(54) Title: SOLUBLE T-CELL ANTIGEN RECEPTOR CHIMERIC ANTIGENS

(57) Abstract

Soluble antigens for efficient production of antibodies against human T-cell receptor epitopes are provided. A preferred antigen polypeptide is a chimeric comprising at least 100 amino acids from a V_β domain with solubility conferred by an immunoglobulin heavy chain C_{H2} or C_{H3} domain fused thereto. Product antibodies and methods for diagnostic and therapeutic uses for both antigens and antibodies are provided.

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SOLUBLE T-CELL ANTIGEN RECEPTOR CHIMERIC ANTIGENS

FIELD OF THE INVENTION

This invention relates generally to the construction of fused gene segments using recombinant DNA techniques to produce T-cell receptor chimeric antigens in secretable form and, more particularly, to improved methods for producing antibodies specifically reactive with T-cell receptor epitopes.

BACKGROUND OF THE INVENTION

The vertebrate immune system is characterized by its ability to respond to an enormously diverse set of antigenic determinants, or epitopes. This response is effected through T and B lymphocytes, commonly referred to as T cells and B cells. The immune system, comprising these specialized cells, recognizes and processes foreign pathogens and macromolecules, typically clearing them from the body. Lymphocytes individually exhibit high specificity in recognition of particular molecular shapes and collectively exhibit great diversity in reacting with, i.e., specifically binding, a broad range of molecular structures. These properties are mediated through both T-cell receptors and immunoglobulins that serve as antigen receptors.

T-cells usually recognize only antigens that are present on the surfaces of other cells. This recognition event generally occurs only when the antigens are correctly presented in combination with polymorphic cell surface molecules encoded by the major histocompatibility complex (MHC). These T-cells can be subdivided into separate functional categories including cytotoxic effector cells (T_c), inducer or helper cells (T_h) and suppressor cells (T_s).

T-cells mature in the thymus, in contrast to B cells which differentiate in the fetal liver or bone

marrow. Among the cell surface molecules which
characterize T-cells are the α and β chains of the T-cell
receptor molecule. General introductory review articles
describing the T-cell receptor include Toyonaga and Mak,
(1987) "Genes of the T-Cell Antigen Receptor in Normal
and Malignant T-Cells" in Ann. Rev. of Immunology 5:585-
620; Kronenberg et al., (1986) "The Molecular Genetics of
the T-Cell Antigen Receptor and T-Cell Antigen
Recognition" in Ann. Rev. of Immunology 4:529-591; and
Davis, (1985) "Molecular Genetics of the T-Cell Receptor
Beta Chain" in Ann. Rev. of Immunology 3:537-560.

T-cell antigen receptor polypeptides are
members of the immunoglobulin superfamily. Members of
the immunoglobulin superfamily are characterized by
similarities in primary and tertiary structure. They
also display characteristic gene segment organization,
ontogeny of expression and diversification. In
particular, the polypeptide chains of the immunoglobulins
typically result from the rearrangement, at the genetic
level, of various variable region (V), joining region
(J), constant region (C) or diversity region (D) gene
segments. Although particular chains may have different
sized repertoires of the various segments, the genetic
rearrangements and combinatorial joining and junctional
diversity all contribute in a characteristic manner to
produce the extraordinary diversity of polypeptide
sequences exhibited by these gene families. See, Hood et
al., (1985) Cell 40:225-22.

A T-cell antigen receptor molecule comprises
two polypeptide chains, generally an alpha chain and a
beta chain, each of which exhibits two extracellular
immunoglobulin-like domains. Such molecules comprise an
amino terminal variable region domain (V) and a carboxy
terminal constant region domain (C), more specifically
designated with α or β when indicating the particular
chain of origin. Each of these domains is normally
stabilized by a disulfide bond between two conserved
cysteine residue pairs on each chain. The two chains are

apparently attached to one another by an inter-chain
disulfide bond near the cell membrane outer surface.
Each chain is anchored on the membrane by a hydrophobic
transmembrane segment which typically spans the entire

5   membrane lipid bilayer. A short carboxy terminal segment
extends into the cytoplasm. Both alpha and beta chains
are normally N-glycosylated at sites near the disulfide
bonds and at a site near the extracellular surface of the
cell membrane.

10   The alpha and beta chains of the T-cell
receptor are encoded by gene segments analogous to the
variable region (V) segment, the joining region (J)
segments, the diversity (D) segment, and the constant
region (C) segments of immunoglobulin genes. Diversity

15   in the T-cell receptor repertoire arises, in part, from
the rearrangement of V, D, and J gene segments and from
the insertion or deletion of nucleotides at the V_β-D and
D-J_β or V_α-J_α junctions. Some cells express T-cell
receptor gamma and delta chains in place of the alpha and

20   beta chains.
             Although specimens of alpha and beta chains of
T-cell antigen receptors from mouse and from human have
been isolated, it has been difficult to raise antibodies
of desired specificity against these polypeptides. T-

25   cell receptors are expressed at very low levels on T-
lymphocytes, with conventional biochemical purification
generally being unfeasible. Thus, physical
characterization studies have been almost non-existent.
In general, the production of antibodies against native

30   T-cell receptors has also been unproductive.
             Previous workers have reported solubilization
of the T-cell antigen receptor (TCR) V_α domain as a
chimeric molecule in either the form V_α-C_{H1}-H-C_{H2}-C_{H3} or the
form $V\alpha C\kappa$, where C_{H1}-H-C_{H2}-C_{H3} and C_κ are, respectively,

35   immunoglobulin heavy and light constant regions. See
Gascoigne et al., (1987) Proc. Natl. Acad. Sci. USA
84:2936-2940; Mariuzza and Winter, (1989) J. Biol. Chem.
264:7310-7316). Gascoigne et al. reported solubilizing

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the chimeric V_α-heavy chain in conjunction with a mouse λ light chain, whereas Mariuzza and Winter reported solubilizing the chimeric $V_\alpha C_\kappa$ light chain homodimers expressed in the absence of heavy chain expression.

Consistently obtaining highly efficient, secreted expression of substantially full length T-cell receptor polypeptide segments remains a major research goal. Although some genetic fusions between T-cell receptor domains and immunoglobulin segments have been constructed, the expression products typically have resulted in insoluble polypeptides. Thus, there exists a need for improved methods for producing large quantities of water-soluble T-cell receptor analogues which will permit the efficient production of antibodies against natural T-cell receptor epitopes. Preferably, when presented to target immune systems the analogues will not induce a significant number of antibodies against epitopes from the non-TCR segments. In particular, polypeptide segments of T-cell receptors which can be effectively reconstituted into conformations highly analogous to their native state would be most preferred.

Furthermore, the absence of a full repertoire of reagents for distinguishing the various T-cell receptor family types has hindered progress in the understanding and treatment of many immunological disorders. The present invention answers these and other needs.

SUMMARY OF THE INVENTION

The present invention provides for the efficient production of antibodies, typically monoclonal antibodies (MAbs), to human T-cell antigen receptor (TCR) epitopes. These antibodies have numerous uses as research, diagnostic, and therapeutic tools for studying or treating immune processes mediated by T-lymphocytes. A pre-requisite to the development of these antibodies is the availability of sufficient amounts of immunogen, in

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this case, proteins comprising human TCR epitopes, in highly pure form.

A preferred soluble chimeric polypeptide of the present invention will have at least 100 amino acids from a V_β domain, typically a complete native human V_β domain. The solubility may be conferred by an immunoglobulin heavy chain C_{H2} or C_{H3} domain fused to the V_β domain. Alternatively, a polypeptide comprising $X1-V_{R1}-C_{R2}-X2-C_{H2}-C_{H3}-X3$ can be prepared, wherein

V_{R1} is a variable region domain of a human T-cell receptor chain;

C_{R2} is a constant region domain of a human T-cell receptor chain;

C_{H2} is a second heavy chain constant region domain of an immunoglobulin gene;

C_{H3} is a third heavy chain constant region domain of an immunoglobulin gene;

each of X1, X2 and X3, if present, is a polypeptide segment comprising less than about 50 amino acids; and

R1 and R2 are each either α or β. R1 may be the same as R2, but need not do so.

Dimers of two such polypeptides may be employed and may be either homodimers or heterodimers. These polypeptides may further comprise a toxin moiety. The invention further embraces nucleic acids encoding and/or expressing such polypeptides.

A preferred method for producing antibodies capable of binding to a V_α or V_β T-cell receptor domain epitope will comprise the step of exposing to a target immune system a soluble hybrid polypeptide comprising a V_α or V_β T-cell receptor domain. Thereafter, one or more cell lines capable of producing the antibodies can be cloned. Alternatively, the antibodies can be produced by exposing to a target the chimeric polypeptide on a cell surface.

The polypeptides and antibodies of the present invention are useful in various therapeutic regimens and

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diagnostic systems. Such antibodies may be employed, for
instance, to diagnose a disease or disorder by
determining specifically what T-cell antigen receptor or
antigen molecules are produced, or, if coupled to a toxin
5   moiety, to substanially eliminate a T-cell population.
The chimeric polypeptides will be useful in interfering
with antigen binding by T-cell receptors characteristic
of that population and in efforts to screen for various
agents capable of binding to T-cell receptors.
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BRIEF DESCRIPTION OF THE FIGURES
        Figure 1 depicts the deletion of CH1 exon from
pMVγ2. Figure 1A is a map of pMVγ2. E_H is the Ig heavy
chain enhancer; C_{H1}, H, C_{H2}, and C_{H3} represent,
15   respectively, the mouse γ2a heavy chain constant region
1, hinge, constant region 2, and constant region 3
domains. Hyg represents the hygromycin resistance gene.
Figure 1B is a schematic description of steps involved in
deleting the CH1 exon. The ΔC_{H1} fragment was first
20   ligated with the adapter oligonucleotide shown, further
gel purified, and then used in the final ligation step
with the vector fragment to generate the construct shown
in Figure 1c, which is a map of pMVγ2(ΔC_{H1}).
        Figure 2 is a map of pCMVγ2(ΔC_{H1}). CMV denotes
25   the ~700 base pair enhancer promoter from human
cytomegalovirus.
        Figure 3 depicts the deletion of trans-membrane
domains from β-TCR. Figure 3A is a hydropathy analysis
of predicted amino acid sequence of β-TCR and its
30   alignment with a restriction endonuclease map of the
cDNA. Figure 3B shows the deletion of trans-membrane
domain from β-chain, and ligation with adapter
oligonucleotide for subsequent cloning into Bluescript
vector. Nucleotides #33 and #34 of this adapter
35   oligonucleotide are modified from the naturally occurring
nucleotides at these positions in the intron so as to
introduce an XbaI site.

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Figure 4 is a map of the pCMVβ8.1(ΔTM)-γ2a(ΔCH1) construct.

Figure 5 represents the amino acid sequence from the amino-terminus of the chimeric Vβ8.1/mouse γ2a protein.

Figure 6 depicts quantitative fluorescence histograms of Jurkat cells. Equal amounts of a reference antibody (αVβ8, line 2 in parts A, B, C, &D) and test antibodies were used to stain 5x10^5 Jurkat cells. The staining patterns were visualized by indirect immunofluorescence using flow cytometry on a Becton-Dickinson FACSmate. The staining pattern produced by the specific antibodies are shown by line 3 in each panel. Line 1 in each panel depicts the background staining profile of the cells. The specific test antibodies resulting from immunization of mice with the chimeric β-TCR are: 9B59.10 (Panel A), 9G47.9 (Panel B), 9A15.6 (Panel C), and 9B47.14 (Panel D).

DETAILED DESCRIPTION

The present invention provides novel nucleic acid constructs which, upon expression, produce polypeptides useful for immunizing target immune systems to raise antibodies specifically reactive with native T-cell receptor epitopes. The antibodies exhibit specific binding to native conformation epitopes to provide powerful diagnostic and therapeutic reagents. The present invention also provides generalized techniques for producing fusion polypeptides which are, typically, both soluble and secreted, and possess structural features exhibiting unusually high epitopic similarity to native T-cell receptor molecular structures.

In accordance with one aspect of the present invention, novel gene fusions are provided which comprise a T-cell receptor extracellular domain, such as the variable and constant domains of a T-cell receptor polypeptide chain, and constant region domains of another immunoglobulin superfamily gene, typically a mouse IgG

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heavy chain lacking a complete CH1 domain. The resulting
gene fusion encodes a hybrid polypeptide than can be
secreted by the producing cell in a soluble form,
preferably with post-translational modifications
5   characteristic of polypeptides naturally occurring in
T-cells. By careful selection of a combination of a
desired T-cell receptor V_α or V_β gene and immunoglobulin
gene domains, as guided by the rules described herein,
the natural conformations of the T-cell receptor domains
10   and the immunoglobulin domains are substantially
obtained.

A preferred embodiment of the present invention
includes the use of these improved antigens to
efficiently produce polyclonal or monoclonal antibodies
15   specific for a selected T-cell receptor molecular
structure, in some instances substantially free of any
immunological response to the non-TCR epitopes or to
unnatural conformational epitopes. The present invention
also includes the antigens themselves, their use in
20   structural and functional characterizations, and the uses
of both antigens and their reactive antibodies as
diagnostic, therapeutic or investigative agents.

General Definitions

25   In order that the invention may be more
completely understood, several definitions are set forth.
As used herein, the term "immunoglobulin" refers to a
protein substantially encoded by one or more
immunoglobulin gene segments. The recognized
30   immunoglobulin genes include κ, λ, α, γ, δ, ϵ, and μ
constant region genes, as well as the myriad
immunoglobulin variable region genes. See, generally,
Hood et al., Immunology, 2nd Ed. (1984) Benjamin Pub.
Co., Menlo Park, CA.

35   The T-cell antigen receptor (TCR) appears to
comprise at least two different polypeptide chains in a
heterodimer complex. This complex normally contains one
alpha polypeptide chain and one beta polypeptide chain.

These two chains are normally held together by an inter-
chain disulfide bond. Each of the alpha and beta chains
contains one segment designated a variable region (V),
and one segment designated a constant region (C).

5   Analogous to the alpha chain, the variable and constant
regions of the beta chain are designated V_β and C_β,
respectively. In addition to the variable and constant
regions, each chain possesses a relatively hydrophobic
transmembrane segment and a short intracellular domain.

