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**BINDING AND FUNCTIONAL PROPERTIES OF NATURAL ANTI-
T CELL RECEPTOR ANTIBODIES IN PATIENTS WITH
RHEUMATOID ARTHRITIS**

by

Ian Forrest Robey

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

**In Partial fulfillment of the Requirements
For the Degree of**

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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A handwritten signature in black ink, appearing to read 'I. Robey', written over a horizontal line. The signature is stylized with a large loop at the top and a long horizontal stroke at the bottom.

Ian Forrest Robey

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DEDICATION

To my dad

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ABBREVIATIONS

APC	antigen presenting cell
BSA	bovine serum albumin
CDR.....	complementary determining region
D.....	diversity
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
ELISA.....	enzyme linked immunosorbent assay
FBS.....	fetal bovine serum
FR.....	framework
GAH	goat anti-human
HCDR.....	heavy-chain complementary determining region
HRP	horse radish peroxidase
Ig	immunoglobulin
J	joining
mAbs	monoclonal antibodies
MHC.....	major histocompatibility complex
MS.....	multiple sclerosis
NSAIDs	nonsteroidal anti-inflammatory drugs
PBS.....	phosphate buffered saline
PBL	peripheral blood lymphocyte
PBMC.....	peripheral blood mononuclear cells
PCR	polymer chain reaction
PI.....	propidium iodide
RA	rheumatoid arthritis
RF.....	reading frame
RFs	rheumatoid factors
scTCR.....	single chain T cell receptor
SLE.....	systemic lupus erythematosus
TCR	T cell receptor
TdT.....	terminal deoxynucleotidyl transferase
V	variable

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ABSTRACT

Natural autoantibodies specific for the T cell receptor (TCR) are present in all human sera. Differences in titer, epitope specificity, and isotype depend on physiological condition, viral infections, or the presence of autoimmune diseases. Individuals with rheumatoid arthritis (RA) make significantly higher titers of IgM isotype autoantibodies demonstrating major reactivity for the CDR1 region of the V β TCR. To establish a more intimate understanding of the role of these antibodies in the immune system we generated B cell hetero-hybridomas secreting monoclonal IgM autoantibodies from the synovial tissue and peripheral blood of RA patients. We report molecular and partial functional characterization of seven IgM anti-TCR monoclonal antibodies (mAbs). These autoantibodies were selected on a recombinant TCR and peptide epitopes and bind JURKAT human T cell lines and a subset of CD3⁺ human peripheral blood mononuclear cells (PBMCs) in flow cytometry experiments. The V regions of these antibodies were generally identical to germline sequences in both the heavy and the light chains and the heavy-chain CDR3 segments did not correspond to known antibody sequence. Three of these anti-TCR mAbs, OR2, OR5, and Syn 2H-11, demonstrated an antigen specific binding property defined as epitope recognition promiscuity. The molecules did not act

as rheumatoid factors. These same mAbs bound to subsets of murine T cells and TCR peptide epitopes. The autoantibodies did not induce apoptosis *in vitro*, but prevented IL-2 production by antigen-specific T cells. These findings describe a unique group of immunoregulatory antibodies and represent a foundation for further investigations and their eventual use as therapeutic agents in human disease.

INTRODUCTION

Natural antibodies

Natural antibodies exist as a unique set of immunological molecules. They represent the immunoglobulins (Igs) that appear in circulation prior to purposeful immunization or primary infection, the repertoire of natural antibodies remains invariable from early childhood through adult life, and they are capable of binding both exogenous and self-determinants (1,2). Natural antibodies are defined by a loose set of parameters. They are typically IgM isotype, but have also been identified in IgG and IgA classes. In healthy adult serum, for example, most natural antibodies are IgG isotype. The mechanisms that drive isotype switching of these antibodies are as yet unknown however, it has been hypothesized that the B cells producing these molecules are capable of making the switch in the absence of T cell help. Other speculation indicates that cross-reactive epitopes that might be recognized by T cells can play a part in driving the class switch (3-7). Natural antibodies can exhibit a broad range of antigen affinities with dissociation constants spanning from 10^{-5} to 10^{-8} M (4, 8-11). The variable region sequences of natural antibodies are mostly in germline configuration with the exception

of the heavy chain V region CDR3 segments. This is the site of junctional diversity where the variable (V), diversity (D), and joining (J) regions of the antibody recombine to form the complete V region sequence. At the site of recombination nucleotides frequently get cleaved by recombinases and replaced by N-nucleotides through the activity of transferal deoxynucleotidyl transferase (TdT). This phenomenon results in the creation of unique sequence segments (12, 13). Heavy chain V region CDR3 diversity, however, is only a property of post-neonatal natural antibodies (14).

Epitope binding properties of natural antibodies

The findings on the binding properties natural antibodies have reported that they tend to exhibit a polyreactive nature. Studies with human sera show that natural antibodies can bind to many epitopes with low affinity. Exogenous antigens recognized by these natural antibodies are usually large complex molecules with repeating structural motifs. Examples of these antigens include both common bacterial and viral structural proteins such as saccharides and lipopolysaccharides. Natural antibodies can also recognize self-derived antigens like hormones, cytoskeletal components, ssDNA, thyroglobulin, albumin, ovalbumin, cytokines, and numerous cellular constituents (15-

19). This occurrence, although a common observation, is not an overall defining characteristic of natural antibodies.

The factor that appears to have the most impact on the relative affinity and specificity of the antibody is the heavy chain CDR3 sequence. This region of the antibody combining site plays a crucial role in both the number of possible binding epitopes and the level of affinity generated for each antigen. Making amino acid substitutions in the heavy chain CDR3 region can profoundly influence the binding site properties of polyreactive antibodies. Using site-directed mutagenesis, substitutions of positively charged arginine residues with neutral residues like serine and alanine in the V_H CDR3 region have been shown to eliminate an antibody's polyreactive properties. Substitution of random arginines however, is not alone sufficient to effect polyreactivity (20). It has been suggested that the presence of hydrophilic residues increases backbone flexibility, and thus confers a greater degree of polyreactivity. The presence of proline residues are thought to incur more restriction in the binding site (21). Other studies have indicated that clusters of glycine, serine, or proline were involved in promoting polyreactivity, but exceptions were noted (22). It is still not known what amino acids or sequences control the degree of polyreactivity or monoreactivity.

The most compelling evidence for the importance of the V_H CDR3 in antibody binding comes from studies demonstrating that changes made in other regions of the Ig sequence were not significant enough to influence antigen reactivity in comparison with changes that were made in the CDR3 region. The V_H CDR3 segment of a polyreactive antibody taken from one V_H family and grafted into the Ig sequence of a different V_H family could form a new hybrid Ig that possessed polyreactive binding qualities. Experiments with chimeric antibodies also showed that exchanging CDR3 sequences from polyreactive antibodies with those of monoreactive antibodies created a new monoreactive species despite what other substitutions were made in any other part of the Ig sequence from FR1 to FR3 (22-24).

Polyreactivity does not mean that the antibody lacks specificity. Moreover, an antibody that appears to bind more than one unrelated epitopes might not bind at all to usual test proteins that might otherwise define it as polyreactive. A term that may be applied to this type of characteristic is epitope recognition promiscuity. It describes an Ig with the ability to bind sequentially and biochemically unrelated epitopes. The affinity for a particular epitope may even be greater than the “cognate epitope” or the specific antigen. Antigens recognized by epitope promiscuous antibodies can occupy the same

binding site in a competitive manner despite sequence and biochemical discrepancies (25, 26).

Natural antibodies in the immune system

Few studies have investigated the exact role of natural antibodies in the immune system and information on the functional properties of these molecules is limited. Some researchers have catalogued natural antibodies as evolutionary artifacts serving no significant role in immune protection. Rational for these claims is based on the low-affinity, polyreactive properties frequently observed with these antibodies. The potent efficacy of the adaptive immune system also tends to overshadow natural antibodies. The more sophisticated immune system enhanced by the property of affinity maturation alludes to a self-sufficient defense network that functions without the aid of such molecules. Compelling arguments, however, can also attest to the functional relevancy of natural antibodies. The degree to which these molecules contribute to the immune system is the unknown factor that remains the true source of controversy.

The idea that an antibody needs to bind to antigen with a high degree of affinity in order to serve the immune system may not be necessarily applicable in the realm of

natural antibodies. These antibodies may function with more impact as a network of molecules where multivalent high avidity is the principle factor in immunological effectiveness (27). Low affinity binding may be sufficient or necessary for generating specific intracellular signaling events. In studies on antibodies specific for the CD47 molecule on leukocytes, for example, it was discovered that low affinity antibodies have high avidity for CD47 clusters on the cell surface which associate with cytoplasmic signaling components (28). With respect to low affinity binding, a useful approach for understanding its relevance is to regard the antibody-antigen interaction as complementary rather than a 'uni-directional' reaction of a paratope with a 'target' epitope (29, 30).

One likely explanation for the existence of polyreactive natural antibodies is that they serve as a first line defense in pre-immunized individuals. The ability to bind many epitopes allows for a preliminary response that can counter a diverse set of various foreign antigens. The detection of antibodies in pre-immunized animals against toxins and bacteria has been described (31, 32). The importance of these molecules has been shown in studies comparing antibody-competent mice with mice lacking natural antibodies. Antibody-free animals infected with various pathogens expressed bacterial and viral organ titers that were 10 to 100 times greater than those mice with circulating

pre-immune antibodies. Antibody-free animals were more susceptible to pathogen dissemination to vital organs while antibody-competent animals possessed enhanced immunogenicity to infections (33). This same method for defense could translate to autoreactive natural antibodies for immunoregulation. For instance, they could be important for the development and physiology of the B cell repertoire (3).

Natural autoantibodies in the immune system

Autoreactive natural antibodies are interesting because they exist in healthy individuals as well as patients with various autoimmune diseases. The presence of these molecules implies that they serve a beneficial function rather than pose a pathologic detriment. Investigations on natural autoantibodies have led to the suggestion of several possible immunological functions. Functions such as cell signaling and repertoire selection have already been discussed, but other proposed functions of natural autoantibodies have been suggested. Some of these functions include antigen presentation to T cells, anti-tumoral surveillance, opsonizing through binding cross-reactive epitopes on pathogens, shielding cellularly expressed antigens that resemble

foreign antigens to avoid immune attack, clearance of aging or dying cells, and anti-inflammatory activity (3).

Autoantibodies against the T cell receptor

Some autoantibodies are known to react with determinants on the T cell receptor (TCR). This was discovered in studies using human sera to examine reactivity to synthetic peptides corresponding to the human TCR β chain sequence (34). The rationale for this study was based on the knowledge that the TCR is an Ig-like molecule and that natural autoantibodies had been earlier discovered to bind idiotypic (35, 36) as well as constant region epitopes of Igs (37). Further investigations revealed that autoantibodies against the TCR were produced in all individuals. These findings were the first indications that every person makes natural autoantibodies against the TCR. Anti-TCR antibodies are found in healthy individuals, patients with retroviral infections, and patients with autoimmune disease. Furthermore, anti-TCR antibodies presented at various titers and to different TCR epitopes depending on the physiological condition of an individual. Normal individuals, for example, generate higher titers of anti-TCR IgM at younger ages (under 40 years) than older individuals. These titers decrease further

with age. Titers of anti-TCR IgG, however, were observed to significantly increase in with age (38). Pregnant women express comparable levels of autoantibodies to the intact α/β TCR, but indicated enhanced reactivity to regions corresponding to the CDR1 of the TCR α chain and the FR3 of TCR β chain. Pregnant women also tended to generate increased amounts of IgG autoantibodies against highly conserved determinants of the CDR3/J region in the TCR β sequence. This type of reactivity was not seen in the non-pregnant controls (39). Heart transplant patients experience increased levels of autoantibodies against the TCR following transplantation then a subsequent decrease over time. Anti-TCR reactivity was mostly generated against the CDR1 region of the TCR V β 6.1, 21.1, and 22.1 gene families. Interestingly, autoantibodies were observed in high titers, and remained high against, the CDR1 region of the TCR V β 7.1 and V β 8.1 gene families in pre-transplant patients (40). Investigations on individuals infected with HIV-1 found that they produced 10-fold higher levels of anti-TCR IgG against the V β CDR1 and FR3 regions than normal individuals. The CDR1 of V β 2.1, 8.1, 10.1, and 22.1 TCR spectratypes were recognized by anti-TCR autoantibodies in AIDS patients. Another process that was uncovered in HIV-1 infected patients was that they made antibodies reactive to a region of the V β FR4 TCR, which was cross-reactive with a portion of the V3 loop on the HIV gp120 protein (41, 42).

Autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) create ideal physiological conditions for large-scale production of anti-TCR autoantibodies. Individuals with SLE make high titers of IgG autoantibodies against numerous epitopes corresponding to the TCR β and α chain. An interesting observation was that SLE patients made autoantibodies against the CDR2 segment of the V α sequence. These kinds of autoantibodies were not found in normal controls (39). Studies of sera taken from patients with RA found that these individuals make high titers of IgM autoantibodies against the V β TCR. Comparison studies with SLE patients, osteoarthritis patients, and asymptomatic individuals reported that RA patients produce at least 7-fold higher titers of IgM against the TCR V β CDR1 region and 50% higher titers against the V β FR3 region. Titers against the TCR V β CDR1 determinant of some patients exceeded serum dilutions of 1 to 6000. Anti-TCR IgM from RA patient sera reacted with many TCR β peptides, but was more restricted on TCR α peptides. Levels of IgG autoantibodies were also decreased in RA individuals (38, 43-45).

A theme observed from these studies is that the CDR1 region of the V β TCR is a major epitope for anti-TCR autoantibodies (and to a lesser degree the V β FR3 region). These regions of the TCR are considered a "public epitopes". The prevalence in autoantibody recognition of these idiotypic sites suggest that anti-TCR autoantibodies are

analogous to anti-idiotypic antibodies and have functional significance for regulation of T cell activity (45). Anti-TCR autoantibody binding sites appear to be capable of targeting V β TCR sequences from specific gene families. In this respect only a restricted population of T cells would be targeted for immunoregulation. This kind of model has been applied in clinical studies using specific TCR V β peptides to immunize patients with multiple sclerosis (MS) in order to upregulate immunoregulatory T cells against autoimmune T cells bearing the antigenic TCR idiotopes (46). There are several possible functions that might be carried out by anti-TCR autoantibodies against the V β CDR1 region. Binding and cross-linking the TCR by anti-TCR autoantibodies might trigger intracellular signaling that could lead to tolerance induction, apoptosis, or activation. Anti-TCR autoantibodies may be involved in clearance of T cells through inert neutralization or removal of senescent T cells. Finally, the binding of autoantibodies to the TCR could inhibit the inflammatory response of antigen activated T cells.

Studying autoantibodies specific for the T cell receptor

Previous investigations have helped identify the existence of anti-TCR autoantibodies, but greater understanding of their binding and functional properties is

limited to speculation without obtaining specific anti-TCR monoclonal antibodies (mAbs) for closer examination. The following studies make use of hybridoma technology to generate anti-TCR mAbs for investigations into their binding characteristics on T cells and TCR epitopes, specific V gene usage, and possible biological function in the immune system. The anti-TCR mAbs used for the following experiments are generated from patients with RA. Based on the results from the subsequent group of experiments, I propose the following hypotheses: 1) the anti-TCR mAbs generated for these studies are naturally occurring molecules and not pathogenic by-products of autoimmune disease, 2) these anti-TCR mAbs have diverse reactivities against TCR epitopes and some demonstrate the property of epitope recognition promiscuity, and 3) the biological function of natural autoantibodies specific for the TCR is immunoregulatory.

MATERIALS AND METHODS

SECTION I

Patients

Peripheral blood and synovial tissue samples were obtained from patients who carried the diagnosis of rheumatoid arthritis according to the American College of Rheumatology (47). Synovial tissue was obtained from a patient undergoing total knee replacement. At the time of joint replacement, the patient had discontinued remittive therapy and was on less than 5mg of prednisone per day. In addition, nonsteroidal anti-inflammatory drugs (NSAIDs) had been discontinued at least 10 days prior to surgery.

Antigens

The following TCR antigens and peptides were used in hybridoma selection and immunoassays. The recombinant human single chain T cell receptor (scTCR) containing the complete VJ α and VDJ β of the JURKAT TCR was prepared as described by Lake *et*

al (48). The β 3 peptide (CKPISGHNSLFWYRQT) represents residues 23-38 corresponding to the complete CDR1 (and part of the FR2) region of the YT35 TCR V β 8.1 sequence. The β 1 peptide (DAGVIQSPREVTEMG) represents residues 1-15 of the human V β 8.1 chain. The V β 20.1 peptide (CTVEGTSNPPLYWYRQA) corresponds to the CDR1 region of the V β 20.1. All peptides were synthesized to 95% purity by Chiron Mimetopes[®] (San Diego, CA).

Generating hybridomas from human lymphocytes

Leukocytes were isolated from anti-coagulated blood by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO) and were > 95% viable. Monocytes and natural killer cells were depleted using 0.05 M L-LME in serum free RPMI medium (49). B cells were purified by negative selection of T cells by using M-450 CD2 Dynabeads (Dynel). B cells were then activated and expanded in a "CD40 ligand" system. Engagement and crosslinking of CD40 on B cells was mediated by mouse fibroblasts (L cells) transfected with mouse CD40 ligand (gp39) (obtained from the Canadian Red Cross) (50). Mouse CD40 ligand stimulates human B cells as effectively as human CD40 ligand (51). Approximately 10^5 B cells were incubated with

10⁴ irradiated L cells (7000 rads) in 1ml Iscove's Modified Dulbecco's Media containing 10% human serum albumin, 0.5% PCS, 5 µg/ml bovine transferrin and bovine insulin, and 10 µg/ml each of IL-4 and IL-10 in 24 well culture plates. The cultures were incubated 5-7 days and fed every 2-3 days. Activated B cells were recovered from the cultures and fused (10⁶-10⁷) with the P3x63-AG8 #653 mouse myeloma fusion partner using PEG 4000 (Merck) at a 1:1 ratio following the procedure described by Lane *et al* (52). The fused cells were plated in 96 well flat bottom culture trays at 20,000 B cells per well. The medium contained 10% hybridoma cloning factor (Origen[®]) and a feeder layer were not used. Culture supernatants were assayed at approximately 14 days by ELISA. Initially, the supernatants were tested for binding to peptides β3 and β8, and to the scTCR protein. Hybridomas were selected from cultures that were positive to β3 and scTCR, but negative for β8. Clones selected for further study were purified by two rounds of limiting dilution.

Synovial tissue was obtained from a patient undergoing knee replacement. A cell suspension was prepared by squeezing the tissue through a coarse metal sieve.

Leukocytes were isolated by density-gradient centrifugation using Ficoll-Paque[®]

(Pharmacia Biotech, Uppsala, Sweden). The cells were incubated overnight in T150

flasks in Iscove's media containing 10% fetal bovine serum (FBS) and 2 ng/ml IL-6. The

non-adherent cells were recovered, plated with L cells expressing CD40 ligand and hybridomas prepared as described above.

Testing for antibody specificity by ELISA

Cell culture supernatants were tested by ELISA. ELISA plates were incubated overnight at either 4°C with 100 µl of 10 µg/ml recombinant single chain 3-domain JURKAT T cell receptor in phosphate buffered saline (PBS), or at 37°C with 10 µg/ml peptide in 0.2 sodium carbonate buffer, pH 9.6. Peptide solutions were dried down overnight. Plates were blocked for 1 hour at room temperature with SuperBlock® blocking buffer in PBS (Pierce, Rockford, IL). Supernatants were applied in 2 fold serial dilutions, starting at neat in the scTCR ELISA or 1/10 in PBS-tween (tween at 0.5 ml/L in PBS) for the β3 peptide ELISA, and incubated at room temperature for 1 hour. After washing, rabbit anti-human IgM heavy chain secondary antibody (Dako, Glostrup, Denmark) conjugated with horse radish peroxidase (HRP), was applied in 1/3000 dilution in PBS-Tween for 1 hour at room temperature. Plates were developed with 30 mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) reagent in citrate buffer, pH 4.0. Color changes on the plates were measured on a plate reader (Titertek Multiskan®)

at wavelength 405nm. Uncoated wells were left on the same plates during the procedure to compare supernatant binding to plastic with antigen. The TCR peptides $\beta 1$ and $V\beta 20.1$ were included as specificity controls.

Testing antibody binding to JURKAT TCR by flow cytometry

JURKAT cells (10^6) that were > 90% viable were incubated in 1 ml of the different supernatants from each clone on ice for 2 hours. Cells were centrifuged at 400 g, washed, and incubated for another hour on ice with a FITC labeled goat F(ab')₂ anti-human IgM secondary antibody (Caltag[®], Burlingame, CA) in cold PBS with 0.5% FBS. This buffer was used in all wash steps. Control groups were treated with FITC labeled mouse monoclonal anti-human CD3 (Caltag[®]), goat F(ab')₂ anti-human IgM alone, or IgM λ and IgM κ isotype controls (Binding site, Birmingham, UK). Commercial antibody concentrations were used at 10 μ g/ml or less. Cells were pelleted after final wash and resuspended in 0.5ml of buffered paraformaldehyde fixative until the cytometric analysis was performed.

Fluorescent staining of the cells was measured using a Becton Dickinson (San José, CA) FACScan at the University of Arizona Cancer Center. This instrument uses a

coherent 90-5 argon laser turned to 488 nm. A 530/30 band pass filter was set, and samples were analyzed at a 100 mWatt log scale. Acquisition and data reduction were analyzed using a Hewlett Packard 340 with Lysys version 2.0 software (Becton Dickinson). Intact cells were gated and 10,000 events were collected. All sample data were collected in triplicates.

Testing antibody binding to human PBMCs

Human PBMCs were isolated from the blood of a normal patient with Ficoll-Paque® (Pharmacia Biotech, Sweden) by density gradient centrifugation. Cells were incubated on ice for 2 hours in 1ml of supernatant from clones Syn 2H-11, OR1, OR2, OR5, anti-human V β 8 (PharMingen, San Diego, CA) and anti-human CD3 at 10⁶ cells/ml, and then processed as described above. Remaining procedure was the same as that which was used for JURKAT cells.

Cloning, Sequencing, and aligning antibody V genes

Polyadenylated RNA was isolated from hybridomas using Micro-Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). AMV reverse transcriptase and oligo dT primers (cDNA Cycle Kit for RT-PCR, Invitrogen) were used to produce cDNA for template in subsequent polymerase chain reactions (PCR). Isolated cDNA was used with a set of immunoglobulin V region primers from Novagen IG-Prime Kit. PCR was performed using a MiniCycler (MJ Research, Inc.) with an in-sample temperature probe under the following conditions: 40 cycles at 95°C for 5 s (denature), 50°C for 15 s (anneal), 72°C for 1 min, and 72°C for 30 min at the end of the 40 cycles. Products were cleaned for ligation with QUIquick PCR Purification Kit (Qiagen, Germany). Products were then ligated overnight into pCR[®] Vector 2.1 from Invitrogen TA Cloning[®] Kit (Carlsbad, CA).

Plasmids were isolated using Quantum Prep[®] plasmid miniprep kit (BioRad, Hercules, CA) and sequenced using the ThermoSequenase[®] radiolabeled terminator cycle sequencing kit (ThermoSequenase: USB, Cleveland, OH). Radiolabeled sequences were run on an acrylamide sequencing gel made with ExplorER gel solution (VWR, Phoenix, AZ). Sequence fragments were aligned, corrected, and translated using AssemblyLign software (Oxford Molecular). V region sequences were compared to the GenBank and Kabat *et al* (53). Assignment of the expressed V-regions to the parental germline gene

was accomplished by searching the V Base directory (©1997 MRC Centre for Protein Engineering). The directory was accessed through the world wide web (www.mrc-cpe.cam.ac.uk/imt-doc/restricted/ok.html). Our gene sequences have been submitted to GenBank with succession numbers to be cited when available.

SECTION II

Antigens and antibodies used for fine specificity mapping analysis

The following synthetic peptides were used for fine specificity mapping and titration analysis of the anti-TCR mAbs: 1) 22 TCR β peptides corresponding to the complete V β 8.1 YT35 TCR (54), 2) 19 TCR α peptides corresponding to the complete V α 1 pY14 TCR (55), 3) 20 Mcg peptides corresponding to the complete Mcg λ light chain sequence (56), 4) 24 V β TCR CDR1 homolog peptides, and 5) 6 V β TCR FR3 homolog peptides (57). The panel of TCR β , TCR α , and Mcg 16-mer peptides overlap each other sequentially by 5 amino acids from the amino (left) to the carboxyl (right) terminus of the complete protein (34) (Table 4, 5, and 6). The V β TCR CDR1 and FR3

16- and 17-mer homologs represent CDR1 (and partial FR2) (Table 7) and FR3 segments (Table 8), reading from the amino (left) to the carboxyl (right) terminus, corresponding to 25 V β gene products. All peptides were synthesized to 95% purity by Chiron Mimetopes[®] (San Diego, CA) or the University of Arizona Biotechnology Center (Tucson, AZ). Whole protein antigens were fetuin, bovine serum albumin (BSA), ovalbumin, thyroglobulin (Sigma, St. Louis, MO) and pooled human polyclonal IgG Gammagard[®] (Baxter, Deerfield, IL) as polyreactivity controls. The antibodies selected for these assays, OR1, OR2, OR5, and Syn 2H-11, were obtained from hybridomas and selection procedures described in *Section I Materials and Methods*. A vital fluid of life sample from an RA individual was used as a positive control for rheumatoid factor (RF) on ELISAs to test reactivity to pooled human polyclonal IgG. ELISA procedure described in *Section I Materials and Methods*.

