A method for production of antibodies to human T-cell receptor β-chain variable regions

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ABSTRACT Mouse T-cell hybridomas bearing human V_β elements were produced by transfection of human/mouse hybrid T-cell receptor β-chain genes into a mouse T-cell hybridoma lacking an endogenous β-chain gene. These hybridomas were entirely mouse in origin except for the human V_β region. These cells were used to immunize mice against human V_β elements. Mouse monoclonal antibodies have thus been generated against human $V_\beta 13.1$ and -13.2. We expect that the method outlined in this paper will be useful in the production of monoclonal antibodies specific for other human V_β or V_α elements.

The receptor for antigen on most peripheral T cells is made of α and β chains. Usually these T-cell receptors (TCRs) engage antigen in the form of peptides, derived from the antigen, bound to cell surface major histocompatibility complex (MHC) molecules (1–4). The specificity of the TCR for this complex ligand is determined by the five variable elements (V_α, J_α, V_β, D_β, and J_β) of the α and β chains as well as non-germ-line-encoded junctional regions (5–8). Exceptions to this rule have been documented by us and others. These are proteins that stimulate T cells bearing the appropriate V_β element almost independently of contributions from other parts of the TCRs borne by the T cells (9–22). Such proteins, called superantigens because they stimulate large numbers of T cells, are encoded by microorganisms including retroviruses, bacteria, and mycoplasma (22–29).

There is considerable interest in the control and consequences of the T-cell repertoire in humans and other species. Obviously a detailed understanding of the T-cell repertoire would require knowledge of all the V_α, J_α, V_β, D_β, and J_β combinations present in a given individual. Such knowledge will not be available in the foreseeable future. However, much has already been learned from studies of the distribution between animals of just one of the TCR variable elements, V_β. Examination of V_β expression in mice has been used to study positive selection, T-cell tolerance, autoimmunity, and the consequences of attack by superantigens (22). Similarly, V_β expression in humans has been used to study the effects of superantigens and also to examine the repertoires of T cells involved in inflammatory and autoimmune disease (18, 19, 29, 30).

Experiments of this type have been done in mice by using an ever increasing collection of anti-mouse V_β monoclonal antibodies, raised, usually, against T-cell hybridomas bearing particular V_β elements (22). Similar experiments in humans have been hampered because so far very few anti-human V_β (hV_β) monoclonal antibodies are available (18), although some important data have been generated with a labor-intensive quantitative polymerase chain reaction (QPCR) (19,

29, 30). Although the majority of hV_β elements have been cloned and sequenced, one of the impediments to the development of a complete battery of anti-hV_β reagents has been the lack of widely available monoclonal sources of human T cells (clones, tumors, or hybridomas) for use as immunogens. To circumvent this problem we have developed a method of expression of hV_β elements on the surfaces of mouse T-cell hybridomas. We show that these transfected T-cell hybrids are excellent immunogens in mice for hV_β, probably because the hV_β is the only xenogeneic material on the surface of the hybrids. We have raised two monoclonal antibodies against $hV_\beta 13.1$ and -13.2 by using these hybrids, and here we illustrate their application for the measurement of T cells bearing $V_\beta 13.1$ or -13.2 in humans.

MATERIALS AND METHODS

Preparation of RNA and cDNA and Amplification by QPCR. Total RNA was prepared from phytohemagglutinin-, anti-$hV_\beta 13.1$-, or anti-$hV_\beta 13.2$-stimulated peripheral blood T cells as described (19). The first strand of cDNA was made from 2 μg of total RNA and was used for QPCR as described (19).

