Fv constructs *(16)* and synthetic peptides duplicating the linear sequence of α and β TCR genes by comprehensive peptide synthesis *(17)*, and by epitope analysis using a set of peptides, each of which corresponded individually to that of a distinct human Vβ gene product *(18)*.

Healthy, asymptomatic humans express low levels of autoantibodies reacting specifically with T-cell receptor Vβ CDR1 epitopes *(17)*, and the levels of these vary with retroviral infection *(14,16,19,20)*, various physiological conditions including aging *(21)*, and autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis *(19,21–23)*. We found that rheumatoid arthritis patients tend to have elevated levels of IgM autoantibodies reactive with CDR1 peptide epitopes, and in one case, we observed a patient with a serum titer of greater than 32,000. The level usually found for normals in our enzyme-linked immunosorbent assay (ELISA) system is a titer of approximately 50 *(19,21)*. Because spontaneously occurring myeloma proteins have been found expressing autoantibody activity against T-cell receptor epitopes *(15)* and a screen of 70 Epstein–Barr virus-transformed B lymphocytes revealed two IgM molecules with such specificity *(24)*, we believed it would be feasible to select for human monoclonal anti-TCRs experimentally. Thus, we hypothesized that it should be possible to produce heterohybridomas between B cells of rheumatoid arthritis patients and appropriate murine myeloma partners in order to generate sufficient human numbers of autoantibodies to enable epitope analysis, binding to cells by flow cytometry, and determination of complete V_H and V_L sequences. In this article, we report the overall profile of

specificity patterns obtained for more than 200 monoclonal autoantibodies generated from either peripheral blood or synovium of rheumatoid arthritis (RA) patients, illustrate detailed properties of specific examples, and compare the V_H and V_L usage with that shown by rheumatoid factors and examples of polyspecific human IgM antibodies.

Methods and Materials

Generation of Heterohybridomas Secreting Human Autoantibodies to TCR Epitopes

To generate human B cells peripheral blood B cells of three RA patients were stimulated to proliferate by interaction with CD40 ligand (gp39) and IL-4 and IL-10 *(25–27)*. Once the B cell population had been amplified approximately 10-fold by in vitro culture under these conditions, the cells were fused with the murine myeloma fusion partner P3X63-AG8.653 *(27)*. The fused cells were plated in a 96-well culture plate at 20,000 B cells per well, and culture supernatants were assayed approximately 14 d later by enzyme–linked immunosorbent assay (ELISA) for activity against CDR1 peptides and recombinant single-chain T-cell receptors containing the appropriate peptide epitopes. The major peptide used in the preliminary screen was a 16-mer duplicating the complete CDR1 and first five residues of the second framework of the Vβ8.1 gene expressed in the Jurkat T-cell line *(17)*. Hybridomas secreting binding antibodies were formally cloned by limiting dilution.

Antigens and Immunoassay

The peptides used to duplicate the set of CDR1 segments of human Vβ gene products are illustrated in Table 1. These were synthesized by Chiron Mimetopes, San Diego, CA, and were generally at levels greater than 90% purity. Recombinant single-chain T-cell receptors were prepared as described by Lake et al. *(28)*. Two were used in this study: the first had complete Vα and Vβ structures corresponding to those in the T-cell Jurkat (Vα1, Jα, Vβ8.1, Dβ, Jβ1.2,), and the second had Vα4 and Vβ2 sequence corresponding to those of a line derived from tumor-infiltrating cells that bound to the MAGE1 peptide when presented on cells expressing HLA-A A1 (Lake, D. F., Salgaller, M. L., Van der Bruggen, P., Bernstein, R. M. and Marchalonis, J. J., unpublished observations).

Immunocytofluorescence in quantitative flow cytometry was carried out using a Becton-Dickinson two-laser FACStar+ machine. The human T-cell Jurkat and the mouse T-cell DO-11.10 that expresses the murine Vβ8.2 gene product were tested *(29)*. The DO-11.10 cell was the gift of Dr. P. Marrack, Howard Hughes Institute, National Jewish Hospital, Denver, CO. Fluorescence was carried out using a sandwich procedure in which FITC-labeled goat $F(ab)_2$ antibodies to human μ-chain (Caltag Labs., Burlingame,

Table 1
Synthetic Peptides Duplicating the Major Human Vβ Gene Family Products[a]

Vβ2.1	C	R	S	L	D	F	Q	A	T	T	M	F	W	Y	G	Q	F
Vβ3.1	C	V	Q	D	M	D	H	E	N		M	F	W	Y	R	Q	D
Vβ4.1	C	Q	V	D	S	Q	V	T	M		M	F	W	Y	R	Q	Q
Vβ5.1	C	S	P	R	S	G	D	L	S		V	Y	W	Y	Q	Q	S
Vβ5.2	C	S	P	K	S	G	H	D	I		V	S	W	Y	Q	Q	A
Vβ6.1	C	D	P	I	S	G	H	T	A		L	Y	W	Y	R	Q	S
Vβ6.5	C	D	P	I	S	E	H	N	R		L	Y	W	Y	R	Q	T
Vβ7.1	C	E	Q	H	M	G	H	R	A		M	Y	W	Y	K	Q	A
Vβ8.1	C	K	P	I	S	G	H	N	S		L	F	W	Y	R	Q	T
Vβ9.2	C	E	Q	N	L	G	H	D	T		M	Y	W	Y	K	Q	D
Vβ10.1	C	V	P	I	K	A	H	S	Y		V	Y	W	Y	R	K	K
Vβ11.1	C	S	Q	T	M	G	H	D	K		M	Y	W	Y	Q	Q	D
Vβ12.1	C	H	Q	T	E	N	H	R	Y		M	Y	W	Y	R	Q	D
Vβ13.1	C	A	Q	D	M	N	H	E	Y		M	Y	W	Y	R	Q	D
Vβ14.1	C	S	Q	N	M	N	H	E	Y		M	S	W	Y	R	Q	D
Vβ15.1	C	S	Q	T	K	G	H	D	R		M	Y	W	Y	R	Q	D
Vβ16.1	C	D	P	I	S	G	H	D	N		L	Y	W	Y	R	R	V
Vβ17.1	C	E	Q	N	L	N	H	D	A		M	Y	W	Y	R	Q	D
Vβ18.1	C	S	P	M	K	G	H	S	H		V	Y	W	Y	R	Q	L
Vβ19.1	C	T	P	E	K	G	H	T	F		V	Y	W	Y	Q	Q	N
Vβ20.1	C	T	V	E	G	T	S	N	P	N	L	Y	W	Y	R	Q	A
Vβ21.1	C	D	P	I	S	G	H	A	T		L	Y	W	Y	R	Q	I
Vβ22.1	C	V	P	I	S	N	H	L	Y		F	Y	W	Y	R	Q	I
Vβ23.1	C	Y	P	I	P	R	H	D	T		V	Y	W	Y	Q	Q	G
Vβ24.1	C	S	Q	T	L	N	H	N	V		M	Y	W	Y	Q	Q	K

[a]For use in mAAb selection and anti-CDR1 spectrotype analysis. The N-terminal Cys (C) is part of Framework 1 and the C-terminal residues beginning with Trp (W) represent Framework 2 (e.g., WYGQF).