Determining antibody concentration

Antibody concentration was determined by capture ELISA. Assay plates were coated with 10 μ g/ml of anti-human λ light chain antibody (Sigma). Known dilutions of human IgM λ myeloma protein (The Binding Site, Birmingham, UK) were used as the

control antigen to plot a standard curve. The slope of the best-fit curve was used to determine the concentration of unknown antibody. Details on ELISA materials and techniques are listed in previous section.

Inhibition assay

Prior to inhibition, assay wells in ELISA plates were pre-blocked overnight at 4°

C. Inhibiting antigen was diluted in PBS-tween with 0.1% BSA and added to the blocked wells. The starting concentrations of inhibiting antigen (50 µg/ml of β3 or β15 peptide and 25 µg/ml of scTCR) were carried out two-fold in six more serial dilutions. The TCR β1 peptide (Table 4) was used as a specificity control for the soluble inhibiting TCR antigens. One set of wells was treated with assay diluent without soluble antigen for the maximum antibody binding measurement. Antibody was added at approximately 2 µg/ml to the wells and the plates were covered and incubated on a rotator overnight at 4°

C. Inhibition reactions were applied to antigen coated plates for ELISA procedure.

SECTION III

Binding to mouse T cells by flow cytometry

The cells used for these experiments were T cell enriched mouse splenocytes from naive, 6 week old BALB/c females and the murine DO-11.10 clone (58, 59). T cells from unimmunized BALB/c mice were isolated by negative selection on a mouse T cell enrichment column (R&D, Minneapolis, MN). Both T cell enriched mouse splenocytes and DO-11.10 cells were generously donated by the E. Akaporiaye Laboratory, Department Microbiology and Immunology, University of Arizona. Cells (10^6 /sample), > 90% viable for the staining procedure, were resuspended in 250 μ l of flow cytometry wash buffer (PBS with 0.5% bovine albumin) with anti-TCR mAbs on ice for 45 min. This buffer was used in all wash steps. Cells were centrifuged at 400 g, washed, and incubated for another hour on ice with a FITC labeled goat F(ab')₂ anti-human IgM secondary antibody (Caltag[®], Burlingame, CA). Control groups were treated with FITC labeled mouse monoclonal anti-mouse V β 8 TCR (BD PharMingen, San Diego, CA), goat F(ab')₂ anti-human IgM alone, or ImmunoPure[®] human IgM (myeloma) isotype control antibody (Pierce, Rockford, IL). Commercial antibody concentrations were used at 10 μ g/ml or less. Cells were prepared for flow cytometry as described in *Section I Materials and Methods*.

Antibodies and mouse T cell antigens

The mAbs chosen for these assays, OR2, OR5, and Syn 2H-11, were obtained from hybridomas and selection assays described in *Section I Materials and Methods*. Five mouse TCR peptides were used for fine specificity mapping of anti-TCR mAbs. The peptides, mu V β 1, mu V β 8.1 HV short, mu V β 8.2 HV short, mu V β 8.2 long, and mu V β 4 (Table 10), represent homologs of the CDR1 segments and part of FR2 of mouse V β gene products (60). Peptides mu V β 8.1 HV short, and mu V β 8.2 HV short are truncated versions corresponding only to the CDR1 region. The amino to carboxyl terminal ends reads from left to right. Mouse peptides were synthesized to 95% purity by Chiron Mimetopes[®] (San Diego, CA). The negative control peptide, β 1 is described in *Section I Materials and Methods*.

Testing antibody reactivity to mouse peptides

Concentrated mAbs (minimum concentration of 400 μ g/ml) from hybridoma culture supernatants were used to test direct binding reactivity to the mouse peptides by

ELISA. Antibody was applied in 2 fold serial dilutions. The smallest starting dilution for one of the mAbs (OR5) was 200 $\mu\text{g/ml}$. The starting dilution was approximately 500 $\mu\text{g/ml}$ for mAbs OR2 and Syn 2H-11. Uncoated wells and wells coated with TCR peptide $\beta 1$ were included as a negative controls. ELISA methods are described in *Section I*

Materials and Methods.

Apoptosis induction experiments

Mouse DO-11.10 T cells ($\geq 90\%$ viability) were diluted to 0.5×10^6 cells in a volume of 1 ml serum free Iscove's Modified Dulbecco's Medium (IMDM). Separate groups of cells were treated with 100 $\mu\text{g/ml}$ of anti-TCR mAb, ImmunoPure[®] human IgM (myeloma) isotype control antibody, 10 mM cytosine β -D-arabino-furanoside (Ara C), or PBS. Cells were left to incubate overnight (approximately 16 hours) at 37° C. Detection of apoptosis was carried out with an Annexin V-FITC Kit (Immunotech, Marseille, France). Samples were washed in cold media after centrifugation at $500 \times g$, 4° C. Cell pellets were resuspended in 200 μl of binding buffer and treated with 5 μl of Annexin V-FITC for 10 min. in the dark on ice. Cells were treated with 2.5 μl of propidium iodide (PI) prior to flow cytometry.

Purification of monoclonal antibodies

Hybridoma cultures were grown to high density and collected for concentration on a 500 kd ultrafiltration membrane (Millipore, Bedford, MA) in a pressurized stirred cell (Amicon, Beverly, MA). IgM fraction of the concentrated supernatants was precipitated in 40% ammonium sulfate for 8 hours at 25° C then centrifuged at 10,000 RPM for 1 hour. Precipitate was resuspended in PBS, pH 7.0. Soluble suspension was separated on a Superose 6 preparative column (Amersham-Pharmacia, Buckinghamshire, UK) by a Waters 625 LC system (Millipore, Bedford, MA) in 0.2M phosphate (pH 7.0) at 0.5 ml/min. Pure fractions were collected and reconcentrated to a minimum working concentration of 1 mg/ml.

IL-2 inhibition

Mouse DO-11.10 T cells $\geq 90\%$ viability were diluted to 1.0×10^6 cells/ml in serum free IMDM. Three sets of DO-11.10 cells were treated with three concentrations of anti-TCR mAb: 200 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, and 50 $\mu\text{g/ml}$. Three sets of DO-11.10 cells

were treated with the same concentrations of ImmunoPure[®] human IgM (myeloma) isotype control antibody: One set of DO-11.10 cells were left untreated as a positive control. The DO-11.10 cells were incubated for 1 hr at 37° C. IL-2 inhibition cultures were conducted in 96-well round bottom tissue culture plates. Each culture well contained the following: 50 µl of 0.4×10^6 cell/ml murine bone marrow derived dendritic cells (E. Akaporiaye Laboratory), 50 µl of 1 mg/ml ovalbumin antigen (Sigma, St. Louis, MO), 50 µl of 10^6 cells/ml DO-11.10 cells after pre-incubation with purified anti-TCR mAb or controls, and 50 µl of RPMI media to bring the final volume in each well to 200 µl. Dendritic cells were prepared by methods described by Fields, *et al.* (61). Ten wells were set up for each pre-incubation and the cultures were tested for IL-2 secretion in the supernatants after a 48 hour period at 37° C.

IL-2 detection

IL-2 detection in 48 hr. culture supernatants was carried out with a Quantikine[®]M Mouse IL-2 Immunoassay (R&D Systems, Minneapolis, MN). A supernatant volume of 100 µl was applied to each ELISA well corresponding to the culture well from the 48 hr. incubation. Internal standards and controls were also used. Samples were left to incubate

at room temperature for 2 hrs. Plates were washed with kit wash buffer then treated with 100 μ l/well of conjugate and left to incubate at room temperature for 2 hrs. Plates were washed and incubated with 100 μ l of substrate solution for 30 min. Development was stopped with 100 μ l of stop solution and plates were read at wavelength 450 nm.

SECTION I: Production and characterization of monoclonal IgM autoantibodies specific for the T cell receptor

Chapter 1 – Introduction

Previous investigations of human sera and purified intravenous immunoglobulin preparations have demonstrated a measurable frequency of natural autoantibodies specific for the CDR1 public idiotope of the α/β TCR β chain (34, 43, 62). These autoantibodies occur in all individuals, but their prevalence has been documented in normal conditions such as aging (38, 63) and pregnancy (39), and also with allograft transplantation (40), autoimmune diseases (34, 38, 43, 63), and retroviral infections (41, 42). The biological role of these autoantibodies is poorly understood, but functional studies suggest that they can serve in immuno-regulation of T cells (64).

A majority of patients with RA produce significantly ($P < 0.01$) higher titers of anti-TCR autoantibody, specifically IgM molecules, by comparison to normal individuals or osteoarthritis patients (38, 43, 44). RA is a systemic disease that is defined by the inflammation and hyperplasia of synovium in joints of an afflicted individual (65, 66). The infiltration of activated lymphocytes to the synovium likens it to the events of a

secondary lymphoid organ, and the organization of T cells and B cells resemble a germinal center (67-71). Because of these processes, much research has focused on antibodies made by RA patients and how they or their production may differ in normal individual.

Many studies in RA focus upon specific trends that occur in antibody rearrangement, in both rheumatoid factors (RFs) and unselected immunoglobulins, with results compared to the events in B cells of normal individuals. Several investigations have focused on heavy (72-74) and light (75) chain gene family prevalence. Some of these studies have indicated that gene rearrangements are non-random, while other findings maintain that gene family sequences are heterogeneous. Other studies have discerned a substantial amount of somatic mutation in the CDR1 and CDR2 regions of both the heavy and light chains from RA patients that was not found in normal individuals (76-79). In studies on light chain sequences from RA patients, a majority were found to exhibit unusually large CDR3 regions due to N-nucleotide insertion (75, 77, 79-81). Overall, these results suggest that immunoglobulin heavy and light chain sequences in RA patients are oligoclonal and antigen driven. There are some exceptions to these findings. A study by Brown *et al* (74) for example, reported a high level of

junctional diversity in the CDR3 regions while the rest of the V_H sequence remained identical to its germline gene.

To characterize anti-TCR autoantibodies and understand their role in autoimmune disease, hybridomas were generated from the peripheral blood or synovial tissue B cell lymphocytes of RA patients. Clones that secrete autoantibodies specific for TCR peptides known to be bound by autoantibodies from RA patients were selected (38,43, 44). Seven clones secreting IgM antibodies were derived in this manner. Sequence analysis of the variable regions of the mAbs demonstrated a consistent homology to their germline matches in both the heavy and light chain sequences, with the greatest diversity occurring in the CDR3 of the heavy chain. The V_H CDR3 sequences were unique and did not correspond to those of characterized rheumatoid factors or known polyreactive human antibodies.

SECTION I

Chapter 2 - Results

Clones generated by their ability to bind β 3 peptide and scTCR

Hybridomas for these experiments were generated from two (RA) patients.

Lymphocytes were isolated from the peripheral blood (OR) of one patient and from the synovium of the other patient (Syn). Culture supernatants were screened for their ability to bind TCR peptide β 3, containing the entire CDR1 region (CKPISGHNSLFWYRQT) and the beginning of the second framework (WYRQT) of the V β 8.1 chain. Another antigen used for selection purposes was a recombinant single V α / β chain T cell receptor (VJ α -VDJ β) containing the cognate sequence and also corresponds to the intact α / β TCR on the surface of JURKAT cells. Figures 1a, b, and c are ELISA plots of clone supernatants and their reactivity to several TCR peptides. Figure 1a shows the reactivity profiles of supernatants against the β 3 peptide. Syn 2F7-2 and Syn IB5 show the highest reactivity to β 3 peptide at the greatest dilution (1/640). The absorbance units at this dilution are greater than 1.0, which is well above the range to generate an accurate titer

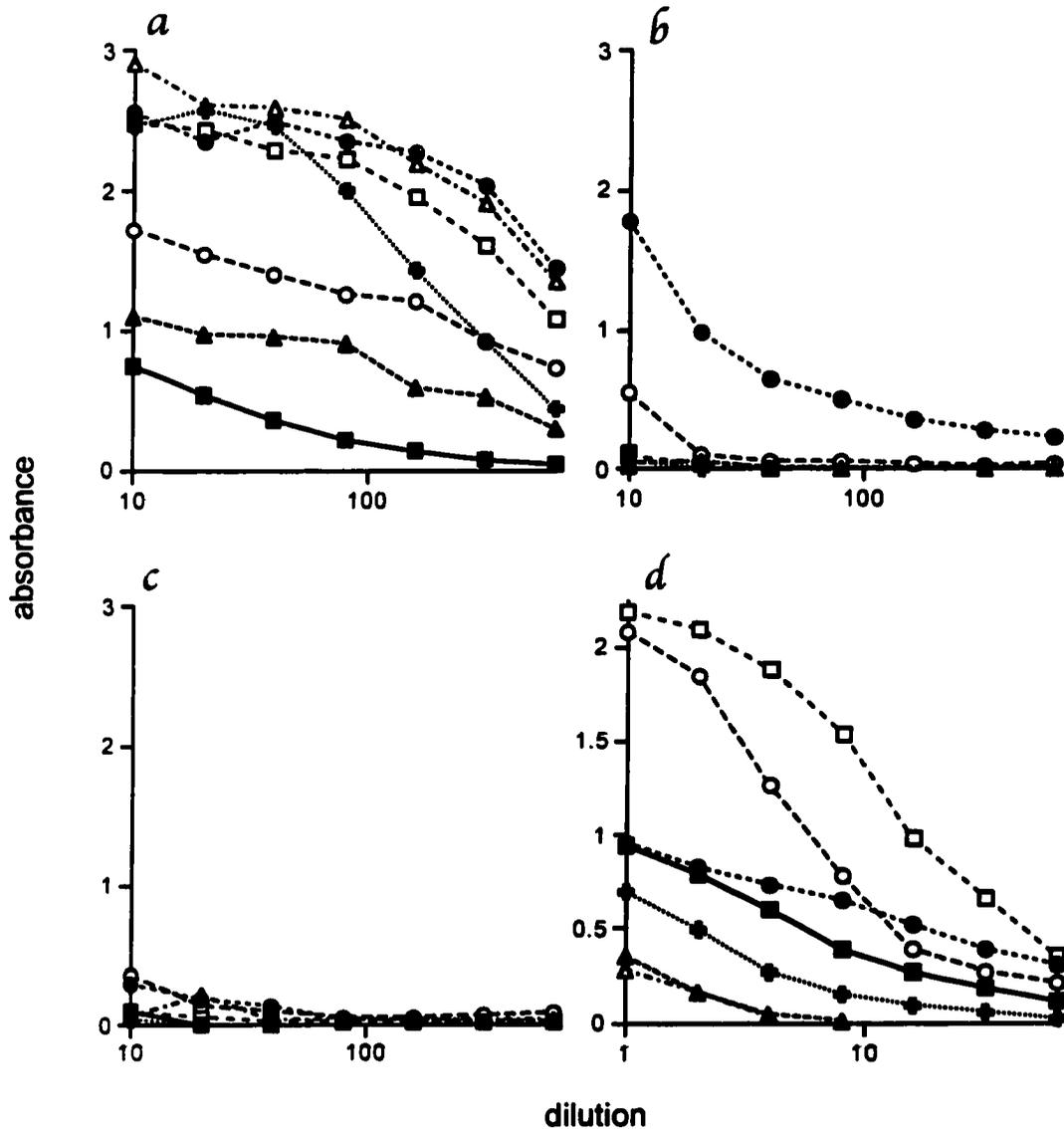


Figure 1. ELISA titration plots of IgM antibodies from the seven clones applied to TCR peptides. Each figure represents a specific peptide or protein used as the coating antigen. The value of binding of the conjugate controls was subtracted point-by-point from real values. The plots are listed as follows: (A) $\beta 3$ peptide, (B) $\beta 1$ peptide, (C) $V\beta$ 20.1 peptide, and (D) single chain TCR. (■) OR5 (▲) OR4 (●) OR2 (+) OR1 (□) Syn 2F7-2. (△) Syn IB5 (○) Syn 2H-11.

curve (normally it is between 0 and 1.0 with 0.5 being close to 50% binding). OR1 demonstrates high reactivity as well, but seems to get weaker than Syn 2F7-2 and Syn IB5 at a lower dilution. OR2, OR4, and Syn 2H-11 have similar curves, and although their reactivity to the $\beta 3$ peptide is considerably weaker than Syn 2F7-2 and Syn IB5, their reactivities still do not 'zero' at their highest dilution. OR5 is the weakest supernatant. At the highest concentration (1/10), its reactivity to $\beta 3$ is lower than Syn 2F7-2 and Syn IB5 at dilution 1/640.

Figures 1b and 1c represent ELISA plots using control peptides $\beta 1$ and V β 20.1. These peptides were used to demonstrate that antibodies produced by the different clones do not react in a non-specific manner to the $\beta 3$ peptide. All supernatants responded poorly to the peptide $\beta 1$. Most supernatant responses to V β 20.1 were also insignificant. Syn 2H-11 was the only exception. This phenomenon may be attributed to possible cross-reactive epitopes shared with the $\beta 3$ peptide. Overall, these combined data give evidence that the selected clones secrete antibodies specific in their capacity to bind the $\beta 3$ peptide.

Figure 1d represents the same assay measuring reactivity of the different supernatants to the recombinant scTCR. Supernatant dilutions here were carried out two-fold from undiluted material to a final dilution of 1/64. All supernatants displayed

positive binding to scTCR relative to their negative and background controls.

"Stickiness" controls (blanks or uncoated, but blocked plates) were routinely included to distinguish between false-positive binding to the uncoated plates and genuine antibody-antigen interaction. The supernatants show a range of titration curves, however, they are not the same in their reactivities to scTCR as they are to $\beta 3$ peptide. Similar to the $\beta 3$ plot, Syn 2F7-2 demonstrates the highest reactivity to scTCR, but in marked contrast, Syn IB5 has the weakest affinity. Syn 2F7-2 binding to scTCR exhibits an absorbance measurement of 1.0 at less than a 1/10 titer. A neat dilution of Syn IB5, however, registers at an absorbance of approximately 0.4 units. Syn 2H-11 demonstrates a stronger reactivity (second only to Syn 2F7-2) than the other supernatants, but its activity drops sharply at 1/16. At a titer of 1/10 it (Syn 2H-11) measures at an absorbance around 1.0. OR2 and OR5 supernatants have similar titer reactivities to scTCR, measuring near 0.5 absorbance units at a 1/10 titer. OR1, OR4, and Syn IB5 supernatants demonstrated the weakest reactivities to scTCR. A 1/2 dilution of OR1 supernatant was sufficient to get an absorbance of 0.5 units and neat dilutions of OR4, and Syn IB5 could only measure an absorbance around 0.4 units on scTCR. Because the hybridomas secrete antibodies at different concentrations, and supernatants are collected somewhat

arbitrarily, a range of titrations should have been expected. It is not possible to gauge and compare affinities between antibodies on the test antigens from these experiments.

Antibodies from clonal supernatants bind to TCR on JURKAT cells

To expand on the ELISA data, the antibody supernatants were tested for their capacity to bind to intact α/β TCR-bearing JURKAT cells in an immunofluorescent flow cytometry experiment. Figure 2 illustrates the results from the fluorescent stains with some of the anti-TCR mAbs (Syn 2F7-2, OR1, OR2, OR5, and Syn 2H-11) compared to the conjugate antibody (negative control) by itself and to a FITC labeled antibody against human CD3 (Fig. 2a and b). The brightness range exhibited by the FITC-labeled anti-CD3 positive control represents a moderate degree of fluorescence. The flow data for the isotype controls was similar to the data for the conjugate antibody used alone on JURKAT cells (data not shown).

The raw supernatants from Syn IB5, Syn 2F7-2, OR1, and OR4 bound weakly on cells or not at all. The weak binding results were thought to be the result of low Ig concentrations in the supernatants. To test this possibility, the supernatants were concentrated approximately 20-fold by dialyzing them in water to precipitate the IgM, and

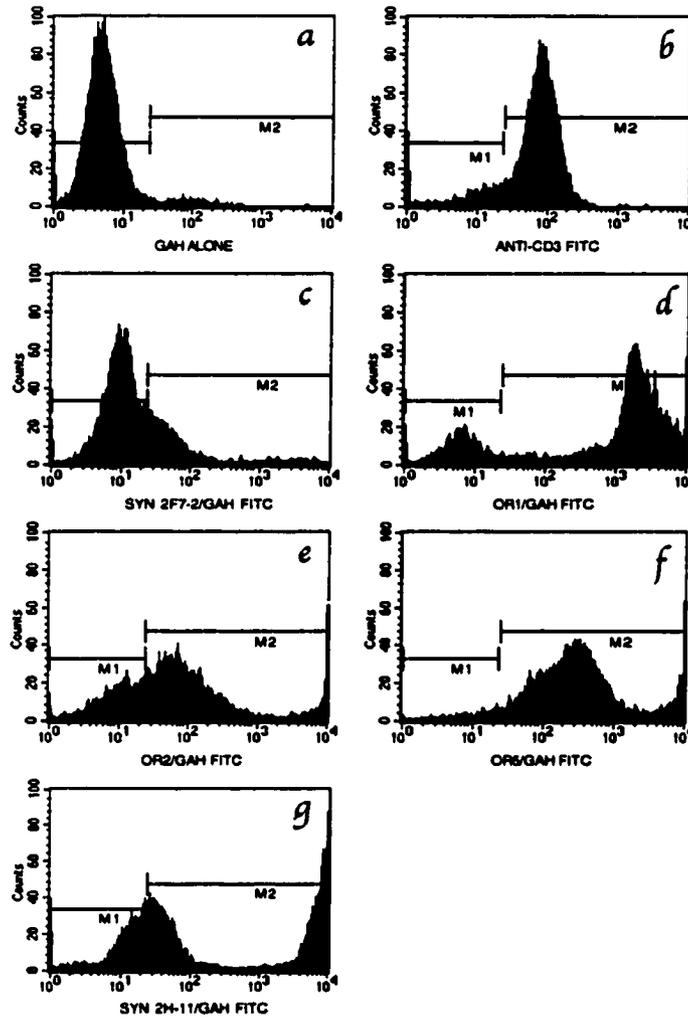


Figure 2. Binding of antibodies from clonal supernatants to the TCR on JURKAT cells. The figure reads as follows: A) negative control with the FITC conjugated goat anti-human IgM antibody alone, B) positive control with FITC conjugated mouse monoclonal anti-human CD3 antibody, C) supernatant from Syn 2F7-2, D) supernatant from OR1, E) supernatant from OR2, F) supernatant from OR5, and G) supernatant Syn 2H-11. The x-axis represents the fluorescence intensity of cells bound by the FITC labeled antibody and the y-axis represents the cell counts. The M1 region designates cells not fluorescing brightly enough to be considered positively bound by antibody and the M2 region indicates the group of cells that fluorescing brightly enough to be considered positively bound by antibody.

then resuspending the pellet in a small volume (between 1 and 2 ml) of PBS. Cells were incubated in 1ml of concentrated material and this effect improved the binding of all the supernatants to JURKAT TCR from at least no fluorescence to weak fluorescence. OR4 (data not shown) and Syn 2F7-2 were examples of concentrated supernatants that were slightly more effective in binding to a greater population of JURKAT T cells.

Concentrated supernatants from OR1 and Syn IB5 (data not shown) yielded profoundly improved the binding results to JURKAT cells. 2F7-2 is an example of weak binding to JURKAT TCR. There appears to be a small group of cells (about 25%) that Syn 2F7-2 does bind at low fluorescence (Fig. 2c). Most JURKAT cells (77%) fluoresced brightly when bound by OR1, but a distinct population of 23% did not exhibit fluorescence. OR1 looked as though it bound to a specific group of JURKAT T cells despite the fact that these cells are clonal (Fig. 2d). Approximately 74% of JURKAT cells are considered positively bound by OR2. OR2 bound in a broad manner from moderate to low fluorescence intensity in one population of cells. Close examination of this plot also indicates a small population of cells fluorescing at greater than 10^4 log units. This population is part of the 74% population considered positive for OR2 binding, but is definitely a distinct group of cells. This population of cells fluoresced too intensely for the histogram to be viewed at the voltage used for these experiments (Fig. 2e). OR5 binding

to JURKAT cells was almost 100%. Most of these cells showed a moderate to high degree of fluorescence intensity. It can also be seen with OR5 that this antibody binds to a small but distinct population of cells brightly in the same fashion OR2 binds to some JURKAT cells (Fig. 2f). Syn 2H-11 is another example an antibody that seems to bind to two groups of the JURKAT clone. It binds dimly to one group and brightly to a second in a profile that may have comparisons with the results obtained from the other antibodies on JURKAT cells. Syn 2H-11 bound positively to approximately 70% of JURKAT cells (Fig. 2g).