Cloning and Sequencing of $hV_\beta 13.3$. First-strand cDNA was prepared from anti-CD3-stimulated peripheral T cells and amplified with oligonucleotide primers 5'HBP34(*Eco*RI) and 3'hC$_\beta$2(*Bam*HI) by PCR. The 5'HBP34(*Eco*RI) primer (5'-GGGAATTCAAGATGGCCATCGGCCTCCTGTGCT-GTGC-3') is part of the leader sequence of the $hV_\beta 13$ family of genes. Oligonucleotide 3'hC$_\beta$2(*Bam*HI) (5'-GGGTGG-GAACACGTTTTTCGGATCCTC-3') is a reverse primer for the human C_β constant region. Amplified products of the PCR were cloned into the pTZ18R vector (31). Multiple clones were sequenced using the Sequenase kit (United States Biochemical). A number of clones were found that had a sequence related to, but not identical to, the $hV_\beta 13.1$ and $hV_\beta 13.2$ gene segments. An oligonucleotide (5'-CTGAAGC-TGATTTATTAT-3') that distinguished this sequence (called $hV_\beta 13.3$) from the other two members of the $V_\beta 13$ family was synthesized and used as a probe to identify more clones containing the gene for the new V_β element. Multiple clones of the new V_β gene with different $D_\beta J_\beta$ regions were sequenced.

Transfection of Mouse T-Hybridoma Cells. An expression vector carrying the appropriate hV_β genes was transfected by electroporation into a mouse T-cell hybridoma (DS23-27.4) that lacked endogenous TCR β-chain genes (31). Transfected cells were selected with G418 (700 μg/ml) as described (31).

Flow Cytometric Analyses. Human peripheral blood T cells or mouse T-cell hybridomas were incubated with purified anti-V_β antibodies H131, H132, and 1C1 (anti-$hV_\beta 5.2/3$; ref.

Abbreviations: hV_β, human V_β; MHC, major histocompatibility complex; QPCR, quantitative polymerase chain reaction; TCR, T-cell receptor.

32) and LC4 (anti-hV$_\beta$5.1; ref. 33). The cells were then stained with fluorescein-conjugated goat antibody to mouse immunoglobulin (Calbiochem) by standard methods (18). The transfectants were also stained with biotinylated H57-597 (anti-C$_\beta$; ref. 34) followed by phycoerythrin-labeled streptavidin (Tago).

RESULTS

Production of T-Cell Hybridomas Expressing hV$_\beta$ Elements. The transfection system used to generate murine T-cell hybridomas expressing a chimeric TCR that includes a hV$_\beta$ element was described previously (ref. 31; see also Fig. 1). In brief, TCR β genes were constructed using a gene fragment encoding the D$_\beta$, J$_\beta$, and C$_\beta$ elements of a mouse T-cell hybridoma β gene (31) and the V$_\beta$ genes encoding human V$_\beta$13.1 or -13.2. The V$_\beta$ genetic elements were created by PCR of the cDNA from human T-cell mRNA, using V$_\beta$-specific oligonucleotides as primers. The complete chimeric β gene was transfected into T-cell hybridomas that were transcribing all the TCR components required for surface expression of TCR, except β. The β gene was encoded on a plasmid that also bore a neomycin-resistance gene, and transfectants were selected using neomycin analogue G418. Transfected cells were monitored for β-chain expression with an anti-mouse C$_\beta$ antibody (ref. 34; see also Fig. 2).