10   T-cell antigen receptor V genes may also be
categorized into categories, either families or
subfamilies. Each subfamily can be characterized by a
consensus sequence and contains similar gene segments
exhibiting greater than about 75% similarity in the DNA

15 sequence. Sixteen mouse V_β sequences, for example, have
been categorized into fourteen different subfamilies by a
proposed arbitrary but simple numerical nomenclature for
the V_β gene segments. See Barth et al., (1985) "The
Murine T-cell Receptor Employs a Limited Repertoire of

20 Expressed V_β Gene Segments" Nature 316:517-523. According
to this nomenclature, members of the same subfamily share
the first digit and differ in second; therefore the $V_{\beta 8.1}$,
$V_{\beta 8.2}$ and $V_{\beta 8.3}$ are all members of the $V_{\beta 8}$ subfamily. A
similar system has been proposed for the human gene

25 segments, and at least subfamilies $V_{\beta 1}$-$V_{\beta 20}$ have been
identified (see, Toyonaga and Mak, op. cit., at pg. 602).

          Similarly, 22 different V_α sequences have been
grouped into 11 subfamilies, and Southern blots have
shwon these families consist of 1 to 10 members each

30 (reviewed in Kronenberg et al., (1986) Ann. Rev. Immunol.
4:529-591). A numerical nomenclature for the mouse V_α
gene segment families has been proposed, analogous to
that described above for the V_β genes. It is understood
that the present invention comprehends additional T-cell

35 antigen receptor variable region subfamilies as they are
discovered and characterized.

          A "soluble" antigen or polypeptide is
characterized by its failure to be sedimented at room

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temperature under low G-force centrifugation out of an
aqueous buffer. Typically, a protein is considered
soluble if the protein, at a temperature greater than
about 5°C, at near neutral pH in the presence of low or
no concentrations of detergent, has a sedimentation value
less than about 10 to 50 svedberg units. Solubility is
particularly important in providing simplicity in
purification of the hybrid T-cell antigen polypeptide
from the producing cell and in presentation of the
purified antigen to an immune system to elicit highly
specific antibodies. Solubility is also important for
use of the fusion polypeptide as a binding receptor for
either ligand specific reagents or cognate antigens.
Secretion of the hybrid immunogen is also important to
maximize ease of purification from producing cells.
Aqueous buffers used in protein chemistry typically have
a buffering compound for providing a pH, typically within
a physiological range of about 5-9, an ionic strength
typically between about 10 mM and about 500 mM, often a
protease inhibitor and a mild detergent. Often a carrier
protein may be added, such as bovine serum albumin (BSA)
or chicken ovalbumin to a concentration of a few mg/ml.
Examples of standard buffers are phosphate buffered
saline, tris-buffered saline or any of a number of
buffers used in protein isolation. See generally,
volumes of Methods in Enzymology; Mahler and Cordes
(1966) Biological Chemistry (2d Ed.) Harper and Row, New
York.

A "natural conformation" for a polypeptide
domain is that secondary and tertiary conformation of the
domain in a hybrid protein exhibiting substantially
identical epitopes to a target immune system as the
native polypeptide would by itself. Such includes,
preferably, antigenic determinants co-translationally or
post-translationally supplied, as by glycosylation or
modification of amino acid residues or other structural
components. Optimally, the tertiary structure of the
hybrid polypeptide closely approximates that of a native

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homologous segment in its natural state, typically in a
natural membrane integrated form. In particular, the
spatial relationship and tertiary and macromolecular
structure of the hybrid domains should retain virtually
identical intra-chain relationships such that the
immunological epitopes eliciting antibodies in a target
immune system are substantially identical to the
structural features of a natural homologous structure.
See Watson et al. (1987) Molecular Biology of the Gene,
Benjamin Pub. Co., Menlo Park, California.

          The term "operably linked" means that a genetic
sequence is positioned on the same molecule so as to
ensure the function of nucleic acid segments or sequences
either upstream or downstream from a given segment.
Those nearby segments typically affect processing or
expression of the given nucleic acid sequence.

          A target immune system is comprised of
essential T-cells and B cells as necessary to
biologically effect the desired immunological response.
Such immune systems will typically be intact animals.
Antigens are presented to such animals by injection, with
or without appropriate adjuvants. Such immunization
should thereby present molecular epitopes to the immune
system in a fashion that allows the T-cells and B cells
to interact to produce the desired response.
Alternatively, such presentation may be performed in
vitro under specifically controlled conditions. See, C.
Reading, J. of Immunol. Methods (1982) 53:261-291.

          In many instances, a target immune system may
be substituted by a library of antibody producing cells.
See, Huse et al. (1989) "Generation of a Large
Combinatorial Library of the Immunoglobulin Repertoire in
Phase Lambda" Science 246:1275-1281. Thus, a broad
repertoire of available gene constructs may be screened
to serve either as sources for producing antigens or for
antibodies against specific antigens. Recombinant
antigens or antibodies may be produced by gene fusion
methods described, e.g., in Cabilly et al., U.S.

4,816,567; Boss et al., U.S. 4,816,397 and Winter, EPO
0239400. These references describe sources for the
various immunoglobulin domains useful in the fusion
proteins of the present invention, along with alternative
5   molecules which may be used as antigens or antibodies.

Finally, in some situations, particularly in
production of antibodies, single chain molecules may be
substituted, as described in, e.g., Huston et al., Proc.
Natl. Acad. Sci. USA 85:423-26 (1988); Bird et al.,
10   Science 323:15-16.

An "accessible" cell surface protein is one
that, as associated with a cellular structure, is
immunogenic when presented to an appropriate target
immune system. Thus, a polypeptide whose epitopes are
15   structurally protected from a target immune system is not
considered immunologically "accessible".

An "immunotoxin" is a molecule which comprises
both a targeting moiety and a toxic moiety. Typically,
the targeting moiety is an antibody binding site. The
20   toxic moiety is toxic to the cell or structure to which
the targeting moiety attaches. Thus, one form of an
immunotoxin is an antibody conjugated to a toxic compound
or reagent, such as ricin, providing a functional highly
specific targeting of the toxin. Immunotoxins are
25   reviewed in I. Pastan et al., Cell 47:641-648 (1986).
Recently, recombinant immunotoxins have been developed
(Chaudhary et al., Nature 339:394-399).

Design Considerations

30   In accordance with the present invention,
genetic constructs have been devised to express
polypeptide immunogens mimicking T-cell receptor
epitopes. These chimeric polypeptides will preferably be
readily secreted in soluble form at high expression
35   levels by cells transformed with the genetic constructs.

An exemplary genetic construct comprises a
chimeric human T-cell receptor (TCR)/IgG construct domain
compound, preferably without a CH1 domain. A chimeric

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protein results from the fusion of polypeptide segments
originating from different proteins. The resultant
protein, also known as a fusion protein, typically
exhibits some biological or physical properties of each
of the proteins from which the chimera is derived. Thus,
fusion proteins of a signal sequence with another protein
may result in secretion of an otherwise cytoplasmic
protein. Such techniques may provide for proteins
exhibiting properties in a single protein characteristic
of each individual parent, often from different species.
Examples of chimeric proteins include chimeric antibodies
providing for molecules exhibiting mouse binding domains
and human constant region domains, and fusion between β-
galactosidase and other proteins (for determining
insertion of cloned segments).

Since hybridoma technology is best understood
in the mouse, the preferred embodiment utilizes a human
$V_\alpha C_\alpha$ or $V_\beta C_\beta$ gene fused with a mouse immunoglobulin gene
fragment. This fusion provides that the human TCR
portion of the chimeric polypeptide serves as the focus
of the immune response when exposed to a target mouse
immune system. It is understood that other target immune
systems and a corresponding immunoglobulin gene may be
substituted for mouse as detailed below.

Because there are inter-chain contact regions
between the variable domain and the constant domain of
the beta chain of the T-cell receptor, the antigens of a
preferred embodiment of the present invention present a
more natural conformation between the variable and
constant domains of the beta receptor by containing both
of these domains as found in a natural beta chain.
Molecular modeling studies have been performed on
immunoglobulin molecules based upon X-ray
crystallographic studies. These studies have indicated
the presence of a ball and socket type of contact between
amino acid residues in V_H and C_{H1} of the antibody heavy
chain. See, Lesk and Chothia, (1988) Nature 335:188-190.
These contact residues have been hypothesized as being

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important for a conformational change occurring upon
antigen binding by the antibody molecule. A homology
search for similar residues at equivalent positions in T-
cell receptor sequences revealed the presence of such

5   residues in the β-TCR sequence, but not in the α-TCR
sequence. Hence in the context of these studies, prior
investigators were forcing a very unnatural juxtaposition
of dissimilar residues between V_α and C_{H1} by substituting
V_α for V_H.

10   Finally, in the preferred constructs of the
present invention, there is no need to produce multiple
chains of T-cell receptors, thus eliminating the
requirement for coordinated processing between different
chains. By appropriate selection of cells for

15 expression, the glycosylation patterns of the alpha or
beta chain may be slightly modified. Moreover, it is
possible to co-transform a construct containing the alpha
chain of the T-cell receptor with another construct
containing the beta chain of the T-cell receptor, thereby

20 providing the possibility for association into homodimers
of alpha and of beta and heterodimers of alpha with beta.
        An exemplary chimeric polypeptide of the
present invention is a chimeric human T-cell receptor
beta chain/mouse IgGγ2a heavy chain protein. The

25 preferred genetic construct contains the human β chain
8.1 subtype (YT35), but any other β T-cell type may be
substituted. The construct is designed to contain:

$$L_\beta-V_\beta-D_\beta-J_\beta-C_\beta(\text{delta TM})-H-C_H2-C_H3,$$
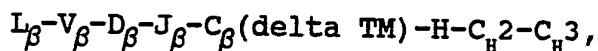
where $L_\beta-V_\beta-D_\beta-J_\beta$ denote one leader peptide, V region, D

30 region and J region of a beta T-cell receptor chain;
C_β(delta TM) denotes the constant region of a beta T-cell
receptor gene with the transmembrane and intracellular
segments deleted (i.e., the 48 carboxy terminal amino
acids of the polypeptide); and $H-C_H2-C_H3$ denote a mouse

35 IgGγ2a heavy chain hinge, second constant region and
third constant regions.
        Alternative chimeric constructs of the present
invention include combinations of V_α, C_α, V_β, and C_β with

constant region domains from immunoglobulins. Thus, alternative constructions include use of less than full length segments of the TCR domains. Thus, instead of using full length V_β or C_β domains, for example, in the described constructs, smaller segments of the domains may be substituted. These segments will ordinarily be at least about 50 amino acids from the appropriate domains, such as the V_β or C_β domains, preferably at least about 80 amino acids and, in preferred embodiments, up to the full length of the domain.

Alternatively, segments from proteins other than immunoglobulins might be used, and may include proteins from other species. Typically, the non-TCR domains are included for the purpose of conferring either secretion or solubility properties to the chimeric protein. Other useful segments such as ovalbumin, serum albumin, and other soluble proteins might be selected. But typically, immunoglobulin gene family domains will be used, more usually constant domains from immunoglobulin genes, from the mouse or other species. Thus, constant region domains from other species may be substituted.

The fusion proteins also may include between each of the specified domains, either additional domains or spacer segments of polypeptide. Such domains will typically be globular domains which will not interfere with the TCR domains. Spacer segments will typically be selected under similar constrains, but will typically be less than about 200 amino acids, preferably less than about 50 amino acids; more preferably less than about 30 amino acids, and in the preferred embodiment, fewer than 10 amino acids.

As the domains, as described, may be less than the entire domain, the hinge region of the CH_2 domain proximate to the TCR domains may be deleted as well. Likewise, the C_α or C_β domains may·be less than the entire domain. In the extreme, particular domains may be totally deleted, for example, one of the constant regions domains may be removed.

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Additions to the described domains may be included. For example, either immunoglobulin or non-immunoglobulin transmembrane or cytoplasmic domains may be added, providing for expression of the antigen on a cell surface. Typical transmembrane domains include those from the TCR proteins or other cell membrane proteins, from the same or other species.

Besides the expression of single chain proteins, the present invention includes multichain antigens. Among the multichain embodiments are combinations of α chain type fusion proteins with β chain type fusion proteins. For example, simultaneous expression, perhaps coordinate, of $V_\alpha C_\alpha C_{H2} C_{H3}$ with $V_\beta C_\beta C_{H2} C_{H3}$ type chains may result in heterodimer formation leading to interchain $V_\alpha C_\alpha$ domains with $V_\beta C_\beta$ domains. Alternatively, an IgG type molecule may result from coexpression of $V_\alpha C_\alpha$ type protein with the fusion protein of $V_\beta C_\beta$ immunoglobulin domains, or the $V_\beta C_\beta$ type protein with a fusion protein of $V_\alpha C_\alpha$-immunoglobulin domains.

The DNA segments will typically further include an expression control DNA sequence operably linked to the chimeric polypeptide coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells and expression therein, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the polypeptides containing the V_α or V_β epitopes.

Human V_α or V_β domain DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably peripheral T-cells. The immunoglobulins and other domains for producing the chimeric proteins of the present invention

will be similarly derived[17] from B cells. They may be
obtained from any convenient mammalian source, including,
mice, rats, rabbits, or other veterbrate capable of
producing antibodies by well known methods. Suitable
5   source cells for the DNA sequences and host cells for
immunoglobulin and other protein expression and secretion
can be obtained from a number of sources, such as the
American Type Culture Collection ("Catalogue of Cell
Lines and Hybridomas," Fifth edition (1985) Rockville,
10   Maryland, U.S.A.). Immunoglobulin domains have been well
defined and particular sequences may be found in, e.g.,
E. Kabat et al., "Sequences of Proteins of Immunological
Interest" U.S. Dept. Health and Human Services (1983).

In addition to the chimeric polypeptides
15   specifically described herein, other "substantially
homologous" modified immunoglobulins can be readily
designed and manufactured utilizing various recombinant
DNA techniques well known to those skilled in the art.
For example, the C_{H2} and C_{H3} domains can vary from the
20   native sequences at the primary structure level by
several amino acid substitutions, terminal and
intermediate additions and deletions, and the like. In
general, modifications of the genes may be readily
accomplished by a variety of well-known techniques, such
25   as site-directed mutagenesis (see, Gillman and Smith,
Gene 8:81-97 (1979) and Roberts, S. et al, Nature
328:731-734 (1987).