CA) were used to detect binding of either monoclonal IgM autoantibodies secreted by the heterohybridomas, or an isotype-matched λμ myeloma protein as a negative control. An additional negative control was assay for binding by the conjugate alone—i.e., no human IgM molecule was used as a first antibody. ELISA assays using peptides and recombinant proteins have been described in detail previously *(14,17,18,24)*. These were carried out in both the direct binding and inhibition modes. The fine specificity of the binding by the mAAbs was determined by ELISA assays, in which a set of 24 peptides duplicating the CDR1/FR2 segments of 24 distinct human Vβ families were used (*see* Table 1). The overlapping peptides, which duplicate the complete covalent structure of T-cell receptor Vβ chains, have been published elsewhere *(30)*. In the studies described here, the complete VDJ domain of TCRβ chains is comprised of overlapping peptides 1–11. The peptides are hexadecamers, and overlap by five residues.

Isolation and Analysis of V_H and V_L Genes Used by the Heterohybridomas

Messenger RNA was isolated using the MicroFast track kit from Invitrogen, Inc. (Carlsbad, CA). Complementary DNA was made by random priming, using the cDNA Cyclekit (Invitrogen), and the cDNA was amplified using TCR with V-region specific primers. Three sets of forward primers derived from leader sequences that cover all six human V_H families, and two other sets specific for Vκ and Vλ genes, were obtained from Novagen (Madison, WI). Amplified products were cloned using the Invitrogen T-vector system and transformed to XL-1 blue cells (Strategene, La Jolla, CA). Double-stranded DNA was prepared and sequenced by the dideoxy-chain termination procedure using Sequenase, USB. The details of PCR, cloning, and sequencing have been reported by previous studies from this laboratory *(14,24,31–33)*.

Analysis of human gene sequences was carried out using the program DNAPlot with local database searching, using the MacVector DNA-analysis package (Oxford Molecular Group, Inc, Campbell, CA). The germline parents have been identified by selecting the sequence with the closest match.

Results

Generation of Monoclonal Heterohybridomas Secreting Human Autoantibodies to TCR Epitopes

We have successfully developed a procedure for production of heterohybridomas secreting human autoantibodies to TCR epitopes by application of the CD40 ligand method *(25)* to amplify the B-cell population followed by fusing with the mouse-myeloma cell line P3X63-AGA.653. In the initial screening, supernatants from wells of 96-well plates were tested against synthetic Vβ-CDR1 and FR3 peptide epitopes, and also against a recombinant single-chain TCR protein containing the complete Vα and Vβ of the T-cell line Jurkat. Table 2 summarizes data comprising more than 200 wells derived from PBL of three RA patients who were positive for reactivity to the CDR1 peptide epitope. The majority appeared to be polyreactive antibodies, but approximately 20% displayed specificity for only the CDR1 epitope and not for the FR3 peptide. All of the mAAbs we have characterized from RA patients thus far are of the IgM isotype. The majority of these autoantibodies that were selected on the CDR1 peptide react only with the peptide and not with the recombinant scTCR or with the T-cell receptor expressed on intact Jurkat T-cells. However, approximately 10% react with the CDR1 peptide alone and with the recombinant scTCR and intact T-cell. Approximately 1% bind both CDR1 and FR3 peptode epitopes as well as the intact protein structures.

Table 2
Reactivity Profiles of the Major Classes of Heteromyeloma Hybridoma Clones Produced from RA Patients[a]

Approximate Frequency of Clones	Screen and Test Antigens			
	CDR1	Fr3	rscTCR	FACS
75%	+	+	—	—
5%	+	+	+	—
10%	+	—	—	—
10%	+	—	+	+
<1%	+	+	+	+

[a]Hybridomas secreting anti-TCR autoantibodies are screened in ELISA using a combination of synthetic and recombinant scTCR Vβ8 antigens. Supernatants positive in ELISA are tested for binding to native TCR by FACS analysis on Jurkat cells. Screening results for fusion experiments using peripheral B cells from 3 RA patients are shown.

Promiscuous and Specific Human IgM Monoclonal Autoantibodies

Figure 1 is a representative example of the ELISA reactivity profile of a hybridoma of the "epitope-promiscuous" type. This monoclonal IgM—clone 4D4—which was derived from peripheral blood of an RA patient is absolutely specific for the CDR1 epitope (peptide #3) of the Jurkat Vβ (Vβ8.1) as demonstrated by reactivity with only this peptide of the set of overlapping peptides modeling the Vβ8.1/D/J β domain (*see* Fig. 1A). It binds only to the scTCR protein, and is negative for ovalbumin (*see* Fig. 1B), as well as several other test proteins, including bovine serum albumin and thyroglobulin (not shown here). As shown in Fig. 2, this monoclonal autoantibody also binds to the Jurkat T-cell line, which expresses the cognate β chain, and the α chain which was used to construct the recombinant molecule and served as the template for the design of the β-chain peptides. The epitope-promiscuous nature of this monoclonal autoantibody is shown by the CDR1 recognition spectratype (*see* Fig. 1C). In addition to reactivity to the Vβ8.1 sequence used in the selection, there is substantial reactivity with CDR1 epitopes including Vβ4.1, 7.1, 10.1, 16.1, and 23.1 gene families. Monoclonal autoantibodies of the monogamous or highly specific type have been isolated from two of the RA patients studied to date. However, our best example of high specificity is illustrated by a monoclonal autoantibody isolated by Epstein–Barr virus treatment of normal B cells from a Burkitt lymphoma patient. This is a κμ autoantibody produced by Clone IARC307 *(24)*. Its binding activity is shown in Fig. 3. The IARC mAb is strictly specific for the CDR1 region of Vβ8.1, and did not react with other CDR1 peptide homologs of various Vβ families or corresponding sequences from TCR, Vα, and immunoglobulin light-chain Vλ sequences (*see* Fig. 3A). In inhibition experiments with related and unrelated pep-