Generation of Monoclonal Antibodies Specific for hV$_\beta$ Elements. The transfectants described above are mouse cells bearing hV$_\beta$ elements on their surfaces; therefore they should be immunogenic in mice for the production of anti-hV$_\beta$ antibodies. The hV$_\beta$13 family is very similar to the mouse V$_\beta$8 family, sharing about 60% amino acid sequence identity. Therefore a mouse strain that lacks V$_\beta$8 genes was used for immunization. SWR mice were immunized as described (35) with transfectants expressing chimeric TCRs with hV$_\beta$13.1 or hV$_\beta$13.2 elements. Sera from immunized animals were screened for their ability to stimulate interleukin 2 secretion by target hV$_\beta$-bearing hybridomas and to immunoprecipitate the TCR complex (34). Spleen cells from seropositive animals were fused with Sp2/0 myeloma cells as described (36). B-cell hybridomas producing anti-V$_\beta$ antibodies were screened by flow cytometric analysis. Transfectants expressing hV$_\beta$ on their surfaces were mixed with the V$_\beta$-negative parents of the transfection, and the cell mixtures were
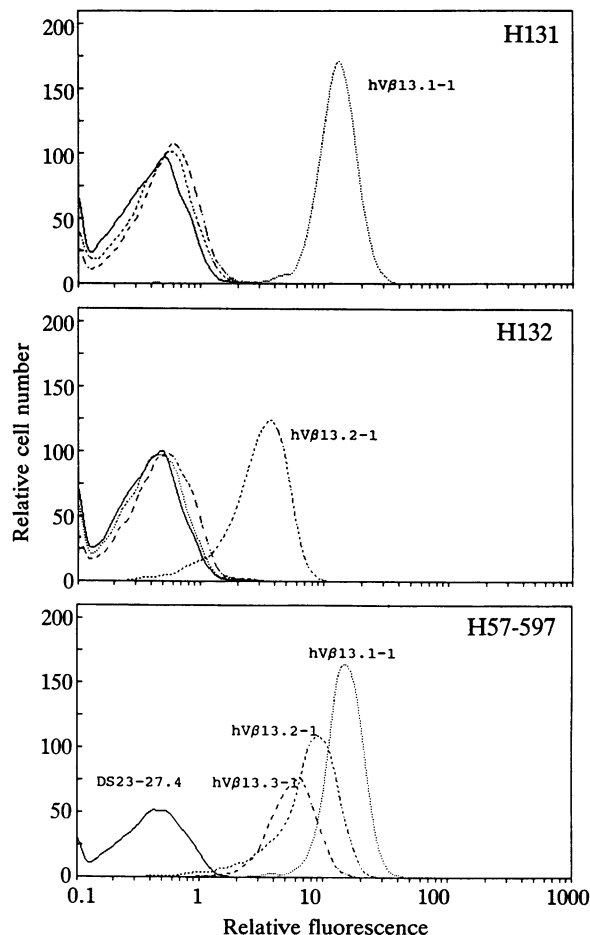

Fig. 2. V$_\beta$-specific staining by monoclonal antibodies H131 and H132. Mouse T-hybridoma cells expressing hV$_\beta$13.1 (hV$_\beta$13.1-1, ‒‒‒), -13.2 (hV$_\beta$13.2-1, ‒‒‒), and -13.3 (hV$_\beta$13.3-1, ‒‒‒‒) were stained with mouse monoclonal antibodies H131 (*Top*) and H132 (*Middle*). DS23-27.4 (—), the transfection recipient, was used as a negative control. The anti-$\alpha\beta$ TCR (H57-597) antibody was used to demonstrate the presence of surface TCR on the transfectants (*Bottom*).

incubated with the supernatants of B-cell hybridomas. After washing, the transfectants were incubated with fluorescein-
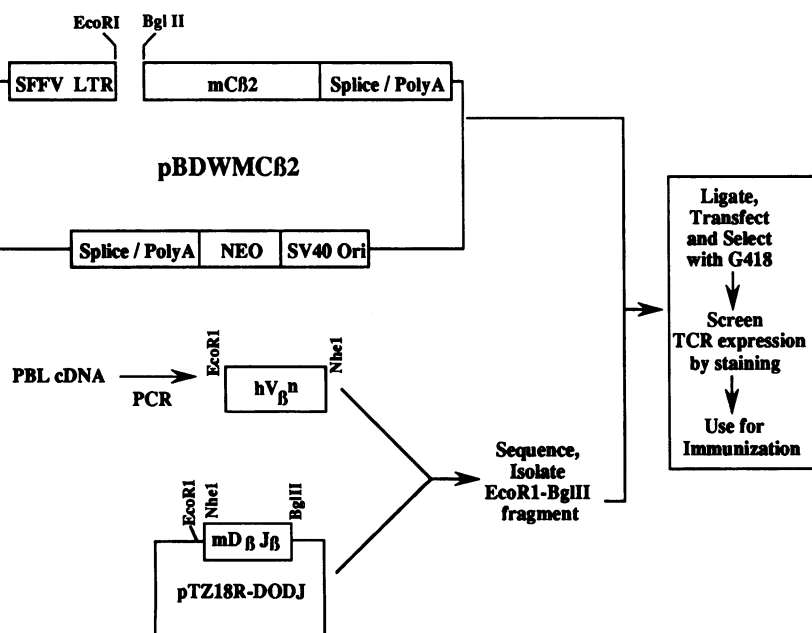