Antibodies from clonal supernatants bind to TCR on human PBMCs

The anti-TCR supernatants were tested on human peripheral blood mononuclear cells (PBMCs). Figure 3 shows dot-plots of CD3⁺ gated cells stained with the goat anti-human IgM FITC conjugate, anti-human V β 8, and four of the anti-TCR mAbs (Syn 2H-11, OR1, OR2, and OR5). Approximately 77.5% of the human PBMCs stained positively for the T cell antigen CD3. Staining with the conjugate control showed that this antibody was unable to bind CD3⁺ cells. About 3% of the human PBMCs were stained by anti-V β 8 commercial antibody. The region (R2) indicated in this plot

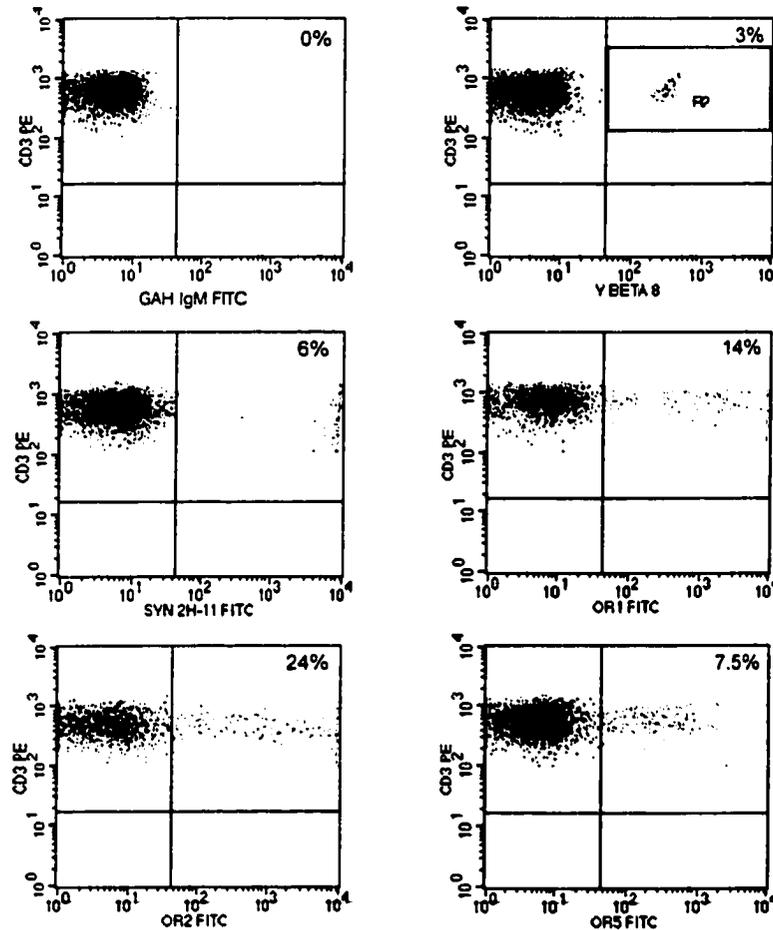


Figure 3. Binding of anti-TCR mAbs to CD3⁺ human peripheral blood mononuclear cells. PBMCs were stained with anti-CD3 PE (y-axis) and gated from CD3⁺ PBMCs. The top left cells are stained with the goat-anti human (GAH) IgM FITC conjugate, top right cells are stained with anti-human FITC V β 8 TCR, middle left cells are stained with Syn 2H-11, middle right cells are stained with OR1, lower left cells are stained with OR2, and lower right cells are stained with OR5 (x-axis). The goat-anti human (GAH) IgM served as the FITC conjugate for anti-TCR mAbs. The region examined for double stained PBMCs is indicated in the top right plot. The axes are measured in log fluorescence intensity units.

designates that the only cells fluorescing in this region were T cells bound by a specific anti-TCR antibody.

Syn 2H-11 bound to about 6% of T cells from peripheral blood. The range of fluorescence intensity goes from dim to bright. In a similar fashion to the JURKAT binding results, there exists a population of cells in this dot-plot that fluoresces so brightly that the voltage would have to be lowered in order to see the population on the plot. About 14% of the peripheral blood CD3⁺ cells were bound by OR1. The pattern in this plot appears similar to that of Syn 2H-11 with a smaller population fluorescing at greater than log 10⁴ units. The dot-plot pattern for OR2 has striking similarities with that of OR1. Approximately 24% of CD3⁺ PBMCs were bound by OR2. OR5 bound to 7.5% of the peripheral blood T cells. The range of fluorescence intensities was more restricted for OR5 with very few cells fluorescing at greater than 10³ log units. These results indicate that the anti-TCR mAbs were not pan-T cell reagents, but reacted selectively with subsets of human T cell receptors. Supernatants from Syn 2H-11, OR1, OR2, and OR5 are featured in this plot.

RNA was isolated from all of the clones and converted to cDNA for sequencing of the expressed V_H regions. A search of the V Base directory revealed that all of the V_H segments belonged to the V_H 3 family except OR2 and OR4 V_H which belong to the V_H 4

family. The OR1 V_H segment aligned with the DP-50 (V_H 3-33) germline sequence, OR2 matched to VIV-4 (V_H 4-4), OR4 with 3d279d+ (V_H 4-61), OR5 and Syn 2F7-2 with DP-54 (V_H 3-7), and Syn IB5 and Syn 2H-11 matched DP-47 (V_H 3-23) (Table 1).

All the sequences (amino acid) from this study were aligned together in their respective V gene families (Fig. 4) to illustrate the differences between sequences in the same family (bold type) and differences from the germline matches (underlined). The V_H regions of OR1, OR4, OR5, Syn 2F7-2, and Syn IB5 were identical to their germline counterparts, while the V_H comparisons of OR2 and Syn 2H-11 differed only in one base pair. The OR2 V_H contained an A to G nucleotide difference in FR3, but this base pair difference represents a wobble mutation retaining the amino acid sequence identical to that of the germline protein sequence (data not shown). The Syn 2H-11 V_H segment had a T to G switch in the FR1 region. This difference caused a single amino acid change from the germline sequence leucine to a valine.

Five of the D_H segments resembled known D_H 3 segments. Syn 2F7-2 and OR5 aligned identically to D_H 3-22 in the second reading frame (RF), OR1 matched closest to D_H 3-3 in RF 1, and OR2 and OR4 resembled D_H 3-3 in RF 3 (Table 2). Syn IB5 and Syn 2H-11 were related to D_H 6-13 in RF 1 (Table 2). The CDR3 regions of the heavy chain sequences were diverse and could not be aligned to any known sequences in the

Table 1. V, D, and J germline family usage of the anti-TCR hybridomas.

Clone name	V_L	Germline gene	J_L	V_H	Germline gene	D_H	J_H
OR1	V _λ 3	IGGLL150	J _λ 2/J _λ 3a	V _H 3	DP-50	D _H 3	J _H 6b
OR2	V _λ 3	IGGLL150	J _λ 3b	V _H 4	VIV-4	D _H 3	J _H 6b
OR4	V _λ 2	2C.118D9	J _λ 2/J _λ 3a	V _H 4	3d279d+	D _H 3	J _H 5b
OR5	V _λ 3	IGGLL150	J _λ 3b	V _H 3	DP-54	D _H 3	J _H 6b
Syn 2F7-2	V _κ 3	DPK 21	J _κ 2	V _H 3	DP-54	D _H 3	J _H 6b
Syn 1B5	V _κ 3	V _g /38K	J _κ 2	V _H 3	DP-47	D _H 6	J _H 4b
Syn 2H-11	V _λ 3	DPL16	J _λ 2/3 _λ a	V _H 3	DP-47	D _H 6	J _H 4b
IARC307	V _κ 3	KV31es	J _κ 2	V _H 3	DP-54	D _H 6	J _H 6c

Table 2. N region diversity of the D_H segments.

Clone	D _H /RF	V-D insertions	V-D deletions	D-J insertions	D-J deletions	Total insertions	Total deletions	Balance
Syn 2F7-2	3-22/2	15	0	0	3	15	3	+12
OR5	3-22/2	15	0	0	3	15	3	+12
OR1	3-3/1	6	3	9	15	15	18	-3
OR4	3-3/3	6	0	3	9	9	9	0
OR2	3-3/3	9	0	15	24	24	24	0
Syn IB5	6-13/1	12	3	3	0	15	3	+12
Syn 2H-11	6-13/1	6	3	15	6	21	9	+12
IARC307	6-19/1	6	9	18	21	24	30	-6

databases or with each other, with the exception of OR5 and Syn 2F7-2 (Fig. 5). Close inspection of the V-D and D-J junctions revealed that both ends of the D and J segments, in most of the sequences, had undergone deletion and insertion of nucleotides. The resulting inclusion of new amino acids contributed to all the sequence diversity of the autoantibodies (Table 2).

The D_H segments, including the flanking junctional nucleotides (N- D_H -N) were examined for common traits because they are the only unique sequence in these autoantibodies. The residues in these sequences were organized by their biochemical properties (e.g. hydrophobicity, aromatic side chains, hydroxyl groups, and acidic and basic side chains). Few commonalties were found within these sequences. There did exist, however, a paucity of amino acids with acidic side chains in these regions. OR1, OR5, and Syn 2F7-2 had 2, and Syn 2H-11 had 1, while the remaining N- D_H -N segments had no amino acids with acidic side chains. Otherwise, there was no common presence or lack of certain kinds of amino acid residues that would provide a recognizable motif. Amongst all these regions, 35% of the residues were hydrophilic, 44% were ambivalent, and 21% were hydrophobic. When the transfer of free energy values (kcal/mol) of these N- D_H -N residues was examined all sequences totaled a negative value. The only exceptions were OR4 (and IARC307, a clone from a previous study used for comparative

	V	N	D	N	J	
OF2	<u>E A R</u>	S H R	<u>Q R R C A V</u> <u>v i i</u>	P P R P R	<u>v y y y y</u> <u>G M D V W G Q G T</u>	J _{H6b}
OF4	<u>E A R</u>	S R	<u>Q R R C A V</u> <u>v i i</u>	A	<u>N W F D P W G Q G T</u>	J _{H5b}
OR1	<u>E A R</u>	G A	<u>Y R R R C A V</u> <u>l l y</u>	P R N	<u>v y</u> <u>Y Y Y G M D V W G Q G T</u>	J _{H6b}
OF5	<u>E A R</u>	E P P T S	<u>Y Y R R C A V</u> <u>Y</u>		<u>Y Y Y Y Y G M D V W G Q G T</u>	J _{H6b}
Syn 2F7-2	<u>E A R</u>	E P P T S	<u>Y Y R R C A V</u> <u>Y</u>		<u>Y Y Y Y Y G M D V W G Q G T</u>	J _{H6b}
Syn 1B5	<u>E A K</u>	G Y R A	<u>Q R R R C A V</u>	F Y I	<u>I D Y W G Q G T</u>	J _{H4b}
Syn 2H-11	<u>E A R</u>	D R	<u>Q R R R C A V</u> <u>Y</u>	H I P S T	<u>F D Y W G Q G T</u>	J _{H4b}
IARC307	<u>E A R</u>	G G	<u>Q Y R R C A V</u> <u>Y</u>	W V	<u>Y Y Y Y M D V W G K G T</u>	J _{H6c}

Figure 5. Alignment of the CDR3 regions. Sequences of the junctional regions were aligned together to illustrate the diversity of the CDR3 segments. All sequences begin with the last three amino acids (boxed) from the V region. The D region sequences display which amino acids resulted from junctional diversity (additions and deletions). The non-shaded, capitalized amino acids are N region additions. The shaded amino acids are conserved from the D_H gene family. The underlined, lower-cased amino acids represent deletions from the D_H gene sequences. The last boxed amino acids in the sequences are the J region. Underlined, lower-cased amino acids in this region represent N deletions from the J_H gene sequences.

analysis) which measured an overall positive free energy transfer from their amino acid side chains (Table 3). The average length of the N-D_H-N sequences was 11.86±2.35. The D_H and J_H sequences were identified from a database search and it was further determined where the deletions and insertions took place on both sides of the D_H region (Table 2). The OR4 clone was related to a J_H 5b and the Syn IB5 and Syn 2H-11 heavy chain sequences were derived from J_H 4b. The remaining J regions were J_H 6b (Table 1).

V region light chain germline sequences of anti-TCR autoantibodies

Light chain sequence analysis revealed five lambda chains (OR1, OR2, OR4, OR5, and Syn 2H-11) and two kappa chains (Syn 2F7-2 and Syn IB5) (Table 1). OR1, OR2, OR5, and Syn 2H-11 were from the V_λ3 gene family and OR4 from the V_λ2 family. The Syn 2F7-2 and Syn IB5 clone belonged to the V_κ3 family (Table 1). Analysis of the V_L and J_L regions from our clones revealed that all of the light chain sequences, except OR4 and Syn IB5, were identical to their germline counterparts (Fig. 4). The entire light chain sequence for OR4 differed by only two nucleotide base pairs in the FR4 of the J segment. These differences occurred between a G and a T at the CDR3-FR4 junction, which resulted in a leucine in place of the valine (Fig. 4). There was also a difference between

Table 3. Hydropathy values for D_H segments. Each amino acid from the D_H segments of all the clones were assessed by their free energy values then totaled.

D_H Hydropathy	
OR2	-25.2
OR4	+4.6
OR1	-24
Syn 2F7-2	-18
OR5	-18
Syn IB5	-4.4
Syn 2H-11	-16.8
IARC307	+10

an A and a T (data not shown) in the last base pair of the third codon, but the sequence still retained its leucine. The Syn IB5 J_L had the most differences from the germline sequence. There are 2 base pair mismatches, one in the last codon of the CDR3, changing the amino acid from a proline to a serine, and one in FR4, changing a lysine to a glutamic acid. Also, two codons for the tyrosine and the threonine from the beginning of the $J_{\kappa}2$ are deleted (Fig. 4). There were some consistencies in the types of J_L sequences that were used by our clones. OR1, OR4, and Syn 2H-11 used the $J_{\lambda}2/J_{\lambda}3a$, OR2 and OR5 used identical $J_{\lambda}3b$ sequences, and Syn IB5 and Syn 2F7-2, used $J_{\kappa}2$ (Table 1). There was little diversity amongst these light chains in terms of CDR3 length. The lengths of the light chain CDR3 segments due to N region insertions were either 8 or 9 amino acids long.

SECTION I

Chapter 3 – Discussion

Here we have analyzed seven IgM anti-TCR mAbs, selected for the ability to bind a 16-mer peptide containing the CDR1 segment of human V β 8.1, produced by clones from either peripheral blood or synovium of RA patients. These mAbs were selected to comprise the subset of peptide binding molecules that react also with the scTCR V α /V β construct, containing this epitope, and with the cognate T cell receptor expressed on intact JURKAT cells. The results from the binding studies indicate that the anti-TCR mAbs are capable of reacting to both the selecting TCR antigens and the intact TCR on human T cells. The flow cytometry results were not, however, an expected outcome of binding on a clonal population of cells. Fluorescence intensities varied and it appeared that most of the anti-TCR mAbs bound differently to 'subsets' of JURKAT cells. Although binding to the JURKAT TCR is considered real, the heterogeneity of binding is difficult to explain on this data alone. We speculate that the various levels of fluorescence intensities are related to the degree of affinity for which the anti-TCR mAbs express against JURKAT T cell receptor. This explanation takes into account that the

selection process used to derive these monoclonal antibodies can be used to derive antibodies with crossreactive/low affinity idiotopes as well as high affinity idiotopes. The existence of dual populations of JURKAT T cells bound by the anti-TCR mAbs may indicate that clones in the cell cycles express various conformational determinants of the TCR on their cell surface. The anti-TCR mAbs may recognize exposed epitopes on the T cells that are not always present. Otherwise, binding heterogeneity to JURKAT T cells may just be a function of IgM molecules. The binding results for the anti-TCR mAbs to CD3⁺ PBMCs were similar to those on JURKAT cells. The data reveal that these antibodies bind to subsets of T cells at various affinities. The findings support the conjecture that natural autoantibodies specific for the TCR are not pan-T cell markers, but rather function as a network of molecules binding to discrete subsets to administer immunoregulatory control.

Analysis of V_H and V_L gene sequences revealed that these antibodies are generated from the natural B cell repertoire with no evidence of somatic mutations in the frameworks or in CDR1 and CDR2. We also consider another human monoclonal autoantibody (IgM κ ; IARC307) which was generated by transformation with Epstein-Barr virus and selected for its specificity against β 3 peptide (37). IARC307 differs from the other seven clones in that it was derived from normal B cells in peripheral blood of a

Burkitt's Lymphoma patient. The V_H segments of all of these antibodies are identical, or only differ by a single nucleotide base pair (in the case of OR2 and Syn 2H-11), to their reported V_H germline sequences. Two sets of the heavy chain sequences share the same germline families (Table 1). Interestingly, the OR5 and the Syn 2F7-2 heavy chain sequences are identical in the flanking junctional nucleotides (N- D_H -N) of the CDR3 which seems unlikely considering these sequences were derived from different patients. This anomaly was re-examined to account for possible cross-contamination using new primers with controls in three separate investigations. The results, however, are unchanged. These findings parallel another study on CDR3 segments in the TCR of patients with RA where the sequences were identical in more than two patients. It should also be noted that the identical TCR CDR3 sequences were not taken from cell lines selected for a specific antigen (83). Four light chain sequences (OR4, Syn IB5, Syn 2F7-2, and IARC307) were only slightly mismatched with their respective germline parent, while the remaining (OR1, OR2, OR5, Syn 2H-11) were identical in their V_L and J_L , including in their CDR3 intervals, to germline alignments. Some of the clones share identical (or nearly identical) light chain sequences, but are coupled to different heavy chains; eg. OR1, OR2, and OR5 (Table 1 and Fig. 4). The essential component that differentiates our clones in terms of sequence is the CDR3 segments of the heavy chains.

Although each one of these clones was selected for their binding specificity to a single epitope, they each contain highly diverse CDR3 regions that can not be exactly matched with each other (with the exception of the OR5 and Syn 2F7-2 V_H sequences). The CDR3 regions do not match up with any other sequences in database and literature searches (Fig. 5).

Most of the heavy chain sequences are from the V_H 3 family with the exception of two (OR2 and OR4), which is in the V_H 4 family. Among the seven human V_H families V_H 3 is the largest and most diverse. V_H 4 is also a large gene family and contains a relatively high level of diversity compared with any other V_H family (84). These families contain the highest percentage of natural autoantibodies in all individuals tested. Moreover, V_H 4 represents the only family that preferentially makes autoantibodies to xenoreactive antibodies (85, 86). A study by Huang *et al.* on V_H genes in RA peripheral blood observed significant elevations of somatic mutation in the CDR1 and CDR2 regions of V_H 3 genes compared to normal individuals. By contrast, an elevated frequency of mutations was not found in comparison of the V_H 4 sequences of RA patients with those of normals (76). The trend towards elevated mutation frequency was not apparent in the V region anti-TCR sequences in these studies. All V_H or V_L sequences were essentially identical to their germline counterparts. These findings

parallel those of Brown *et al.* who investigated heavy chain V regions from synovium derived hybridomas of RA patients. Their study reported that the sequences examined were in germline configuration, but expressed considerable diversity in the CDR3 segments. An important difference to note is that Brown *et al.* did not select for the types of antibodies produced. Not one of their unselected monoclonal antibodies, with the exception of one rheumatoid factor, was autoreactive (74).

The HCDR3 segments appear to be major contributors to the specificity of the anti-TCR mAbs, although it should be considered that the contributions of the various light chains to the formation of the unique antigen combining sites. Because of these important features and the fact that the anti-TCR clones have been selected for a specific epitope, these autoantibodies exist as a potentially valuable model for studying the physical properties of the D_H region and the characteristics of polyreactivity. These findings were compared with that of Clausen *et al.*, who published D_H segments from clones sequenced from RA patient synovial tissue that share some similarities with our D_H segments. Consistent with these results, the results from this study found a prevalence of D_H 3 segments. More specifically, the authors noted a significantly enriched utilization of D_H 3-3 and D_H 3-22. Five out of seven of the clones from this study are D_H 3. Two are D_H 3-22 and three are D_H 3-3 (Table 2). The remaining clones were D_H 6. It is important to note

that Clausen *et al.* (73) also observed D_H 6 utilization in their cloned sequences from synovial tissue of RA patients, but its representation was not considered significant.

The two factors that contribute to the specificity of CDR3 regions are diversity of amino acid side chains and peptide length. Investigation into these traits found a general trend of hydrophilic and neutral residues being more prevalent than hydrophobic. This parallels the findings by Clausen *et al.* (73) who reported that D_H sequences in RA patients contained more hydrophilic residues than hydrophobic residues. The CDR3 sequences in these studies were either normal in size or larger due to N-insertions (Table 2). N region addition is typically known to cause a higher proportion of G and C nucleotides in V-D and D-J junctions (60% or higher) (87, 88). All the sequences except for Syn IB5, which had a G/C content of 46.7% in the V-D and D-J regions, fit this criterion.

The J_H segment contributes a portion of the CDR3 and should be considered an important component of an antibody's binding characteristics. There were 3 types of J_H segments found in the clones from this study, five J_H 6, two J_H 4, and one J_H 5 (Table 1). So far, there is no evidence of J_H prevalence in RA. These findings also resemble those of Brown *et al.* (74), who reported J_H 4, J_H 5, and J_H 6 in their hybridoma sequences from RA synovium. The expression of J_H segments in these sequences does not appear to be a random occurrence.

With the exception of OR4, which used a $V_{\lambda}2$, all of the V_L sequences were either $V_{\lambda}3$ or $V_{\kappa}3$ (Table 1). The $V_{\lambda}3$ family is the largest V_{λ} family (89, 90) and is frequently found in autoantibodies from patients with RA (91-94). The fact that all the V_{κ} segments are from the $V_{\kappa}3$ family is not surprising in that it is a highly conserved and large family (89) and contributes the most to the structural repertoire diversity (95). One feature of the V_L sequences that warranted investigation was the possibility of unusually long CDR3 consistent with other findings on light chains expressed in RA patients (75,77,79-81). The enzyme TdT is active in light chain rearrangements as well as in heavy chain rearrangements (79,81,96-98) and can play a critical role in N region diversity in the light chains. A study on unselected light chains from RA synovium (about 43-50%) and peripheral blood (40-45%) reported a prevalence of V_{λ} chains with abnormally large CDR3 regions over that of normal individuals (23-43%). Similar results were also found in V_{κ} sequences from patients with RA in comparison with normal individuals, but to a significantly lesser degree (81). The lengths of the CDR3 interval in the V_L sequences were 8 or 9 amino acids. This amino acid interval (8, 9, or 10) is considered normal in light chain CDR3 segments. Eleven amino acids for a CDR3 in a light chain sequence are considered unusually long (53). The clones sequenced from the other studies report CDR3 regions up to 16 amino acids long (81).

Somatic mutations have been reported to occur at a higher frequency in the CDR1 and CDR2 segments of the light chains from RA patient synovium and PBLs than is found in light chains of normal individuals (77, 79). In this study only two of the V_L sequences in do not align identically with their germline matches in both the V_L and J_L segments (OR4 and Syn IB5). Moreover, the sequence from the clone IARC307 does not align perfectly with its germline parent. OR4 contained a single nucleotide mismatch in its germline sequence in the first codon of its J_L segment, changing its amino acid from a germline valine to a leucine (Fig. 4). These side chains have similar chemical properties, so it would be difficult to assume that this difference has an altering effect on the binding region of this antibody. Syn IB5 contrasted with its germline alignment in eight nucleotides. Two codons were completely deleted in the $V_{\kappa} 2$, omitting the first two amino acids (tyrosine and threonine). One base pair mismatch occurred in the last three codons of the CDR3, changing a proline to a serine, and a base pair mismatch in FR4 changed a lysine to a glutamic acid (Fig. 4). This light chain sequence has the most striking germline deviations in the CDR3 and most likely has an altered combining site structure. IARC307 had eight nucleotide mismatches compared with its germline best match. Five of the mismatches occur in the last CDR3 codons, altering the $V_{\kappa} 3$ - $J_{\kappa} 2$ junction to read WPHFGQG in place of the germline LYTFGQG sequence. The other

three base pair mismatches were found in the V_x region. One occurs in the CDR2, where a germline isoleucine is a threonine, another in the FR3, where an alanine is changed to an aspartic acid, and the last discrepancy occurs in the CDR3 (data not shown) which did not effect on the amino acid outcome (Fig. 4). This sequence had the most outstanding differences from its germline match compared to the rest of our V_L sequences. This mAb is specific only for the $\beta 3$ peptide.

Although the sequences in this study belong to V_H and V_L families that have been reported to occur in a greater frequency in patients with RA, their usage is not unique to RA patients. In fact, the exact light chain sequences from OR2 and OR5 were present in mRNA from peripheral blood of normal individuals (81). IgM autoantibodies to TCR V-domain epitopes are produced in significantly higher titer in RA patients than in healthy individuals or patients with osteoarthritis. Furthermore, anti-TCR antibodies of distinct isotypes and specificities are found in greater concentrations in other types of clinical disorders (43,44). Interestingly, the anti-TCR mAbs appear to be a natural part of the antibody sequence repertoire, rather than the products of antigen driven selection, suggesting their general role in immunoregulation, as opposed to a specific pathologic dysfunction, for these ubiquitous molecules. These data reinforce previous findings that some B cells play a natural role in the immune system by producing polyclonal

autoantibodies and evade the negative selection processes that typically lead to their deletion from the B cell repertoire (99-103).

The mAbs studied here express V-regions of naive germline origin with no evidence to date of somatic hypermutation characteristic of antigen driven selection. This finding is consistent with them performing a basic immunomodulatory role via interaction with V β -defined public idiotopes. This suggestion gains further credence from the observations that sharks, which have only circulating IgM antibodies and lack the capacity for isotype switching and affinity maturation, naturally express antibodies possessing anti-TCR specificities comparable to those of mammals (104). Studies of both IgM (105, 82) and IgG (106) mAbs reactive with TCR V-domain epitopes show that some demonstrate reactivity for a wide range of peptide determinants, while others bound only a small panel of epitopes or just the β 3 peptide. This variation in binding specificity may serve as an important factor in determining the extent of antibody binding reactivity.