The nucleic acid sequences of the present
invention capable of ultimately expressing the desired
30   chimeric polypeptides can be formed from a variety of
different polynucleotides (genomic or cDNA, RNA,
synthetic oligonucleotides, etc.) and components (e.g.,
J, D, and C regions), as well as by a variety of
different techniques. Joining appropriate genomic
35   sequences is presently the most common method of
production, but cDNA sequences may also be utilized (see,
European Patent Publication No. 0239400).

As stated previously, the DNA sequences will be
expressed in hosts after the sequences have been operably
linked to (i.e., positioned to ensure the functioning of)
an expression control sequence. These expression vectors
are typically replicable in the host organisms either as
episomes or as an integral part of the host chromosomal
DNA. Commonly, expression vectors will contain selection
markers, e.g., tetracycline or neomycin, to permit
detection of those cells transformed with the desired DNA
sequences (see, e.g., U.S. Patent 4,704,362).

Cell Transformation with the Gene Fusions

Having constructed gene fusions, according to
design considerations as described, expression of the
antigen products is desired. Appropriate vectors and
cell types may be selected which will produce an antigen
or reagent which most closely parallels the desired
product. For immunogen purposes, it is expected that
glycosylation may be one of the primary criteria upon
which a cell type selection will be based. Thus,
particular cell types may be chosen for their expression
properties. In other circumstances, efficiency in
secretion or some other cellular property may be more
desirable.

Although microorganisms may be used, eucaryotic
cells are preferred to express and produce the
polypeptides of the present invention (see, Winnacker,
"From Genes to Clones," VCH Publishers, N.Y., N.Y.
(1987)). Mammalian cells are actually most preferred,
because a number of suitable host cell lines capable of
secreting intact immunoglobulin-like proteins have been
developed in the art, and include the CHO cell lines,
various COS cell lines, HeLa cells, myeloma cell lines,
etc., but preferably transformed B-cells or hybridomas.
Expression vectors for these cells can include expression
control sequences, such as an origin of replication, a
promoter, an enhancer (Queen, et al., Immunol. Rev.
89:49-68 (1986)), and necessary processing information

sites, such as ribosome binding sites, RNA splice sites,
polyadenylation sites, and transcriptional terminator
sequences. Preferred expression control sequences are
promoters derived from immunoglobulin genes, SV40,
adenovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of
interest (e.g., the V_β domain and C_{H2} domain encoding
sequences and expression control sequences) can be
transferred into the host cell by well-known methods,
which vary depending on the type of cellular host. For
example, calcium chloride transfection is commonly
utilized for prokaryotic cells, whereas calcium phosphate
treatment or electroporation may be used for other
cellular hosts. (See, generally, Maniatis, et al.,
Molecular Cloning: A Laboratory Manual, Cold Spring
Harbor Press, (1982)).

Once expressed, the chimeric polypeptides of
the present invention can be purified according to
standard procedures of the art, including ammonium
sulfate precipitation, affinity columns, column
chromatography, gel electrophoresis and the like (see,
generally, Scopes, R., Protein Purification, Springer-
Verlag, N.Y. (1982)). Substantially pure polypeptides of
at least about 90 to 95% homogeneity are preferred, and
98 to 99% or more homogeneity most preferred, for
pharmaceutical uses. Once purified, partially or to
homogeneity as desired, the polypeptides may then be used
therapeutically (including extracorporeally) or in
developing and performing assay procedures,
immunofluorescent stainings, and the like. (See,
generally, Immunological Methods, Vols. I and II,
Lefkovits and Pernis, eds., Academic Press, New York,
N.Y. (1979 and 1981)).

Antibodies

Once the antigens of the present invention have
been constructed and expressed, thereby producing soluble
and/or secreted proteins for use as antigens, antibodies

may be produced by presenting the hybrid protein to a
target immune system. For production of polyclonal
antibodies, an appropriate target immune system is
selected. Typically a mouse is used, of the appropriate
5   strain, usually producing heavy chains of the type
selected for the fusion domains of the hybrid antigen.
The substantially purified antigen is presented to the
immune system in a fashion determined by the mouse strain
and other parameters well known to immunologists.
10   Typical sites for injection are in the footpads,
intramuscularly, intraperitoneally, or intradermally. Of
course, different animals may be substituted for a mouse.
        Typically, an immunological response is assayed
by use of an immunoassay. Normally such immunoassays
15   involve some purification of a source of antigen, for
example, produced by the same cells and in the same
fashion as the antigen was produced. The immunoassay may
be a radioimmunoassay, an enzyme-linked assay, a
fluorescent assay, or any of many other choices, most of
20   which are functionally equivalent but may exhibit
advantages under specific conditions.
        Monoclonal antibodies with affinities of 10^8 M^{-1}
preferably 10^9 to 10^{10}, or stronger will typically be made
by standard procedures as described, e.g., in Harlow and
25   Lane (1988), Antibodies: A Laboratory Manual, Cold Spring
Harbor Laboratory or Goding (1986), Monoclonal
Antibodies: Principles and Practice (2d edition) Academic
Press, New York. Briefly, appropriate animals will be
selected and the desired immunization protocol followed.
30   After the appropriate period of time, the spleens of such
animals are excised and individual spleen cells fused,
typically, to immortalized myeloma cells under
appropriate selection conditions. Thereafter the cells
are clonally separated and the supernatants of each clone
35   are tested for their production of an appropriate
antibody specific for the desired region of the antigen.
Note that because the heavy chain regions of the hybrid
antigen can be selected to be non-immunogenic in the

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target immune systems, there is inherently an advantage in the screening of the resultant monoclonal antibody supernatants since clones producing antibodies specific for the immunoglobulin heavy chains should be rare.

5   It will be appreciated by those of skill in the art that the binding regions of the antibody molecules contain critical amino acid residues for contact and binding in epitope recognition. Thus, these complementarity determining regions (CDR's) which produce

10   specificity of binding are the structural features conferring specificity in binding. For the binding functions of these antibodies, molecules which exhibit sufficiently similar spatial relationship of these CDR's will be functionally equivalent molecules. Gene fusions

15   or deletions leading to molecules which provide CDR's based on antibodies of the present invention will obviously serve, in many cases, to provide equivalent functions.

20   Selecting Specific V$_\beta$ Antibodies

One important objective of this invention is the development of an array of antibodies capable of distinguishing the various forms of the T-cell receptor molecules. Thus, it is desirable to produce antibodies

25   analogous to anti-idiotypic antibodies of immunoglobulin genes.

A large array of antigens might be produced from different V$_\beta$ chains against which each of a large number of different T-cell receptor specific antibodies

30   might be tested. By comparing which antibodies are specific for a single or few subfamilies or specific V$_\beta$ chains, those antibodies will become diagnostic for particular receptor subfamilies or chains. Antibodies which show broad specificity for most or all subfamilies

35   find different uses in diagnostic or therapeutic contexts as described below.

Diagnostic, Therapeutic and Investigative Utilities

The present invention provides means for the
efficient and economical production of antibodies
directed to each of the various T-cell receptor chains.
By providing the means for producing enormous amounts of
a soluble and secreted antigen which elicits,
specifically, only antibodies reactive with the desired
structural domains, the immunization of target immune
systems is dramatically simplified. This allows the
generation of a large repertoire of antibodies specific
for binding to individual members of T-cell types,
families, subfamilies or combinations.

The hybrid polypeptides of the invention are
also useful in structural and functional studies on the
T-cell receptor. Besides serving as substrates or
binding domains of specific forms of receptor, these
analogues may serve in conformational studies which
approximate the native configurations as subjects. Such
studies provide approximate structural or functional data
which might be easily verified with smaller amounts of
native TCR chains.

The solubilized chimeric receptors also serve
as binding competitors to block normal cellular binding
of particular ligands, thus serving to clear the ligand
or to compete with the physiological binding by cells.
The effective amount of such a receptor sufficient to
interfere with the binding of an antigen to a T-cell
antigen receptor will depend, for instance, upon the
particular affinity of the receptor for the ligand in
question. A likely effective dose of receptor to be
administered is between 100 μg to 100 mg daily for a
human subject. Thus, these antigens which exhibit
binding domains with the native protein binding
specificity may be used in a fashion equivalent to
standard antibodies for certain purposes. Qualitative
and quantitative assays for ligand binding are well known
to those skilled in the art. See, for instance, E.

Harlow and D. Lane, (1988) Antibodies: A Laboratory
Manual (Cold Spring Harbor).

These antigens may also be used to simulate the
native binding domain to investigate what ligand may
5   serve as an agonist or antagonist for the selected fusion
polypeptide. Thus, the binding domain is used as a
screen to select those ligands which will bind to the
encoded alpha or beta chain variable region.

With the antibodies of the present invention, a
10   repertoire of antibodies may be generated which can
easily distinguish the particular type, family or
subfamily of a given cell. Thus, if a clonally
overproduced cell type is produced, as may occur in, for
example, a leukemia condition, it is possible to
15   determine the type of cell involved, and then readily
produce a targeted immunotoxin for substantially
eliminating those cells. By substantially eliminating,
it is meant that the cell number is reduced by 50%,
preferably 80%, most preferably 90% or more.

20   Determination of the cell type can also serve
as a diagnostic tool for the purpose of investigation
into the problems of, or the treatment of a condition.
The use of antibodies raised against T-cell receptors
have been successful in treating experimental allergic
25   encephalomyelitis (EAE). See, e.g., Acha-Orbea et al.
(1988) Cell 54:263-273; and Urban et al. (1988) Cell
54:577-592.

In addition, the antibodies or binding proteins
of the present invention are useful as idiotypic
30   antibodies to determine specifically what T-cell receptor
or antigen molecules are expressed within an organism.
Such may be important in, among other disorders or
diseases, T-cell malignancies, autoimmune diseases,
transplant/graft rejection reactions, host versus graft
35   disease, cancers, solid tumors, allergies and various
infectious diseases. Quantitative or qualitative
measurement of types of expressed T-cell antigen
receptors allow diagnosis and monitoring of a patient

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with multiple infections or a disease involving a
plurality of responses. It is understood that both
qualitative and quantitative measurements are embraced by
the present invention. By use of the term diagnosis is
5   also meant the monitoring of the severity or progression
of a disease or disorder or the recovery of a subject
from a disease or disorder.

Accordingly, patient samples are tested for
expression of characteristic T-cell receptors. Test
10   samples may comprise biological fluids including but not
limited to blood, plasma, serum, saliva, urine, spinal
fluid, synovial fluid, amniotic fluid and cranial fluid.
Quantitation may be performed by comparison to known
standard samples.

15   The following examples are offered by way of
illustration, not by limitation.

EXPERIMENTAL

Vector

20   1.   Deletion of C_{H1} exon from the expression vector.
The antibody domains chosen for incorporation
into the chimeric β-TCR construct were derived from the
vector pMVγ2. This vector was itself derived by
replacing the XbaI-BamHI fragment of PVγ1 (Queen et al.
25   (1989), Proc. Natl. Acad. Sci. USA 86:10029-10033)
containing the human constant region with an XbaI-BglII
fragment containing the mouse γ2a constant region, a ClaI
linker being used to connect the BamHI and BglII ends.
A map of pMVγ2 along with pertinent structural features
30   and relevant restriction sites is presented in Fig. 1A.
The vector contains the full mouse gamma 2a constant
region downstream of a unique XbaI cloning site. The
first modification of the vector involved deletion of the
C_{H1} exon. This was accomplished as outlined in Fig. 1B.
35   Specifically, 50 μg of pMVγ2 were digested with XbaI and
ClaI to produce a 6.7 kb "vector" fragment, and a 4.1 kb
fragment containing the full mouse gamma 2a constant
region. These fragments were recovered by electroelution

25

following fractionation on a preparative 0.9% agarose
gel. In order to delete the C_{H1} exon from the γ2a
fragment, the 4.1 kb XbaI/ClaI fragment was subsequently
digested with StuI. StuI cuts in the intron between the

5   C_{H1} and Hinge (H) exons, 47 nucleotides downstream of the
intron donor site. Thus, the purified 4.1 kb γ2a
fragment was further digested with StuI, and the
resultant 1.6 kb XbaI/StuI "C_{H1} fragment" and 2.3 kb
StuI/ClaI fragments recovered following a second round of

10   agarose gel fractionation and electroelution. The 2.3 kb
StuI/ClaI fragment, which contains the Hinge (H), and
second and third heavy chain constant region exons (C_{H2},
C_{H3}), will be henceforth referred to as the ΔC_{H1} fragment.
The ΔC_{H1} fragment was ligated with the following synthetic

15   adapter oligonucleotide to restore an XbaI site at its 5'
end:

            5' CTAGAGGTTCACAAGTGATTAGTCTAAGG 3'
            3'   TCCAAGTGTTCACTAATCAGATTCC 5'
20

the sequence of this adapter oligonucleotide is derived
from the naturally occurring sequence in the intron
between the C_{H1} and H exons for 28 of the 29 nucleotides
upstream of the StuI site in this intron. The first

25   nucleotide of this oligonucleotide, shown underlined, was
modified from the naturally occurring nucleotides so as
to reconstitute the desired XbaI site. The naturally
occurring nucleotide at this position in the intron is a
G. The first three nucleotides of the hexanucleotide

30   StuI site are highlighted in boldface.
            The adapter ligated 2.3 kb ΔC_{H1} fragment was
purified away from unligated excess adapter by
fractionation on an agarose gel and recovered by
electroelution. Finally, this was religated to the 6.7

35   kb XbaI/ClaI "vector" fragment from step 1 above to
reconstitute a ΔC_{H1} version of the original vector,
referred to as pMVγ2(ΔC_{H1}), shown in Fig. 1C. Preparative
amounts of this first modification of the vector were

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obtained by transformation of <u>E.coli</u> DH1 competent cells
with the products of ligation of the 6.7 kb "vector"
fragment and the 2.3 kb adapter ligated ΔC_{H1} fragment.
Transformed colonies were positively identified by their
Amp^r phenotype, and the ΔC_{H1} modified vector was purified
in preparative quantities using conventional plasmid
purification procedures (Sambrook et al., (1989) in
<u>Molecular Cloning: A Laboratory Manual</u> (Second Edition),
Cold Spring Harbor Laboratory). The sequence of the
artificial junction between the XbaI and StuI sites was
verified by DNA sequence analysis of the plasmid.