FIG. 1. Scheme for the expression of hV$_\beta$ elements on murine T-hybridoma cells. hV$_\beta$13.1, -13.2, and -13.3 genes were amplified from peripheral blood lymphocyte (PBL) cDNA by using primers containing *Eco*RI and *Nhe* I restriction enzyme sites. hV$_\beta$13.1 and -13.2 were amplified with primers as described (31). hV$_\beta$13.3 was amplified with the primers 5'-GGGAATTCAAGATG-GCCATCGGCCTCCTGTGCTGTGC-3' [5'HPB34(*Eco*RI)] and 5'-TAACTGCTAG-CACAGAAGTACACAGATGTC-3'. PCR products were cloned in a pTZ18R vector carrying the mouse D$_\beta$ and J$_\beta$ (mD$_\beta$J$_\beta$) gene segments. The *Eco*RI–*Bgl* II fragment carrying the hV$_\beta$/mD$_\beta$J$_\beta$ gene was inserted into vector pBDWMCβ2 between the spleen focus-forming virus (SFFV) long terminal repeat (LTR) promoter and the mouse C$_\beta$2 (mC$_\beta$2) constant-region gene. pBDWMCβ2 also carries the simian virus 40 origin (SV40 Ori), mRNA splicing and poly(A)-addition signals, and the neomycin-resistance gene (NEO) to allow selection with G418. Transfectants expressing human/mouse chimeric TCR β chain were screened by staining with anti-mouse TCR C$_\beta$ (H57-597) antibody (34).

ated goat anti-mouse immunoglobulin. The cell mixtures were then analyzed on a flow cytometer, and B-cell hybrids picked as positive if their supernatants yielded two separate peaks (one negative and one positive) in the analysis. With this screening method, we could easily eliminate B-cell hybrids making antibodies against antigens (autoantigens, viral antigens, etc.) common to both the β transfectants and their parents.

About 1000 B-cell hybridomas were screened from each fusion. Supernatants from 3 B-cell hybridomas stained specifically transfectants expressing $hV_\beta 13.1$, and the supernatants from 2 B-cell hybridomas stained transfectants bearing $hV_\beta 13.2$ (Fig. 2). To determine whether these antibodies recognized the hV_β elements or some combination of the hV_β and other elements of the mouse TCR, culture supernatants from these B hybridomas were used to stain normal human T cells. Antibodies specific for a hV_β should react with a significant proportion (>0.5%) of normal human T cells, whereas anti-idiotypic reagents would not. Of the 5 B-cell hybridomas, 2 stained a significant portion of normal human T cells (Fig. 3). These hybridomas were cloned by limiting dilution and named H131 for $hV_\beta 13.1$ and H132 for $hV_\beta 13.2$.

Specificity of H131 and H132. The specificities of H131 and H132 were established by two criteria. First, H131 and H132 were used to stain transfectants expressing $hV_\beta 13.1$, $hV_\beta 13.2$, and $hV_\beta 13.3$ (a new member of the $hV_\beta 13$ family described below). H131 recognized specifically cells bearing $hV_\beta 13.1$, and H132 stained only the transfectant expressing $hV_\beta 13.2$ (Fig. 2).

Second, H131 and H132 were used to stimulate human peripheral T cells (19). RNA was made from the T-cell blasts so created and was analyzed by QPCR for sequences specific for different human V_β elements (19). cDNA made from RNA of cells stimulated with H131 was enriched for sequences that could be amplified using a $V_\beta 13.1$-specific oligonucleotide (ref. 19; Fig. 4). Amplification of the same cDNA with other V_β primers was relatively inefficient, suggesting that H131 specifically stimulated T cells bearing $hV_\beta 13.1$ (Fig. 4). Similar results were observed with a $V_\beta 13.2$-specific oligonucleotide and RNA made from T cells stimulated with H132 (Fig. 4).