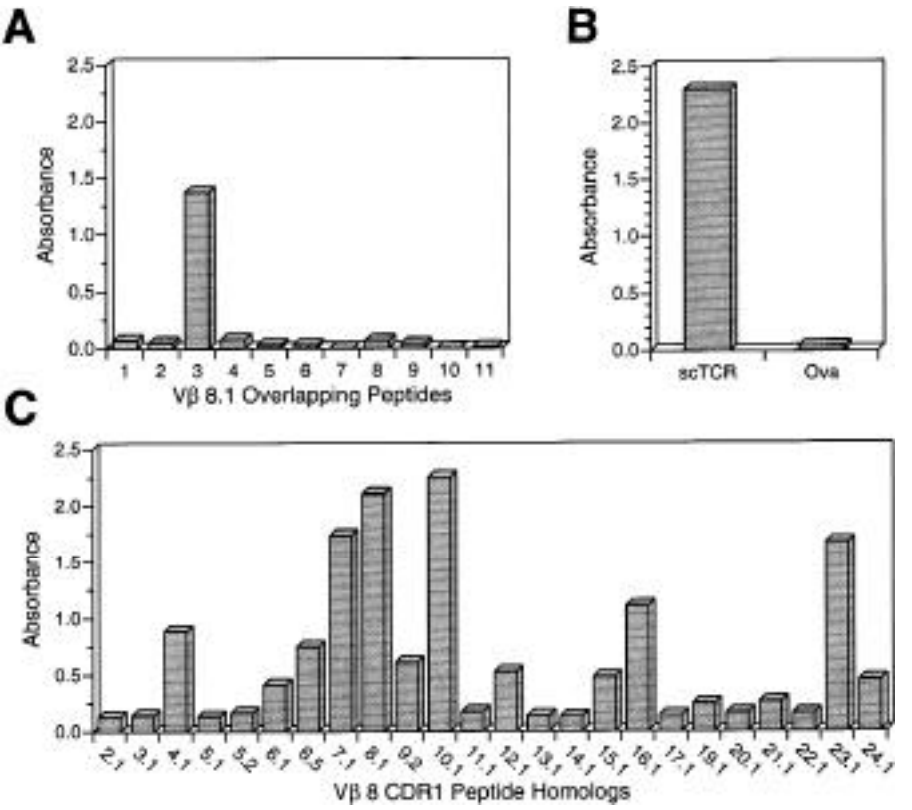

Fig. 1. Reactivity profile of hybridoma 4D4 derived from the peripheral blood of an RA patient. (A) The specificity of the monoclonal was assessed by determining in ELISA binding to 11 overlapping 16-mer peptides corresponding to the V-region of Vβ8.1. The IgM fraction was partially purified and concentrated fivefold from tissue culture supernatant by ammonium sulfate precipitation. The preparation was tested at a dilution of 1/100. (B) Binding to recombinant scTCR. The antigen used in this assay was a recombinant single-chain construct consisting of the alpha and beta chain TCR V regions isolated from the Jurkat T-cell line. Recombinant protein was produced by expression in bacteria. (C) CDR1 recognition spectratype. To determine the fine specificity of the anti-CDR1 monoclonal autoantibody, a set of 24 homologous 16-mer peptides modeling the CDR1 segment of a broad range of different Vβ family germline-gene sequences were tested in ELISA.

tides (*see* Fig. 3B), only the appropriate Vβ8-CDR1 peptide blocks the binding. Under the experimental conditions used, 50% inhibition was obtained with 20 nmol of peptide.

Epitope Analysis of Two Monoclonal Autoantibodies Derived from Synovial B Cells of a RA Patient

Figure 4 presents ELISA titers obtained for serial dilutions of culture fluid of two λμ monoclonal autoantibodies secreted by heterohybridomas formed from B cells, derived from the synovium of a RA patient (not the

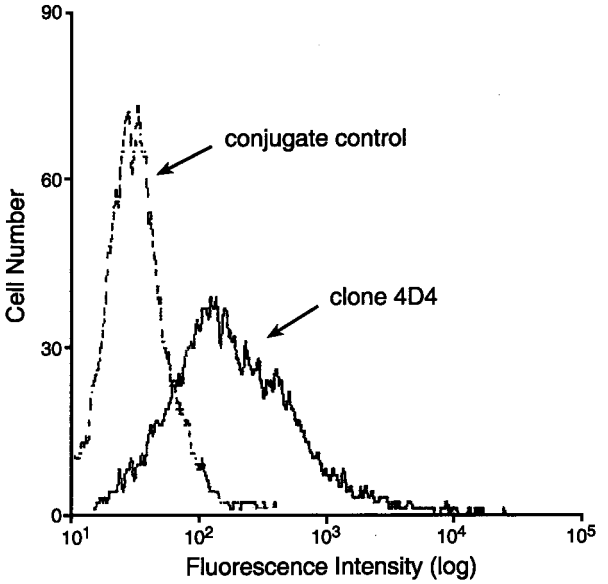

Fig. 2. FACSCAN profile showing binding of a human IgM monoclonal anti-TCR autoantibody (clone 4D4) to Jurkat T-cells (solid line). The IgM fraction was partially purified and concentrated 10-fold by ammonium sulfate precipitation. The dotted line here represents the conjugate control (fluorescein-labeled antihuman IgM antibody). Pooled normal IgM (not shown) is comparable to the conjugate control.

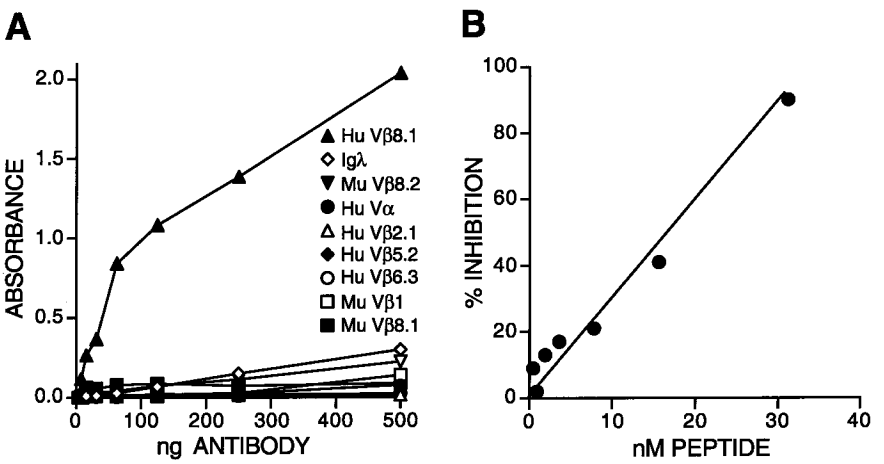

Fig. 3. **(A)** Binding of IARC307 autoantibody in ELISA to synthetic peptides of CDR1 region from several TCR Vβ families of man and mouse and to peptides of the same region of TCR Vα (PY14) and Igλ (Mcg). **(B)** Inhibition of IARC307 autoantibody binding to CDR1-region peptide of Vβ8.1 with free peptide. Peptide inhibitions with the other peptides are not shown, since they were all negative. [Fig. adapted from Dedeoglu et al., *(24)*.]