2. Replacement of Immunoglobulin heavy chain
promoter/kappa enhancer with the human cytomegalovirus
enhancer/promoter.

Comparative studies carried out at Protein
Design Labs on relative strengths of promoter enhancer
combinations leading to highest levels of expression of a
desired gene product have established the human
cytomegalovirus (HCMV) enhancer/promoter as superior to
the immunoglobulin heavy chain enhancer/κ promoter
combination for the production of humanized antibodies in
myeloma cells. Since we wished to achieve the highest
possible levels of expression of the chimeric Vβ8.1/mouse
γ2a protein, we further engineered the pMVγ2(ΔC_{H1}) vector
by replacing the immunoglobulin heavy chain enhancer/κ
promoter with the HCMV enhancer/promoter.

The HCMV enhancer/promoter was isolated as an
approximately 600 bp FnuD2 fragment extending from
position -522 to position 72 (numbered according to
Boshart et al. (1985) <u>Cell</u> 41:521-530), and an EcoR1
linker was added at the 5' end and an XbaI linker at the
3' end.

The deletion of the immunoglobulin
enhancer/promoter from pMVγ2(ΔC_{H1}) was effected by
digestion of 8 μg of pMVγ2(ΔC_{H1}) with XbaI and HindIII.
This digest generated a 4.5 kb fragment containing the
γ2(ΔC_{H1}) and Hygromycin^r genes (referred to as fragment A)

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and a 4.4 kb fragment containing the Ig promoter/κ enhancer, pBR322 ori sequences, and a SV40 polyadenylation fragment (referred to as fragment B). Following size fractionation by agarose gel

5   electrophoresis and recovery by electroelution, fragment A was set aside for later use, and fragment B was subjected to secondary digestion with EcoRI. This produced three fragments: a 2.64 kb EcoRI/HindIII fragment containing pBR322 ori sequences, and the SV40

10   fragment polyadenylation signal; a 1.13 kb EcoRI/XbaI fragment containing the Ig heavy chain promoter; and a 0.7 kb EcoRI fragment encoding the κ enhancer. The 2.64 kb EcoRI/HindIII fragment was recovered by electroelution following agarose gel fractionation, and treated with

15   alkaline phosphatase from calf-intestine to prevent self ligation and polymerization in subsequent steps.

The 4.5 kb XbaI/HindIII fragment (fragment A), 2.6 kb phosphatased EcoRI/HindIII pBR322 ori, SV40 fragment, and 600 bp EcoRI/XbaI HCMV enhancer/promoter

20   encoding fragments were combined in a triple ligation experiment to produce the desired vector, designated pCMVγ2(ΔC_{H1}), shown in Fig. 2. This vector was recovered by transformation of competent E.coli with an aliquot of the triple ligation reaction mixture. Potential colonies

25   containing the desired vector construct were selected on the basis of their Amp^r phenotype, and plasmid mini-preps from Amp^r colonies were purified and analyzed by diagnostic restriction endonuclease digests to identify those colonies containing the correct vector construct,

30   as predicted from the map in Fig. 2.

3. Deletion of the Trans-membrane Domain from the β-TCR constant region.

A full length cDNA insert containing a

35   functionally rearranged human Vβ8.1 T-cell receptor sequence was obtained from the clone YT35 (Yanagai et al., (1984) Nature 308:143-149) as an XbaI/SphI fragment,

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and transferred into a Bluescript vector (Stratagene) and designated pBSβ8.1.

Hydropathy analysis (Chou and Fasman, (1978) Adv. in Enzymol. 47:45-147) of the predicted amino acid sequence encoded by this cDNA serves to identify a putative membrane spanning region at the carboxy terminal end of this protein. A unique StuI site is situated just upstream of the trans-membrane (TM) and cytoplasmic domains of the β T-cell receptor, and hence, serves to delineate them from the extra-cellular V,D,J, and C domains (Fig. 3 A). Analytical digests of pBSβ8.1 plasmid isolated from DH1 strain of E.coli, with StuI showed the plasmid refractory to digestion with this enzyme. This was discovered to be due to a methylation modification of a cytosine residue contained within the StuI site by dcm methylase, which rendered the site non-cleavable by StuI (New England Biolabs, 1988-1989 product catalog, p.143). Hence, the plasmid pBSβ8.1 was transfered into E.coli HB101, which is dcm modification negative. Thus, 112 μg of pBSβ8.1, isolated from HB101 transformed cells, were digested with XbaI and StuI. The 817 bp β-TCR insert deleted of its trans-membrane domain (ΔTM) was recovered by electroelution after fractionation in 1% agarose gel.

The purified β(ΔTM) fragment was ligated with a synthetic 37-mer oligonucleotide, as outlined in Fig. 3B, to restore the StuI site abutting the fragment, and reintroduce an XbaI site at its 3' end. The first 15 nucleotides of this oligo were derived from the Cβ sequence to preserve the β-TCR residues immediately extracellular to the plasma membrane. The remaining 22 nucleotides of this oligo-nucleotide are derived from the intron donor site and the first 16 nucleotides downstream of it, in the intron between the C_{H1} and H exons from the vector pMVγ2. Nucleotides #33 and #34 of this adapter oligonucleotide are modified from the naturally occurring nucleotides at these positions in the intron so as to introduce an XbaI site, to facilitate cloning of the

transmembrane deleted insert fragment into the (ΔC_{H1})
modified vector (see <u>Vector</u>, above, and Fig. 4 below).

The adapter ligated β-TCR(ΔTM) fragment was
purified from unligated adapter oligo by fractionation on
an agarose gel and recovered by electroelution. It was
then introduced into the XbaI site of the Bluescript
vector (Stratagene) for amplification by preparative
isolation of plasmid from transformed bacteria. This
plasmid was designated pBSβ8.1(ΔTM).

<u>Generation and Verification of the Final Construct</u>

Generation of the final construct is outlined
below, and a map of the construct is presented in Fig. 4.
For generation of the final construct, the vector
pCMVγ2(ΔC_{H1}) was linearized by digestion with XbaI,
followed by phosphatasing of 5' termini with calf
intestinal alkaline phosphatase. Meanwhile, the ~880 bp
β8.1(ΔTM) fragment was purified by electroelution of XbaI
digested pBSβ8.1(ΔTM) fractionated on an agarose gel.
The β8.1(ΔTM) insert was then ligated into the XbaI
linearized and phosphatased vector and the products of
the ligation reaction used to transform competent E.
coli.

Transformed colonies were selected on the basis
of resistance to ampicillin, and plasmids analyzed by
diagnostic restriction digestion of "mini-prep" DNAs
purified from small scale cultures. The mini-prep DNAs
were digested with XbaI to verify presence of an insert
fragment of the expected size (880 bp). Finally,
orientation of insert relative to promoter was determined
by digestion of the DNAs with StuI. Those clones with
insert in the same orientation as the promoter were
chosen for final sequence verification of relevant
portions of the molecule. The results from DNA sequence
analysis of the constructs at the 5' end of the insert
and at the 3' insert/vector junction containing the
artificial splice donor site, across the XbaI and StuI
sites, and into the C_{H1}-Hinge intron, agreed exactly with

30
the expected sequences at these junctions, and verified
the correctness of the the construct. Following sequence
confirmation of the identity of the constructs, the CMV
enhancer/promoter construct was designated pCMVβ8.1(ΔTM)-
5   γ2a(ΔC_{H1}).

Expression of Chimeric T-Cell Receptor/Immunoglobulin Protein

1.   Transfection of Cells and Detection of Expressing
10 Cell Lines.

For expression of the CMV enhancer/promoter
driven chimeric Vβ8.1 T-cell receptor/immunoglobulin
heavy chain construct, the construct [pCMVβ8.12(ΔTM)-
γ2a(ΔC_{H1})] was linearized with BamHI. The linearized DNA
15 was extracted with phenol and chloroform, ethanol
precipitated, and resuspended in 1X PBS at a
concentration of 1 μg/μl (measured by A_{260}). The
constructs were transfected by electroporation into two
myeloma cell lines, Sp2/0, and 25-1. Sp2/0 is a B-cell
20 tumor line which does not secrete endogenous
immunoglobulins, while 25-1 is an Sp2/0 derived cell line
generated at Protein Design Labs, Inc., Mountain View,
CA, which secretes high levels of a humanized anti-TAC κ-
light chain as Bence-Jones homodimers (Queen et al.,
25 (1989) Proc. Natl. Acad. Sci. USA 86:10029-10033).

Hence, 10^7 cells in 1 ml PBS from each of these
lines were transfected by electroporation (Chu et al.,
(1987) Nucl. Acids Res. 15:1311-1326) with 20 μg of the
BamHI linearized construct from above using a Bio-Rad
30 Gene Pulser, and transformed cells were selected on the
basis of resistance to hygromycin. Hygromycin selection
was carried out on a daily basis, starting 24 hr after
transfection, by feeding the cells with media
supplemented fresh daily with 700 μg/ml ($\pm$10%)
35 hygromycin. This daily feeding was continued for 10-12
days, after which surviving cells were allowed to reach
confluence in the 96-well micro-titre plating dishes.
Cells were expanded in selective media by passage from

the 96-well plates into 24-well plates, and supernatant
aliquots were assayed for presence of chimeric β-TCR/γ2a
protein by ELISA assay once the culture reached
confluency in 24-well plates.

5   The ELISA assay for detection of the chimeric
β-TCR protein was carried out as follows: ELISA plates
(Immulon) were coated with 200X dilution of goat anti-
mouse IgG (Heavy + Light chain, Tago Immunochemicals) in
1X PBS at 4°C for overnight to several days. Following
10   blocking of non-specific protein binding sites in the
plate with 1% Bovine Serum Albumin (BSA), 0.1% Tween-20,
0.01% sodium azide in 1X PBS, for at least 2 hr at 4°C,
the plates were incubated with test antigens
(supernatants from confluent 24-well plate cultures of
15   hygromycin resistant transfected cell lines), in
duplicate. The plates were incubated with antigen for 2
hr at room temperature, and then washed 3X with 200
μl/well of 1X phosphate buffered saline (PBS)/0.1%
TWEEN-20. The plates were then incubated for 1 hr at
20   room temperature with 200X dilution of horseradish
peroxidase (HRP) conjugated rabbit anti-mouse Igγ2a
(Zymed Immunochemicals) in 1X PBS as the detection
reagent. Positive cell lines were identified by virtue
of a green color reaction upon addition of HRP substrate
25   (Biorad).

          Table 1 below summarizes the number of
expressing primary cell lines obtained from transfection
of Sp2/0 and 25-1 cells with the construct pCMVβ8.1(ΔTM)-
γ2a(ΔCH1).

30

Table 1. Ratio of expressing cell lines transfected with
Construct

Cell Line	pCMVβ8.12(ΔTM)γ2a(ΔC_{H1})
Sp2/0	4/10
35 | 25-1 | 32/32 |

32

The results show that the frequency of expression of the
chimeric protein was significantly higher in 25-1 cells
(>95%) than in naive Sp2/0 cells (~35%).

5   This difference in frequency of expression
between 25-1 and Sp2/0 cells was paralleled by the level
of expression of the chimeric β-chain (as measured by
ELISA assay using mouse anti-TAC as a concentration
standard). The highest level of expression observed from
the Sp2/0 primary transfectants was 90 ng/ml for the CMV

10   enhancer/promoter driven construct. By contrast, the
highest level of expression observed in 25-1 primary
transfectants was 200 ng/ml for the CMV enhancer promoter
driven construct. Thus, comparing cell lines, 25-1 was
superior to Sp2/0 in both the frequency of expression as

15   well as the overall level of expression.

        In summary, the data presented above from ELISA
assays of supernatants from primary transfectants
establish the following three facts: (1) the frequency
of expression from transfection of the chimeric β-TCR

20   construct is greater in 25-1 cells than in Sp2/0 cells;
(2) the overall level of expression attained is greater
from 25-1 cells than from Sp2/0 cells; and (3) the
transfectants observed are secreting a protein which
contains mouse immunoglobulin-γ2a determinants.

25

2. Immunochemical Characterization of the Secreted
Protein.

        The ELISA data presented above established, at
a preliminary level, the secreted expression of a protein

30   containing mouse immunoglobulin-γ2a determinants, as
evidenced by the immuno-crossreactivity with the HRP-
conjugated 2° reagent in the assay. In order to further
characterize the biochemical properties of the secreted
molecule, and thereby more firmly establish its identity,

35   we analyzed the secreted protein by SDS-PAGE following
immunoprecipitation of metabolically labelled proteins
from ELISA positive cell lines.

33

For this analysis, two ELISA positive cell
lines were selected at random, and cells arising from the
primary transfection were expanded into 10 ml cultures
for metabolic labelling of proteins by incubation with

5   ^{35}S-methionine. The metabolic labelling and subsequent
immunoprecipitation were carried out essentially as
described in Harlow and Lane, (1988) in Antibodies, A
Laboratory Manual, Cold Spring Harbor, New York.
Briefly, one confluent plate each of cells (0.5-1x10^7

10   cells) from experimental (transfected) and control
(untransfected) lines were harvested by centrifugation at
1000 rpm in a benchtop clinical centrifuge, per labelling
reaction. The cell pellets were washed 2X with sterile
1X PBS (37°C), 1X with methionine free media (37°C), and

15   then resuspended in 1 ml each of methionine free media
(Dulbecos Modified Eagle Medium, without L-methionine,
without L-glutamine, GIBCO) supplemented with dialyzed
10% fetal calf serum (FCS, Hyclone), and 2 mM glutamine.
The cells were incubated at 37°C for 30-45 min to deplete

20   endogenous methionine pools. Protein labelling was then
carried out by addition of 100-200 μCi of ^{35}S-methionine
(Amersham, ≥1,000 Ci/mmol), and a further incubation of
the cultures at 37°C for 5-7 h. The metabolically
labelled cells were harvested as described above, and the

25   supernatant fractions saved for analysis of secreted
proteins by immunoprecipitation as described below. In
order to analyze intracellular synthesis, the cells were
lysed by resuspending the cell pellets in 0.9 ml ice cold
Tris buffered saline (TBS, 50 mM Tris-Cl, pH 7.5, 150 mM

30   NaCl), and lysed immediately by addition of 5% nonidet-
P40 (NP40) to 0.5% final concentration. The protease
inhibitor, phenylmethylsulonyl flouride (PMSF) was added
to a final concentration of 1 mM, and the lysate(s)
incubated on ice for 20 min. Cellular debris/insoluble

35   material was removed from the cell lysate by
centrifugation at 12,000 x g for 10 min at 4°C. The
supernatant from this step, containing cell lysate, was
recovered and saved for further analysis.