Analysis of $hV_\beta 13.1$ and $hV_\beta 13.2$ Usage in Peripheral Blood T Cells. H131 and H132 were used to study the usage of $V_\beta 13.1$ and $V_\beta 13.2$ on peripheral blood T cells in normal unrelated persons. $V_\beta 13.1^+$ T cells were about 4% of the total

FIG. 4. The anti-V_β antibodies (Ab) H131 and H132 stimulate human T cells expressing human $V_\beta 13.1$ and -13.2, respectively. Peripheral blood T cells from normal human donors were stimulated with H131, H132, or phytohemagglutinin. RNA was isolated from these cells and subjected to QPCR as described in *Materials and Methods*. The amplified products were electrophoresed in 2% agarose gels, dried, and exposed to x-ray film. +, H131 or H132 stimulation; −, phytohemagglutinin stimulation.

T cells in the peripheral blood of most people (Fig. 3). The percentages of T cells bearing $V_\beta 13.1$ were not very different among the individuals tested in this study. Similar findings were observed for T cells bearing other V_β sequences that could be detected with preexisting monoclonal antibodies, $V_\beta 5.1$ and -5.2/3 (Fig. 3). By contrast, the $V_\beta 13.2$ element was used by quite different percentages of T cells among the same individuals (Fig. 3). $V_\beta 13.2^+$ T cells varied from 0.5% to 4% in peripheral blood.

The usage of $V_\beta 13.2$ in some of the individuals tested was also analyzed by QPCR. The QPCR value of $V_\beta 13.2$ use was proportional to the percentage of T cells staining with H132 in peripheral blood (data not shown). This result strengthens the validity of QPCR for analysis of expression of V_β elements for which specific antibodies are not available.

Isolation of a Third Member of the $hV_\beta 13$ Family. When a human $hV_\beta 13.1$ probe is used to analyze human genomic DNA in a Southern blot, four hybridizing bands can be identified (6). Two of these bands are the genes for $V_\beta 13.1$ and -13.2. The gene for $V_\beta 13.2$ is 70% identical to that for $V_\beta 13.1$. A third member of the $hV_\beta 13$ family was isolated by PCR with a 5′ primer common to the leader sequence of $hV_\beta 13$ and a 3′ primer specific for the hC_β gene, as described in *Materials and Methods*. Multiple clones with different $D_\beta J_\beta$ sequences were sequenced to eliminate the possibility that the new sequence was due to a PCR artifact. The sequence of the $V_\beta 13.3$ gene is compared with those of $V_\beta 13.1$ and -13.2 in Fig. 5. $HV_\beta 13.3$ has 11 and 19 amino acid differences with $hV_\beta 13.1$ and $hV_\beta 13.2$, respectively. A transfectant expressing $hV_\beta 13.3$ (generated as described above) was not recognized by the antibodies H131 and H132, however (Fig. 2).
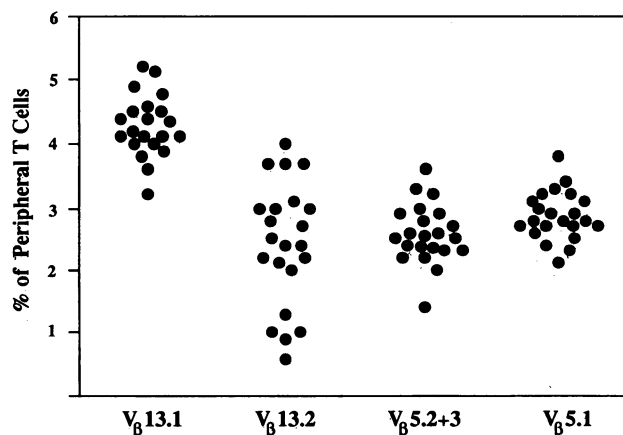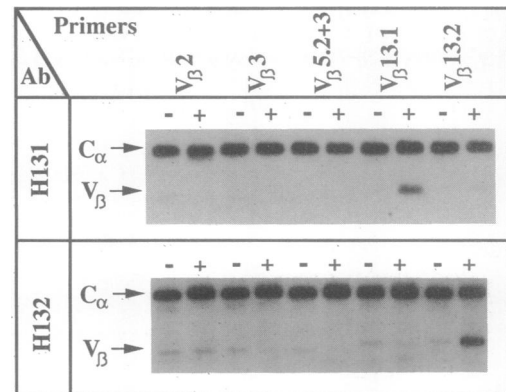

FIG. 3. V_β expression on human peripheral T cells. Peripheral blood T cells were stimulated with phytohemagglutinin (3 μg/ml) for 3 days, washed, and cultured for another 24 hr with recombinant human interleukin 2 (25 units/ml) to allow regeneration of potentially modulated receptors. The cells were then analyzed with antibodies to CD3 or one of four monoclonal antibodies to hV_β (H131 to $hV_\beta 13.1$, H132 to $hV_\beta 13.2$, 1C1 to $hV_\beta 5.2+3$, and LC4 to $hV_\beta 5.1$).