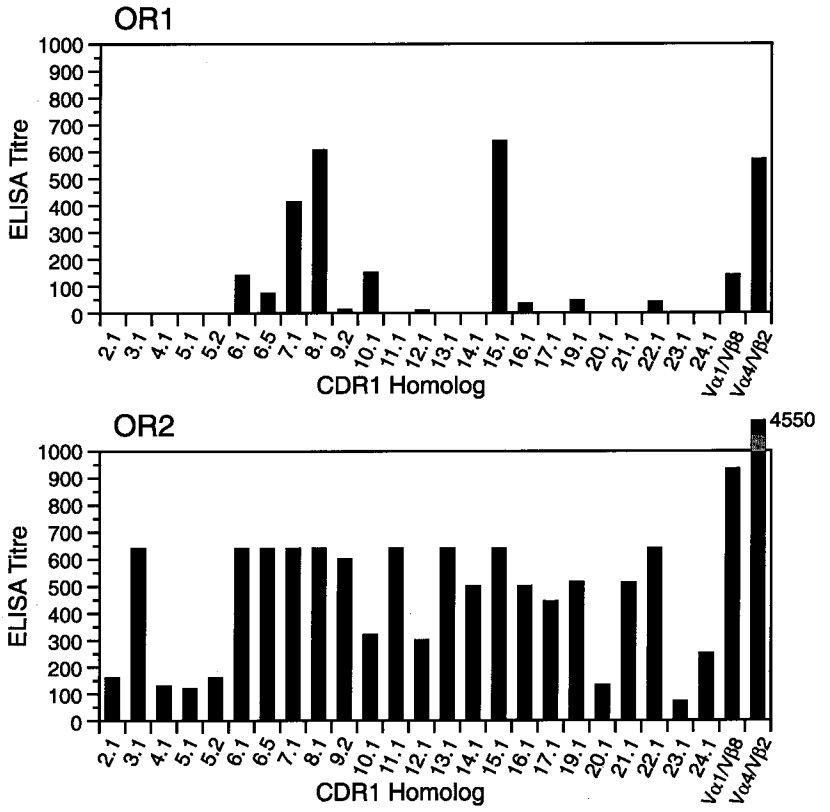

Fig. 4. Fine-specificity analysis of heterohybridomas formed from synovial B cells of a RA patient. Culture supernatants were tested in ELISA against a set of peptide CDR1 epitopes representing 24 human Vβ families. Also included is reactivity against recombinant single-chain Fv TCR constructs. The reactivity of OR1 is shown in the top panel and OR2 in the bottom panel. The titer of OR2 against the Vα4/Vβ2 scTCR protein is off the graph scale, as indicated by the extended bar, and the actual titer (4550) is indicated.

individual from whom 4B4 was derived). Both monclonals show substantial titers against the recombinant single-chain T-cell receptors comprised of either the Jurkat Vα1/Vβ8 gene products or a second scTCR containing Vα4 and Vβ2 gene products. Although the selection for these clones was based upon binding to the CDR1 peptide of Jurkat and the Jurkat scTCR, both λμ products showed higher titers to the Vα4/Vβ2 single-chain product than for that used in the direct selection. The CDR1 peptide spectratype analysis provides an interesting comparison between the two. The OR1 product binds best to the CDR1 peptide epitopes of human Vβ8.1 and Vβ15.1, with substantial reactivity to the Vβ7.1 peptide. Some activity at a lower degree is obtained for several other peptides. By contrast, the OR2 product shows titers of at least 600 against 10 of the Vβ CDR1 epitopes tested. In addition, ELISA titers of at least 500 were observed to another

Table 3
Strategy to Define Peptide Epitopes of Vβ CDR1

	CDR1
	⟷
pep β2	VTEMGQEVTLRCKPIS
pep β3	CKPISGHNSLFWYRQT
pep β4	WYRQTMMRGLELLIYF
pep β8.1	KPISGHNSLF
pep β3R	GGPISGHNSLFWYRQT

three of the test peptides. Thus, the OR1 IgM shows relatively restricted CDR1 epitope specificity, whereas the OR2 product shows substantial epitope promiscuity. By comparison with Fig. 1 for the monoclonal IgM autoantibody 4D4, it is apparent that the peptide epitope spectratype of each monoclonal is characteristically different.

Definition of Peptide Epitopes of Vβ CDR1 Segments

The preceding data indicate that the selected mAAbs bind to both intact T-cell receptor Vα/Vβ regions and to peptide epitopes corresponding to a public idiotypic marker. Table 3 illustrates a strategy to define peptide epitopes of the Vβ CDR1 segment. This consists of determination of the binding to overlapping peptides duplicating the linear sequence of FR1/CDR1 (pepβ2), CDR1/FR2 (pepβ3), and the FR2 segment (pepβ4). In addition, pepβ8.1 contained only the segment defined as CDR1. The peptide β3R had the N-terminal cysteine (C) of peptides β2 replaced by two glycines. The purpose of this peptide is to maintain the length of the original peptide, and to determine whether the presence of the N-terminal cysteine was essential to retain antigenicity as detected by the two monoclonals. Figure 5 illustrates ELISA titrations of the IgM autoantibodies of hybridoma clones OR2 and OR1 to these sets of peptides. The OR1 molecule binds with virtually identical high-titration profiles to the original pepβ3 and to the N-terminal-derived molecule. It does not bind to any of the other peptides. The OR2 IgM autoantibody likewise binds with identical strong titration behavior to the pepβ3 and pepβ3R. Moreover, it shows substantial binding to pepβ4, which contains the first five residues of FR2. It also binds to the internal 10-mer peptide consisting only of the CDR1 segment. The binding to pepβ2 containing the first five residues of CDR1 is negligible.

Because the peptides overlap by five residues, these data indicate that the residues crucial to the recognition of the CDR1 epitope of this molecule are GHNSLF in both cases. However, the short 10-mer peptide is not bound at all by OR1, indicating that the complete hexadecameric length is required for recognition of the peptide epitope. This suggests the existence of conformational structure by this peptide. The most obvious conjecture is that the peptide forms a hairpin loop with the N-terminal cysteine in close proximity to the tryptophan (W) just as it is in the loop structure

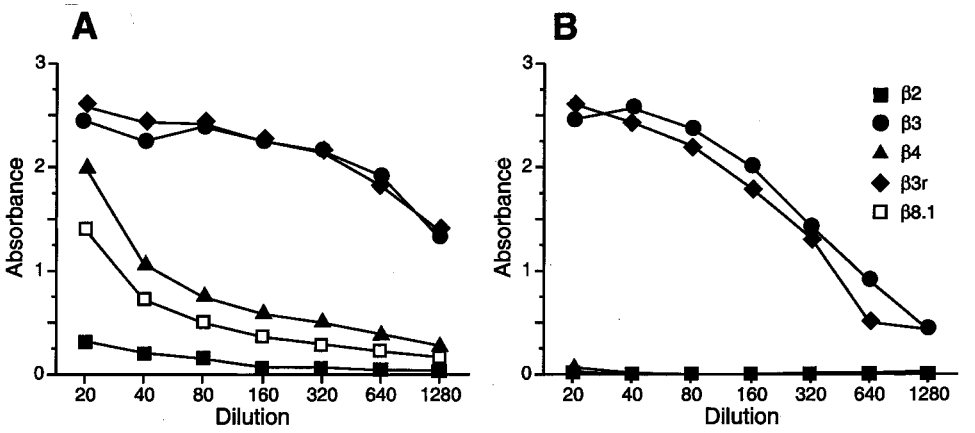

Fig. 5. Definition of peptide epitopes of Vβ CDR1 segments. To determine the precise epitope recognized by OR1 **(B)** and OR2 **(A)**, reactivity was measured against a set of peptides duplicating the linear sequence of the Vβ CDR1 region. The peptides used are described in Table 3.

of the intact Vβ domain *(34)*. The results with the OR2 autoantibody are consistent with this finding, although this molecule shows binding capacity with the internal 10-mer peptide as well. Part of the capacity of OR2 to bind a large set of pepCDR1 epitopes may result from its property of binding to the β4 peptide containing the shared motif WYRQ. Both autoantibodies give results consistent with a lack of importance of the N-terminal cysteine to formation of the peptide epitope.