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Preliminary immunoprecipitations with this
crude lysate and supernatant fractions revealed a high
level of background, presumably from non-specific
adsorption of metabolically labelled proteins to the
5   antibody conjugated agarose beads employed in the
experiment. Hence, these two fractions were routinely
pre-cleared by overnight adsorption to a rabbit IgG-
agarose beads (Sigma, 100 µl beads/ml lysate or
supernatant), which served as a non-specific adsorbent.
10   The cleared supernatants were recovered following a brief
centrifugation to remove the non-specific adsorbent, and
used in subsequent immunoprecipitation experiments
described below.

Immunoprecipitations of radiolabelled protein
15   were carried with 100 µl aliquots from pre-cleared cell
lysates and supernatants prepared as described above.
The samples were immunoprecipitated directly with both
rabbit IgG-agarose and protein-A agarose, using 5 µl
protein conjugated agarose resin/100 µl sample, at 4°C
20   for 1-3 hr with constant agitation using a TOMY micro
tube mixer. Alternatively, the samples were also
immunoprecipitated in a two step procedure using a
purified 1° antibody (1 µl/100 µl sample) at 0 - 4°C for
1-3 hrs, followed by protein A-agarose as described
25   above. The samples were then prepared for washing by a
brief centrifugation at 12,000g for 15 - 30 seconds. The
radioactive supernatant was removed by aspiration. The
immunecomplex coated agarose beads were washed 3X with 1
ml/wash of a solution containing 0.5% NP-40, 0.2% sodium
30   deoxycholate, and 0.1% SDS in 1X TBS (NDS solution),
followed by a final wash with 1X TBS (to remove
detergents). The immune complex coated agarose beads
were air dried overnight at room temperature prior to
SDS-PAGE analysis of the immunoprecipitated product.
35   For polyacrylamide gel analysis of the
immunoprecipitated products, the samples were
resolubilized in Laemmli sample buffer (2% SDS, 10%
glycerol, 10 mM DTT (dithiothreitol), and 60 mM Tris-Cl

pH 6.8) in a boiling H_2O bath for 10 min. For analysis on
non-reducing gels, the samples were resolubilized in
Laemmli buffer minus DTT. Aliquots of the resolubilized
samples were then loaded onto 10% or 12% polyacrylamide
5   gels containing 0.1% SDS prepared and run according to
the method of Laemmli, (1970) Nature 277:680-688
described in Harlow and Lane, (1988) in Antibodies, A
Laboratory Manual, Cold Spring Harbor, New York. Two-
dimensional gel analysis of the samples was carried out
10   essentially as described in O'Farrell (1975) J. Biol.
Chem. 250:4007-4021. Analytical experiments using broad
pI range test IEF gels established the pI of the
immunoprecipitated protein was in the neutral pH range.
Consequent fine pI range titration further established a
15   pH range in the IEF gels of 6-8 as optimal for maximal
resolution of the proteins isoforms.   Hence, the
isoelectric focusing gels contained 2% (vol/vol)
ampholytes pH range 4-10, and 4% (vol/vol) pH 6-8
ampholytes (BioRad). Following electrophoresis, gels
20   were fluorographed, dried, and the results visualized by
autoradiography.

Immunoprecipitation of cell lysates or
supernatants with protein-A agarose revealed the
exclusive presence of a major polypeptide band of 68 kD
25   apparent molecular weight in both the intracellular and
supernatant fractions of material from transfected cells,
and its complete absence from untransfected cells. A
protein of identical molecular weight is also
immunoprecipitated with a two step procedure using either
30   affinity purified goat anti-mouse IgGγ (GαmIgγ) as the 1°
antibody followed by protein A-agarose, or goat anti-
human κ (GαHuκ) as the 1° antibody followed by protein A-
agarose.

Hence, the immunoprecipitation data confirmed
35   the secreted expression by transfected cells only (25-1,
as well as Sp2/0), of a protein bearing mouse IgGγ2a
determinants. The data furthermore extend the
observations from ELISA assay and document the

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intracellular expression of this protein by transfected
cells only.   The apparent molecular weight of this
protein, 68 kD, agrees favorably with its predicted
molecular weight, 54 kD, based on knowledge of its

5   predicted primary structure.   The 14 kD difference can be
accounted for by post-translational modifications, e.g.
glycosylation, which are known to occur on antibody
molecules.   A major site for N-linked glycosylation is in
the CH2 domain of the molecule, which is retained in our

10   chimeric construct.

A second major conclusion from the one-
dimensional SDS-PAGE analysis of immunoprecipitated
products is that the putative chimeric β-TCR/γ2a protein,
a chimeric Ig heavy chain, is expressed in the absence of

15   any requirement for assembly with light chain prior to
its secretion from the cell.   This is evidenced by two
observations.   The first observation is that
immunoprecipitation of radiolabelled proteins from
transfected 25-1 cells by either protein A-agarose alone

20   or with Gαmγ followed by protein A-agarose failed to co-
immunoprecipitate a polypeptide in the known size range
of Ig light chains (~25 kD).   The cell line 25-1 was
derived from Sp2/0 by transfecting it with a humanized
anti-TAC κ light chain construct.   Thus, it is expressing

25   high levels of a humanized κ light chain.   The second,
and more conclusive piece of evidence in support of the
notion that the chimeric β-TCR/γ2a heavy chain is
secreted in the absence of any requirement of prior
assembly with a light chain, is its expression in Sp2/0

30   cells.   Sp2/0 cells do not secrete any endogenous light
chain, yet this protein is immunoprecipitated from both
lysate and supernatants of transfected cells only, by
either protein A-agarose alone, or Gαmγ followed by
protein A-agarose.

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3. The Putative Chimeric β-TCR/γ2a Heavy Chain is
Secreted as a Homodimer.

The hinge domain in antibody molecules contains
two important cysteine residues which are responsible for
5   inter-chain disulfide linkages between immunoglobulin
heavy chains. The formation of these interchain
disulfide bonds is important to the structural integrity
of native antibody molecules. We were interested in
determining whether these sites would retain their native
10 properties, i.e. serving to cross-link heavy chain
monomers into dimers by disulfide linkages, in the
chimeric molecules encoded by our construct. Thus, in
order to obtain information regarding the quarternary
structure of the protein, we analyzed its electrophoretic
15 properties under non-reducing conditions.

For this analysis, radiolabelled proteins from
transfected and untransfected cells were
immunoprecipitated as described. Subsequent to drying,
the immunoprecipitated products were resolubilized in
20 Laemmli buffer lacking the reducing agent DTT. The
solubilized immunoprecipitated products were analyzed by
SDS-PAGE on 6% polyacrylamide gels. The pattern of
immunoprecipitated products is similar to that observed
earlier. Both protein A-agarose alone, and GαmIgγ
25 followed by protein A-agarose, specifically
immunoprecipitate a readily detectable product from
transfected cell lysates and supernatants. A major
difference, however, between the results observed in the
absence of reducing agent compared with those in the
30 presence of reducing agent is in the apparent molecular
weight of the immunoprecipitated products. The apparent
molecular weight of the immunoprecipitated product under
reducing conditions (where both intra- and inter-chain
disulfide bonds are reduced) was 68 kD. Under non-
35 reducing conditions, the mobility of this protein in the
supernatant fraction, shifts to approximately 110 kD.

Immunoprecipitation of the intracellular
fraction revealed a doublet of bands, both of higher

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apparent molecular weight than observed under reducing conditions. One of these two bands is of similar apparent molecular weight as that observed in the supernatant fraction. The second band is ~96 kD apparent
5   molecular weight. One explanation, given that it is present only in the intracellular fraction of transfected cells, is that it corresponds to an unglycosylated form of the putative chimeric β-TCR heavy chain. The plausibility of this explanation arises from two facts.
10   First is that the sites for N-linked glycosylation in the CH2 domain are contained within the chimeric molecule. Second, glycosylation was demonstrated to occur and indeed was required for the secreted expression of a chimeric $V_\alpha C_\kappa$ light chain Mariuzza and Winter, (1989) J.
15   Biol. Chem. 264:7310-7316. Thus, the apparent molecular weight of the non-reduced, presumably unglycosylated protein, is approximately double its predicted molecular weight of 54 kD derived from its primary structure.

Therefore, the data from analysis of
20   immunoprecipitated products under non-reducing conditions established that the chimeric TCR/heavy chain is cross-linked via disulfide linkages to form a homodimer. Although we do not have direct evidence, the most probable site for this is cross-linking is via the
25   cysteine residues encoded in the hinge domain of the Ig portion of the molecule.

4.   The Chimeric Protein Secreted from Cells Transfected with pVβ8.1(Δtm)-γ2a(ΔC$_{H1}$) Contains β-TCR Determinants.
30   A commercially available antibody that cross-reacts with common determinants on human β-TCR chains, βF1 (T-Cell Sciences), was used to demonstrate, by western blot analysis, the presence of human β-TCR determinants on the chimeric protein secreted by
35   transfected cells. For this analysis, a 25-1 primary cell line expressing the putative chimeric β-TCR/heavy chain protein was cloned by limiting dilution. Cell lines expressing higher levels of the chimeric protein,

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as assayed by the ELISA method described above, were
identified. One of these was expanded further and used
as a source for preparative isolation of chimeric β-TCR
from large scale tissue culture supernatants.

5   For purification of preparative amounts of the
protein, the clonal cell line was first acclimatized to
growth under serum-free media conditions.
Acclimatization to serum free media conditions was
carried out initially on a gradual basis over the course
10 of approximately 7-10 days. A dense 10 ml culture of
cells (~10^6 cells) growing in DME-High Glucose media
(Irvine Scientific) supplemented with 10% FCS was
passaged daily by splitting the plate 1->2, and fed with
DME-High Glucose media supplemented with 1% Nutridoma NS
15 (Boehringer Mannheim), such that the vol/vol ratio of the
final media after feeding increased from 9.0% FCS
supplemented media/0.10% Nutridoma supplemented media on
day one following the initial split, to 0% FCS
supplemented media/1% Nutridoma supplemented media at the
20 end. Once a cell culture had been established and
reached a vigorous growth state in serum free media, the
culture was expanded to preparative scale for isolation
of the chimeric β-TCR. Initially, 0.5 - 0.6 liter cell
cultures were employed as starting material. The cells
25 were maintained in 1.0 l. flasks as 100 ml cultures for
2-5 days, and then harvested. Secreted proteins in the
supernatants were then concentrated using an Amicon
concentrator (model no 8200), and either PM10, PM30, or
XM50 filtration membranes. The starting supernatants
30 were concentrated to 15-20 ml final volume. The chimeric
β-TCR protein was purified from this concentrated
supernatant by a one-step affinity purification on a
recombinant protein-A column (Repligen) essentially as
described in Harlow and Lane, (1988) in _Antibodies, A_
35 _Laboratory Manual_, Cold Spring Harbor, New York.
Briefly, binding of the ligand to the column was achieved
by two passages of the concentrate over the column, and
the column was washed with 10 volumes of 1X PBS. The

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ligand was eluted from the column with 5 volumes of 50 mM
Glycine (pH 2.5), 150 mM NaCl. The eluant was collected
in 1 or 2 ml fractions (depending on the volume of the
column), and the pH immediately raised to neutral by
addition of a pre-titrated amount of 1 M Tris-Cl (pH
9.0). The protein peak in the eluant was identified by
A_{280} measurement, which also served as an initial
estimation of the yield of total protein.

The presence of β-TCR determinants in the
putative chimeric protein were established by western
blot analysis. For this experiment, 1-2 μg of total
protein eluted from the protein-A column was loaded,
following denaturation in reducing Laemmli buffer, onto a
preparative well (1 mm x 8 mm x 4 mm), and
electrophoresed into a 12% polyacrylamide gel. The
proteins were then electrophoretically transferred onto
nitrocellulose membrane for 2-3 hr with a Transphor model
TE-50 electroblotter (Hoeffer Scientific Instruments) as
described in Harlow and Lane, (1988) in Antibodies, A
Laboratory Manual, Cold Spring Harbor, New York.
Following transfer, the membrane was marked to assign
polarity with respect to the original gel, and then
mounted in a PR150 mighty small deca probe western blot
staining apparatus (Hoeffer Scientific Instruments), and
incubated in 2-3 ml/lane of 3% BSA, 1X PBS for 1 hr at
room temperature to block non-specific protein binding
sites on the membrane.

The presence of β-TCR determinants in the
affinity purified secreted protein from transfected cells
was documented using a three-step reagent system. The
reagents were: 1°-100X diluted βF1 MAb (overnight); 2°-
biotin conjugated goat anti-mouse kappa (Fisher Biotech)
affinity purified antibody (1-2 hr); and 3°-1000 X
diluted alkaline phosphatase conjugated strepavidin
(Sigma, 1/2-1 hr). All reagents were diluted in 1% BSA,
1X TBS, 0.1% NaN3, and the blot washed between addition
of subsequent reagents with 1X TBS, 0.1% Tween-20.
Following the 3° reagent, the blot was washed with 1X

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TBS, and then incubated with alkaline phosphatase
substrate (Nitro Blue Tetrazolium + 5-Bromo-4-chloro-3-
indoyl-phosphate, Sigma, as described in Harlow and Lane,
Supra) to visualize antibody-antigen complex. In control

5   experiments, it was established that the 3* reagent alone
did not cross react with the test antigen when diluted at
least 100X, and the 2* reagent plus 3* reagents alone did
not cross react when the 2* reagent was diluted at least
1000X.

10   The results of this experiment established that
at a 1000X (or higher) dilution of the 2* reagent, an
immuno-crossreactive product is observed only when the
blot was incubated with the 1* reagent, βF1.
Furthermore, the apparent molecular weight of this

15   product, 68 kD, agrees precisely with that of the
protein-A immunoprecipitated products described above.
Therefore, this data established the presence of β-TCR
determinants in the secreted product expressed by the
transfected cell lines.