**SECTION II: Specificity mapping of anti-T cell receptor monoclonal
natural antibodies: defining the property of epitope recognition
promiscuity**

Chapter 4 – Introduction

Immunoglobulins (Igs) serve as the principal molecular components of the humoral immune system. Comprised of protein structures that form multivalent binding sites, they function as a first line of defense, coupled with long-term protection, against tissue damage and infection through the neutralization of toxic and pathogenic determinants. A remarkable feature of Igs is that each one possesses an antigen binding site that can discriminate between discrete molecular structures (107). The existence of such a comprehensive binding motif network, capable of responding to millions of diverse antigens, is dependent on a highly complex combinatorial immune system providing an extreme level of variability amongst the Ig pool (108).

Studies on antibody specificity have yielded many discoveries about the nature of antigen binding. The variable (V) region sequences determine what epitope and how tightly the antibody will bind, but to what extent can not be predicted. The basic model for

the antibody to antigen interaction consists of a specific binding to a single epitope at a relatively high affinity. This notion was deduced from the clonal selection theory proposed by Burnet in the 1950's (109). Since that time investigations have revealed that this concept is too simplified to represent all antibody/antigen interactions. New terms were required to account for antibody binding to non-cognate antigen. 'Cross-reactivity', for example, describes an interaction between an antibody and epitopes that are antigenically related. Binding affinity differences for related structures are dependent on the spatial orientation of functional groups of the antigenic determinant (110). This can be seen at the protein and peptide level. 'Polyreactivity' describes a very different, but not unusual quality of antibody binding to many large, complex, and commonly occurring proteins or antigens with repeating structural patterns. Typically, these antibodies are naturally occurring IgM molecules that exist in the serum prior to an immune priming event. Polyreactive antibodies are generally known to bind to multiple determinants or molecular patterns with low affinity (8,17). Epitope recognition promiscuity is a binding characteristic that differs from the mainstream definition of polyreactivity in that the antibody can specifically recognize distinct antigenic epitopes at a different or similar affinity, and the epitopes compete for the same binding site. These kinds of antibodies are

restricted in binding to a limited set of epitopes and are not thought to bind determinants in a non-specific manner (25, 26).

Epitope recognition promiscuity is a characteristic observed in IgM natural antibodies and IgG antibodies (106, 111, 112). Although its existence stems from the earliest origins of the combinatorial immune system, based on findings of epitope promiscuous antibodies in shark serum, there is limited speculation on its functional purpose (26, 104, 113). Epitope promiscuity may be explained as an evolutionary occurrence that was unadjusted by selective processes. However, it seems more likely that these kinds of antibodies carry out equally important (and perhaps diverse) immunological functions as their mono-specific counterparts. With respect to natural antibodies, it may be that epitope promiscuity enhances the functions of these molecules allowing them to target multiple antigens in a specific manner.

Natural antibodies express variability of the amino acid sequence in the CDR3 V region heavy chain. These unique sequence segments are products of the imprecise joining events between the variable (V), diversity (D), and joining (J) region of the heavy chain, and subsequent N region additions, that occur during V gene rearrangement. The rest of the antibody is usually identical to the germline sequence. It is believed that heavy chain CDR3 diversity is the crucial factor that determines if an antibody will be

polyreactive epitope promiscuous, or monospecific for antigen (114, 21, 16, 115, 23, 116, 117).

We report from studies determining the specificities for defined synthetic peptides of monoclonal natural antibodies selected for the ability to bind to the same human T cell receptor (TCR) antigens, that epitope recognition promiscuity is a genuine property distinguishable from the phenomenon of polyreactivity. Our findings demonstrate that natural antibodies with diverse heavy chain CDR3 segments, but otherwise in germline sequence, are capable of binding specifically to sequentially non-related peptide epitopes, but not to test proteins typically recognized by polyreactive antibodies. We further show that epitope recognition promiscuity can be expressed in various degrees and affinities to different TCR epitopes.

SECTION II

Chapter 5 – Results

Anti-TCR mAbs demonstrate differential binding profiles on TCR and Ig light chain peptides

In *Section I* we reported the generation and characterization of seven clones from RA patients, each with different variable region heavy and light chain sequences, but selected on the same TCR antigens. Four of these mAbs, OR1, OR2, OR5, and Syn 2H-11, were tested for binding to three sets of sequentially overlapping peptides, approximately 16 amino acids in length, corresponding directly to the complete pY14 α TCR, YT35 β TCR, and the Mcg Ig λ chain sequence. The anti-TCR mAbs were tested on peptides corresponding to antibody light chain because the Mcg λ sequence is structurally homologous to the TCR β sequence, and thus helpful for assessing the degree of cross-reactivity between these peptides. A minimum of three ELISAs was conducted (at separate time intervals) for each anti-TCR mAb on the different peptide groups. The reciprocals of the supernatant dilutions at which the absorbance measurement (OD 405) was 0.5 units were compiled and expressed as a geometric mean titer to designate relative

antibody reactivity. This method allows for comparison of data from different assay dates, but excludes tenuous and false positive assay results.

As indicated in Figure 6, relative antibody binding profiles differed significantly on the TCR β peptides. Anti-TCR mAb OR1 bound only to β 3. This is the CDR1/FR2 peptide on which all the anti-TCR mAbs were selected. OR1 tested on the TCR α peptides reacted only with α 3 and α 4 (Fig. 7). It did not bind with any of the Mcg Ig λ peptides (Fig. 8). A comparison between the β 3 peptide in Table 4 and α 3 and α 4 peptides in Table 5 reveals evident sequence homologies, specifically in the carboxyl end of α 3 and the amino end of α 4. The shared sequence WYVQY is similar to the WYRQT sequence in the carboxyl end of the β 3 peptide. From these results it is uncertain if OR1 could yet be defined as an epitope promiscuous antibody. With respect to the shared binding reactivity between peptides of homologous sequences, it is arguable that the binding is made possible by cross-reactive epitopes.

The mAb OR2 illustrates a very different quality observed amongst the anti-TCR mAbs. When examined on the TCR β peptides OR2 reacted substantially with 5 other peptides besides β 3. These peptides were β 8, β 11, β 14, β 15, and β 20. OR2 bound to peptides that corresponded to the FR3 (β 8) region of the V β TCR, parts of the J and constant (C) region (β 11), and at least two different segments of the C region (β 14/ β 15

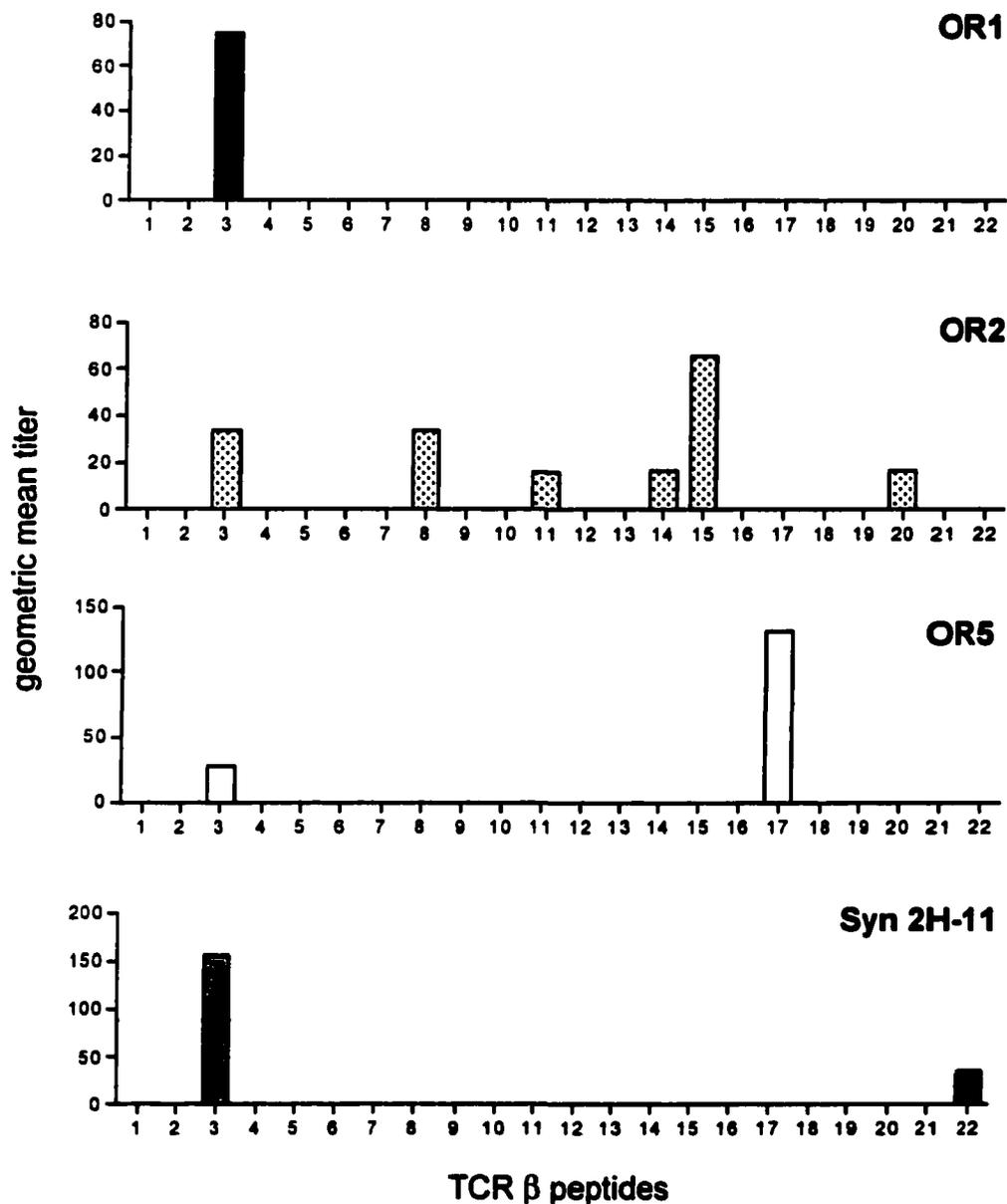


Figure 6: Anti-TCR mAbs binding to TCR β peptides. Binding reactivity, as indicated by geometric mean titer units on the y-axis of each bar plot, was measured on the individual TCR β peptides listed on the x-axis. The peptides were numbered by how they would correspond from the amino to the carboxyl terminus of the complete protein sequence. OR1) OR2) OR5) Syn 2H-11) .

Table 4. TCR β peptides.

	TCR β peptides ^a
$\beta 1^b$	D A G V I Q S P R H E V T E M G
$\beta 2$	V T E M G Q E V T L R C K P I S
$\beta 3^c$	C K P I S G H N S L F W Y R Q T
$\beta 4$	W Y R Q T M M R G L E L L I Y F
$\beta 5$	L L I Y F N N N V P I D D S G M
$\beta 6$	D D S G M P E D R F S A K M P N
$\beta 7$	A K M P N A S F S T L K I Q P S
$\beta 8$	K I Q P S E P R D S A V Y F C A
$\beta 9$	V Y F C A S S F S T C S A N Y G
$\beta 10$	S A N Y G Y T F G S G T R L T V
$\beta 11$	T R L T V V E D L N K V F P P E
$\beta 12$	V F P P E V A V F E P S E A E I
$\beta 13$	S E A E I S H T Q K A T L V C L
$\beta 14$	T L V C L A T G F F P D H V E L
$\beta 15$	D H V E L S W W V N G K E V H S
$\beta 16$	K E V H S G V S T D P Q P L K E
$\beta 17$	Q P L K E Q P A L N D S R Y C L
$\beta 18$	S R Y C L S S R L R V S A T F W
$\beta 19$	S A T F W Q N P R N H R F C Q V
$\beta 20$	F R C Q V Q F Y G L S E N D E W
$\beta 21$	E N D E W T Q D R A K P V T Q I
$\beta 22$	P V T Q I V S A E A W G R A D

^a The β peptides are derived from the complete V β 8.1 YT35 TCR sequence referenced in Yanagi *et al.* (54).

^b The $\beta 1$ peptide, representing the amino terminal end of the FR1 V β region of the human YT35 TCR sequence, was the peptide antigen used as a specificity control for assays involving the OR2 anti-TCR mAb.

^c The $\beta 3$ peptide, representing the complete CDR1 and part of FR2 V β region of the human YT35 TCR sequence, was the peptide antigen used to select all of anti-TCR secreting hybridomas (*Section I Materials and Methods*).

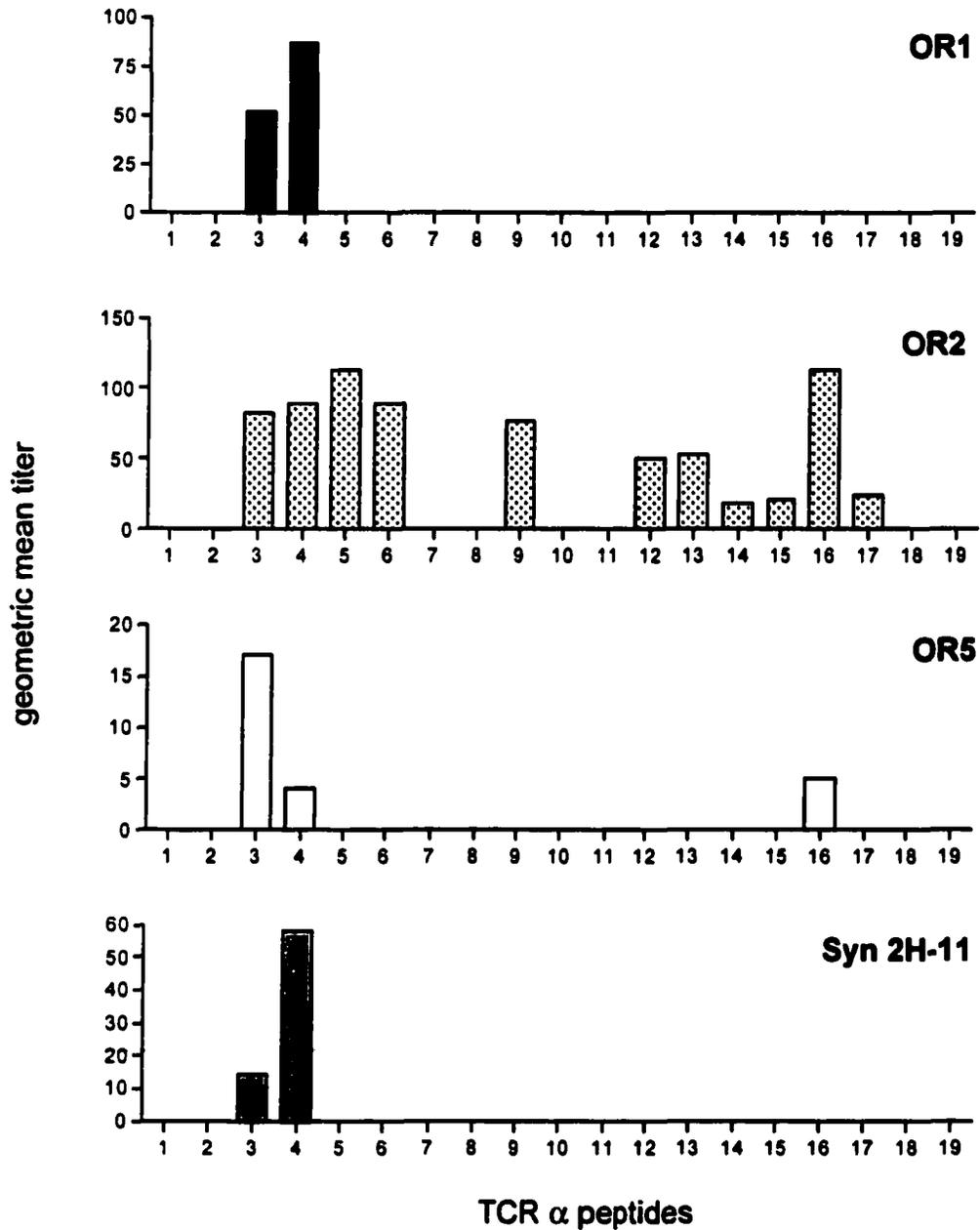


Figure 7: Anti-TCR mAbs binding to TCR α peptides. Binding reactivity, as indicated by geometric mean titer units on the y-axis of each bar plot, was measured on the individual TCR α peptides listed on the x-axis. The peptides were numbered by how they would correspond from the amino to the carboxyl terminus of the complete protein sequence. OR1) OR2) OR5) Syn 2H-11) .

Table 5. TCR α peptides.

TCR α peptides ^d	
$\alpha 1$	Q S V T Q L G S H V S V S E G A
$\alpha 2$	V S E G A L V L L R C N Y S S S
$\alpha 3$	N Y S S S V P P Y L F W Y V Q Y
$\alpha 4$	W Y V Q Y P N Q G L Q L L L K Y
$\alpha 5$	L L L K Y T S A A T L V K G I N
$\alpha 6$	V K G I N G F E A E F K K S E T
$\alpha 7$	K K S E T S F H L T K P S A H M
$\alpha 8$	P S A H M S D A A E Y F C A V S
$\alpha 9$	F C A V S D L E P N S S A S K I
$\alpha 10$	S A S K I I F G S G T R L S I R
$\alpha 11$	R L S I R P N I Q N P D P A V Y
$\alpha 12$	D P A V Y Q L R D S K S S D K S
$\alpha 13$	S S D K S V C L F T D F D S Q T
$\alpha 14$	F D S Q T N V S Q S K D S D V Y
$\alpha 15$	D S D V Y I T D K T V L D M R S
$\alpha 16$	L D M R S M D F K S N S A V A W
$\alpha 17$	S A V A W S N K S D F A C A N A
$\alpha 18$	A C A N A F N N S I I P E D T F
$\alpha 19$	P E D T F F P S P E S S C D

^d The α peptides are derived from the complete V α 1 pY14 TCR sequence referenced in Yanagi *et al.* (55).

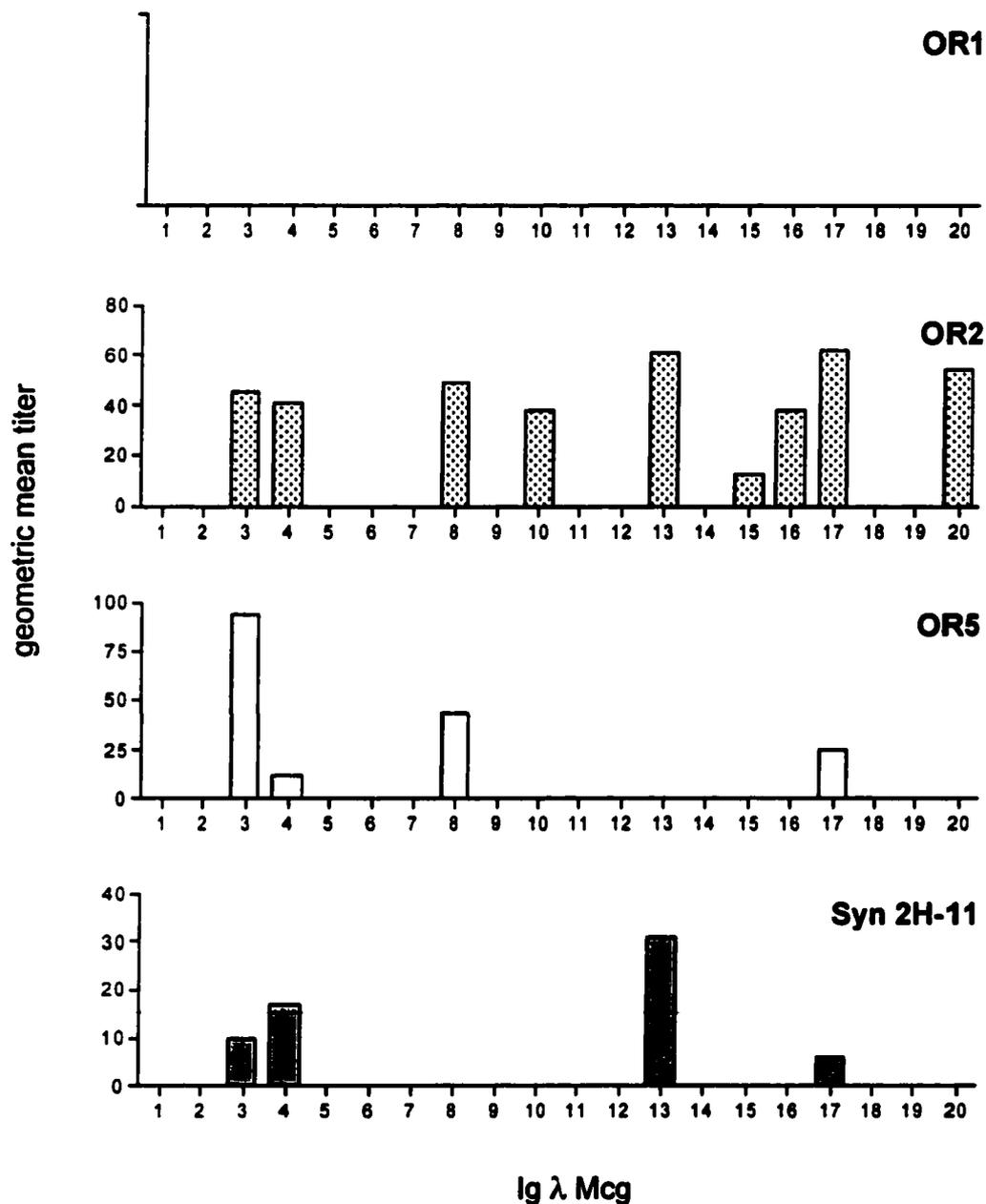


Figure 8: Anti-TCR mAbs binding to Ig λ Mcg peptides. Binding reactivity, as indicated by geometric mean titer units on the y-axis of each bar plot, was measured on the individual Ig λ Mcg peptides listed on the x-axis. The peptides were numbered by how they would correspond from the amino to the carboxyl terminus of the complete protein sequence. OR1) OR2) OR5) Syn 2H-11) .

Table 6. Mcg Ig λ peptides.

	Ig λ Mcg peptides ^a
Mcg 1	Q S A L T Q P P S A S G S L G Q
Mcg 2	G S L G Q S V T I S C T G T S S
Mcg 3	T G T S S D V G G Y N Y V S W Y
Mcg 4	Y V S W Y Q Q H A G K A P K V I
Mcg 5	A P K V I I Y E V N K R P S G V
Mcg 6	R P S G V P D R F S G S K S G N
Mcg 7	S K S G N T A S L T V S G L Q A
Mcg 8	S G L Q A E D E A D Y Y C S S Y
Mcg 9	Y C S S Y E G S D N F V F G T G
Mcg 10	V F G T G T K V T V L G Q P K A
Mcg 11	G Q P K A N P T V L L F P P S S
Mcg 12	F P P S S E E L Q A N K A T L V
Mcg 13	K A T L V C L I S D F Y P G A V
Mcg 14	Y P G A V T V A W K A D G S P V
Mcg 15	D G S P V K A G V E T T K P S K
Mcg 16	T K P S K Q S N N K Y A A S S Y
Mcg 17	A A S S Y L S L T P E Q W K S H
Mcg 18	Q W K S H R S Y S C Q V T H E G
Mcg 19	V T H E G S T V E K T V A P T E
Mcg 20	T V E K T V A P T E C S

^a The Mcg peptides are derived from the complete Mcg λ light chain sequence referenced in Edmundson *et al.* (56).

and β 20). There is little if any comparative sequence homology between these peptides.

OR2 appeared to demonstrate the same kind of multi-reactive qualities on the TCR α peptides (Fig. 7). OR2 bound similarly with OR1 to α 3 and α 4 and also the α 5 and α 6 peptides. OR2 also demonstrated reactivity to the α 9 peptide, which is apart of the V region CDR3 sequence, and TCR α peptides 12 through 17, which all correspond to the TCR α constant region sequence. OR2 bound to the set of Ig λ Mcg peptides corresponding to the CDR1 region (Mcg 4 and 5), the FR3 and CDR3 region (Mcg 8), the J region (Mcg 10), and the constant region (Mcg 13, 15 through 17, and 20) (Fig. 8).

Anti-TCR mAbs OR5 and Syn 2H-11 demonstrated more restrictive binding patterns to the test peptides. Besides the β 3 peptide, OR5 and Syn 2H-11 bound to one other β peptide corresponding to the constant region. OR5 bound to the β 17 peptide and Syn 2H-11 bound to the β 22 peptide (Fig. 6). On the TCR α peptides, OR5 and Syn 2H-11 reacted to α 3 and α 4 as was observed with OR1 and OR2. OR5 bound to the α 16 peptide of the TCR α constant region, but Syn 2H-11 reacted with no other α peptides (Fig. 7). OR5 and Syn 2H-11 bound to Mcg CDR1 region peptides 3 and 4, similar to OR2, and two other Mcg peptides each. OR5 reacted to Mcg 8 and 17 and Syn 2H-11 reacted to Mcg 13 and 17 (Fig. 8).

Geometric mean titers from these studies ranged from 5 to 150. These numbers relate the various degrees of binding reactivity that the anti-TCR mAb supernatants demonstrated on the test peptides. The differences seen in the titer reactivities are related to antibody affinity for peptide. Relative binding affinities of mAb can not be accurately compared, however, because of the different concentrations of mAb expressed in the recovered supernatants.