Binding of the OR2 Monoclonal Autoantibody to Murine TCR Epitopes

Analysis of the T-cell receptor β and α genes of humans and mice indicates that these are orthologous structures specified by genes that arose in evolution prior to the ancestral divergence between primates and rodents *(35,36)*. Figure 6 compares titers of OR1 and OR2 monoclonal autoantibodies to CDR1-associated peptide epitopes of humans and mice. In this analysis, the OR1 molecule has an appreciable titer only to the human Vβ8.1 16-mer. By contrast, the OR2 IgM binds well to the 16-mers of both human Vβ8.1 and human Vβ3.1. These represent distinct families, with the human Vβ3.1 showing some similarity to the murine Vβ8 family *(35)*. The human Vβ8 family shows an orthologous relationship to the murine Vβ11 family *(35)*. We tested the capacity of purified OR2 λμ autoantibody to react with a monoclonal mouse T-cell line (DO-11.10) that expresses the Vβ8.2 gene product by flow cytometry in Fig. 7. The conjugate control binds to fewer than 5% of the murine T-cells. An isotype-matched (λμ) control human protein (the myeloma protein Frymac) binds to less than 10% of the cells. By contrast, the OR2 IgM protein binds to approximately one-half of the murine T-cells. Thus, the epitope-promiscuous human mono-

VβCDR1	Peptide sequence	TITER OR1	OR2
Hu 8.1	CKPISGHNSLFWYRQT	924	>1,280
Hu 3.1	CVQDMDHENMFWYRQD	0	640
Mu Vβ1	EQHLGHNAMY	0	131
Mu Vβ8.1	MQTNNHDYMY	0	443
Mu Vβ8.2	NQTNNHNNMY	0	257

Fig. 6. Comparison of binding to human and mouse orthologous CDR1 structures. Identities between mouse and human sequences are shaded. The titers of OR1 and OR2 culture supernatants were determined in ELISA.

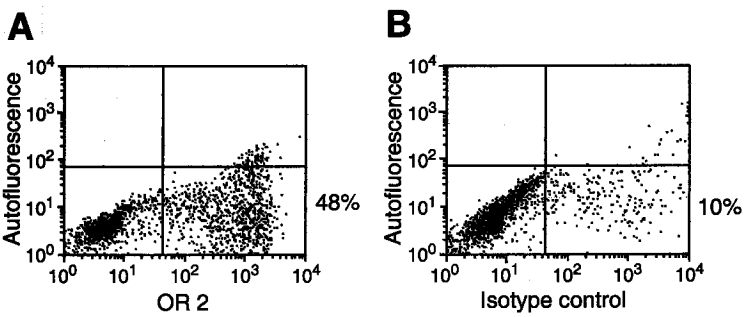

Fig. 7. Flow cytometric analysis of binding by OR2 to the mouse T-cell line DO- 11.10. Binding of OR2 and an isotype-matched IgM control are plotted against autofluorescence. Numbers represent the percentage of positive cells in the lower right quadrant.

clonal autoantibody OR2 binds not only to a set of human TCR epitopes, but to at least some murine T-cells as well.

Molecular Analysis of OR1 and OR2 V_H and V_L Usage

cDNA specifying V_H and V_L products of OR1 and OR2 were prepared from mRNA by RT-PCR using primers designed to select for the set of V_H and V_L gene products. Both OR1 and OR2 used Vλ 3 gene products that were identical to the germline sequence hsigll150 *(37)*. The Vλ sequences of OR1 and OR2 differed from each other by only a single residue in the third hypervariable region. As of this time, we have generated and partially characterized 10 anti-TCR autoantibodies from the peripheral B cells of this patient, and all of them have used λ light chains. Figure 8 compares the sequence of the OR2 λ light chain with that of a rheumatoid factor derived from RA synovial B cells by other workers *(38)*. This monoclonal rheumatoid factor (RFSJ2) uses a λ chain with a Vλ1 sequence. Although both of these synovial B-cell-derived λ chains utilize the same J segment (Jλ2), it is apparent that there are substantial sequence differences throughout in the direct comparison between the two λ light chains.

OR1—the relatively restricted monoclonal autoantibody to TCR—has a V_H3 sequence that is extremely similar to the V_H3 sequences used by the