20   The purity of these affinity purified protein
preparations was assessed by quantitative one and two-
dimensional SDS-PAGE, and by these criteria, typical
purity of the chimeric β-TCR from batch to batch varied
between 95-99%. The major contaminant between batches

25   was usually the humanized κ-light chain (migrating at ~20
kD apparent molecular weight), which is also expressed by
the transfected cell line used for the purification of
the chimeric β-TCR.

             The purity of chimeric β-TCR batches was

30   assessed by silver-staining of one-dimensional SDS-PAGE.
Silver staining was carried out using a kit from
Stratagene, and the instruction provided by the
manufacturer. This analysis revealed the presence of a
major polypeptide band of 68 kD apparent molecular weight

35   as the primary elution product from the protein-A column.
This corresponds precisely with the size of the
polypeptide immunoprecipitated by protein-A agarose, as
well as the βF1 immuno-cross reactive product, in the

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experiments described above. (A faint band constituting
1-5% of the total yield was detectable in the 20 kD size
range. This corresponded to the humanized κ light
chain). This data established that a 68 kD protein is
the predominant constituent in affinity purified protein
from supernatant derived from transfected cells, and in
conjunction with the evidence described above, this
molecule bears both T-cell receptor, and immunoglobulin
heavy chain determinants.

A more critical assessment of the purity of a
protein preparation is its electrophoretic profile on
two-dimensional SDS-gels (Harlow and Lane, (1988) in
Antibodies, A Laboratory Manual, Cold Spring Harbor, New
York). Thus, in order to further characterize the
protein heterogeneity of the 68 kD polypeptide band
observed in the one dimensional gels, we analyzed the
affinity purified protein preparation by two dimensional
SDS-PAGE. In the interest of brevity, the results of
this analysis will only be summarized here, as they
simply confirmed the conclusions discussed above. Two-
dimensional analysis of the affinity purified chimeric
Vbβ.1/mouse γ2a protein revealed it to be comprised of at
least six isoforms, all of which had the same apparent
molecular weight, and differed in their isoelectric
points. The pI of the chimeric protein isoforms ranged
from pH 7-7.5. Furthermore, all the isoforms of the
affinity purified chimeric protein also possessed β TCR
determinants by virtue of their cross-reactivity to the
βF1 antibody in a western blot analysis.

We conclude, therefore, that all the
isoelectric variants observed in the affinity purified
chimeric protein contain both immunoglobulin heavy chain
determinants, as well as β-TCR determinants. Possible
contributors to the iso-electric heterogeneity of
immunoglobulin molecules which have been documented
include post-translational modifications, e.g.
glycosylation (Williamson, (1978) in Handbook of
Experimental Immunology (D.M. Weir, ed.), pp 9.1-9.31,

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Blackwell Scientific Publishers, Oxford, England). This
is consistent with evidence presented earlier regarding
the difference between predicted and apparent molecular
weight of the chimeric protein, and the observation of a

5   lower molecular weight form exclusively in the
intracellular fraction when analyzed by non-reducing SDS-
PAGE.

     5.   Evidence That The Chimeric β-TCR/Heavy Chain Protein
10   is Vβ8.1 in its T-cell receptor domain.
          The immuno-crossreactive data described above
     provide very convincing evidence that the protein
     secreted from cells transfected with the pVβ8.1 construct
     contains both β T-cell receptor determinants, and
15   immunoglobulin heavy chain determinants, as expected. In
     order to obtain direct evidence of this fact, and to
     provide final verification of the identity of the
     chimeric protein as Vβ8.1 specific, we subjected the
     purified protein to N-terminal amino acid sequence
20   analysis.
          Accordingly, 18 μg/~260 pmoles of affinity
     purified chimeric protein, isolated as described above,
     were concentrated further by overnight precipitation at
     4°C in 5% trichloroacetic Acid (TCA). The precipitated
25   protein was concentrated by centrifugation at 12,000 X g,
     washed 2X with 90% ethanol, and dried in a speed-vac
     concentrator. The pellet was subjected to amino acid
     sequence analysis. The results from the sequenator are
     summarized in Fig. 5, which shows a comparison of the
30   predicted amino acid sequence of Vβ8.1 deduced from the
     cDNA sequence, and the amino-terminal sequence determined
     from the affinity purified chimeric β-TCR protein. Amino
     acid residues whose identity could not be ascribed with
     100% confidence are indicated in parentheses in Fig. 5.
35   The sequence of the purified protein agrees exactly with
     the predicted sequence of Vβ8.1 for the first 10 residues
     sequenced (residues #2-11), and for 12 of the 14 residues
     sequenced. The identity of the first residue, gly,

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although in agreement with the predicted sequence, cannot be ascribed with confidence due to a technical limitation.

The yield per residue at each cycle of the sequenator is a critical parameter in protein sequence determination. The magnitude of this parameter is useful in ascribing the degree of confidence to the sequence obtained as originating from the major/sole polypeptide in the preparation as opposed to originating from a minor contaminant in the protein preparation (due to, e.g., a blocked amino-terminus of the major constituent). Typically this value is highest for the first few cycles, and drops at each progressive cycle thereafter, for technical reasons. The yield per residue for the first two amino acids determined (V and I), was 122 pmoles, and 130 pmoles, respectively. Based on the estimated initial amount of protein submitted for sequence analysis, 260 pmoles, this represents a yield per residue of 45-50%. Hence, we can be confident that the sequence determined was from the major polypeptide in the preparation, as opposed to a minor contaminant in the preparation.

The results from sequence analysis show that the amino-terminus of the purified protein expressed by the transfected cells agrees precisely for the first 10 residues, with that of Vβ8.1, starting one residue downstream of the predicted leader peptide cleavage site. This evidence establishes two important facts. First, the identity of the chimeric β-TCR/immunoglobulin heavy chain can be assigned positively and unambiguously as Vβ8.1 in its T-cell receptor domain. Second, it shows that the β-TCR leader peptide is correctly processed in myeloma cells, a heterologous cell line.

Therefore, we conclude that myeloma cells transfected with pCMVβ8.1(ΔTM)-γ2a(ΔCH1) express a soluble protein which is a chimeric Vβ8.1/mouse γ2a molecule into the extracellular media.

6.   Production of Monoclonal Antibodies Targeted to Native β-TCR determinants from Mice Immunized with Solubilized Chimeric Vβ8.1/mouse γ2a Protein.

Preliminary experiments carried out to test the feasibility of using the solubilized chimeric β-TCR for generating antibodies to native determinants indicated that the solubilized form would be an effective molecule for this purpose. In the discussion below, the native form of the TCR refers to the α- and or β-chain as it occurs on the surface of T-lymphocytes in association with the CD3 complex.

Immunization regimens were initiated in mice to determine the optimal immunization strategy which would lead to the desired antibodies in the shortest time period using the least amount of antigen. Two basic parameters were investigated in this regard: the adjuvant (complete Freund's versus RIBI (RIBI Immunochemical, Hamilton, MT)), and the immunization route (intraperitoneal versus footpad).

The immunocompetence of the mice for fusion was assessed by monitoring serum antibody levels via two assays. The serum titre levels were determined by the ELISA assay described above using chimeric β-TCR coated plates, and the quality of the immune response was measured by the ability of immune sera to immunoprecipitate β-TCR specifically from cell surface radio-iodinated proteins (Marchalonis, (1969) Biochem. J. 113:299-305) from Jurkat cells. Radio-iodinated cell surface proteins from MOLT4 cells, which do not express TCR/CD3 on their cell surface, served as negative controls in this experiment. Immunoprecipitations were carried out using 5μl of immune sera (the antibody) from mice (diluted 100X in 1X PBS) plus 10^6 c.p.m. of I^{125}-labeled lysate (the antigen) from Jurkat versus MOLT4 cells, in a total volume of 50-100μl. The antibody/antigen mixtures were incubated at 4-8°C for ~90 min. The immune complex was precipitated using 5μl of goat-anti mouse IgG (Sigma) with agitation for a further

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60 min at 4°C. The immunoprecipitates were washed as
described earlier, and analyzed by one-dimensional
SDS-PAGE. Serum from non-immunized mice served as a
negative control, and rabbit IgG-agarose was the
5   non-specific immunoadsorbent. The quantity of antigen
used for immunization was determined to not be a critical
factor. Thus, it typically ranged between 5μg - 50μg in
the immunizations described below.

It was determined that all immunizations led to
10   a detectable immune response against the solubilized
chimeric β-TCR as measured by the ELISA assay. However,
only serum titre levels in excess of about 1:2,000 had
antibodies which were also immunocompetent in the
immunoprecipitation assay. Intraperitoneal immunization
15   with Freund's adjuvant required a 3-4 month interval
between primary immunization and first boost in order to
reliably and reproducibly elicit these strong titres.
Two to four week rest periods between primary
immunizations and subsequent boosts via this route using
20   Freund's adjuvant led to highly variable immune
responses. By contrast, RIBI adjuvant proved superior to
Freund's in consistently eliciting strong immune
responses (serum titres ≥ 1:5,000 + immunoprecipitation
competent sera) in a shorter period between the primary
25   immunization and first boost (2-4 weeks) when the mice
were immunized intraperitonealy.

Immunization via the footpad route (Holmdahl et
al., (1985) J. Imm. Methods 83:379-384; Mirza et al.,
(1987) J. Imm. Methods 105:235-243), using RIBI adjuvant,
30   proved to be of superior utility with regard to the two
important parameters of quantity of antigen required and
the length of immunization necessary. For footpad
immunizations, popliteal lymph nodes served as the donor
organ of lymphocytes for fusion with a myeloma fusion
35   partner. With respect to the time factor, the fusions
were carried out 11 days following the primary
immunization. The experiment yielded an abundance of
independently derived hybridoma lines (n=32) which were

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secreting antibodies targeted against CD3 exposed
determinants on the TCR β-chain. Hence, as detailed
below, this immunization regimen proved to be superior to
all others tested for the generation of anti-TCR
antibodies, while requiring the least amount of antigen.
The details of this, and the identification of the
relevant hybridoma lines are presented below.

Four mice were immunized with ~6μg of antigen
in 50μl RIBI adjuvant in each of their rear footpads
following anesthesia. The degree of the anesthesia was
monitored by the loss of the leg withdrawal reflex upon a
pinch stimulus of the footpad (mice were not injected
with antigen until this reflex had completely
disappeared). Following immunization, the mice were
monitored on a daily basis for the next four to seven
days for signs of motor impairment as a consequence of
the immunization. They exhibited no overt signs of
impairment or physical discomfort. The mice were killed
11 days subsequent to the single immunization, and their
popliteal lymph nodes were dissected for fusion with the
myeloma fusion partner P3X63Ag8.653 (ATCC CRL1580).
Fusion of lymphocytes with myelomas was carried out
essentially as described in Harlowe et al. (Harlow and
Lane (1988), in Antibodies, a Laboratory Manual, Cold
Spring Harbor, NY), using the method of fusion by
stirring with the modifications noted below: The ratio
of lymphocyte:myeloma employed was 1:2, and the cells
were plated following fusion at a density of 1x10^5
lymphocytes in 50μl HAT media per well (onto a 24 hr old
feeder layer of 2X10^4 peritoneal exudate derived mouse
macrophage cells per well), in 96-well plates. The
peritoneal exudate was obtained as described (Fazekas
DeSt.Groth and Scheidegger, (1980) J. Imm. Methods 35:1-
21). Following harvesting, the exudates were washed 1X
in HAT media (DMEM, 20%FCS, pen and strep, 1X OPI, 1X
HAT, 5x10^{-5}M β-mercaptoethanol, 5mM L-glutamine), and
resuspended to the appropriate density for plating in
50μl HAT media per well. Fused hybridoma cells were left

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undisturbed at 37°C in 7.5% CO$_2$ atmosphere for seven days following plating.

The cells were fed with 100 μl/well HAT media on day 8. The spent media was aspirated off and the cells were fed 200 μl/well fresh HAT media on day 12. By day 14, individual wells showing active hybridoma growth were easily discernable by visual examination, and were screened as soon as the media in individual wells just began turning yellow. Screening consisted of a two step procedure, a preliminary ELISA assay to rapidly identify anti-β TCR antibody secreting lines, followed by a flow cytometric analysis using indirect immunofluorescence to identify that subset of anti-β TCR antibody secreting lines whose antibodies were targeted to CD3 exposed determinants of the β-chain. Those lines which tested negative in the initial ELISA assay were discarded.