With respect to the binding profiles observed on the TCR and Ig λ peptides, it might be likely OR2, OR5, and Syn 2H-11 are polyreactive antibodies. Similar ELISA studies were conducted with the anti-TCR mAbs on whole protein test antigens. These experiments also helped to determine if the IgM mAbs derived from RA patients were rheumatoid factors (RF). The anti-TCR mAbs were examined on fetuin, bovine serum albumin, ovalbumin, thyroglobulin, and pooled human polyclonal IgG. Reactivity to all test proteins by the anti-TCR mAbs was non-existent even at the most concentrated titers. The titer of the human RA plasma control to polyclonal human IgG at an OD of 0.5 carried out to 1 in 12,000.

Anti-TCR mAbs display diverse binding properties on TCR CDR1 spectratype peptides

The anti-TCR mAbs were tested on a set of V β homolog peptides corresponding to the V β CDR1 sequence (CDR1 homologs). In the set of CDR1 homologs the β 8.1 peptide is the designated nomenclature for the selecting β 3 peptide. The CDR1 peptides range from 33% (11.1) to 80% (6.5) in homology to β 3 (Table 7). OR1, defined tentatively as a monospecific antibody, bound to 10 of the 24 CDR1 homologs. Mean titers to peptides 10.1, 16.1, and 21.1 were the highest (between 50 and 100 geometric mean units) and suggest a greater binding affinity to these peptides (Fig. 9). Sequence examination of these peptides show that all contained a PIXXH motif where those that did not were unable to generate measurable titers. A notable exception is peptide β 6.1, which contains the PIXXH motif and is 69% homologous to β 8.1, but was not bound by OR1. Presumably this lack of binding is attributed to inhibition residues not expressed in the other peptides. Alternatively, some peptides (β 15.1 and β 19.1) lacking the PIXXH motif could still elicit a small, but measurable titer (Table 7). Although distinct patterns are apparent in the peptides that elicited a strong OR1 response, the definitive impact of other key residues not identified in these observations must be considered crucial for the degree of binding.

The reactivity of OR2 on the CDR1 homologs was the most extreme among the mAbs. It bound to 20 of the 24 CDR1 homologs in mean titer ranges from 4 to over 400

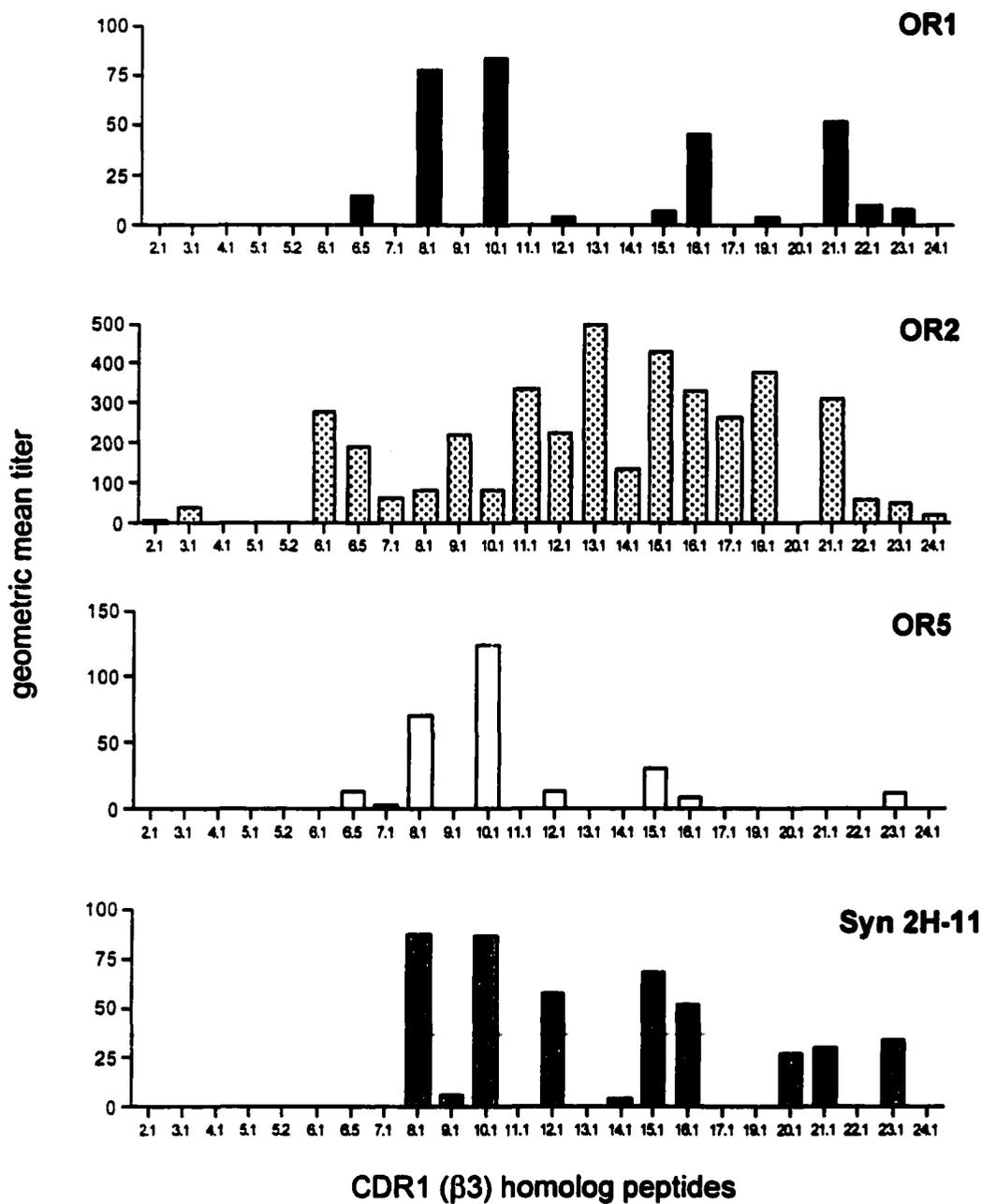


Figure 9: Anti-TCR mAbs binding to CDR1 (β 3) homologs. Binding reactivity, as indicated by geometric mean titer units on the y-axis of each bar plot, was measured on the individual CDR1 peptides listed on the x-axis. OR1)  OR2)  OR5)  Syn 2H-11)  .

Table 7. V β 8.1 CDR1 (β 3) homologs.

	CDR1 (β 3) homologs ^{f,g}														
β 2.1	R	S	L	D	F	Q	A	T	T	M			G	F	
β 3.1	V	Q	D	M	D		E	N	-	M				D	
β 4.1	Q	V	D	Q	V	T	M	-	M					Q	
β 5.1	S	R	S	D	L			-	V	Y			Q	S	
β 5.2	S	K	S	D	T			-	V	S			Q	A	
β 6.1	D			S	T	A		-		Y				S	
β 6.5	D			E		R		-		Y					
β 7.1	E	Q	H	M		R	A	-	M	Y			K	A	
β 8.1 ^h	K							-							
β 9.1	E	Q	N	L		D	T	-	M	Y			K	D	
β 10.1	V		K	A		S	Y	-	V	Y			K	K	
β 11.1	S	Q	T	M	G	D	K	-	M	Y			Q	D	
β 12.1	H	Q	T	E	N	R	Y	-	M	Y				D	
β 13.1	A	Q	D	M	N	E	Y	-	M	Y				D	
β 14.1	S	Q	N	M	N	E	Y	-	M	S				D	
β 15.1	S	Q	T	K		D	R	-	M	Y				D	
β 16.1	D			S	D	H		-		Y			R	V	
β 17.1	E	Q	N	L	N	D	A	-	M	Y				D	
β 19.1	T	E	K			T	F	-	V	Y			Q	N	
β 20.1	T	V	E	G	T	S	P	N		Y				A	
β 21.1	D					A	T	-		Y				I	
β 22.1	V					S	H	-	V	Y				L	
β 23.1	Y		P	R		D	T	-	V	Y			Q	G	
β 24.1	S	Q	T	L	N		V	-	M	Y			Q	K	

^f Classification for the V β nomenclature is referenced from Arden, *et al.* (57).^g Shaded letters designate amino acids that share sequence homology with the β 8.1 peptide.^h Peptide β 8.1 is the nomenclature for the β 3 peptide in the CDR1 peptide set.

units. OR2 bound to 13 of the peptides with a greater titer measurement ($\beta 6.1$, $\beta 6.5$, $\beta 9.1$, $\beta 10.1$, $\beta 11.1$, $\beta 12.1$, $\beta 13.1$, $\beta 14.1$, $\beta 15.1$, $\beta 16.1$, $\beta 17.1$, $\beta 19.1$, and $\beta 21.1$) than the selecting $\beta 8.1$ peptide (Fig. 9). The diverse binding profile of this antibody on the CDR1 homologs may be enhanced by its epitope promiscuous nature. Because of this unpredictable property it is impossible to distinguish where cross-reactivity and heteroclitic reactivity overlap. Key residues are not present in finite sequence motifs, but rather interchange such that specific binding interactions are not disrupted.

The mAbs OR5 and Syn 2H-11 demonstrated a range of binding reactivities that were very similar to that of OR1. OR5 bound similarly to $\beta 6.5$, $\beta 10.1$, $\beta 12.1$, $\beta 15.1$, $\beta 16.1$, and $\beta 23.1$ with the highest mean titer for $\beta 10.1$. Syn 2H-11 also bound to $\beta 10.1$, $\beta 12.1$, $\beta 15.1$, $\beta 16.1$, and $\beta 23.1$ with the highest mean titer for $\beta 10.1$, but also notably high (greater than 50 mean titer units) for $\beta 12.1$, $\beta 15.1$, and $\beta 16.1$. In addition, Syn 2H-11 bound to $\beta 9.1$, $\beta 14.1$, $\beta 20.1$, and $\beta 21.1$ (Fig. 9). Again the motifs of the peptides recognized by OR5 and Syn 2H-11 share the common PIXXH sequence implying that these peptides may be more permissive to binding by the different anti-TCR mAbs despite variable sequences in the heavy and light chain antigen combining site.

Examining binding reactivity of epitope promiscuous OR2 on TCR FR3 spectratype peptides

The anti-TCR mAbs were also tested on a set of peptides homologous to $\beta 8$, which corresponds to the FR3 region of the human V β TCR. The 5 peptides examined ranged from 25% to 62.5% in sequence homology to $\beta 8$ (Table 8). The results from these experiments revealed that only OR2 was capable of binding to the FR3 homologs. Examination of the titers indicated that the strongest binding interactions occurred on $\beta 5.2$ and $\beta 17.1$, which were close to 400 mean titer units. The sequence comparisons between these two peptides bear little resemblance except for the **AXYXCA** in the amino terminal end corresponding to the $\beta 8$ peptide. The titer data suggest, however, that this motif has little impact on the binding interaction between OR2 and the FR3 homologs, given the relatively low mean titers generated against the other peptides (Fig. 10).

Non-related peptides share the same binding site in epitope promiscuous anti-TCR mAb

OR2

In the studies on the property of epitope recognition promiscuity we focused on mAb OR2 due to its diverse binding profile on the TCR and Ig λ peptides. As mentioned previously, the anti-TCR mAbs were selected on the recombinant single chain T cell

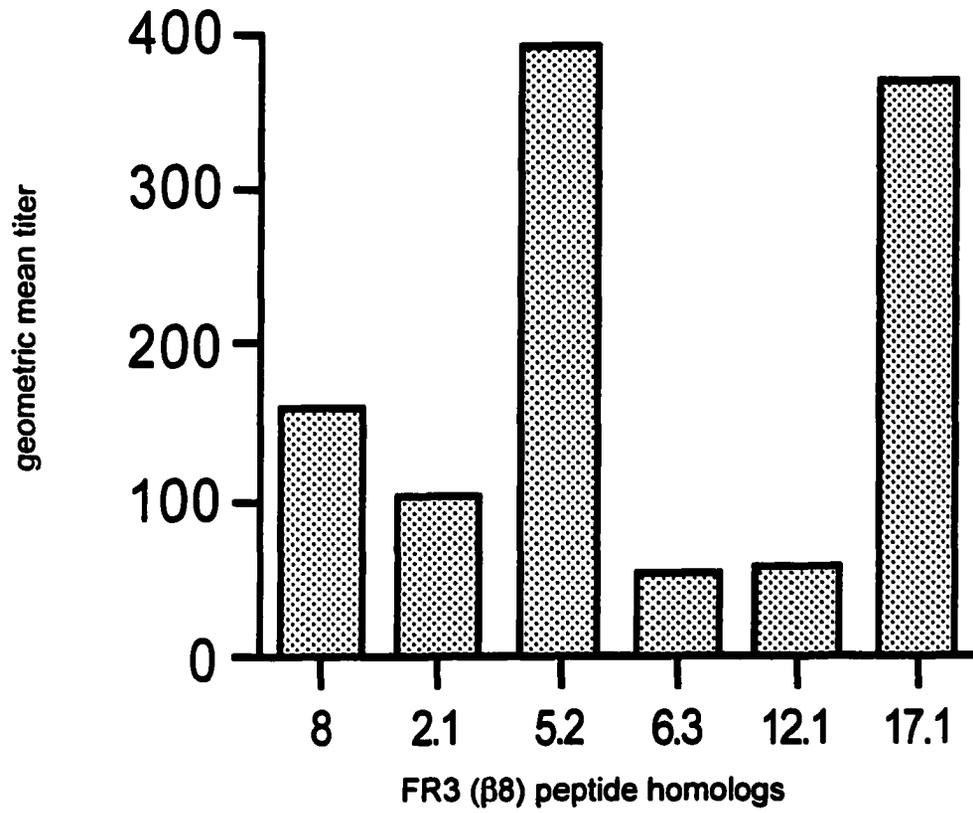


Figure 10: OR2 anti-TCR mAbs binding to the FR3 (β8) homologs. Binding reactivity, as indicated by geometric mean titer units on the y-axis of each bar plot, was measured on the individual FR3 peptides listed on the x-axis.

receptor (scTCR) and the $\beta 3$ peptide. The following experiments examined the nature of the OR2 binding site by using selected TCR antigens to inhibit its binding in competition ELISAs. The constant antibody binding site concentration in these assays was estimated at 2.0×10^{-8} M. The first set of experiments, illustrated in Figure 11, show that OR2 binds to these antigens in a specific manner. The $\beta 3$ peptide and the scTCR interact with the OR2 combining site to the effect that increasing the antigen concentration can inhibit antibody binding. In Figures 11b and c it was examined if inhibitions with soluble antigens could be carried out against the same coating antigens. In estimation from these experiments a 0.34×10^{-6} M concentration of scTCR and an 8.5×10^{-6} M concentration of $\beta 3$ were sufficient to block OR2 binding by 50%. In repeated experiments increasing molar amounts of the $\beta 1$ control peptide were unable to inhibit the level of binding of OR2 to immobilized $\beta 3$ (Fig. 11b). The scTCR and the $\beta 3$ peptide could also cross-inhibit against alternated immobilized antigens. An approximate scTCR concentration of 0.6×10^{-6} M was sufficient to block OR2 binding to $\beta 3$ coated plates by 50% (Fig. 11a) and 21.0×10^{-6} M of $\beta 3$ was sufficient to block OR2 binding to scTCR coated plates by 50% (Fig. 11d).

Among the TCR β peptides, OR2 reacted with the highest titer to $\beta 15$, indicating that the OR2 binding site has a greater specific affinity for $\beta 15$ than it does for the

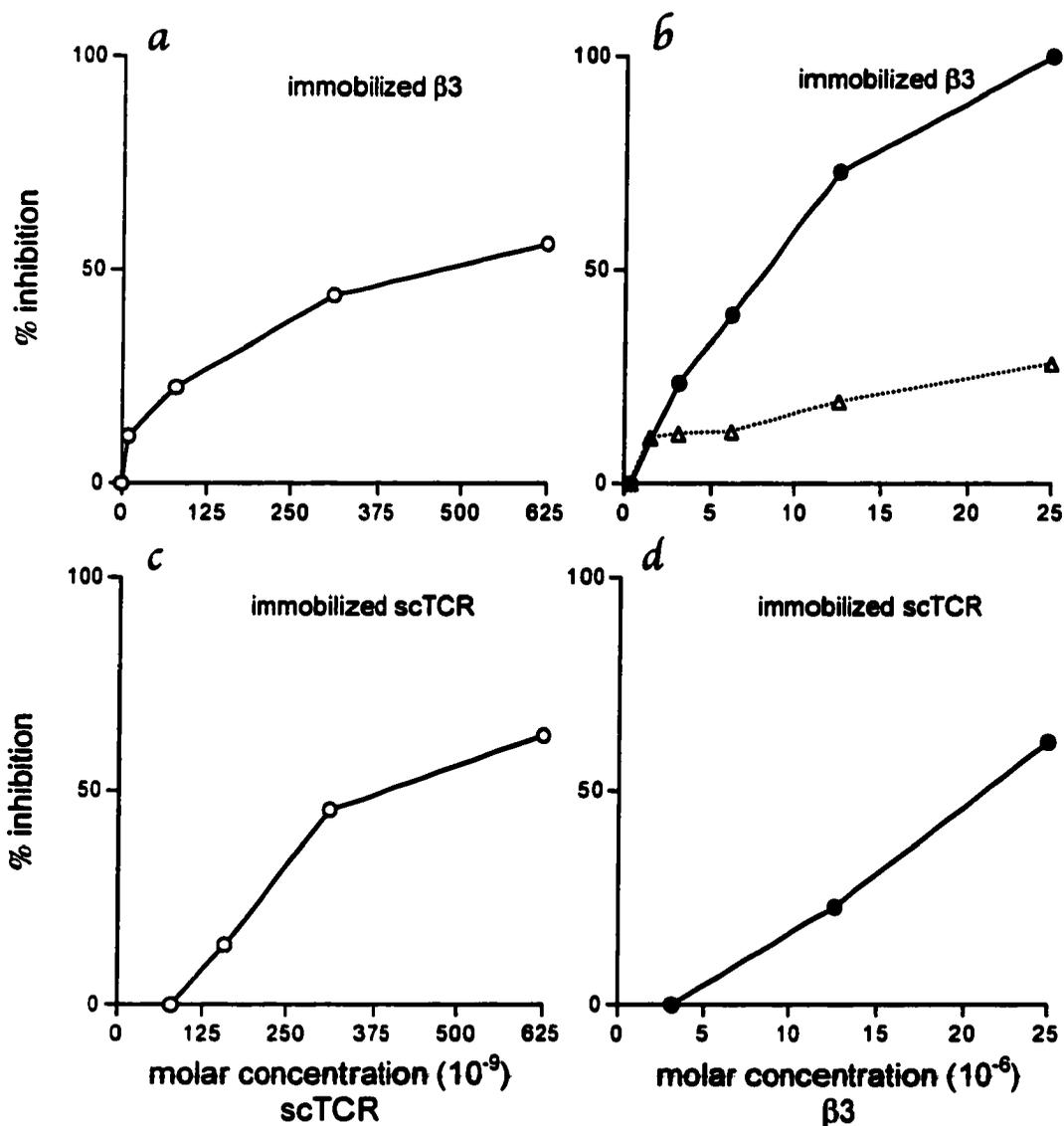


Figure 11: Inhibition of OR2 binding to immobilized $\beta 3$ peptide and scTCR in ELISA with soluble $\beta 3$ and scTCR. Increasing molar amounts of soluble antigen (y-axis) were mixed with an invariable antibody dilution (2.0×10^{-8} M). Concentration of immobilized peptide and scTCR was 2.5×10^{-6} M and 3.0×10^{-8} M. Inhibition of antibody binding to immobilized antigen on ELISA plate was measured as a percentage (x-axis). A) Soluble scTCR (O) on $\beta 3$ coated ELISA, B) soluble $\beta 3$ (●) and $\beta 1$ negative control peptide (Δ) on $\beta 3$ coated ELISA, C) soluble scTCR on scTCR coated ELISA D) and soluble $\beta 3$ on scTCR coated ELISA.

selecting peptide (Fig. 6). Similar inhibition experiments therefore, were conducted with the TCR β 15 peptide. The β 15 peptide however is completely unrelated to β 3 sequentially (Table 4). On β 15 coated ELISAs a concentration of approximately 0.3×10^{-6} M of scTCR was capable of inhibiting OR2 binding by 50%. A molar concentration of about 1.25×10^{-6} M of soluble β 15 was sufficient to accomplish the same level of OR2 inhibition on β 15 coated plates (Fig. 12a). The β 1 specificity control peptide was unable to inhibit OR2 binding to β 15 coated plates (Fig. 12b). The inhibition results for soluble β 15 on plates coated with β 3 and scTCR were similar. A molar concentration of 3.1×10^{-6} M of β 15 was required to attain 50% inhibition on β 3 coated plates and 1.0×10^{-6} M of β 15 could block 50% OR2 binding to scTCR coated ELISAs (Fig. 12c and d). In assays using soluble β 3 to inhibit OR2 binding to β 15 coated plates no OR2 inhibition could be measured. These results clearly demonstrate that the anti-TCR mAb OR2 binds to the cognate or selecting antigens (scTCR peptide and β 3) in a specific manner, and that the binding site is shared by these two antigens. Also, OR2 binds specifically to the non-cognate, non-selected antigen β 15. These data are representative of the overall results obtained from the inhibition studies.

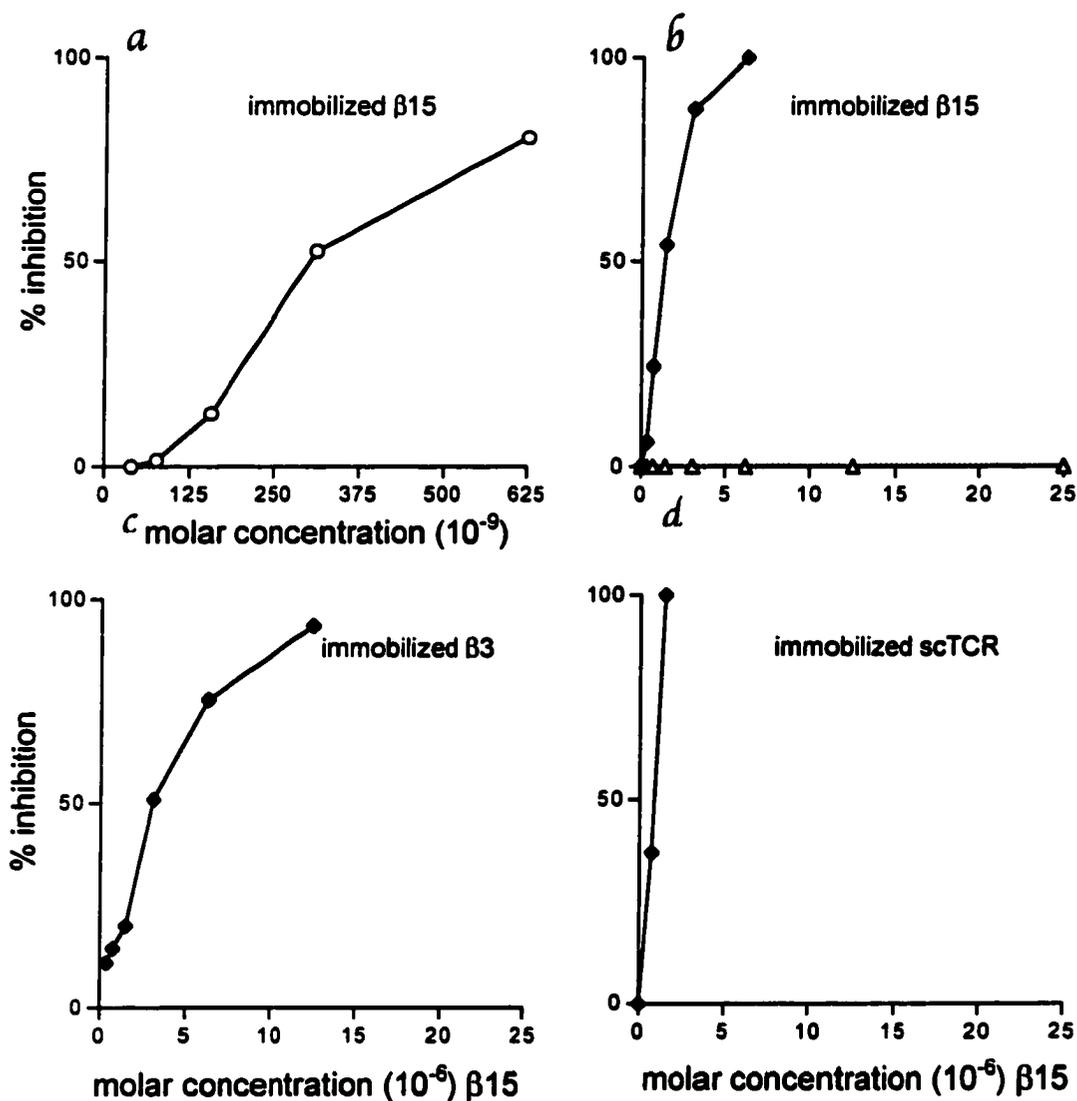


Figure 12: Inhibition of OR2 binding to immobilized $\beta 15$ peptide and scTCR in ELISA with soluble $\beta 15$ and scTCR. Increasing molar amounts of soluble antigen (y-axis) were mixed with an invariable antibody dilution (2.0×10^{-8} M). Concentration of immobilized peptide and scTCR was 2.5×10^{-6} M and 3.0×10^{-8} M. Inhibition of antibody binding to immobilized antigen on ELISA plate was measured as a percentage (x-axis). A) Soluble scTCR (O) on $\beta 15$ coated ELISA, B) soluble $\beta 15$ (\blacklozenge) and $\beta 1$ negative control peptide (\triangle) on $\beta 15$ coated ELISA, C) soluble $\beta 15$ on $\beta 3$ coated ELISA, and D) soluble $\beta 15$ on scTCR coated ELISA.

