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T CELL RECEPTOR-γ AND -δ GENES PREFERENTIALLY UTILIZED BY ADULT THYMOCYTES FOR THE SURFACE EXPRESSION[1]

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To assess the diversity of γδ receptors expressed on adult thymocytes we characterized the γ- and δ-genes used in a panel of T cell hybridomas expressing a TCR-γδ-CD3 complex on the cell surface. DNA cloning and sequencing analysis were necessary to determine the used genes because of the presence of multiple rearrangements in these hybridomas. Among eight hybridomas analyzed, five used $V_{\gamma 4}$, two used $V_{\gamma 7}$, and one used $V_{\gamma 6}$ for γ-genes, and four used $V_{\delta 5}$, two used $V_{\delta 4}$, and two used $V_{\delta 7}$ for δ-genes. The last δ-gene is documented for the first time. V_γ and V_δ appear to pair randomly only restricted by the frequency of the utilization of individual V_γ and V_δ segments. These γ- and δ-genes contained N region addition between the V and J region in most cases. Both $D_{\delta 1}$ and $D_{\delta 2}$ regions were used in the most δ-genes as was seen previously. These results show that certain γ- and δ-gene segments that are rarely used for the expression on fetal thymocytes are preferentially used by adult thymocytes. Also the repertoire of the γδ receptors on adult thymocytes is much greater than that on fetal thymocytes. BW5147 specific V_4-C_1 γ-gene was expressed in all of the adult hybridomas. Inasmuch as this gene was not expressed in BW5147 cells or in fetal thymocyte hybridomas expressing V_5 or V_6 γ genes, there exists a novel V gene segment-dependent control of transcription in the γ-gene system.

In vertebrate's immune systems, Ag are recognized by cell-surface receptors on B and T cells. The TCR has been characterized and found to be a heterodimeric protein composed of α and β-subunits, each containing a V, J, and C region like Ig, its counterpart on a B cell (1). During the search for the genes coding for TCR α- and β-subunits, a third gene, termed γ, was isolated, which carries the characteristics similar to those of α- and β-genes including T cell-specific expression and gene rearrangement (2, 3).

Recently the γ-gene product has been demonstrated to form a heterodimer with another polypeptide, δ-chain, and this γδ-heterodimer was shown to be associated with a monomorphic CD3 molecule as is the αβ-heterodimer (4–8). Furthermore, the γδ/CD3 complex has been identified on a subset of both thymic and peripheral T cells, which express neither CD8 (Lyt-2) nor CD4 (L3T4) differentiation Ag i.e., DN[4] cells (5–10). The genome of a typical inbred strain of mouse such as BALB/c or C57BL/6 carries three pairs of J_γ- and C_γ-gene segments and seven V_γ-gene segments (3, 11–13) (see Fig. 1). The δ-gene segments are located within the cluster of α-gene segments (14). The δ-cluster consists of two D, two J, one C and an unknown, but an apparently small (~10) number of V gene segments (15, 16).

Rearrangement and RNA expression of γ- and δ-genes were previously studied by analyzing the DNA, RNA, and cDNA clones isolated from populations of fetal and adult DN thymocytes and their hybridomas (11, 15, 16). These studies revealed interesting age-dependent utilization of specific γ- and δ-gene segments. However, because only less than 5% of DN thymocytes actually express γδ-receptors on the cell surface (6, 17), the profiles of DNA rearrangement and RNA expression obtained by these studies may not reflect those of the γδ-bearing cells. To circumvent the shortcomings we recently prepared a number of γδ-bearing hybridomas by fusing the thymoma BW5147 with DN thymocytes isolated from timed embryos and newborn mice. Analysis of these hybridomas revealed fetal age-dependent utilization of γ- and δ-gene segments for the surface expression of γδ-receptors (18).

In the present study we analyzed a series of γδ-bearing hybridomas prepared from 5-wk-old adult mice. The results, when combined with out earlier ones, show a dramatic switch in the use of γ- and δ-gene segments for the surface expression as the thymus develops from the embryonic state to the adulthood. One feature of this switch is that the expressed repertoire of the receptor is substantially increased during the development. In addition we report a novel V_γ-gene segment-specific control of γ-gene transcription.