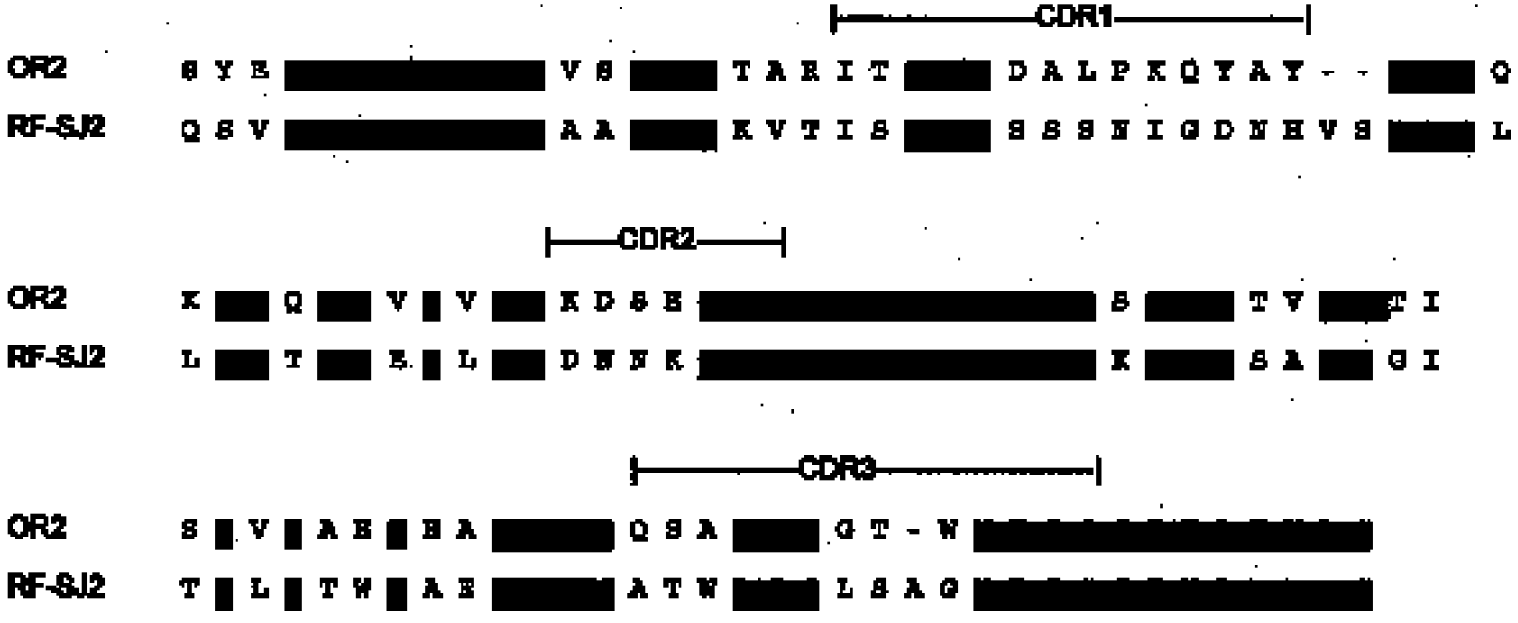

Fig. 8. Vλ sequences of OR2 (Vλ3) compared with Vλ1 of a monoclonal rheumatoid factor (RF-SJ2) . Complete VJ sequences as aligned. Shading indicates identities.

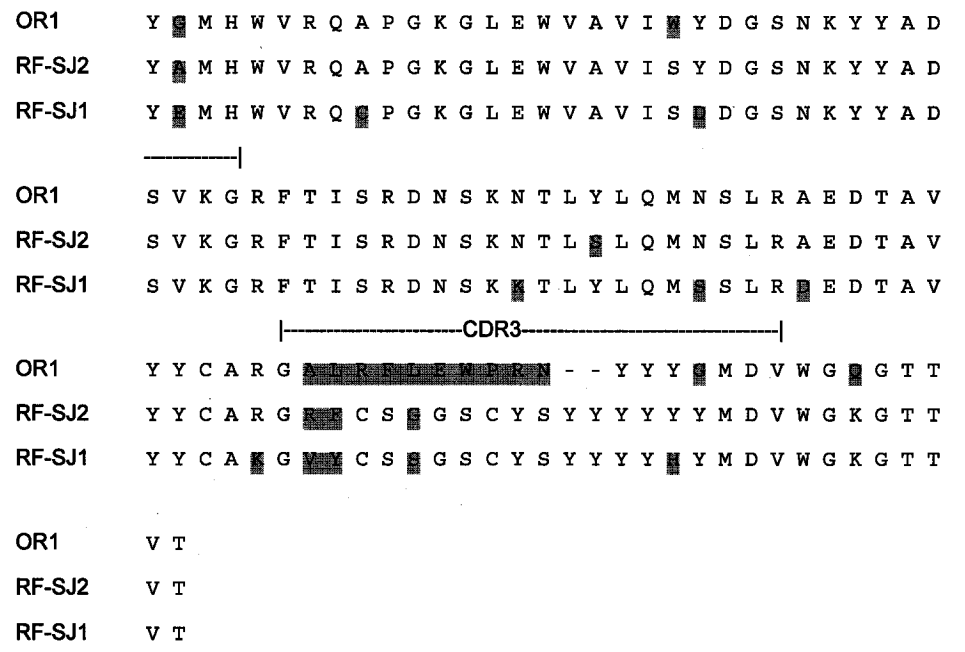

Fig. 9. Comparison of OR1 V_H3 sequence with that of synovium-derived rheumatoid factors *(38)*, using variants of germline V_H DP-49. Complete VDJ sequences are aligned. Shading indicates differences.

synovium-derived rheumatoid factors of Randen et al. *(38)*, as illustrated in Fig. 9. The OR1 V_H3 sequence differs from that of the germline prototype DP-49 in only a single residue, tryptophan (W) instead of serine (S) in CDR2 position #3. In comparisons among V_H3 segments of OR1 and the DP-49 derived RFs, there are no sequence differences in the first framework, one difference in the first complementarity-determining segment, one difference with respect to RFSJ1 in the second framework, two differences in CDR2, and five differences in the third framework. The CDR3 that is formed from D and J segments shows substantial differences, although both utilize a J segment (S) incorporating large numbers of tyrosines (Y).

The markedly epitope-promiscuous monoclonal autoantibody OR2 utilizes a V_H4 sequence identical to that of the germline prototype VIV-4 *(39)*. This is shown in Fig. 10, which includes a comparison with a variant of V_H4-21 that specifies anti-I and anti-i binding specificities and expresses the shared idiotype of cold agglutinins *(40)*. Substantial sequence differences—six residues—occur in the first framework. Two residues are different in the CDR1; one difference in FR2; three substitutions in CDR2; and only a single difference in the third framework. However, the CDR3s are totally distinct, not only from the V_H4 molecule 48u-43, but from a V_H4-21 variant that was selected because of the similarity in the joining segment. Of the seven V_H4-21 monoclonal variants derived *(40)*, six expressed Vk