DISCUSSION

The study of TCR V_β usage has been a powerful tool for the understanding of the processes of T-cell development and the pathogenesis of some T-cell-mediated autoimmune diseases. For example, in experimental autoimmune encephalomyelitis (EAE), it was shown that the mouse $V_\beta 8$ element was used by the TCRs of most pathogenic T-cell clones, and it was even possible to prevent or reverse the progression of EAE by using an antibody specific for mouse $V_\beta 8$ (37). Likewise, V_β analysis has shown that endogenous superantigens can cause deletion (9–14) or anergy (38, 39) of the cells bearing target V_β elements. Presumably much is yet to be learned
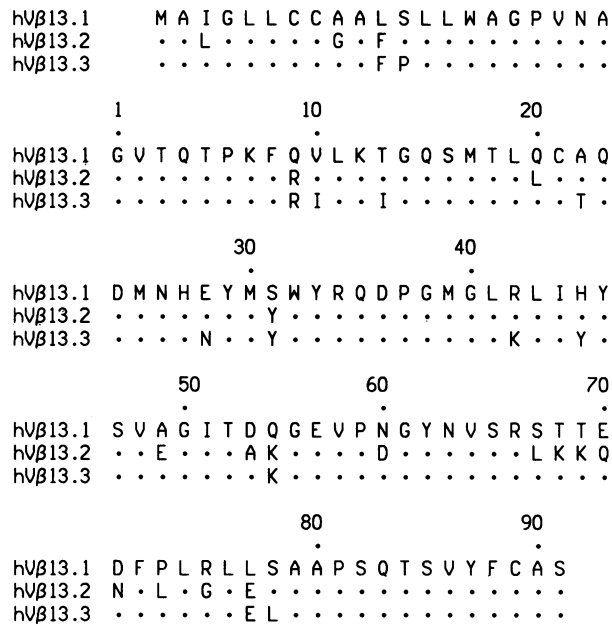
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hVβ13.1   M A I G L L C C A A L S L L W A G P V N A
hVβ13.2   · · L · · · · · G · F · · · · · · · · · ·
hVβ13.3   · · · · · · · · · · · F P · · · · · · · · ·

          1          10             20
          ·          ·              ·
hVβ13.1   G V T Q T P K F Q V L K T G Q S M T L Q C A Q
hVβ13.2   · · · · · · · · R · · · · · · · · · · L · · ·
hVβ13.3   · · · · · · · · R I · · I · · · · · · · T · ·

              30                40
              ·                 ·
hVβ13.1   D M N H E Y M S W Y R Q D P G M G L R L I H Y
hVβ13.2   · · · · · · · · Y · · · · · · · · · · · · · ·
hVβ13.3   · · · · N · · Y · · · · · · · · · K · · Y ·

              50        60              70
              ·         ·               ·
hVβ13.1   S V A G I T D Q G E V P N G Y N V S R S T T E
hVβ13.2   · · E · · · A K · · · · D · · · · · · L K K Q
hVβ13.3   · · · · · · · K · · · · · · · · · · · · · · ·

                  80          90
                  ·           ·
hVβ13.1   D F P L R L L S A A P S Q T S V Y F C A S
hVβ13.2   N · L · G · E · · · · · · · · · · · · · ·
hVβ13.3   · · · · · · E L · · · · · · · · · · · · ·
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FIG. 5. Sequence comparison of $hV_{\beta}13.3$ with $hV_{\beta}13.1$ and -13.2. Amino acid residues identical to those of $hV_{\beta}13.1$ are marked as dots.