MATERIALS AND METHODS

Animals. C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

mAb. mAb were produced as culture supernatant from the following hybridomas: anti-CD4 (L3T4) was from RL174.2 supplied by H. R. MacDonald (Ludwig Inst. for Cancer Research, Lausanne, Swit-

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[4] Abbreviation used in this paper: DN, double negative.

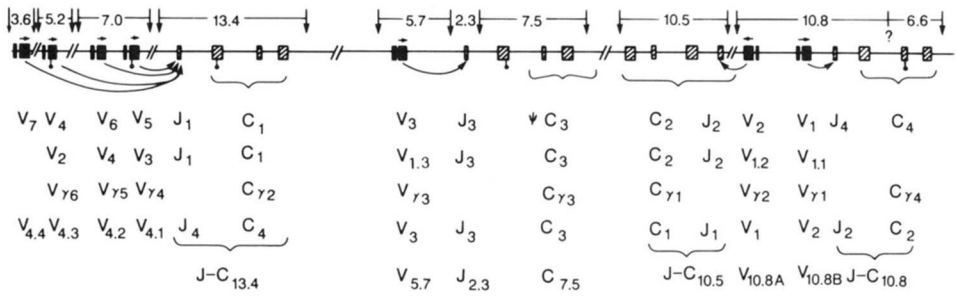

Figure 1. Organization of the γ-genes and their nomenclature. The relative position of γ-genes are indicated according to Pelkonen et al. (13) without proportionality to the actual length. Nomenclatures listed are: first row nomenclature according to the order of findings, used in this paper and elsewhere (10, 44); second row from Garman et al. (11); third row from Iwamoto et al. (12); fourth row from Pelkonen et al. (13); and the last row from Hayday et al. (3). *Shaded box* indicates Constant region exons and *black box* indicates V region exons. The *horizontal arrows* above the V gene segments indicate direction of transcription. The *curved arrows* indicate the conventional V-J rearrangements. The numbers between the *vertical arrows* indicate the sizes (in kb) of the *Eco*RI DNA fragments.

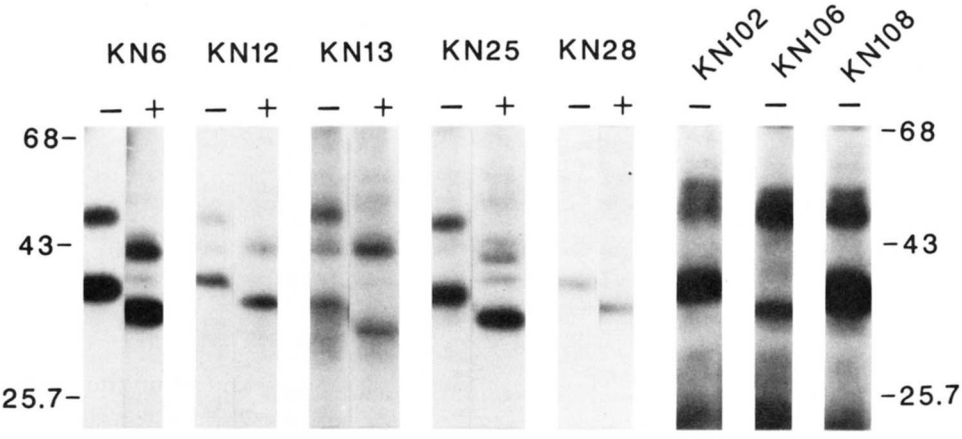

Figure 2. Immunoprecipitation of hybridoma lysates with the anti-γ mAb. Radioiodinated hybridoma cells were lysed with 1% Nonidet P-40 and immunoprecipitated with protein A-coupled Sepharose beads and anti-γ mAb KN365. N-glycosidase treatment was done for the lanes marked +. Molecular weight markers are indicated in kDa.

zerland), anti CD8 (Lyt-2) was from AD4.15 supplied by F. W. Fitch (Dept. Pathol., Univ. Chicago), and anti murine CD3 was from 2C11 supplied by J. A. Bluestone. Anti-γ-mAb, KN365, was raised against a polypeptide produced in *Escherichia coli*, which consisted of the downstream 30 amino acids of the $V_{\gamma 2}$-encoded region, the complete $J_{\gamma 2}$, and most of the $C_{\gamma 2}$ region encoded by the pHDS203 γ-gene (2) (N. Nakanishi, Ph.D. thesis, University of Tokyo, Tokyo, Japan).