The ELISA assay was carried out using chimeric β-TCR coated plates as the capture agent. Specifically, ELISA plates were coated with 100 μl/well of affinity purified chimeric Vβ8.1/mouse γ2α protein at a concentration of 1.5 μg/ml (diluted in 1X PBS) for at least 24 hr at 4°C. The plates were then blocked for at least 24 hr with 200 μl/well of ELISA buffer. Following washing with 1X PBS, 0.1% Tween-20, test antibodies (i.e. supernatants from HAT resistant hybridoma cells) were applied to the wells and the plates incubated at room temperature for at least 2 hours. Next, the plates were incubated with 100 μl/well of 2000X diluted, biotin conjugated GαMIgκ (Fisher Biotech) for 1 hr at room temperature. Finally, the plates were incubated with a 3° reagent, 200X - 500X diluted horseradish peroxidase conjugated strepavidin (Sigma) at room temperature for 30 - 60 min. The plates were washed in between reagents with three changes of 1X PBS, 0.1% Tween-20, using 200 μl/well. Following the 3° reagent, the plates were washed 4X with 1X PBS alone. Positive samples were visualized by application of HRP substrate (BioRad). Hybridoma lines were screened using typically 100μl of supernatant

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from the wells in which they were growing in the 96-well
microtitre plates. Those lines which tested positive out
of the 96-well plates were expanded to 24-well plates (1
ml cultures), whereupon they were retested as soon as
5   their media again just began to turn yellow. In this
second round of screening, triplicate aliquots of
supernatant were removed from the wells for retesting by
the ELISA assay, as well as for their ability to stain
Jurkat cells and MOLT4 cells by flow cytometry. For cell
10   staining, 5 X 10^5 Jurkat or MOLT4 cells (in 50 - 100 μl 1X
PBS, 0.01% sodium azide) were incubated with hybridoma
supernatant at 4°C for 30 min. The cells were washed 1X
with 250 μl/well PBS/azide, and incubated with 25μl/well
FITC conjugated GaMIgG for another 30 min as above.
15   Following a final wash, the cells were fixed by
resuspending in 50-200μl 1% paraformaldehyde, and
analyzed by flow cytometry on a Becton-Dickinson FACS
scan. The level of background/non-specific staining
(control) was established using media alone, and positive
20   controls for staining intensity included the following
three antibodies: OKT3 (Ortho Diagnostics), WT31 (sold
as TCR-1 Becton-Dickinson, Cambridge, MA), αVβ8 (T-Cell
Sciences). The specificity of these antibodies is
targeted to the CD3 complex on T-lymphocytes (Kung et al,
25   (1980) Proc. Natl. Acad. Sci. USA 77:4914-4917), a shared
determinant between TCRα/β and the CD3 complex (Van der
Griend et al., (1988) J. Immunology 140:1107-1110), and
the Jurak β-TCR chain (Tian, et al., (1989) FASEB J.
3:A486 Abstracts), respectively.
30   Those lines which tested positive by the ELISA
assay, positive for Jurkat staining and negative for
MOLT4 staining were expanded further into 3 ml cultures.
Only two out of 34 lines which showed positive cell
surface staining profiles on Jurkat cells also showed
35   weakly positive profiles on MOLT4 cells. These cell
lines were retested a third time, This time by flow
cytometry alone. At this stage of their expansion, in
addition to the Jurkat and mMOLT4 lines, all psoitive

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hybridoma lines were tested against three additional TCR
negative T-lymphoma cell ines, namely MOLT3, CEM and
HuT102. With the exception of the two lines which had
tested positive against MOLT4 cells, the remaining 32
5   lines again were negative in their staining profiles on
these additional T-cell lines. Those lines which tested
Jurkat+/MOLT- a second time were expanded further into 10
ml cultures, and the lines were either frozen away or
cloned immediately by limiting dilution. The results of
10   the numbers from the fusion are summarized in Table 2
below.

Table 2: Summary of results from fusion of lymph node
derived lymphocytes with a Myeloma cell line for the
15   generation of hybridomas producing antibodies targeted to
native determinants of the β-TCR chain.

Description	Number
Total wells plated	672
No wells plated	467
No ELISA positive	214
No Jurak (+)/MOLT4(-)	32
No Jurak (+)/MOLT4(+)	2

In addition to the ELISA and cell staining
25   criteria for identifying hybridoma lines described above,
the identity of the antigen which the antibodies produced
by these lines were targeted against was further
confirmed by their ability to immunoprecipitate β-TCR.
Supernatants from ELISA+/Jurkat+/MOLT4- hybridoma lines
30   were used to immunoprecipitate cell surface
radio-iodinated proteins from Jurkat and MOLT4 lines.
The results revealed all hybridomas tested
immunoprecipitated the TCR β-chain from Jurkat cell
lysates.
35   All hybridoma lines which passed each of these
criteria were either frozen for later analysis or cloned
immediately for further characterization. Seven lines
were selected for cloning by limiting dilution for

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further characterization. Subsequent to cloning of
positive cell lines, four lines were chosen on the basis
of their Jurkat staining profiles for antibody
purification. For this purpose, clonal lines were grown
under serum free media culture conditions and expanded to
100 ml stage. The cultures were maintained at this level
until ≥75% of the culture consisted of dead cells. At
this time, the culture was harvested and the supernatant
was concentrated to ~20 ml in an Amicon concentrator
using a P10 membrane. Antibody was purified from the
concentrate using FPLC based protein-A affinity
chromatography. As the four lines selected to purify
antibody from were secreting heavy chains of the γ1
isotype, the salt was adjusted to 1M NH_4SO_4 in order to
facilitate binding of the antibody to the column. Purity
of the antibody preparations was verified by SDS-PAGE.
Silver stain visualization of the acrylamide gels
revealed homogeneous preparation of antibody in all four
cases, consisting of just a heavy chain and a light chain
band, with no minor contaminants detectable. Antibody
yields were quantitated by A_{280} measurements, and by
quantitative ELISA assay using a goat anti-mouse IgG
coated plate. Quantitative FACS analyses were carried
out using precisely matched amounts of the reference
antibody (αVβ8) and the four purified antibodies.
Results of the FACS analysis using the purified
antibodies to stain Jurkat cells are shown in Fig. 6.
The results show that three of the four antibodies
produce histograms identical to the reference antibody
αVβ8, while the fourth antibody, 9B47, yields a slightly
brighter level of staining than the reference antibody.
This feature is a consistent hallmark of this antibody,
in that it was observed at every level of analysis from
initial identification through cloning, and ultimately
with the purified antibody itself. The brighter staining
observed with this antibody indicates that 9B47 is
targeted to a different epitope than the reference
antibody.

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Thus, the solubilized chimeric β-TCR/IgG heavy
chain molecule is a completely native molecule,
particularly with respect to the TCR domains, on the
basis that antibodies raised against the soluble form
5   cross react with native form of the receptor. As such,
it represents a proven and viable antigen for generating
antibodies targeted to native determinants of any given
member of the TCR family.

Although the present invention has been
10   described in some detail by way of illustration and
example for purposes of clarity and understanding, it
will be apparent that certain changes and modifications
may be practiced within the scope of the claims.

WHAT IS CLAIMED IS: 53

1.   A composition comprising a substantially pure
soluble chimeric polypeptide having at least 100 amino
acids from a V_β domain and at least about 50 contiguous
amino acids from an immunoglobulin superfamily protein.

2.   A composition of Claim 1, wherein the polypeptide
comprises a complete native V_β domain.

3.   A composition of Claim 1, wherein the V_β is a human
V_β.

4.   A composition of Claim 1, wherein the solubility is
conferred by an immunoglobulin heavy chain CH2 or CH3
fused to the V_β domain.

5.   A substantially pure chimeric polypeptide comprising
$X1-V_{R1}-C_{R2}-X2-C_{H2}-C_{H3}-X3$, wherein
        V_{R1} is a variable region domain of a human
T-cell receptor chain;
        C_{R2} is a constant region domain of a human
T-cell receptor chain;
        C_{H2} is a second heavy chain constant region
domain of an immunoglobulin gene;
        C_{H3} is a third heavy chain constant region
domain of an immunoglobulin gene;
            each of X1, X2 and X3, if present, is a
            polypeptide segment comprising less than about
            50 amino acids; and
            R1 and R2 are each either α or β.

6.   A chimeric polypeptide of Claim 5, wherein the
variable region domains and constant region domains are
selected from the group of $V_{\alpha 1}$ to $V_{\alpha 11}$ or the group of $V_{\beta 1}$
to $V_{\beta 20}$.

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7. A chimeric polypeptide of Claim 5, wherein at least one of the heavy chain constant regions is a mouse heavy chain constant region.

8. A chimeric polypeptide of Claim 5, wherein the chimeric polypeptide is soluble in an aqueous buffer.

9. A chimeric polypeptide of Claim 5, wherein the V_β is the V_β 8.1 T-cell receptor chain.

10. A chimeric polypeptide of Claim 5, further comprising a toxin moiety.

11. A chimeric polypeptide of Claim 10, wherein the toxin moiety is covalently linked to the chimeric polypeptide.

12. A soluble polypeptide comprising human V_α-C_α or V_β-C_β domains fused to mouse immunoglobulin constant region, wherein the mouse immunoglobulin constant region has an incomplete CH1 domain.

13. A dimer of two polypeptides, wherein each polypeptide is a chimeric polypeptide of Claim 5.

14. A nucleic acid encoding a polypeptide of Claim 1, 5 or 12.

15. A composition comprising a nucleic acid encoding a polypeptide of Claim 1, 5 or 12, said nucleic acid operably linked to a heterologous promoter or enhancer region.

16. A method for producing antibodies capable of binding to a V_α or V_β T-cell receptor domain epitope comprising the step of exposing to a target immune system a soluble hybrid polypeptide comprising a V_α or V_β T-cell receptor domain.

17.   A method of Claim 16, wherein the V_α or V_β domain is a human V_α or V_β domain.

18.   A method according to Claim 16, further comprising the step of cloning one or more cell lines capable of producing the antibodies.

19.   A method for producing antibodies capable of binding to a human V_α or V_β T-cell receptor domain epitope, said method comprising the step of exposing to a target immune system a chimeric polypeptide of Claim 5 or a V_α or V_β chimeric polypeptide on a cell surface.

20.   A method according to Claim 19, further comprising the step of cloning cells secreting antibodies reactive with the human V_α or V_β domain epitope.

21.   An antibody produced by a method of Claim 16.

22.   An antibody of Claim 21 which is a monoclonal antibody.

23.   A method of diagnosing a disease or disorder of a mammal comprising the steps of:
        mixing an antibody of Claim 21 with a sample from the mammal; and
        detrmining whether the antibody binds to the sample, thereby determining whether a T-cell antigen receptor or antigen molecule is produced in the mammal.

24.   A method of treating a mammal comprising administering to the mammal an effective amount of a polypeptide of Claim 5 sufficient to interfere with the binding of an antigen to a T-cell antigen receptor characteristic of a T-cell.

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25. A method of treating a mammal comprising administering to the mammal an amount of a polypeptide of Claim 10 effective to substantially eliminate a T-cell.

5   26. A method of Claims 24 or 25, wherein the mammal clonally overproduces the T-cell.

27. A method of Claims 23, 24, or 25, wherein the mammal is a human.

10

28. A method of screening for an agent capable of binding a T-cell antigen receptor, comprising the steps of:

        adding a polypeptide of Claim 5 to a sample
15 containing the agent; and

        determining the amount of the agent bound to the polypeptide.
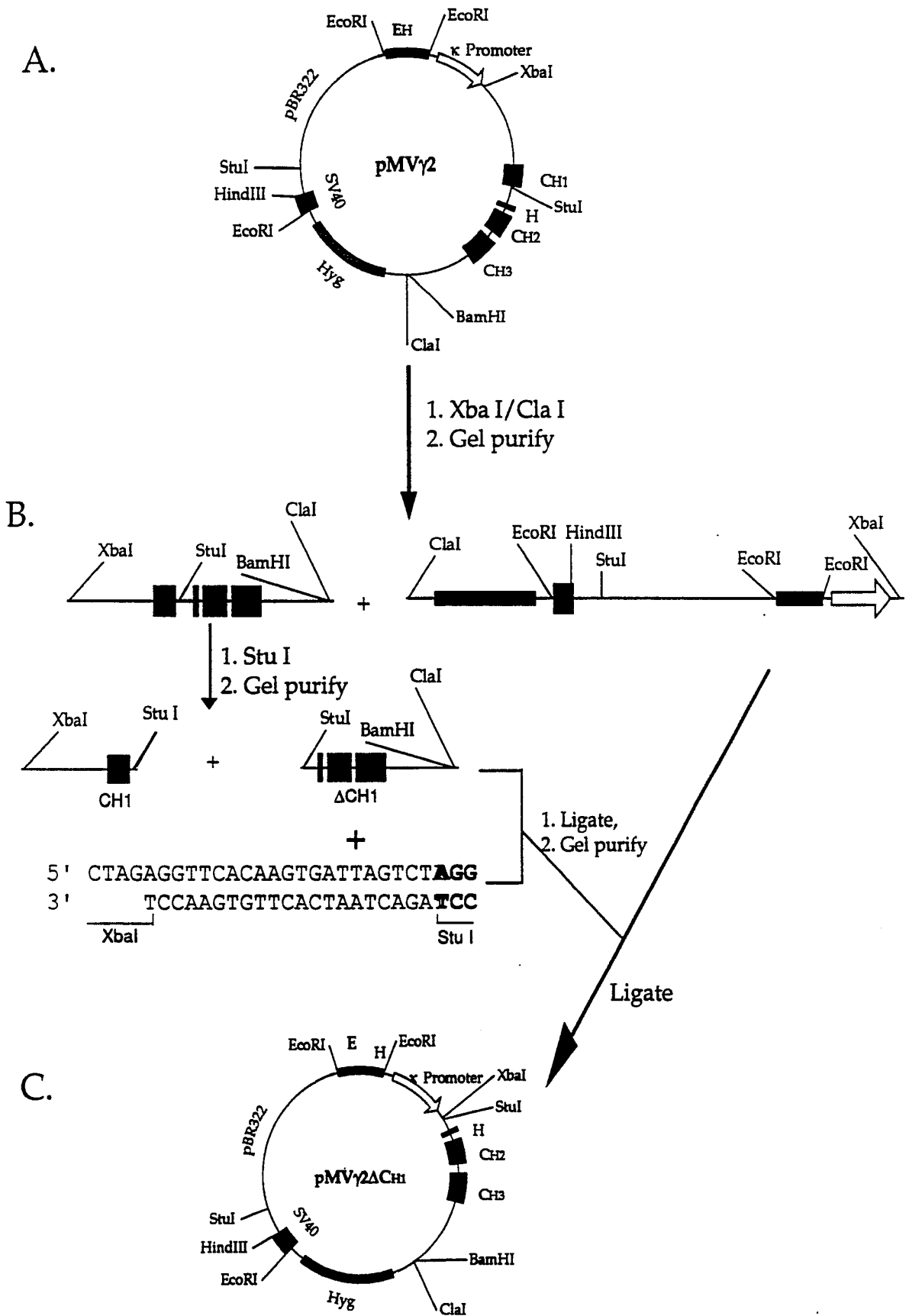
20

A.

EcoRI   EH   EcoRI
                κ Promoter
PBR322   XbaI

pMVγ2

StuI   CH1
HindIII   StuI
EcoRI   SV40   H
                              CH2
         Hyg   CH3

                    BamHI
        ClaI

1. Xba I / Cla I
2. Gel purify

B.

XbaI   StuI   BamHI   ClaI   ClaI   EcoRI HindIII   StuI   EcoRI   EcoRI   XbaI

        ▪ ▪▪ ▪   +   ▪▪▪▪   ▪▪

1. Stu I
2. Gel purify

XbaI   Stu I   StuI   BamHI   ClaI

       ▪   +   ▪▪▪
      CH1   ΔCH1

                      +

5' CTAGAGGTTCACAAGTGATTAGTCT**AGG**
3'   TCCAAGTGTTCACTAATCAGA**TCC**

    ‾‾‾‾   ‾‾‾
    XbaI   Stu I

1. Ligate,
2. Gel purify

Ligate

C.