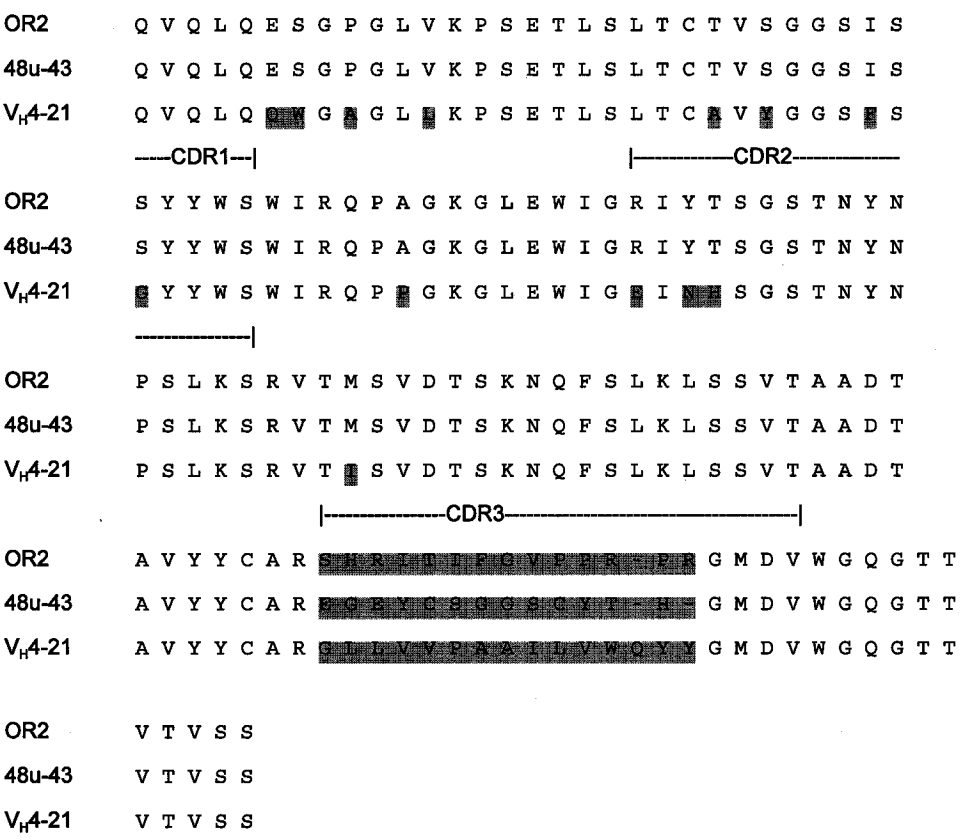

Fig. 10. Comparison of V_H4 sequence of OR2 with an identical V_H4 sequence [Clone 48u-43, Wang and Stollar, unpublished, GenBank Accession number AF062203] and a V_H4-21 used in a monoclonal cold agglutinin (FS-8) *(11)*. Shading indicates differences.

gene products. The single Vλ molecule used the Vλ1 subgroup in association with the Jλ1 mini-gene.

Discussion

These results support our hypothesis that IgM monoclonal autoantibodies to TCR epitopes can be derived from B cells from RA patients. The salient findings of the study are: 1) approx 10% of the monoclonal antibodies selected on the basis of binding to peptides bind to both individual peptide epitopes and to intact Vα/Vβ structures in recombinant single-chain constructs and of receptors expressed on the surface of T cells. 2) There is a range of fine specificity as defined by binding to panels of peptide epitopes, with some monoclonals showing extremely restricted reactivity (monogamous), and others showing a high degree of binding to distinct peptide-defined epitopes (epitope promiscuity). 3) In addition to

the expected conformational determinants of intact TCR, monoclonal autoantibodies can detect apparent conformational features of 16-mer peptides containing the crucial CDR1 segments. 4) Not only can epitope-promiscuous autoantibodies bind to distinct human TCR, they can bind to certain orthologous products of murine T-cells. 5) Although epitope-promiscuous autoantibodies frequently occur in the V_H3 family, our best example to date arises in the present study and uses a V_H4 gene product identical to the germline gene VIV-4.

We use the term "epitope-promiscuous" to describe the reactivity of our monoclonal autoantibodies here because they are specific in their reactions with T-cell receptor constructs, but not with usual test proteins including thyroglobulin, ovalbumin, and bovine serum albumin. Likewise, typical polyspecific antibodies are IgM molecules, but they can react with the range of protein and even nucleic-acid determinants. Our MAbs to TCR Vβ CDR1 epitopes resemble the induced murine MAbs to HIV-1 P24 that binds strongly to the inducing antigen, but shows binding specificity to three sets of distinct peptide epitopes *(12,13)*. The OR1 and OR2 molecules characterized in detail here use variable-region gene products strongly similar to the germline prototypes. The OR1 V_H3 segment differs from the germline gene DP-49 in only a single residue. There is one interchange—a tryptophan for a serine in the second complementarity determining region. The V_H4 used by OR2 is identical to the germline prototype VIV-4. Both OR1 and OR2 use Vλ3 genes. The OR1 sequence is identical to that of the germline gene *hisgll150*, and the sequence of the OR2 differs by only one residue in the third hypervariable region. Thus, the gene usage at this initial stage resembles that for polyspecific antibodies because the antibodies generated use the μ chain, and the V domains tend to be in germline configuration *(10,41)*. Considerable variability, however, occurs in the third hypervariable region, which is formed of D and J segments as compared to V_H4 autoantibodies to red-cell epitopes *(40)*, or monoclonal rheumatoid factors *(38)*, or unselected monoclonal immunoglobulins *(42)* derived from synovial B cells of RA patients.

The V_H CDR3 segments of both OR1 and OR2 are relatively long and complex, comprising 18 residues. This is consistent with the expectation that broadly specific antibodies tend to have large CDR3s, and may have few identifying patterns shown in comparison among different molecules of comparable specificities *(10,11)*. The OR1 V_H CDR3 loop meets the prediction of having a relatively large percentage of aromatic side chains, notably tyrosines *(14,41)* contributed by the JH segment. The two monoclonal rheumatoid factors studied by Randen et al. *(38)* have CDR3s that clearly meet the expected criteria because these are 20 residues in length and are approximately one-third tyrosines. The V_H of highly epitope-promiscuous OR2, however, has a CDR3 that is lacking in tyrosines and tryptophans. In other studies, we have found that an epitope-promiscuous murine monoclonal autoantibody to the same set of peptides tested here

has an extremely short CDR3 segment *(14)*. Thus, our results are consistent with the conclusions of Ramsland et al., *(10,11)* indicating that clear-cut definitions of the molecular basis of polyspecificity or epitope promiscuity have not yet been established.

Our prior studies established that all individuals have low levels of autoantibodies against T-cell receptors, and that these can be found in the IgG as well as the IgM class *(17,22)*. The levels and contribution by the different isotypes vary with normal physiological conditions such as pregnancy and aging, as well as viral infections and the generation of autoimmune disease. We speculate that the function of the autoantibodies directed against public idiotopes of T-cell receptor variable regions is essentially regulatory because they function to modulate the levels of particular Vβ gene products expressed in response to infection or possible autoimmune disease. The range of epitope recognition as illustrated by the spectratype analysis using peptide epitopes duplicating the CDR1 segments of 24 distinct human Vβ gene families illustrates that individual monoclonal autoantibodies can either have very restricted specificity, or can react appreciably to as much as one-half of the set of Vβ molecules. Cross-reactions are not unexpected—there is at least 40% identity among the set of test peptides used. However, we would suggest that the epitope-promiscuous autoantibodies would act in a relatively broad-spectrum manner resembling that of an "internal superantigen" in regulating the appearance or suppression of relatively large blocks of Vβ receptor sets. Evidence consistent with this has been obtained in studies of monoclonal autoantibodies to Vβ CDR1 epitopes derived from retrovirally infected mice that act synergistically with superantigens in activating appropriate populations of T-cells *(14)*. Administration of the commonly recognized VβCDR1 peptide epitope pepβ3 to mice immunosuppressed by either retroviral infection *(43)* or advanced age *(44)* has resulted in a significant restoration of the balance of TH1 and TH2 immunity and the capacity to reject infection by opportunistic parasites *(45)*.

The molecules themselves provide challenging models for deciphering the basis of epitope promiscuity, which can be studied using both synthetic peptides and recombinant single-chain VαVβ constructs. Furthermore, these human autoantibodies may prove to be candidates for direct immunotherapy or use in immunomodulation, because they are already of human origin and do not need molecular engineering to adapt them for use in therapy.