Cell preparation and cell fusion. DN thymocytes were isolated from 5-wk-old adult mice as described by Fowlkes et al. (19). Briefly, thymocytes were depleted of CD4- and CD8-bearing cells by C-mediated lysis. After removal of dead cells through Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation, DN thymocytes were cultured for 4 days with the mixture of IL-1 (Hoffman-La Roche, Nutley, NJ), IL-2 (Ajinomoto, Tokyo, Japan), rat T cell monoclone (Collaborative Research, Lexington, MA), and WEHI-3 culture supernatant. These DN thymocyte blast cells (4×10^7 cells) were fused with AKR thymoma cell line (1×10^7 cells), BW5147, in 0.5 ml 45% polyethylene glycol (1500, Sigma Chemical, St. Louis, MO) for 2 min. After diluting out with 20 ml DMEM containing 10% FCS and culturing for 1 day, cells were subjected to HAT selection. Subcloning was done with the addition of irradiated peritoneal macrophages from BALB/c mice.

Cytofluorimetric analysis of cells. Cell were incubated first with the anti-CD3 antibody and subsequently with FITC-labeled goat anti-mouse Ig (Cooper Biomedical Inc., West Chester, PA). Epix-5 was used for analysis.

Immunoprecipitation of the γδ-complex and SDS-PAGE. Cells were labeled with ^{125}I (New England Nuclear, Boston, MA) by using glucose oxidase/lactoperoxidase method (8, 20). ^{125}I labeled cells were washed three times in PBS and were lysed with 0.5% NP-40 or 1% digitonin buffer (9). Immunoprecipitation, peptide N-glycosidase F (N-glycosidase, Genzyme, Boston, MA) treatment, and SDS-PAGE analysis were as described before (10).

Southern and Northern blot analysis. Cellular DNA was extracted as described (21), digested with *Eco*RI or *Hin*dIII restriction enzymes (New England Biolabs, Beverly, MA), and electrophoresed through 0.75% agarose gel. Total cellular RNA was extracted using the guanidinium thiocyanate-CsCl gradient method (22), and electrophoresed through formaldehyde (2.2%) containing 1.0% agarose gel (23). The DNA or RNA was transferred to a nitrocellulose filter (Nitroplus 2000, MSI, Westboro, MA) and hybridized with random primed probes by standard procedures (24–26).

cDNA library. Poly A^+ RNA was purified by absorption onto oligo (dT) cellulose (type 7 from Pharmacia) as described (27), and ds cDNA was synthesized according to Gubler and Hoffman (28). Blunt ended ds-cDNA was ligated with a synthetic adaptor, 5′ (HO-) AATTCTA-GAGTCGAC-3′ 3′-GATCTCAGCTG-(P)5′ and size fractionated by Bio-gel A50m filtration column. After ligation with *Eco*RI-cleaved λgt-10, DNA was packaged using Giga-Pak Gold (Stratagene, La Jolla, CA). The libraries were screened with both V and C probes using the standard procedure (29).

Genomic library. For the isolation of $V_{\gamma 4}J_{\gamma 1}$ genomic DNA clones *Hin*dIII digested DNA was size fractionated by 0.75% agarose gel, and 1.3 ~ 1.5 kb DNA was eluted from the gel. This was, then, blunt-ended using Klenow enzyme, ligated with cDNA adaptor described above, gel purified, and ligated into *Eco*RI cleaved λgt-10. The library was screened with both $V_{\gamma 4}$ and $J_{\gamma 1}$ probes. For cloning X from KN106 or Y from KN108, *Eco*RI-digested DNA was fractionated by 0.75% agarose gel, and 5 ~ 6 kb DNA from KN106 or 3.8 ~ 4.4 kb DNA from KN108 was ligated directly into λgt10. The libraries were screened with the $C_{\gamma 4}$ genomic probe. The cosmid library was constructed by partial digestion of KN6 DNA by *Mbo*I and ligation into pWE15 cosmid vector (30).