Determination of the dissociation constant of OR2 on unrelated TCR peptides and scTCR

The inhibition studies carried out with OR2 give an indication of the affinity this mAb has for specific TCR epitopes, which can be roughly determined in several ways. If a specific molar concentration of soluble antigen can block the binding of OR2 to an antigen coated well to the degree that the optical density units are 1/2 the absorbance of an equivalent OR2 concentration in control wells without soluble antigen, then it could be considered theoretically that 1/2 of the total OR2 binding sites occupied. This can be directly applied to the Scatchard equation (118):

$$r/c = 1/K_D(n-r)$$

where x is the total antibody binding site concentration (n) minus the free antibody site concentration (i). At 50% inhibition n is $2 \times r$. The amount of soluble antigen is designated as c . Given that $i_0 - r = r$, then a working dissociation constant (K_D) (based on the assumption that 50% of the binding sites of OR2 are occupied) can be determined by the value of c . Table 9 presents the approximate molar values of soluble antigen required to inhibit OR2 binding to immobilized antigen in ELISA by 50%. The working K_D

Table 9. Estimated molar concentrations of soluble antigen required to inhibit OR2 binding immobilized antigen by 50%.

		Soluble antigen		
		$\beta 3$	$\beta 15$	scTCR
immobilized antigen	$\beta 3$	$8.5 \times 10^{-6} \text{ M}$	$3.1 \times 10^{-6} \text{ M}$	$0.6 \times 10^{-6} \text{ M}$
	$\beta 15$	no inhibition	$1.3 \times 10^{-6} \text{ M}$	$0.3 \times 10^{-6} \text{ M}$
	scTCR	$21.0 \times 10^{-6} \text{ M}$	$1.0 \times 10^{-6} \text{ M}$	$0.34 \times 10^{-6} \text{ M}$

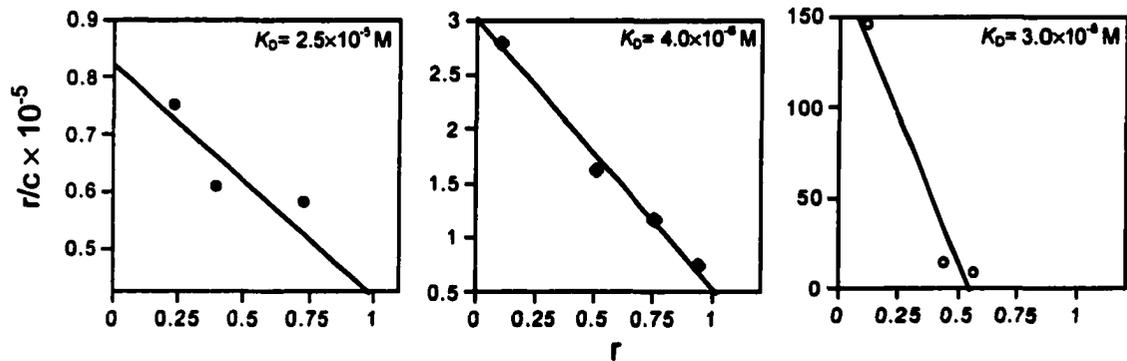


Figure 13: Scatchard plots of the binding of inhibiting soluble antigens $\beta 3$, $\beta 15$, and scTCR to OR2 on $\beta 3$ coated ELISA. Data from the inhibition experiments was used to K_D values for OR2 binding to $\beta 3$ (●), $\beta 15$ (◆), and scTCR (○) (119).

values of OR2 for the TCR antigens vary slightly depending on coating antigen, but the differences are not significant (or cannot be determined). OR2 appears to have a very weak affinity for the $\beta 3$ peptide, but has about a 20-fold greater affinity for the $\beta 15$ peptide and a 70-fold greater affinity for scTCR if determining the working K_D from inhibitions conducted on scTCR (cognate antigen) coated ELISAs. Although $\beta 3$ peptide is a specific epitope for OR2 it does not appear to be the cognate epitope based on relative affinities.

Inhibition ELISAs have been used to determine affinity constants of antibodies in previous studies. The results were comparable with those obtained from fluorescence transfer and immunoprecipitation experiments. The values of free versus bound antibody sites were translated from comparing absorbance measurements of antibody with soluble antigen to those of antibody without inhibiting antigen (119). This system could be applied to the inhibition studies carried out in these experiments. Scatchard plots were generated from inhibition experiments conducted with soluble $\beta 3$, $\beta 15$, and scTCR on $\beta 3$ coated ELISAs (Fig. 13). Relative dissociation constants for OR2 were comparable to the working K_D values obtained for 50% binding in that the plots demonstrate the lowest antibody affinity for $\beta 3$, followed by $\beta 15$, and the highest affinity for scTCR.

SECTION II

Chapter 6 – Discussion

These studies confirm that epitope recognition promiscuity is a property of some natural antibodies and as seen from the results, can manifest in various degrees in monoclonal natural antibodies. Epitope promiscuity can be distinguished by several features: 1) reactivity to one or more epitopes that are unrelated to the cognate antigen, 2) non-reactive to proteins that often serve as antigens for antibodies defined as polyreactive, and 3) antigens compete for the same binding site, but affinities can vary. The four mAbs examined in these studies were selected for on the same TCR antigens, but all possess unique V region sequences particularly in the CDR3 of the heavy chain (Fig 5). This characteristic is the proposed driving force behind the property of epitope recognition promiscuity. Reactivity against the overlapping TCR β , α , and Mcg λ peptides was displayed in very unique patterns ranging from monospecific binding (OR1), to restricted epitope promiscuous binding (OR5 and Syn 2H-11), to highly epitope promiscuous binding (OR2).

In the discussion we examine the differences between OR1 and OR2. These mAbs differ somewhat from affinity purified natural IgG molecules isolated by binding to peptide β 3 or scTCR. Although these likewise bind to β 3, the other major peptide epitopes recognized are peptide β 8 from the FR3 of the V region and peptide β 17 from the C β domain (34). These are loop structures that are spatially contiguous in the 3-dimensional models, suggesting the capacity of the antibodies to perceive conformational determinants with residues contributed by portions of the intact proteins distant from one another in linear sequence.

OR1 reacted in a classical sense to the 'cognate' antigen used in the selection procedure by binding in a restricted fashion. OR1 also bound to the α 3 and α 4 peptides, but not to the homologous region of the Mcg λ chain. The property of epitope promiscuity must be challenged in this scenario due to the existence of shared sequence between the β 3 (WYRQT) and the α 3 and α 4 peptides (WYVQY). This conjecture is supported in the molecular models of the complete TCR α and TCR β chain Figure 14. The regions highlighted in yellow represent the peptide sequences to which OR1 reacted. The structural motif of the V α / β TCR to which OR1 binds spans from the first N-terminal β strand through the exposed loop representing the CDR1 back through the second β strand and part of the next α helix turn representing part of the FR2. It is more conceivable, based

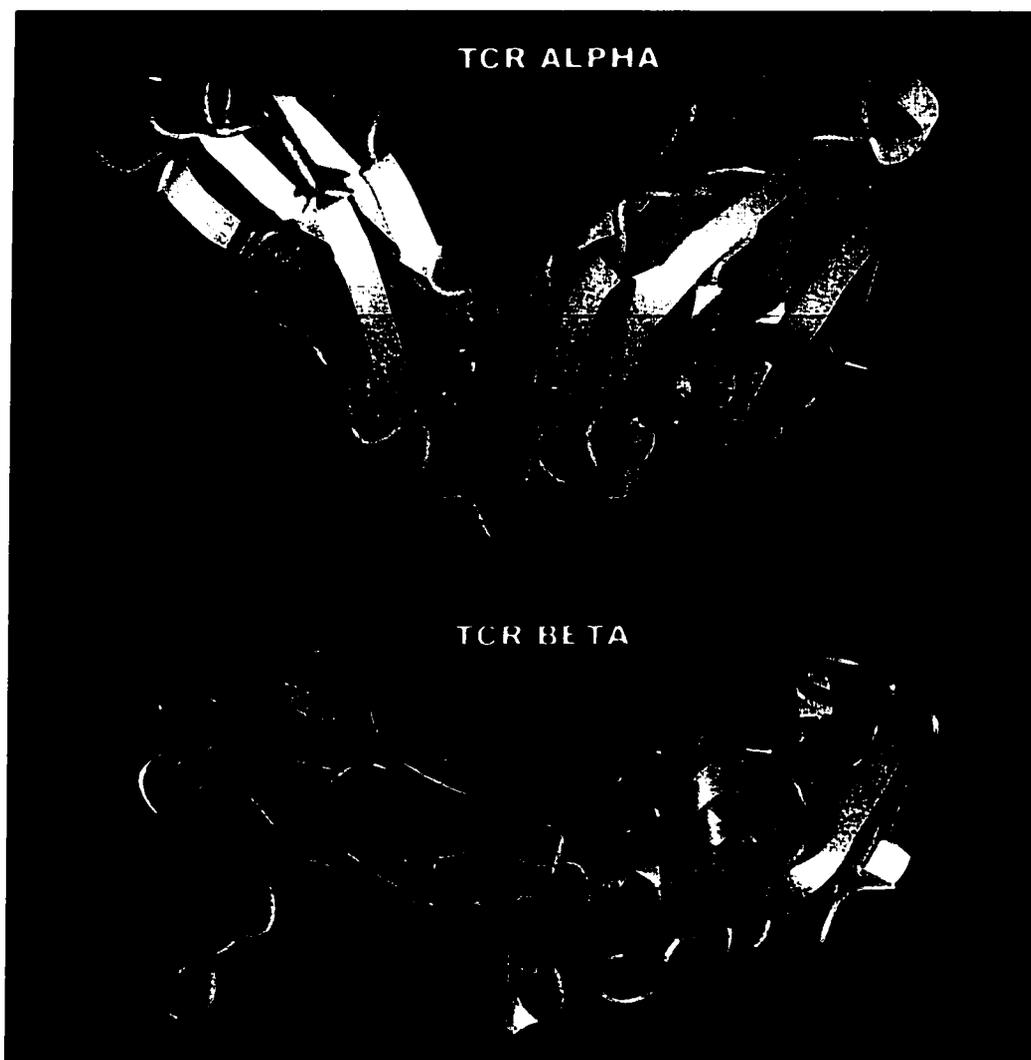


Figure 14: Molecular models of the complete TCR α and β chains with highlighted regions of OR1 binding. Segments on the three-dimensional structures corresponding to 16-mer peptides bound to OR1 antibody. Atomic coordinates are taken from the Protein Data Bank (PDB) for examples of a TCR α chain (PDB#1ao7) and a TCR β chain (PDB#1bec). Ribbon models are drawn with the program MOLMOL (122). Secondary structures are represented by directional arrows (β -strands) or spirals (helices). Putative epitopes representing peptides bound by OR1 are highlighted in yellow. They correspond to complete CDR1 and N-terminal parts of the contiguous FR2 segments.

on the sequence homologies and the models, that the binding interaction of OR1 to the α peptides is a property of cross-reactivity. OR1 possesses a sequence motif (RFLEW) in its heavy chain CDR3 (Fig. 4 and 5) that has been described in 4 of 50 known polyreactive antibodies and none of the 2,500 human antibodies believed to be monoreactive (20). These findings suggest that OR1 may exist as an exception to the current research and moreover represents a caveat in assessing binding reactivity according to sequence motifs.

OR2 represents the most extreme example of epitope recognition promiscuity in these studies. This mAb reacted to non-related TCR peptides often with what appeared to be comparable affinities (based on direct binding studies). Figure 15 shows the same TCR α and β model and a model for the complete Mcg λ chain with highlighted regions of OR2 binding. The structure of Mcg was determined crystallographically (120) and was used previously as a template to model the structure of the YT35 β chain (121). OR2 reacted to two conspicuous regions in the variable portions of all the structures highlighted in yellow and cyan. These regions correspond to the CDR1 and part of the FR2 (yellow) and the C-terminal end of the FR3 to the CDR3 (cyan). Additionally, OR2 reacted to the J region (FR4) of the Mcg λ chain highlighted in royal blue. The major constant region structures to which OR2 bound are highlighted in violet, green, and orange. OR2 bound to the various unrelated secondary structures in the constant regions of the models. This

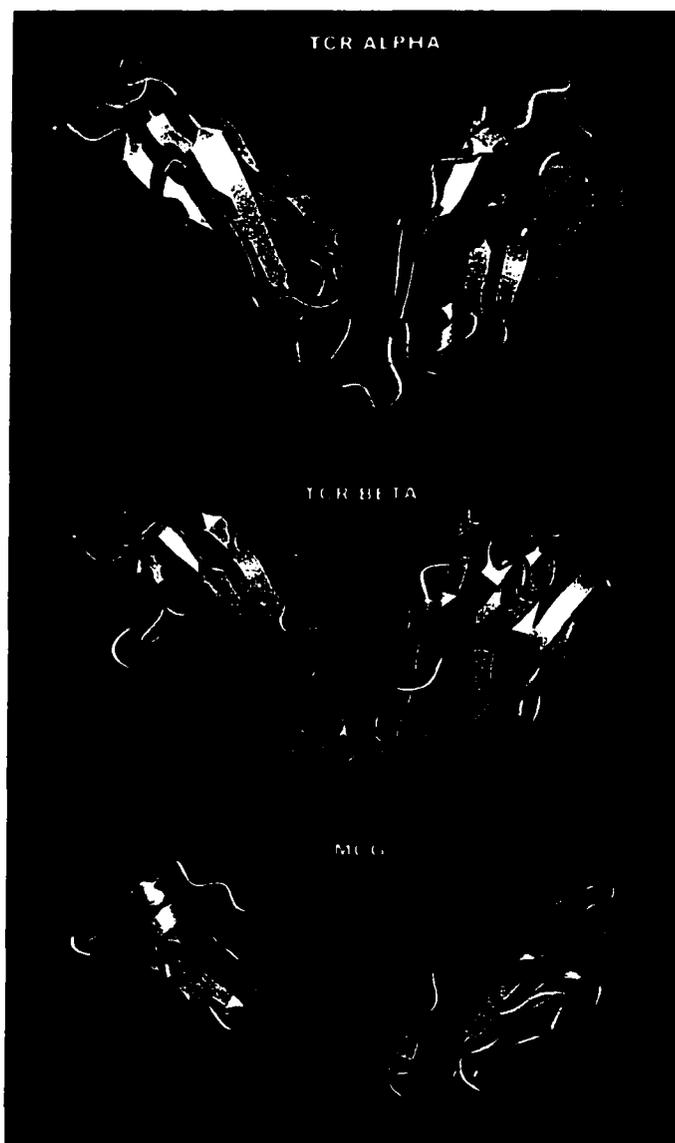


Figure 15: Molecular models of the complete TCR α and β chains, and the complete Mcg λ chain with highlighted regions of OR2 binding. Segments on the three-dimensional structures corresponding to 16-mer peptides bound to OR2 antibody. Atomic coordinates are taken from the PDB for examples of a TCR α chain, a TCR β , and the human Ig λ chain (Mcg, PDB#2 Mcg). Putative epitopes representing peptides bound by OR2 are highlighted in yellow, cyan, royal blue, violet, green, and dark orange. They correspond to the complete CDR1 and N-terminal and parts of the contiguous FR2 region, the C-terminal end of the FR3 to the CDR3, the J region (FR4) of the Mcg λ chain, and to constant region portions of the TCR α and β and Mcg λ chains.

observation is supported by the lack of recognizable homology between the TCR α , TCR β , and Ig λ peptide sequences corresponding to the different constant regions.

In summary, OR2 binds to several different regions of the constant and variable TCR and Mcg light chain, and some of the regions to which it binds these structures are structurally similar. These are mainly the CDR1/FR2 and FR3/CDR3 regions of the TCR Ig light chains. Examination of the peptides corresponding to these regions reveals shared sequence motifs (Tables 4, 5, and 6). This pattern can also be seen in the geometric mean titer plots (Fig. 6, 7, and 8). The distinction between cross-reactivity and epitope promiscuity is blurred for OR2 binding to these regions, but becomes obvious when examining OR2 binding to the TCR α , β , and λ chain constant regions. This is even more evident when examining unrelated peptides that correspond to different structural components of the individual TCR and Ig light chain models.

The idea that the heavy chain CDR3 region is a key factor driving these unusual binding interactions is partly evidenced by the fact that OR2, an epitope promiscuous mAb, shares the same light chain with OR5. The OR1 light chain is only different from the OR2 and OR5 light chain by one amino acid difference in the CDR3 (Fig. 4). The dynamics of epitope promiscuous binding such as that seen with OR2 on the TCR α , β , and Mcg λ chain peptides is inexplicable without crystallographic evidence.

Experiments investigating the binding of the anti-TCR mAbs on the CDR1 (β 3) and FR3 (β 8) homologs expand on the epitope mapping studies. These data provide two important details about the anti-TCR mAbs used in these experiments and the nature of epitope recognition promiscuity. First, they support the results from *Section 1* that binding to human CD3⁺ PBMCs occurs only on restricted V β subsets (binding to V α subsets is unknown). Even the epitope promiscuous mAb OR2 did not bind to every CDR1 homolog (Fig. 9). Secondly, the degree of epitope promiscuity may have a profound effect on the level of cross-reactivity an antibody can demonstrate against homologous epitopes. It is conceivable that these properties can synergize to enhance the binding reactivity such as what was observed with OR2. Not only does OR2 react to at least a minimal degree with approximately 80% of the CDR1 peptides, it is also the only anti-TCR mAb from these studies that reacts with the panel of FR3 homologs (Fig. 10). This does not necessarily mean that the degree of cross-reactivity is the driving determinant of epitope promiscuity. If that were the case we would expect to see a greater amount of binding reactivity to the CDR1 peptides by OR5, a mAb believed to be epitope promiscuous. Alternatively, epitope promiscuity could not be the driving determinant of cross-reactivity. This is evidenced by the CDR1 homolog binding results seen with monoreactive OR1 (Fig. 9).

OR2 became the focus in these preliminary investigations to characterize the nature of epitope recognition promiscuity. Clearly OR2 best epitomizes this type of molecular dynamic. In competition ELISA studies OR2 bound to the epitopes on which it was selected ($\beta 3$ and scTCR) as well as an unrelated epitope ($\beta 15$) in a specific manner. From the analysis of these inhibition studies it is believed that all three of the antigens compete for the same binding site in OR2. Interestingly, the inhibition studies reveal that OR2 appears to have a very low affinity for the selecting $\beta 3$ peptide. Considerably larger molar concentrations of $\beta 3$ were required to block OR2 binding to coated ELISAs. These findings suggest that the binding to peptide $\beta 3$ used in the selection represents a cross-reaction, and that the major specificity of OR2 is directed towards a different epitope. In contrast to the inhibitions using $\beta 3$, the 3-domain constant and variable scTCR could inhibit OR2 binding to antigen coated wells at far smaller molar concentrations than the $\beta 3$ and $\beta 15$ peptides (Fig. 11, 12, and Table 9). The sequence for $\beta 3$ is contained in the scTCR sequence, but it appears that OR2 reacts against another epitope(s) with greater affinity. Most likely it shows preference for conformational determinants of the intact construct. OR2 is also inhibited at lower concentrations of $\beta 15$ than $\beta 3$. The two peptides however are unrelated sequentially and biochemically. The estimated pI for $\beta 3$ is 9.96 while the estimated pI for $\beta 15$ is 5.18. The $\beta 3$ peptide contains 31.25% non-polar residues,

50% polar residues and 18.75% basic residues, while the $\beta 15$ peptide contains 37.5% non-polar residues, 25% polar residues, 18.75% acidic residues, and 18.75% basic residues (Table 4).

We estimated the dissociation constant (K_D) for OR2 on the $\beta 3$ peptide, $\beta 15$ peptide, and the scTCR using the data from the inhibition studies. This method has been used in previous studies with monoclonal IgG molecules (119). The results obtained from this study appear to be representative of what was expected. OR2 demonstrated a very low binding affinity K_D for its selecting antigen the $\beta 3$ peptide ($\sim 2.5 \times 10^{-5}$ M). The affinity of OR2 for $\beta 15$ ($\sim 4.0 \times 10^{-6}$ M) was significantly higher, but still considered to be a relatively low antibody binding dissociation constant. OR2 demonstrated an affinity for the scTCR ($\sim 3.0 \times 10^{-8}$ M) that is considered to be relatively high for antibody binding. To obtain these values the method for K_D calculation could only be applied to OR2 competition assays performed on $\beta 3$ coated ELISAs. These results could not be sufficiently derived from any of the other competition assays. This is most likely due to the fact that OR2 is a multivalent molecule and demonstrates a low affinity for its antigens compared with the types of K_D values found with IgG molecules. Generally, the difficulty in obtaining accurate K_D values lies in the fact that the change in free antibody concentration declines at a greater order of magnitude with increasing amount of soluble antigen. Additionally the

molar concentration of antigen is much larger than the number of antibody sites to affect the free ligand value. The Scatchard plots as a result have positive slopes.

These studies make the distinction that the degree of identity among epitopes is sometimes indicative of the degree of binding by the antibody and in other interactions there is no definitive relationship. This complex property of epitope recognition promiscuity introduces a new perspective on epitope binding interactions for TCRs as well as Igs. It is apparent therefore that new algorithms are required to successfully predict the binding relationship that would occur between an idiotope and its epitope(s), even in the well controlled case of synthetic peptides and mimetopes. Epitope recognition promiscuity is a property of antibodies found in all species of vertebrates from sharks to humans and is recognized as a conserved feature of the combinatorial immune system including TCRs and Igs (26). The variation in Ig gene products ranging from monospecific to polyreactive is not considered as a random recombinatorial phenomenon. Although the antibodies from these studies may possess low affinity for their respective epitopes, the binding interactions they carry out are still specific. Low affinity antibodies are important because they can discriminate among antigens better than high affinity antibodies (107). Moreover, binding specificities for different epitopes by antibodies with different V region sequences on the

same molecular structures represent further attestation to the overlapping functionality of the immune system.

SECTION III: Human monoclonal natural autoantibodies against the T cell receptor inhibit IL-2 production in murine T cells

Chapter 7 – Introduction

Natural antibodies represent any type of immunoglobulin present in the serum of an individual in the absence of purposeful immunization or infection. These molecules are typically IgM Igs of which the variable sequences may exhibit diversity in the region where recombination between the variable (V), diversity (D), and joining (J) chains occurs, but they are not known to undergo affinity maturation for antigen. Thus, natural antibodies are Igs with V region sequences in germline configuration (1, 123, 124).

Natural antibodies generally bind antigen with low affinities, but this is not the rule as notable exceptions have been observed (3). Another characteristic exhibited by natural antibodies is the ability to bind non-related epitopes in a specific manner. This is defined as epitope recognition promiscuity. Epitope promiscuity, as opposed to polyreactivity, describes a specific and measurable binding to more than one epitope, but negative reactivity with usual test proteins including thyroglobulin, ovalbumin, and bovine serum albumin (25).

Since their discovery, the role of natural antibodies has been unclear. The existence of antibodies against toxins and bacterial determinants described in unimmunized animals, however, implies these molecules may act as components of the innate immune system (32, 125, 126). Evidence for this has been effectively demonstrated in comparison studies between animals with and without circulating natural antibodies. It was determined that these molecules play an essential role in impeding the spread of various pathogens during primary infections (33, 127). In addition to antigen-specific properties, other possible roles for natural antibodies include antigen processing and presentation through B cell Fc receptors (125, 126), clearance of lipopolysaccharides (128), and immunoregulation (1, 3, 108).

Reactivity with self-antigens is a common feature of many natural antibodies. Natural autoantibodies occur in healthy individuals as well as patients with autoimmune disease and react with a wide range of evolutionarily conserved cell surface, intracellular, and circulating antigens (3). These determinants were originally thought to be apart of the spectrum of antigens to which an individual should be tolerant. 'Tolerance', however, implies that these kinds of antibodies should not be apart of a healthy functioning immune system, whereas it appears more likely natural autoantibodies represent a group of molecules that have been overlooked as important immuno-

regulatory elements. This is evidenced in findings of germline sequences from the fetus coding for self-reactive antibodies, which suggests evolutionary selection of natural autoantibodies. The neonatal B cell repertoire is selected for recognition of self during the fetal period, resulting in serum concentrations of natural autoantibodies of which external antigens could have little or no influence upon during immunological development (129). Furthermore, the repertoire of natural autoantibodies in healthy individuals is observed to remain conserved throughout the lifetime of each individual (3).

Suggested functions for natural autoantibodies include removal of metabolic waste and senescent cells, clearance of soluble immune complexes, anti-tumoral surveillance, anti-inflammatory activity, and control of autoreactivity and immune homeostasis (3). Antibody functions would be dependent, of course, on immunoglobulin isotype, the specific antigen(s), and epitope binding affinity. The TCR represents one such self-antigen targeted by natural autoantibodies. Previous findings document serum activity specific for the CDR1 public idiotope of the α/β TCR β chain in all individuals tested (34, 43, 62). A prevalence of anti-TCR antibodies, however, has been reported in such conditions as aging (38, 63), pregnancy (39), allograft transplantation (40, 130), retroviral infections (41, 42), and autoimmune diseases (34, 38, 43, 63).