about disease from studies of this type. Unfortunately, however, this kind of analysis in human disease has been hampered by the lack of anti-hV_{β} antibodies. Although V_{β} use by human T cells can be studied by QPCR (19, 29, 30), this method cannot be used with ease, and moreover QPCR cannot be used to easily detect target T cells *in vivo* or expand them *in vitro*, whereas monoclonal anti-V_{β} antibodies can. We have therefore set out to develop a method that will allow investigators to produce anti-hV_{β} antibodies reliably and relatively easily. We made use of knowledge, acquired in a previous set of experiments (31), that a chimeric hV_{β}/mouse D_{β}, J_{β}, C_{β} gene transfected into a mouse T-cell hybridoma can give rise to a product that can be expressed functionally as a β chain on the surface of the transfectant. These transfectants have all the advantages of mouse T hybridomas. They can be easily maintained, grown in large amounts, and used for structure–function analyses (31). Because mouse T hybridomas have been successfully used in the past to generate many different monoclonal antibodies specific for mouse TCR V_{β} elements, we reasoned that the transfectants described in this manuscript would have similar properties, with the added advantage that the hV_{β} element would be the only non-mouse component on the surface of the cells. This prediction proved correct. Using these cells as immunogens, we generated monoclonal antibodies to $hV_{\beta}13.1$ and -13.2.

The method described here does seem to have some limitations, however. Transfectants bearing $hV_{\beta}2$ expressed the protein product of the transfected β-chain gene poorly, and we have not succeeded in raising a monoclonal anti-$hV_{\beta}2$ antibody by using the transfectants as immunogens. Perhaps there is some structural incompatibility between $hV_{\beta}2$ and the D_{β}, J_{β} or α elements used in the recipient cell, a possibility that can be tested by transfection of human $V_{\beta}2$-containing β genes into other mouse T-cell hybridoma recipients.

H131 and H132 are each specific for their target V_{β} elements, and neither crossreacts with human $V_{\beta}13.3$. This complete specificity will be very useful in comparisons of V_{β} usage by T cells among individuals or between tissues of the same individual.

In the preliminary studies reported in this paper, we were particularly struck by the wide variation in percentages of T cells bearing $V_{\beta}13.2$ between normal individuals. Such differences could be due to several phenomena. The TCR genes

themselves may vary from one individual to another. For example, polymorphism of the $hV_{\beta}6.7$ gene has been shown to determine the percentage of $hV_{\beta}6.7^+$ cells in peripheral blood (40). In a preliminary experiment, however, we did not find polymorphism by analysis of the $hV_{\beta}13$ loci on Southern blots after digestion with a limited set of restriction enzymes (data not shown). Nevertheless, it is still possible that there are sequence differences between the $V_{\beta}13.2$ genes of the individuals tested and that these sequence differences affect the level of $hV_{\beta}13.2^+$ T cells in the periphery.

Alternatively, the differences may be controlled by the MHC types of the people concerned or by other background genes expressed in the various individuals. With this in mind, it is interesting that the 10-fold differences in $V_{\beta}13.2$ usage seen between some individuals is quite similar to that found in the usage of a number of V_{β} elements in laboratory and wild mice (9–14, 22, 41). In mice, V_{β} expression by peripheral T cells is controlled predominantly by MHC molecules and superantigens, encoded by endogenous murine mammary tumor viruses (9–14, 22–28, 41). Although human retroviruses closely homologous to the murine mammary tumor viruses have not been found, it is tempting to speculate that superantigen-encoding endogenous retroviruses may exist in humans. The availability of a monoclonal antibody against a V_{β} element that varies widely in use between different people will certainly help in tests of this hypothesis. Further experiments with more individuals and family studies are needed to clarify the matter. In the future, other anti-hV_{β} monoclonal antibodies can be used to study variations in the expression of other human V_{β} elements, previously noted in QPCR measurements (19).

Finally, it remains to be shown that the method described in this paper can be applied to the production of anti-hV_{α} antibodies. In practice, it has been more difficult to raise antibodies against mouse or human V_{α} than against V_{β}. This may be because the TCR α polypeptide is more heavily glycosylated than the β polypeptide, or because of some structural asymmetry in the TCR. Anti-V_{α} monoclonal antibodies will undoubtedly be useful in studies of the human TCR repertoire in health and disease, however, and their production by methods such as those described here should be attempted.

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