Probes. $J_{\gamma 1}$ probe was excised out of an in-frame $V_{\gamma 4}$-$J_{\gamma 1}$ genomic DNA clone from KN6, and was a *Sty*I-*Hin*dIII fragment (1.1 kb) containing $J_{\gamma 1}$ and a part of the flanking intron. $V_{\gamma 2}$ probe was a *Pst*I-*Ava*I fragment (400 bp) from pHDS 203 (2). $V_{\gamma 4}$ probe was a 5′-*Cla*I fragment (320 bp) from the clone FT-2 (31). $V_{\gamma 6}$ probe was a *Sph*I-*Sph*I fragment (750 bp) from a *Pvu*II-*Hin*dIII subclone (2.4 kb) of clone 13 supplied by Raulet and co-workers (11). $V_{\gamma 7}$ probe was a *Pst*I-*Bst*NI fragment (300 bp) isolated from a $V_{\gamma 7}$ containing cosmid clone (L. Helman, unpublished data). $C_{\gamma 4}$ probe was either a *Bam*HI-*Eco*RI fragment (1.6 kb) from the genomic clone $V_{10.8}$ (3) for Southern hybridization or an *Ava*I-*Ava*I fragment (400 bp) from the clone 5/10-13 1.2 supplied by P. S. Ohashi and T. W. Mak (12). $J_{\delta 1}$-probe was an *Ava*I-*Sac*I fragment (800 bp) from a genomic $V_{\delta 5}$-$J_{\delta 1}$-clone isolated from KN6. $V_{\delta 4}$-probe was the 5′-*Eco*RV fragment (390 bp) from a TCR-δ cDNA clone from KN12. $V_{\delta 5}$-probe was an *Ava*I-*Ava*I fragment (300 bp) from a KN6 cDNA clone, and $V_{\delta 7}$-probe was 5′-*Dde*I fragment (375 bp) from KN25 cDNA clone.

Oligonucleotide probes and primers. Oligonucleotide probes and primers were synthesized in Autogen 6500 (Milligen, Bedford, MA) using β-cyanoethylphosphoramidite and 5.5-min program. Because of the high efficiency of the synthesis, the oligonucleotides were