It has been proposed that natural autoantibodies against the TCR may serve in immunoregulation of T cells (64). This theory seems plausible given the nature of the specificity of these molecules and the understanding that antibodies play a critical role in neutralizing antigens through the interaction of the antibody combining site with specific antigen. To test this premise, monoclonal natural autoantibodies against the TCR are necessary to perform the requisite assays for determining their functional capacities. We have proposed, therefore, to study this question by generating B cell hybridomas from human patients with the capacity to produce autoantibodies specific for the CDR1 determinant of the α/β TCR.

We generated seven hetero-hybridomas expressing anti-TCR antibodies from two patients with RA. Hybridomas were produced from peripheral blood lymphocytes of one patient and from synovium tissue lymphocytes of a second patient. The antibodies were IgM, almost completely in germline sequences except for the heavy chain CDR3 region, which were unique due to extensive N-region diversity. Although these mAbs were unrelated in sequence, they were positive in ELISA for binding to a recombinant single chain JURKAT TCR construct and a 16 amino acid peptide corresponding to the entire CDR1 and part of the FR2 of the V β 8.1 TCR sequence. The anti-TCR mAbs also bind to JURKAT T cells and to subsets of CD3⁺ human PBMCs in flow cytometry

experiments (Fig. 2, 3, and 4). Further analysis of some of these mAbs, OR2, OR5, and Syn 2H-11, revealed that they are capable of binding to murine T cells and the T cell clone DO-11.10 by flow cytometry, and have distinct and varied binding profiles against peptides corresponding to the mouse V β TCR CDR1 region. These data were auspicious for conducting subsequent *in vitro* experiments. We report evidence that the anti-TCR mAbs tested may serve an immunomodulatory role since they inhibit production of IL-2 by antigen activated DO-11.10 murine T cells. These findings, however, do not support evidence that these anti-TCR mAbs are capable of inducing apoptosis through cross-linking TCRs on the T cell surface.

SECTION III

Chapter 8 – Results

Anti-TCR mAbs bind to purified mouse T cells

Previously, we reported positive binding to the JURKAT human T cell line and CD3⁺ PBMCs by flow cytometry with several of the anti-TCR mAbs used in the current studies (Fig. 2 and 3). In view of those findings and the significant homology between human and mouse TCRs, we sought to test by flow cytometry the anti-TCR mAbs for binding to murine T cells enriched from a splenocyte preparation. These cells were also examined for normal T cell markers such as CD4, CD8, CD3, and CD95 (Fas). Overall, the T cells from this preparation exhibited normal levels of CD4 (40%) and CD8 (30%) (Fig. 16b), CD3 (80%) (Fig. 16c), and Fas (75%) (Fig. 16d).

We tested the anti-TCR mAbs OR2, OR5, and Syn 2H-11 on these cells with a PE labeled anti-human IgM conjugate and FITC labeled antibodies to mouse CD4 and CD8. About 8% of the cells were bound by OR2 divided between CD4⁺ and CD8⁺ T cells.

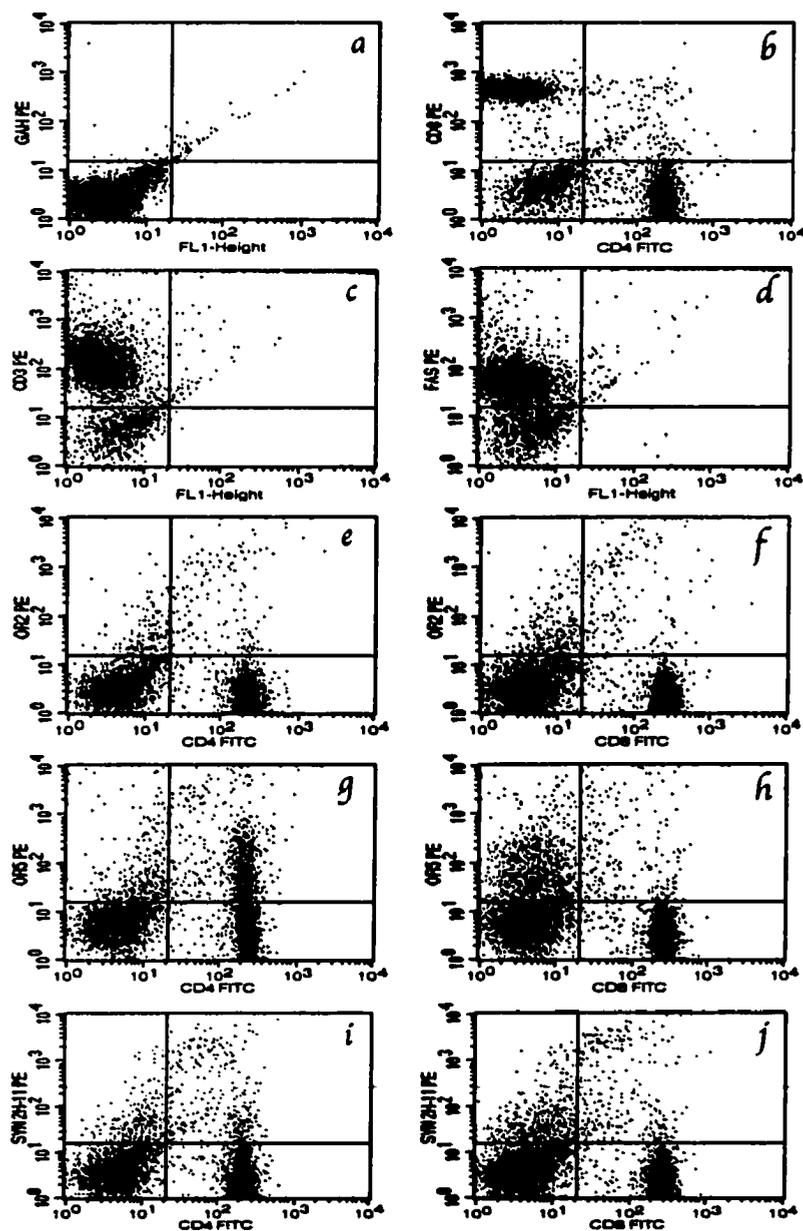


Figure 16: Binding of anti-human TCR monoclonal antibody to purified naive mouse T cells in flow cytometric analysis. Cells were either single stained with PE (y-axis) conjugated antibodies or double stained with FITC (x-axis) and PE conjugated antibodies on various T cell markers. Cells were labeled with the following antibodies: A) Purified IgM isotype control antibody with a goat anti-human (GAH) PE conjugate. B) Double label with anti-mouse CD4 FITC and anti-mouse CD8 PE. C) Single label with anti-mouse CD3 PE. D) Single label with anti-mouse Fas PE. E) Double label with anti-mouse CD4 FITC and OR2 anti-human TCR with GAH PE conjugate. F) Double label with anti-mouse CD8 FITC and OR2 anti-human TCR with GAH PE conjugate. G) Double label with anti-mouse CD4 FITC and OR5 anti-human TCR with GAH PE conjugate. H) Double label with anti-mouse CD8 FITC and OR5 anti-human TCR with GAH PE conjugate. I) Double label with anti-mouse CD4 FITC and Syn 2H-11 anti-human TCR with GAH PE conjugate. J) Double label with anti-mouse CD8 FITC and Syn 2H-11 anti-human TCR with GAH PE conjugate.

Fluorescence intensities ranged from 20^1 to 10^4 (Fig. 16e and f). OR5 bound to 28% of mouse T cells with approximately 20% being CD4⁺ (Fig. 16g). The brightness level for OR5⁺ CD4⁺ cells was moderate ranging from 20^1 to 10^3 . OR5 appeared at most to bind about 4% of CD8⁺ T cells (Fig. 16h). Syn 2H-11 exhibited a similar binding pattern to mouse T cells as OR2, but to a greater percentage of cells. Approximately 15% of the mouse T cells were bound by Syn 2H-11. There were three distinct populations of Syn 2H-11 positive cells; a double positive bright population ($\geq 10^3$) which was 1% of CD4⁺ and CD8⁺ cells, a double positive dim population which was 2% of CD4⁺ and CD8⁺ cells, and a single positive dim population which was 5% of CD4 stained cells and 9% of CD8 stained cells.

Binding to mouse T cells by the anti-TCR mAbs was restricted to a sub-population of cells depending on which mAb was tested. This was evidenced when a comparison was made to the conjugate control (Fig. 16a) and the isotype control (not shown) where $\leq 1\%$ of the total cells fluoresced in the lower right and upper right quadrant. Each anti-TCR mAb OR2, OR5, and Syn 2H-11, reacted in a unique manner to human peptide epitopes. It is most likely that these human anti-TCR mAbs cross-react with specific V β and V α subsets of the murine TCR repertoire.

Anti-TCR mAbs demonstrate positive binding to mouse DO-11.10 T cells

The results of the anti-TCR mAbs binding to mouse T cells indicate that these molecules may function in murine models. Preliminary experiments were necessary to determine if these anti-TCR mAbs could bind to the TCR on DO-11.10 murine T cells. This cell line is useful for prospective *in vitro* functional assays because it is specific for a known antigen (58, 59). We tested OR2, OR5, and Syn 2H-11 on the DO-11.10 mouse T cell clone by flow cytometry. Figure 17 shows binding of OR2, OR5, and Syn 2H-11, to DO-10.11 cells accompanied with the results from a negative control stain with FITC-labeled goat anti-human (GAH) IgM F(ab')₂ and a positive control demonstrating binding to > 90% cells with an antibody against the mouse V β 8 TCR. The DO-11.10 cells examined for this study are represented within the region indicated in Figure 17a. These cells generally typify the population of large, round, healthy cells in log growth phase.

The anti-TCR mAb OR2 demonstrates binding to DO-11.10 cells in a bi-phasic histogram (Fig. 17d). OR2 appeared to bind at a low fluorescence intensity (20¹) to one population of DO-11.10 cells and a high fluorescence intensity (60³) to another

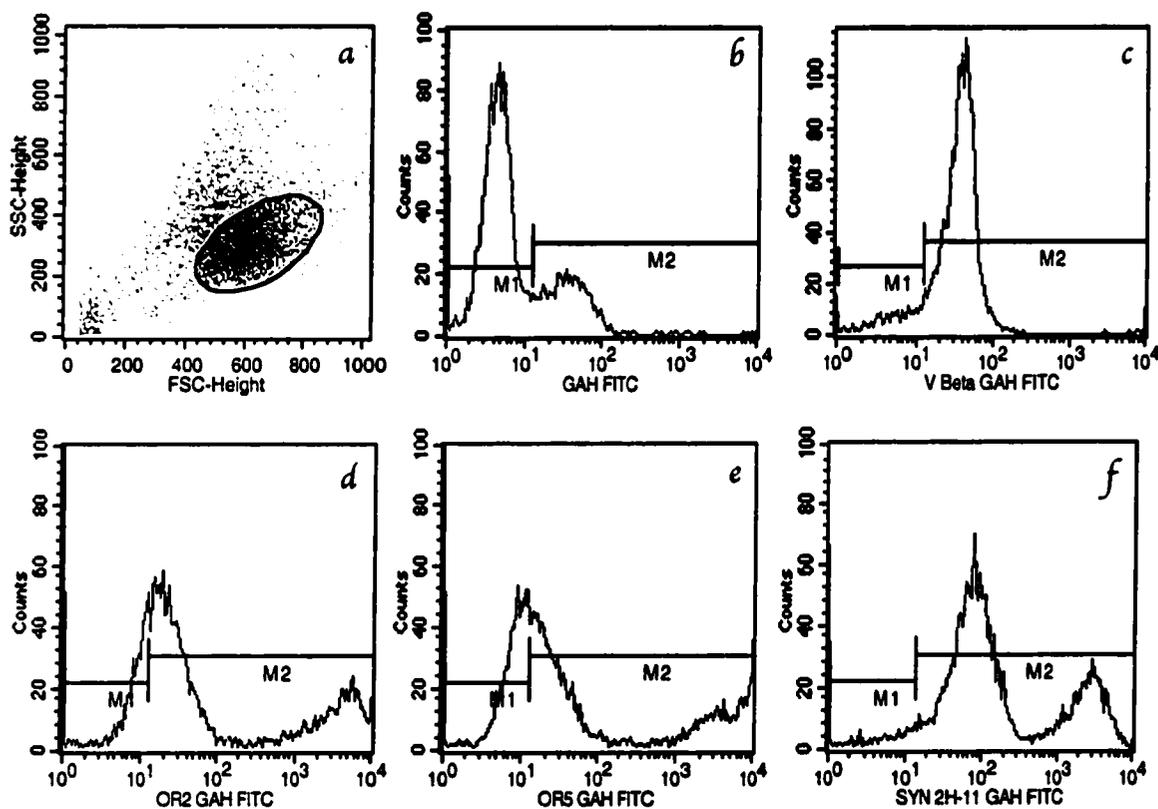


Figure 17: Binding of anti-human TCR monoclonal antibody to mouse DO-11.10 T cells by flow cytometric analysis. All cells were single stained with a FITC label and presented on a FACS analysis histogram designating the fluorescence intensity (x-axis) and the number of event counts (y-axis). The plot reads as follows: A) Forward scatter vs. side scatter dot plot of DO-11.10 cells with region designating the cell population examined for anti-TCR mAb binding. B) Goat anti-human (GAH) IgM conjugate background binding control. C) FITC labeled anti-mouse V β 8 positive control. D) OR2 anti-TCR with GAH FITC conjugate. E) OR5 anti-TCR with GAH FITC conjugate. F) Syn 2H-11 anti-TCR with GAH FITC conjugate.

population of DO-11.10 cells. The number of cells that fluoresced at the lower voltage was approximately 3 times larger than the high fluorescence intensity population. According to the markers set up to distinguish positive from negative fluorescence, approximately 76% of the gated cells (M2 region) were bound by OR2. The remaining 24% fluoresced too weakly to be considered 'positive' within the defined parameter. The histogram profile for OR5 was very similar to that of OR2. OR5 bound to two populations of DO-11.10 cells, a dim population and a bright population. Up to 62% of the cells fluoresced in the M2 region when treated with OR5. The dim population (10^1) of OR5 positive cells was also 3 times smaller in cell numbers than the bright population (10^4) (Fig. 17e). Syn 2H-11 (Fig. 17f) bound to > 95% DO-11.10 cells. Just as with OR2 and OR5, Syn 2H-11 binding to DO-11.10 cells generates a distinguishable bi-phasic histogram plot. One population of cells fluoresced at moderate to moderate-high brightness (10^2), which was twice as large as the more intensely fluorescing population at 30^3 . These results and the data on T cells from mouse spleens confirm that OR2, OR5, and Syn 2H-11 bind to murine T cells.

Anti-TCR mAbs demonstrate binding activity to mouse peptides

Since the anti-TCR mAbs bound subsets of mouse T cells, we determined whether these antibodies reacted with murine V β peptide homologs to the CDR1/FR2 segment used in their selection (Table 10). Mouse peptides mu V β 1, mu V β 8.1 HV short, and mu V β 8.2 HV short are 10 amino acids in length. These peptides correspond only to the CDR1 region of the V β TCR, whereas the longer peptides, mu V β 8.2 long and mu V β 4 include parts of the FR2 sequence (58). Binding of the anti-TCR mAbs to mouse peptides was determined by ELISA. Figure 18 shows binding to mouse TCR peptides by the anti-TCR mAbs, OR2, OR5, and Syn 2H-11. The absorbance readings in optical density units range from 0 to 1.0 in which the degree of antibody binding reactivity to peptide is ascertained. Stock concentrations of OR2 and Syn 2H-11 were approximately 1.0 mg/ml and OR5 was 0.4 mg/ml. Antibody dilutions were carried out two fold from 1/10 to 1/640 and 1/100 to 1/6400.

OR2 demonstrated binding reactivity to 4 out of 5 of the mouse TCR peptides with the highest absorbance measurement on mu V β 4. Titers on this peptide carried out past dilution 1/5000, which was less than 0.5 μ g/ml. OR2 exhibited significantly weaker, but detectable titers against mu V β 8.2 HV short, mu V β 8.1 HV short, and mu V β 8.2 long. The highest absorbance reading at a titer of 1/200 (0.5) for these peptides was the same for the measurement on mu V β 4 at a titer of about 1/6500. OR5 also reacted to 4

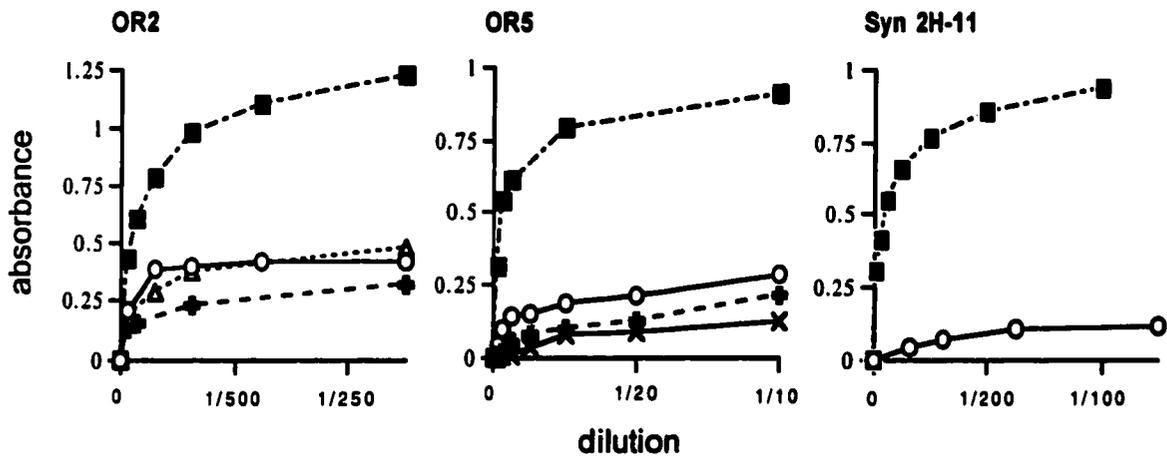


Figure 18: Binding of human anti-TCR mAbs to peptides specifying the CDR1 segments of murine V β gene products. OR2, OR5, and Syn 2H-11 anti-TCR mAbs were examined by ELISA for binding to peptides corresponding to the mouse V β 8 CDR1 region. Binding of titrating antibody dilutions (x-axis) was measured by absorbance (y-axis) signal generated by enzyme-linked secondary anti-human IgM conjugate. (■) ms V β 4, (O) ms V β 8.1 HV short, (Δ) ms V β 8.2 HV short, (+) ms V β 8.2 long, (X) ms V β 1 HV.

Table 10: Synthetic V β CDR1 mouse sequences used in the study compared to the human β 3 peptide sequence.

	Murine Vβ CDR1 homolgs^a
β 3 ^b	C K P I S G H N S L F W Y R Q T
mu V β 1	E Q H L G H N A M Y
mu V β 8.1 HV short	M Q T N N H D Y M Y
mu V β 8.2 HV short	N Q T N N H N N M Y
mu V β 8.2 long	C N Q T N N H N N M Y W Y R Q D
mu V β 4	C E Q Y L G H N A M Y W Y R Q

^a Classification for the V β nomenclature is referenced from Arden, et al. (60)

^b Peptide β 3 consists of residues 23-38 (Kabat numbering system) of the YT35 (48) gene product that represents the V β 8S1 family in the old nomenclature and TRBV 12-3 in IGMT nomenclature. This peptide was identified as a major epitope recognized by anti-TCR autoantibodies of humans (34) and mice (64). The murine peptides are homologous to this sequence.

out of 5 of the mouse TCR peptides. The titer for OR5 on mu V β 4 carried out past dilution 1/300 which was less than 2 μ g/ml. OR5 had weaker titers against three other mouse peptides, mu V β 1, mu V β 8.1 HV short, and mu V β 8.2 long. The highest absorbance readings for OR5 on these peptides ranged between 0.1 and 0.25 at titers of 1/10, whereas a dilution of approximately 1/400 was required to equal the same absorbance for OR5 on mu V β 4. Syn 2H-11 also exhibited the highest binding reactivity to peptide mu V β 4. The titer carried out past dilution 1/2000 or less than 1 μ g/ml. Syn 2H-11 reacted weakly to only one other mouse peptide, mu V β 8.1 HV short. The absorbance measurement at a dilution of 1/100 for Syn 2H-11 on this peptide was slightly higher than 0.1.

Negative controls were established in each ELISA to distinguish between antibody/antigen binding and false positive signals. These controls include non-antibody (assay diluent only) treated wells to account for background signal, uncoated wells to check for antibody non-specific binding to plastic, and the β 1 negative control peptide. All titers reported in this study represent the signal generated by antibody binding to antigen at various dilutions with background and false positive signals subtracted out. These results show that some of these human anti-TCR mAbs were cross-reactive against

epitopes, although they may vary between each monoclonal, on the mouse V β TCR CDR1 region.

Anti-TCR mAbs do not induce apoptosis

The IgM is a pentameric, multivalent structure with 5 times the number of antigen binding sites as an IgG molecule. The ability to bind multiple TCRs, and thus cross-link receptors, on the cell surface could induce intracellular signaling leading to apoptosis. We investigated this possibility with anti-TCR mAbs, OR2, OR5, and Syn 2H-11. DO-11.10 mouse T cells at a concentration of 0.5×10^6 cells/ml were treated for 16 hours with up to 100 μ g/ml of soluble antibody and then stained with Annexin V and PI to determine the level of cell death by apoptosis. The cells were incubated in media alone (Fig. 19a) and 100 μ g/ml of soluble IgM isotype matched control (Fig. 19b) to account for background cell death measurements. The pro-apoptotic compound Ara C (10 mM) served as the positive control for these experiments. Background cell death from the negative controls, including single positive Annexin V and PI stains and double positive Annexin V/PI stains, ranged between 30-40%. The amount of cell death induced by Ara C during a 16 hour incubation was at least 90%. We were unable to ascertain a

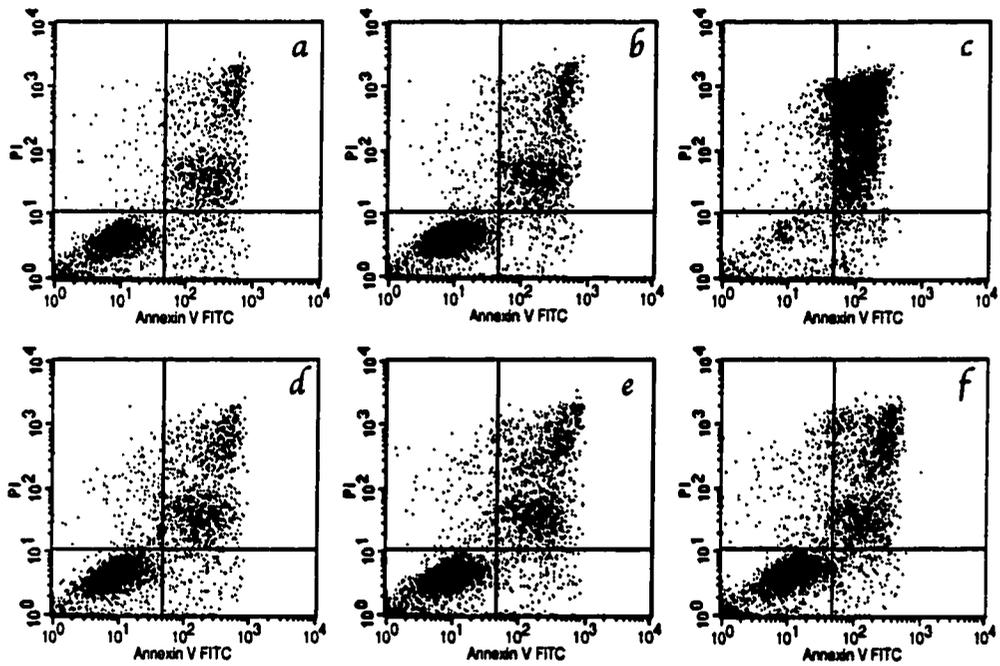


Figure 19: Annexin/PI staining of DO-11.10 T cells after 16 hour treatment with soluble anti-TCR monoclonal antibodies. Cells were treated with Annexin V (x-axis) and peridinium iodide (PI) (y-axis) after 16 hour incubation with A) media alone, B) IgM isotype control, C) Ara C, D) OR2, E) OR5, or F) Syn 2H-11 and examined by flow cytometric analysis for comparison of the level of apoptosis and necrosis between each group.

significant difference in the levels of Annexin V staining or Annexin V/PI staining within this viability range where there should have been an increase if the anti-TCR mAbs were capable of inducing cell death. The level of Annexin V/PI staining for OR2, OR5, and Syn 2H-11 were 27%, 30%, and 29% (Fig. 19d, e, and f) respectively, which corresponds to the 30% cell staining by Annexin V/PI in the isotype control (Fig. 19b). Single stains with Annexin V alone were always about 4% for every group.

These experiments were conducted on naive mouse T cells from spleens, DO-11.10 cells, and human JURKAT T cell lines, using immobilized and soluble antibody (data not shown). The results for all of these experiments were comparable and not significant in marginal cases. Overall, the data suggest that the anti-TCR mAbs used in these experiments were not capable of inducing apoptosis or cell death through interaction with TCRs. Such results, however, provide indirect evidence to the nature of natural autoantibody functions.