Acknowledgments

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Discussion

Kohler: What is the function of these antibodies? I think it is striking that these antibodies are IgM. There must be a reason why you don't have affinity maturation.

Marchalonis: That's a double question. The first question deals with function. We believe that initially these antibodies are regulatory. If you have a superantigen effect and an infection, and you generate a very high level of T cells with a particular Vβ receptor, the job of the antibodies is to shut off the production of these cells by apoptosis and other mechanisms. In autoimmune diseases such as rheumatoid arthritis and lupus, the control mechanisms are gone, so the antibodies pile up. Concerning your second question—in fact, some of the antibodies are IgGs. We have had myeloma IgG and polyclonal IgGs from HIV-infected humans with the antireceptor activity. We just haven't made any hybridomas yet, but I believe there will be affinity maturation. This ties in with the problem of what autoantibodies do in general—there are pathologic autoantibodies and there are regulatory ones. Our prediction is that the highly mutated ones will be pathologic.

Kohler: Would you say that this is just a part of the polyclonal polyspecific response you get every time you have strong antigen presentation and response?

Marchalonis: Again, every time you immunize or infect, you get the equivalent of an idiotypic network operation. Also, our donors are a very heterogeneous group of people. But networks are unpopular in the United States and are not easily funded by the National Institutes of Health, even though everybody knows networks exist. Every time you immunize with a strong antigen, if it is a T-cell-dependent antigen, you're going to get these antibodies to the T-cell receptor, much as you see an anti-IgG response. Both types of antibodies are ways of damping out the system, which helps get it back to ground state.

Tribbick: At our company we have the luxury of making lots of peptides. We found that for many antibodies the epitopes are quite short, although some antibodies bind long epitopes. Have you done any mapping to establish the length of the critical residues for binding? Secondly, in doing this sort of work, it's very important to try to understand—in our experience anyway—what the proper control is for a particular binding activity. One thing we found useful is to reverse the peptide sequence.

Marchalonis: With respect to the size of the peptide, we can get it down to essentially an 8-mer and still see binding. One of my colleagues, Doug Lake, is using a different approach. He is selecting the sequence from recombinant peptide libraries containing thousands of individual peptides. He can get down to 6-mers and 7-mers that will bind specifically. With respect to controls, you are right about their importance. We haven't done the reverse peptides but we've done hundreds of different peptides. When we get binding, as I showed that we do in inhibition studies, we can affinity-purify the λ-globulins. In many cases, we couple the peptide onto a large protein carrier. This makes it easier to get reproducible inhibition results.

Paul: With respect, your controls may not be good because you're dealing with promiscuous activity. If you invert the peptide or use completely unrelated peptides and still find binding, I'm not sure how bad that is.

Marchalonis: I'd like to comment on that. Not only did we test many peptides, but we studied two distinct recombinant T-cell receptors. Some of our monoclonals only bound one receptor, and some bound both. So there's more to it than just the peptides. The promiscuity is a real thing. Every monoclonal has its own characteristic signature if you go through enough peptides. There was a paper in *Cell* by a German group in which they studied hundreds of peptides for binding to a mouse monoclonal antibody against gp41. They ended up with three classes of peptides that bound with essentially the same affinity, but were unrelated in sequence.

Zouali: I have a comment and a question. It has been known for some time that if you inject normal immunoglobulins into virgin or naive mice, you see an effect on the T-cell repertoire of these mice. Your observations may account for these effects. Now to the proliferation question. You have found that these antibodies have no effect on the stimulation index of the T cells when they are incubated alone—but did you test for other effects like cal-

cium influx and phosphorylation? The antibodies may have subtle effects that could be important for the T cells, like inducing anergy.

Marchalonis: That is a very important question. We have not done those experiments yet, but I think you're right. We should look at other indicators and at receptor crosslinking. If antibodies bind, they probably have a platter of effects, and the more we look, I think the more we'll find.

Sinha: What is the extent to which the various peptides bind to the same monoclonal antibody?

Marchanolis: We have done competitions. We haven't done it with all of them, but we've found that we can get better than 50% competition. The binding constants and slopes are roughly the same.

Vijayalakshmi: Do you have any idea of the binding site with reference to different diseases? For example, if you have to classify the different antibodies in different autoimmune diseases, do they have shared binding sites, or is it different?

Marchalonis: There are some differences. Pregnant women tend to have high levels of IgG against different epitopes. Pregnancy, of course, is a normal process. Many antibodies are against an epitope around the junction of the J and the C domains. In rheumatoid arthritis, the antibodies are similar to the IgM antibodies that arise spontaneously in healthy humans. We have been trying to look at differences in retroviral infections. In AIDS, the antibodies are predominantly IgG, and there are some IgA. These seem to be almost like the antibodies in lupus. The AIDS antibodies recognize a larger set of peptides than the rheumatoid arthritis antibodies or the normal background IgM. I think it will vary with disease. We have preliminary results that multiple sclerosis patients tend to have antibodies binding predominantly to Vβ5 and Vβ6 as opposed to Vβ8.

Paul: Dighiero from Pasteur feels says that it is the rule rather than the exception that myeloma paraproteins are autoreactive. I noticed that some of your slides referred to the same phenomenon. Would you agree that this is the rule?

Marchalonis: On one hand, I'd agree that we clearly have found good autoantibody activity with high-affinity binding in some myeloma protein. Dighiero finds it in 40-60% percent of myeloma proteins. We'd be down under 20%. People have looked at the idiotypes of myeloma proteins and they found that T cells of myeloma patients are turned on by the idiotypes of their own myeloma proteins. One reacts with the other, but which is the binder and which is the antigen is not clear. Clearly, a subset of myeloma proteins has this activity, but I don't know if it is the dominant subset.

Rao: Would you comment on the V3 loop of HIV-1?

Marchalonis: We tend to use that as a negative control in our studies. However, my colleague, Doug Lake, published in *PNAS* four years ago some

very interesting studies in which antibodies against the V3 loop can cross react with the T-cell-receptor Jβ-joining segment. Even though it is a short stretch of amino acids, it has essentially the signature of glycine, and then a positively charged residue. It's got the GPGR sequence also found in the one V3 loop. Some antibodies from HIV-positive individuals isolated by V3 loop binding react with synthetic peptides corresponding to T-cell receptor joining segments. This suggests a cross-reaction between the viral protein V3 loop and the joining segment of T-cell receptors.

Unidentified: Question: Why are the antibodies restricted only to IgM and IgG? Is it because of the limitations of the technique used?

Marchalonis: If we affinity-purify from serum, normal people don't have much of the other classes. In HIV infection, the titers could be up around 1/10,000 or 1/20,000. In fact, one of our rheumatoid arthritis patients had a titer of over 1/30,000 against the receptor peptides. The results depend on the source of our material. We collaborated with a group in Heidelberg, and in that study found IgM, IgG, and IgA antibodies to the receptor in HIV-positive individuals.