EcoRI   E   H   EcoRI
                  κ Promoter   XbaI
PBR322   StuI
                                H
pMVγ2ΔCH1   CH2
                                CH3
StuI
HindIII   SV40
EcoRI   BamHI
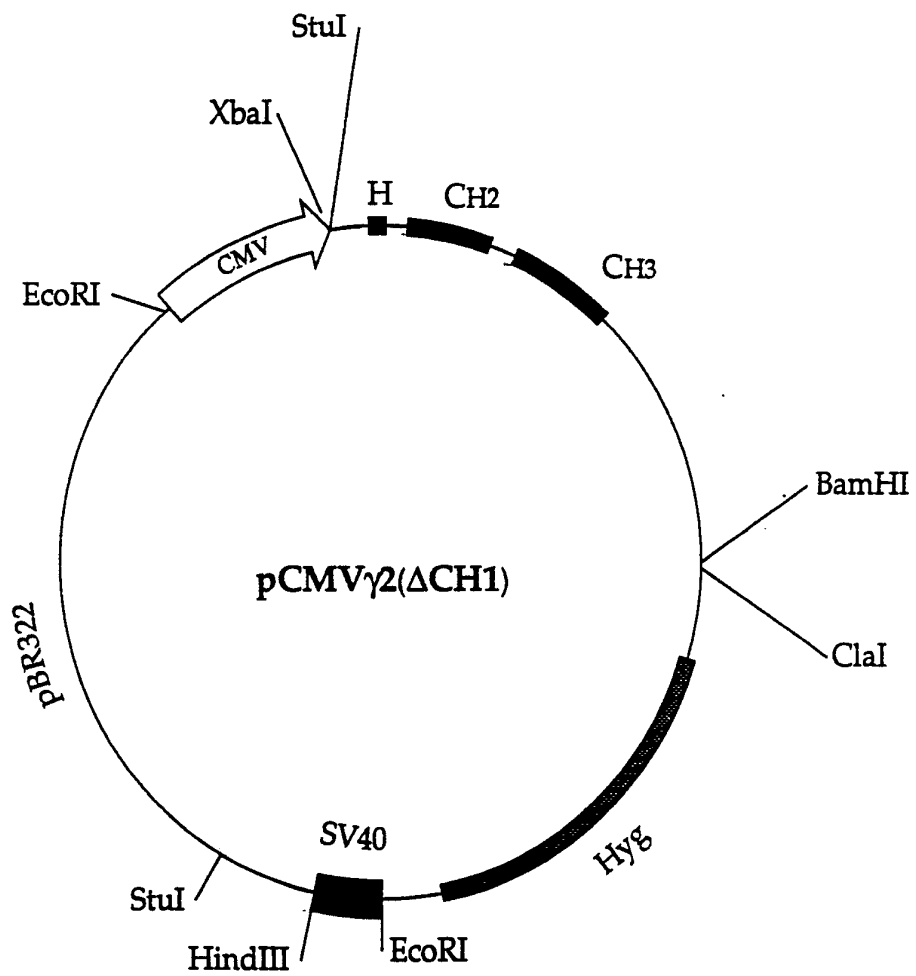   Hyg   ClaI
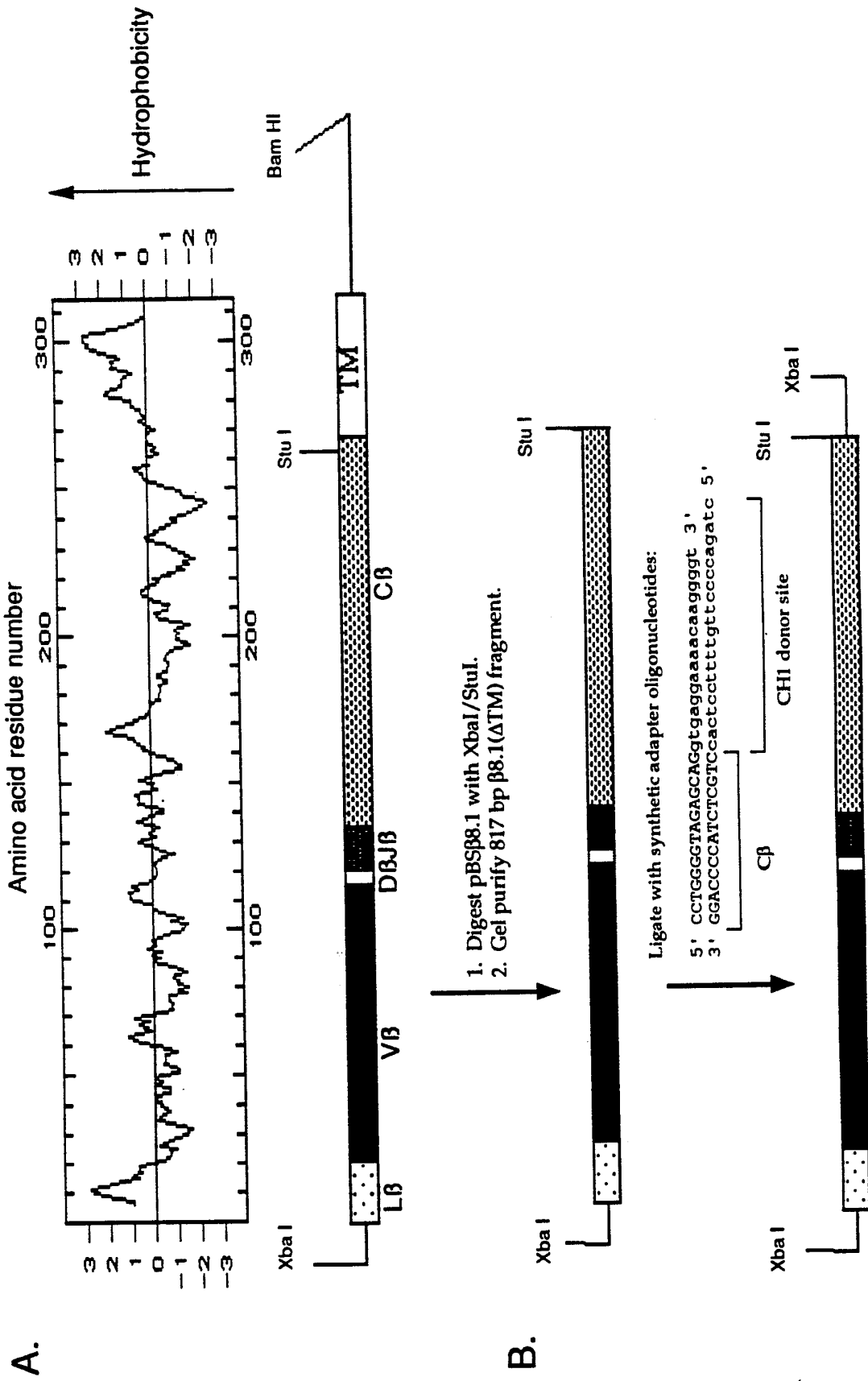
Figure 1

Figure 2. Map of pCMVγ2(ΔCH1)

Figure 3. Deletion of trans-membrane domain from ß-TCR

Figure 4. Map of pCMVß8.1(ΔTM)-γ2a(ΔCH1)

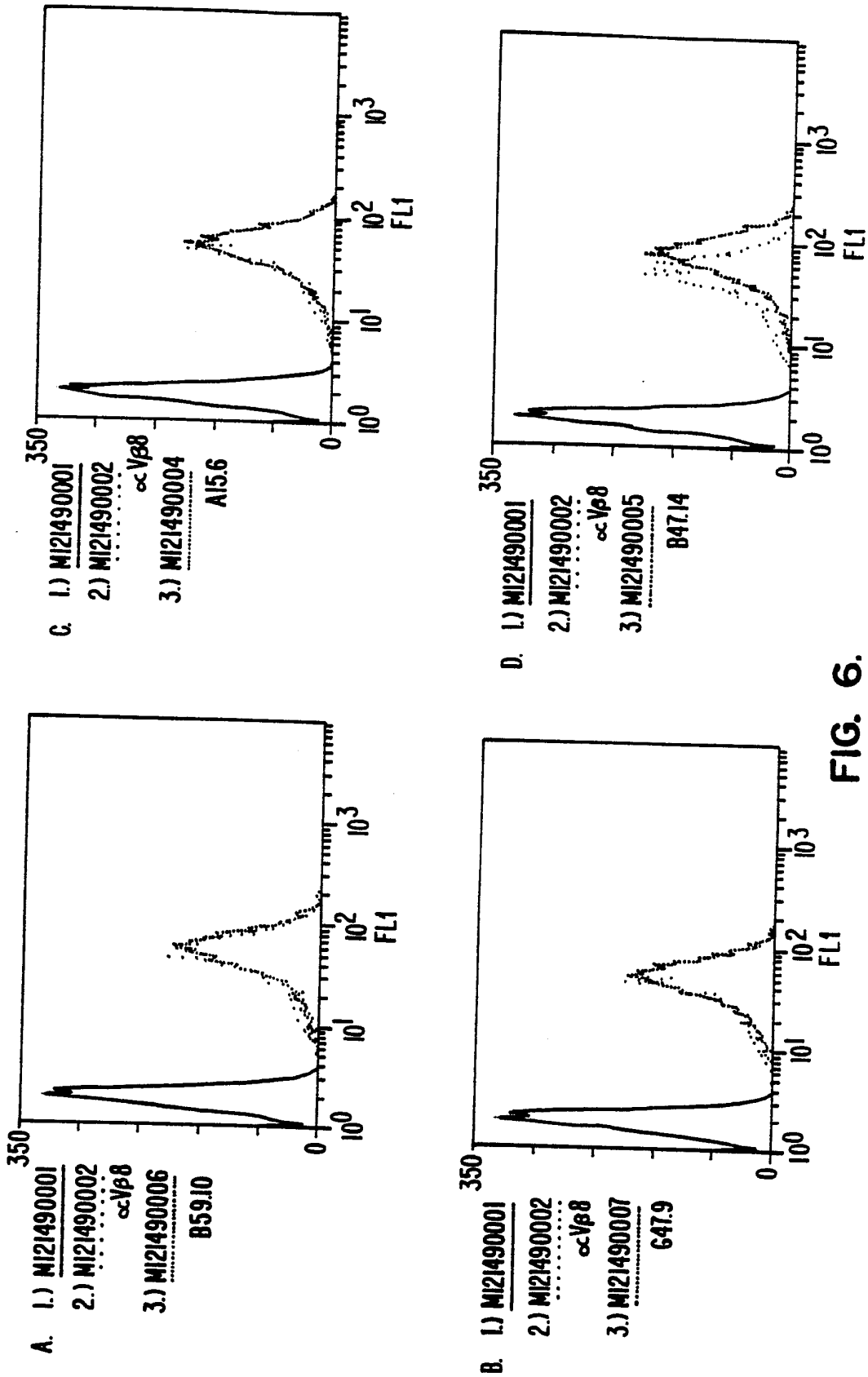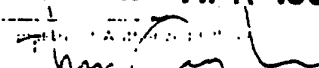**ß8.1 Leader Peptide** | **Vß8.1**

Predicted ß8.1 Sequence:

-21 M D S W T F C C V S L C I L V A K H T D A | 1 G V I Q S P R H E V T E M G E 15

Sequence of Protein Affinity Purified from Tissue Culture Supernatant: (G) V I Q S P R H (E) V T (T) M (G) (Q)

Yield per residue (p moles): 122 130

Figure 5. Amino acid sequence from amino-terminus of affinity purified chimeric protein.

FIG. 6.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):A61K 35/14,37/00;C07K 3/00,13/00,15/00,17/00;C08H 1/00;A01N 37/18
U.S. CL.: 530/387,389,402,403; 514/2; 435/7.1,69.1, 69.3

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	530/387, 389, 402, 403; 536/27; 514/2; 424/88; 435/7.1, 69.1, 69.3

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched

Automated Patent System, DIALOG Databases

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	IMMUNOLOGICAL REVIEWS, No. 89. Issued 1986. C. Queen et al.. "Cell-Type Specific Regulation of a Kappa Immunoglobulin Gene by Promoter and Enhancer Elements". pages 49-68. see entire document.	1-28
X Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA). volume 84. issued May 1987. N.R.J. Gascoigne. et al. "Secretion of a Chimeric T-Cell Receptor-Immunoglobulin Protein", pages 2936-2940. See entire article.	1 1-28

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

25 February 1991

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

24 APR 1991

Signature of Authorized Officer

Thomas M. Cunningham, Ph.D.

ebw

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, ¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No ¹³
Y	CELL, volume 54, issued 15 July 1988, H. AchaOrbea, et al., "Limited heterogeneity of T-Cell receptors from Lymphocytes Mediating Autoimmune Encephalomyelitis Allows Specific Immune Intervention", pages 263-373. see especially pages 269-270.	1-28
Y	CELL, volume 54, issued August 1988, J.I. Urban et al., "Restricted use of T Cell Receptor Genes in Murine Autoimmune Encephalomyelitis Raises the Possibility of Immune Intervention", Pages 577-592. See especially pages 588-589.	1-28
Y	JOURNAL OF EXPERIMENTAL MEDICINE, volume 168, issued December 1988, M. Owhashi, et al. "Protection from Experimental Allergic Encephalomyelitis Conferred by a Monoclonal Antibody Directed Against A Shared Idiotype on Rat T Cell Receptors Specific for Myelin Basic Protein". Pages 2153-2164. see entire article.	1-28
Y	EUROPEAN JOURNAL OF IMMUNOLOGY, volume 19, issued 1989. J. Chluba et al. T Cell Receptor B Chain Usage in Myelin Basic Protein Specific Rat T Lymphocytes". pages 279-284, see entire article.	1-28
Y,P	SCIENCE, volume 248, issued 25 May 1990, K.W. Wucherpfenning, et al. "Shared Human T Cell Receptor VB Usage to Immunodominant Regions of Myelin Basic Protein". See pages 1016-1019.	1-28