Anti-TCR mAbs can inhibit IL-2 secretion by DO-11.10 T cells

T cell activation is triggered by the formation of the TCR/MHC complex between the T cell and an APC. This interaction typically leads to the expression of IL-2. If

TCR/antigen binding to the MHC is prevented by the introduction of a soluble anti-TCR antibody, IL-2 levels should be significantly reduced as a result. We tested this concept with anti-TCR mAbs OR2, OR5, and Syn 2H-11. DO-11.10 mouse T cells were pre-treated for one hour at 37° C with antibodies and controls then distributed to cultures with bone marrow derived murine DCs and OVA antigen. Cultures were incubated for 48 hours at final antibody concentrations of 50, 25, and 12.5 µg/ml. Supernatants from these cultures were examined for levels of IL-2 by cytokine ELISA. These experiments were repeated with both purified (Fig. 20) and non-purified anti-TCR mAbs. There was no significant variation between these antibody preparations. Increasing amounts of TCR specific antibody were capable of blocking IL-2 expression in DO-11.10 T cells. These results were significant when compared with the isotype controls (Fig. 21).

All three of the anti-TCR mAbs tested were capable of inhibiting by at least 50% IL-2 expression by DO-11.10 T cells at a range of about 25 µg/ml. According to Figure 21b, OR5 at a concentration of 50 µg/ml could block up to 90% IL-2 expression and suppressed greater than 50% IL-2 expression at the lowest concentration (12.5 µg/ml) used for these experiments. The results for OR2 (Fig. 21a) were similar to the highest concentration blocking greater than 80% IL-2 expression and the lowest concentration preventing approximately 50% IL-2 secretion. Our results for Syn 2H-11 (Fig. 21c)

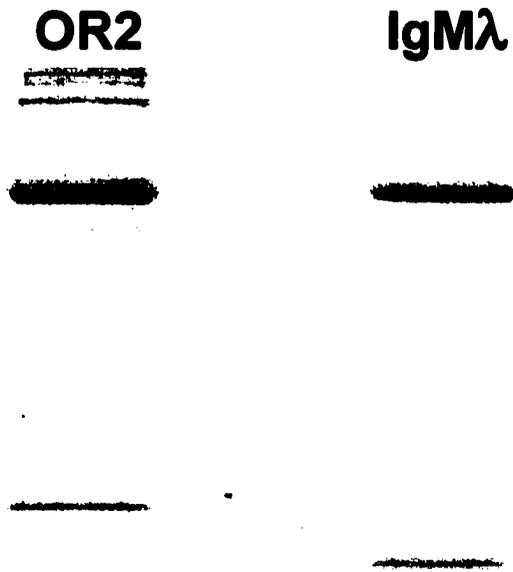


Figure 20: Purified OR2. SDS PAGE of purified OR2 and IgMλ myeloma protein.

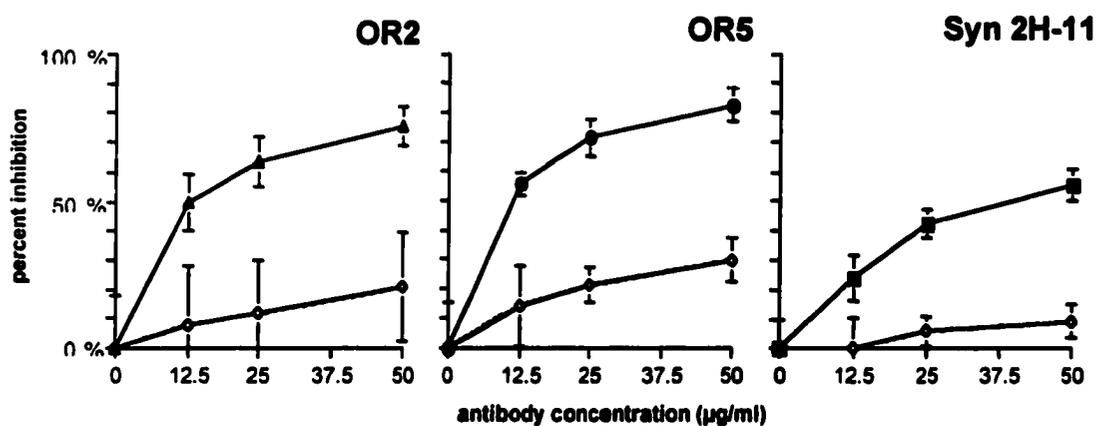


Figure 21: Inhibition of IL-2 secretion by DO-11.10 mouse T cells by anti-human TCR monoclonal antibodies. IL-2 secretion was measured by capture ELISA and plotted as percent inhibition (y-axis) vs. antibody concentration (x-axis). Inhibition of IL-2 with anti-TCR mAbs were compared with an internal isotype control at the same concentrations.

indicate that inhibition of IL-2 expression was lower than that for OR2 and OR5. The maximum concentration of Syn 2H-11 blocked up to 60% IL-2 secretion and the lowest concentration blocked near 30%. All plots in Figure 21 were representative from a collection of separate experiments. The IL-2 inhibition experiments were conducted with each anti-TCR mAb and an internal isotype matched control independently and therefore, can not be accurately compared for relative potency.

These experiments provide evidence that some natural autoantibodies specific for the TCR may function as anti-inflammatory molecules. We were able to demonstrate with various concentrations of antibody that inhibition of IL-2 expression was dose dependent and significant compared with the same concentrations of isotype control (Fig. 21). Although the isotype control does appear capable of blocking IL-2 secretion to a minor degree, we believe this is a function of stickiness that is often encountered when using high concentrations of IgM antibody.

SECTION III

Chapter 9 – Discussion

The goal of these experiments is to establish a possible functional role of human natural autoantibodies specific for TCR variable domains. The procedure used to determine this include assessing the degree of cross-reactivity at which anti-TCR mAbs react against the mouse TCR and its epitopes, followed by studies on how these molecules can influence T cell behavior in a TCR-specific and dose dependent manner. The use of murine T cell lines serves as a viable route for obtaining immediate and interpretive functional data and establishes a foundation for future studies with anti-TCR mAbs in animal models.

Anti-TCR mAbs OR2, OR5, and Syn 2H-11 bound only to a restricted number of mouse T cells by flow cytometry (Fig. 16). About 8% of the murine T cells tested were bound by OR2 which is about half the number of the CD3⁺ human PBMCs bound by this antibody (Fig. 3). Syn 2H-11 bound to about 15% of the murine T cells (Fig. 16i and 16f) in a similar pattern to that of OR2 (Fig. 16e and 16f). This may be explained by the degree of cross-reactivity between human and mouse TCR epitopes, the level of

expression of certain V β subsets available for binding in the human compared with the level of expression of homologous mouse V β subsets, or both.

The most unusual results came from OR5 binding to mouse T cells. OR5 could bind up to 28% of mouse T cells examined by flow cytometry. These data significantly contrasted with the results from flow cytometry on CD3⁺ human PBMCs, which was only about 5% (Fig. 3). This is the highest percentage of a polyclonal T cell population bound by any of the anti-TCR mAbs tested. Even more striking is the prevalence of CD4⁺CD8⁻ murine T cells that were positively bound by OR5. We estimate $\geq 80\%$ OR5 positive murine T cells were also CD4 single positive (Fig. 16g and 16h). This phenomenon may be largely attributed to the fact that the CD4 receptor on T cells can physically associate with the TCR. CD4 association with the TCR is dependent on a specific binding interaction between the TCR and its ligand. When this occurs a conformational change takes place in the TCR. The binding interaction between the TCR and the MHC II, for example, induces a conformational change in the TCR leading to CD4 recruitment (131). Some anti-TCR antibodies specific for V region epitopes possess a similar quality (132, 133). OR5 binding to murine T cells demonstrates a 'smeared'-like population on a bivariate plot ranging from low to high fluorescence intensity (Fig. 16g and 16h). As with OR2 and Syn 2H-11, we believe this is due to the availability of V region epitopes

on each TCR. The anti-TCR mAbs we are working with most likely bind with higher affinity to some TCR epitopes than others and because not all TCRs possess identical V region sequences most of these receptors will not interact with these molecules.

The α/β TCR on the DO-11.10 mouse T clone is identical on every cell, therefore, the anti-TCR mAbs used for these studies should bind 100% of the cells if the antibody binding sites cross-react with murine TCR epitopes. The flow cytometric analysis supports this conjecture despite the unusual profiles observed. If the regions set up to designate positive and negative binding are considered arbitrary to establish a reference to the controls (given they were neither 0% or 100% in terms of negative or positive binding) then it could be stated that the 'negative' shoulder (M1 region) of the low fluorescence histograms for OR2 and OR5 are brighter than that of the negative control and thus, can arguably be considered as fluorescing dimly. Otherwise, the histogram shoulders of OR2 and OR5 occupying the M1 region may represent a group of cells at a stage of the cell cycle that will not permit binding of the IgM anti-TCR monoclonals.

Human anti-TCR mAbs OR2, OR5, and Syn 2H-11 cross-reacted with epitopes corresponding to the murine $V\beta$ TCR CDR1 region. These region sequences bear various degrees of homology to the human $V\beta$ TCR CDR1 segment (Table 10) on which the anti-TCR producing hybridomas were selected. Since we did not test the entire

spectrum of murine V β CDR1 homologs it is not possible to deduce the exact epitopes involved except for the conclusion that murine homologs are recognized by the human autoantibodies. Furthermore, the participation of conformational determinants can not be excluded because these antibodies reacted to the recombinant single chain TCR and the cognate α/β TCR exposed on the T cell surface (Fig. 1).

The active induction of T cell apoptosis is a viable possibility for antibodies directed against the TCR. This has been demonstrated before using monoclonal antibody against the TCR β chain. The process can be measured in 16 hours and operates through a Fas-dependent mechanism (134). Given the anti-TCR mAbs for these studies are IgM isotype, we examined the likelihood that multivalent binding sites on these molecules might cross-link multiple TCRs and induce intracellular signaling events leading to apoptosis. These experiments were tested using naive murine T cells DO-11.10 cells, and JURKAT human T cell lines, with plate-bound and soluble antibody at various concentrations. The results were consistently negative for apoptosis by comparison to the controls.

The binding interaction of OR2, OR5, and Syn 2H-11 to the TCR may be sufficient alone to control T cell responses. These molecules significantly inhibit IL-2 secretion of antigen activated DO-11.10 murine T cells by in a dose dependent manner

(Fig. 21). These results implicate natural autoantibodies against the TCR as anti-inflammatory, thus potentially designating them as immunoregulatory molecules. Experimental proof for natural autoantibodies as immunoregulatory agents has been demonstrated in animal models with mAbs to treat experimental autoimmune encephalomyelitis (EAE) (135), non-obese diabetes (136), and experimental autoimmune myasthenia gravis (137). In our own studies with murine mAbs to TCR V β epitopes we documented that such antibodies acted synergistically with superantigens in the activation of T cell subsets (64). Furthermore, studies using therapeutic preparations of purified human IgG immunoglobulins document the immunoregulatory role of natural autoantibodies in a variety of human diseases with an anti-TCR specificity beneficial in Kawasaki's disease (108).

We have obtained evidence for a functional role of natural autoantibodies to the human TCR that was made possible by their recognition of cross-reactive murine TCRs. The capacity of these molecules to suppress production of IL-2 suggests that they may serve as anti-inflammatory agents possibly to hold aggravated autoimmune Th1-type responses in check. Taking into account that the mAbs used for these investigations are derived from individuals with RA, we maintain that these antibodies are not adverse products from autoimmune disease. A strong piece of evidence supporting this

conclusion is that the V region sequences of these autoantibodies are in the un-mutated germline configuration as opposed to those of most pathogenic autoantibodies, which show considerable somatic mutation (138). Furthermore, the epitope recognition profiles are similar to those of affinity-purified anti-TCR natural autoantibodies from normal Igs (26). In closing, the generation of antigen specific monoclonal antibody secreting hybridomas from human B cells serves as a valuable tool for obtaining humanized antibodies for clinical use. We envision that human anti-TCR mAbs may serve as potential agents for therapies in cancer, organ transplantation, and autoimmune disease.

DISCUSSION

Generation of human monoclonal antibodies from patients with autoimmune disease

Since the development of hybridoma technology many new innovations have emerged to improve on methods for rapid production of non-immunogenic and high affinity Igs valued for passive immunotherapy or tumor therapy. Some of these technologies include transgenic animals for production of humanized antibodies and phage display technology for production of recombinant antibodies or antibody fragments (139). Prior to these latest developments the method for generating human mAbs from hetero-hybridomas was a promising advancement for producing therapeutic antibodies, especially after the discouraging results from preliminary clinical trials using murine-derived antibodies (140). The risks and limitations of non-human antibodies administered to patients were thought to be overcome by this method. Unfortunately, there were several problems with this technology mostly concerning production and efficacy. In theory it is conceivable to derive any antibody from humans. The problem most encountered was the strenuous difficulty in sequestering the desired antibodies through the limit dilution process. This is compounded by the difficulty in obtaining

large numbers of lymphocytes from patients. The most readily available source is from the peripheral blood, but this is also the least ideal considering that B cells are poorly represented in circulation when compared to either the spleen or the lymph node. The antibodies obtained from the peripheral blood were also considered less desirable because 80-90% of the hybrids secreted IgM molecules. Moreover, the affinities these antibodies expressed for antigen was often lower than 10^{-7} M. The debilitating obstacles encountered in the human hetero-hybridoma method ultimately led researchers to conclude that unless new advances were improvised the use for the current technology would go under (141).

Despite the difficulties in obtaining high affinity antibodies the generation of mAbs from hetero-hybridomas is still potentially effective for immunotherapy and a valuable tool for immunological research. One area of study where this technology may prove to be important is in systemic autoimmune diseases. Rheumatologic disorders such as SLE and RA are distinguished by a marked production of Igs in circulation and tissues. Often these antibodies are specific for various self-determinants including cell surface molecules and cytokines. Patients with SLE, RA, and similar diseases generally possess high levels of activated B cells. The increased frequency makes the B cells more accessible and contributes to the ease in isolating and deriving many kinds of mAbs.

Autoantibodies (or the V regions of autoantibodies) derived from these patients may potentially serve as therapeutic modalities for tumor therapy, suppression of transplant rejection, or control of the inflammatory response. The derivation of mAbs from these individuals could also be useful for studies to better characterize the functional dynamics of specific systemic autoimmune diseases.

Natural autoantibodies specific for the TCR from patients with rheumatoid arthritis

One of the aims of the project that entails these studies is to gain a better understanding on why individuals with RA make high titers of autoantibodies against the CDR1 region of the TCR. Several insights have been gained from this research. All the mAbs generated from these patients are categorized as natural autoantibodies. These molecules are isotype IgM with V_H and V_L genes in germline configuration. The mAbs share in common the ability to bind to the CDR1 determinant of the human TCR and a recombinant scTCR. These antibodies are not, however, all derived from a single V_H or V_L gene family, nor do they possess V_H CDR3 sequences (the crucial epitope binding region) that compare in sequence or biochemical properties.

The conspicuous elevation of anti-TCR autoantibodies in RA individuals suggests a meaningful role for these molecules in either pathogenicity or regulation of immune function in response to the disease process. Most of the evidence from these findings favors the latter possibility. Affinity maturation of V region genes is a consistent feature of pathogenic autoantibodies. The sequences from these studies clearly have not undergone this process. An antibody that has undergone somatic mutation is regarded as the product of an antigen activated T cell dependent B cell. Affinity matured antibodies, which are mostly IgG, can have affinities 100 times greater than IgM molecules with the same specificities (138). These products of the adaptive immune response are generally thought of as molecular recruits in a systematic effort to eradicate a specific antigen as aggressively and quickly as possible.

Examination of the V region genes from some of the mAbs in these studies has revealed that the germline sequences are expressed in healthy individuals (81). Although a limited number of clones were characterized, the only two V_H gene families represented in our findings were V_H3 and V_H4 . This was not surprising because these are the largest and diverse V_H gene families and both are known to code for autoantibodies (84-86). The same trend was observed in the V_L sequences. The anti-TCR mAbs from these studies expressed light chain sequences mostly from the $V_{\lambda}3$ and $V_{\kappa}3$ families (OR4, a $V_{\lambda}2$ was

the only exception). These sequences are from both the largest of the light chain V gene families and are known to code for sequences of autoantibodies (89-94). The results for the sequencing experiments are not significant, but noteworthy. They suggest that the anti-TCR mAbs are standard components of the antibody repertoire in healthy individuals as well as those with autoimmune disease.

Finally, there is striking diversity amongst the anti-TCR mAbs investigated in these studies, particularly in the heavy chain CDR3 sequences expressed by each clone. It is likely that more unique sequences would be discovered with the generation of new clones even if they were derived from the same individual. The reason for this type of sweeping redundancy is not exactly known. The selecting β 3 peptide from these studies is most likely a cross-reactive or a heteroclitic epitope and the true or cognate epitope for each anti-TCR mAb is distinct determinant which exists in a subset of T cells. Through this perspective it is attractive to speculate that regulation of the recombinatorial immune system is founded on an ancient and conserved antibody repertoire designed to maintain a delicate degree of control over T cell functions.

Binding characteristics of anti-TCR natural autoantibodies

The results from the TCR (intact and peptide) binding studies were varied and complex. The percent of binding to a T cell clone (JURKAT) ranged from 20-90% for the mAb and the cells fluoresced at various intensities. In the data where anti-TCR mAbs bind to JURKAT T cells at negative to low fluorescence, it may be reflective of low affinity interactions. These findings are indicative of the monoclonal selection process in that low affinity and cross-reactive antibodies can be derived as easily as high affinity antibodies for the cognate antigen. In most of the JURKAT T cell binding results it is evident that the anti-TCR mAbs bind to different groups of the same clone. Similar results were observed on the murine DO-11.10 cell line. This is a phenomenon that cannot be explained without further investigation. We propose several interpretations. The immortal lines were not growing in log phase when they were tested for binding by anti-TCR mAbs. At certain stages of the cell cycle the TCR may have epitopes exposed that are not otherwise available during other stages. There is reason to believe that this could occur when a cell is unhealthy or about to die. Another explanation may be that the IgM isotype with its multivalent and polyreactive binding sites reacts in an unpredictable manner in flow cytometric assays.

The binding results for the anti-TCR mAbs on human CD3⁺ PBMCs showed that every antibody tested bound to a percentage of polyclonal human T cells. The smeared

fluorescence pattern demonstrated by all of the anti-TCR mAbs indicated that the antibodies exhibited various levels of affinity for the different T cell subsets. These findings are also supported by the ELISA data on the TCR V β CDR1 (and FR3) peptide homologs. All of the monoclonals tested cross-reacted to several of the V β homologs at various titers. There was a variation in binding reactivities to the different homologs attributed to the unique binding site of each anti-TCR mAb. These combined results suggest that natural autoantibodies to the TCR are not pan-T cell markers, but rather function to interact with discrete T cell subsets.

The anti-TCR mAbs from these studies revealed a diverse set of antigen binding properties as determined through the epitope mapping analysis. Unsurprisingly, the types of binding interactions observed range from monoreactive to polyreactive. These findings were observed in previous studies on natural autoantibodies. Moreover, the affinities expressed by those autoantibodies ranged from low ($K_D = 10^{-5}$ M) to high ($K_D = 10^{-10}$ M) (142). Further investigations on the nature of one of our polyreactive antibodies (OR2) demonstrated that it was capable of binding to different epitopes in a specific manner. The term used to define this type of 'specific polyreactivity' is epitope recognition promiscuity. This definition is a more accurate because it describes an intentional rather than the random or coincidental binding interaction often associated

with the term 'polyreactive'. OR2 bound to a number of unrelated peptides corresponding to the TCR and Ig λ chain. It did not, however, react at even the highest titers to test proteins that polyreactive antibodies are known to bind. In addition, OR2 (or any of the anti-TCR mAbs) did not bind to human IgG, confirming that these antibodies were not RFs. Finally, unrelated peptides could compete for the binding site of OR2. The results from the inhibition studies suggest that OR2 bound to its epitopes specifically despite sequential and molecular incongruities. The concentration of antigen required to bring about OR2 inhibition, however, was not the same for each antigen. From these studies we were able to derive estimated dissociation or affinity constants. It was shown from these experiments that epitope promiscuous antibodies express low and high affinities simultaneously for different antigens. Such results dispel the notion that polyreactivity and low affinity are mutually inclusive.

Ruminations on epitope recognition promiscuity

The property of epitope recognition promiscuity is a genuine binding reaction observed in both IgG and IgM isotypes (106, 111, 112). The fact that epitope promiscuity occurs in natural antibodies implies that it is not a coincidental property

brought on by random rearrangements in the V region genes. The crucial component that appears to drive epitope binding reactivities is the heavy chain CDR3 region. This is evidenced in these studies as well as others (22-24). The heavy chain CDR3 region is the site of junctional diversity due to nucleotide deletions and additions that take place during recombination. Nucleotide additions are carried out primarily by the enzyme TdT, which is thought to function by adding random nucleotides (random collisions) to the V(D)J junctions and create unique and diverse amino acid sequences in this region (13). The notion that complete antibody and TCR V regions are produced in a stochastic manner, however, renders little confidence that this system could be the foundation of a fully organized and competent combinatorial immune system. It is more conceivable that TdT functions in concert with a highly organized complex of nuclear proteins that maintain constant control over the direction of the recombinatorial process. Evidence for this concept was reported recently indicating the Ku80 DNA-binding protein as a necessary recruiting molecule for TdT. It was suggested that the Ku80 protein conducts TdT into association with other active DNA proteins present during recombination (143).

There is limited concrete data to elucidate the biological significance of an epitope promiscuous idiotope. Through speculation, however, it could be understood why this property might be extremely crucial. A likely explanation is that it provides

antibody binding sites with a greater hit-rate against various antigenic determinants. This property would be extremely valuable in a primitive immune system unable to make adaptive responses to every single antigen. The same could be true for the autoreactive natural antibody repertoire. These antibodies could target new V region idiotopes that emerge in the course of evolution, thus keeping in step with the developing immune system and maintaining the same degree of immunomodulatory control.

Possible functional properties of natural autoantibodies specific for the TCR

As mentioned previously, evidence from these studies suggests that natural autoantibodies specific for the TCR play a role in immunoregulation of T cell responses. The TCR is both a binding and signaling molecule. The type of signal transmitted through the TCR is dependent on its interaction with the ligand and other surface molecules recruited in the interaction. Taking into account the anti-TCR antibodies from these studies are IgM isotype, they may possibly have a significant signaling affect due to their multivalent structures. The cross-linking of receptors is often an important event in intracellular signaling. We investigated the possibility that the anti-TCR mAbs from these studies could activate T cells and induce proliferation. Alternatively, we tested to

see if these antibodies could induce apoptosis. Repeated experiments were unable to furnish sufficient evidence that these anti-TCR mAbs could influence either process.

Another functional possibility with IgM molecules is the ability to induce lysis through the complement cascade. The experiment did not yield satisfactory results, but this may have been attributed to the use of human complement proteins on human cells. Human cells are resistant to human complement proteins. Similar experiments have obtained more success using rabbit complement to lyse human cells with human IgM mAb (144).

The results from the studies with some these anti-TCR mAbs suggest that they can bind to the TCR and inhibit further signaling interactions with APCs. T cell interaction with APCs triggers activation and proliferation. The introduction of a ligand that can inhibit this process would prevent T cell activity including receptor upregulation, receptor clustering, and cytokine secretion. If the T cell were a Th1 helper cell then an inhibiting anti-TCR antibody could ultimately prevent T cell proliferation and effector functions. Such a role could be envisioned for the conserved natural autoantibody repertoire to target specific T cells and regulate in a non-depleting fashion.

Therapeutic potential for natural autoantibodies specific for the TCR

Natural autoantibodies have been proven successful in treatment of human autoimmune and inflammatory disease in clinical studies (Table 11). Many other ongoing trials are also showing to be effective. The adverse effects from this type of treatment occur in less than 5% of patients. Side effects are often treatable and usually decrease after subsequent infusions (108, 145).

Use of natural immune globulin has been shown to prevent or decrease the activity of autoimmune T cells in animal disease models such as EAE and autoimmune uveitis. The CD4⁺ T cells from these animals could be transferred to other animals and prevent disease induction without having been treated with immune globulin. In both cases a sharp reduction in autoreactive T cell activity and IL-2 and interferon- γ production was observed indicating specific immunoregulation of Th1 type mediated responses. The findings support the evidence that natural autoantibodies can restore the balance between Th1 and Th2 type T cells (146, 147).

It is too early to confirm from these data if the anti-TCR mAbs from these studies possess real therapeutic value. More studies involving animal model are required. These studies indicate that natural autoantibodies against the TCR are not T cell activating or killing antibodies. These molecules probably target very specific T cell subsets and regulate their activity as a part of a delicate and conserved system of checks and balances.

Table 11. Autoimmune and inflammatory diseases treated by intravenous infusion of human immune globulin containing natural autoantibodies.

Autoimmune and inflammatory diseases treated by natural immune globulin in established control clinical trials*

Idiopathic thrombocytopenic purpura
Guillain-Barré syndrome
Chronic inflammatory demyelinating polyradiculoneuropathy
Myasthenia gravis
Multifocal motor neuropathy
Corticosteroid-resistant dermatomyositis
Kawasaki's disease
Prevention of graft-versus-host disease
Antineutrophil cytoplasmic-autoantibody- positive vasculitis
Autoimmune uveitis
Multiple sclerosis

* Referenced from review by Kazatchkine and Kaveri (108).

The studies have demonstrated that anti-TCR antibodies can inhibit the production of IL-2 by antigen activated T cells, designating them as anti-inflammatory molecules. Such antibodies may prove to be valuable in certain types of therapy. These therapies may include prevention or treatment of T cell cancers, graft-versus-host disease and Th1 mediated autoimmune diseases such as multiple sclerosis, insulin-dependent diabetes mellitus, and rheumatoid arthritis.

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