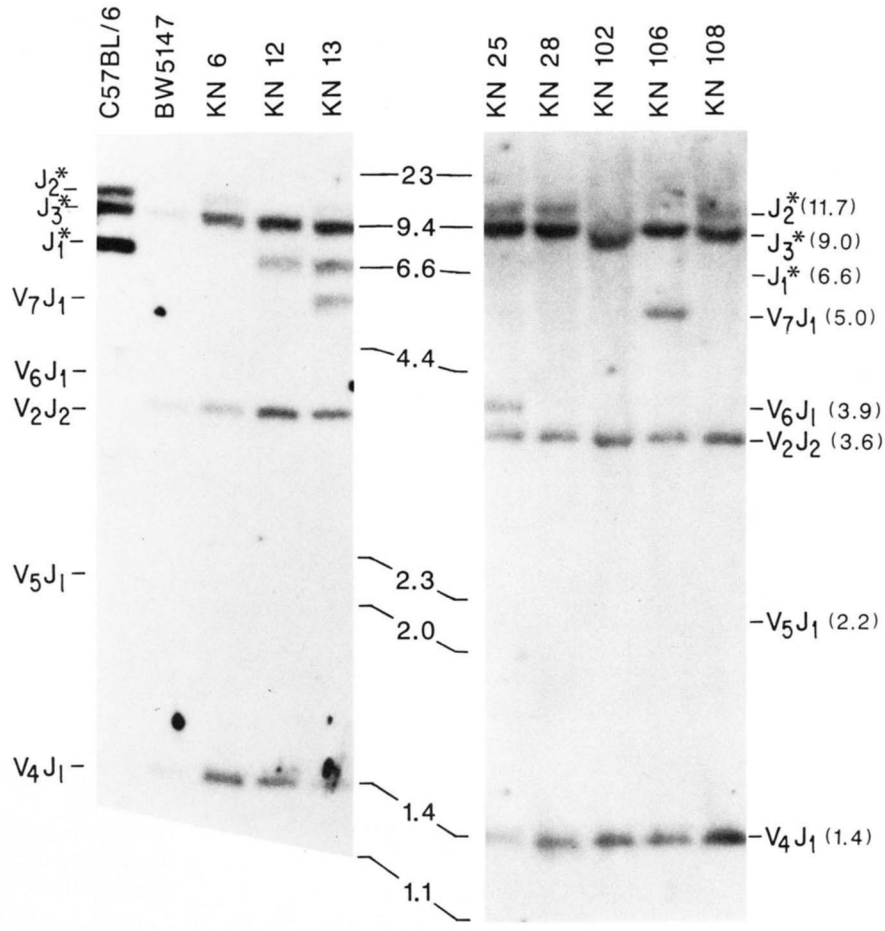

Figure 3. Southern blot analysis of γ-gene rearrangements-*Hin*dIII digest. *Hin*dIII-digested DNA (8 μg) was electrophoresed in 0.75% agarose gel. The probe used for the hybridization is a radom primed $J_{\gamma 1}$-probe. The assignment of each band is according to Pelkonen et al. (13) for the germline J's, V_7-J_1, V_2-J_2, and V_4-J_1. The assignments were in part confirmed by each V probe. *Hin*dIII-cut λ-size markers, and *Hae*III cut φX174 size markers are indicated in the middle in kb. The sizes of the germline or rearranged γ-gene segments are indicated in kb in parentheses at right. *Asterisks* indicate J gene segments in germ-line configuration. Because the 5′ end of $J_{\gamma 1}$-gene segment is 1.2 kb upstream of the nearest downstream *Hin*dIII site, any V-$J_{\gamma 1}$-rearrangement should be detected in *Hin*dIII-bands larger than 1.2 kb.

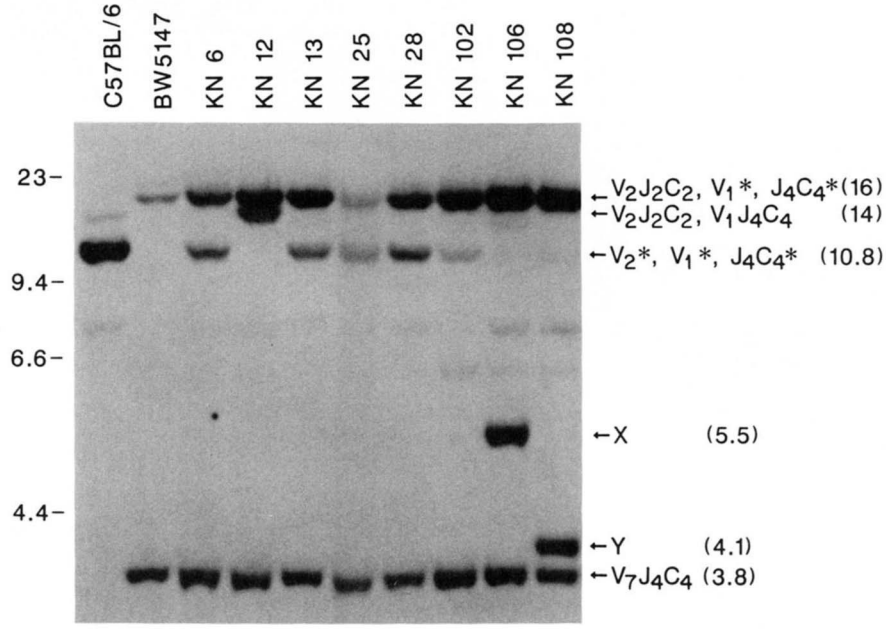

Figure 4. Southern blot analysis of γ-genes-*Eco*RI digests, with the $C_{\gamma 4}$-genomic probe. The 16-kb band corresponds to the rearranged $V_2J_2C_2$ with V_1 and J_4C_4 in the germ-line configuration and the 14-kb band corresponds to the DNA fragment that contains two rearrangments, $V_2J_2C_2$ and $V_1J_4C_4$ (see Fig. 1). The 10.8-kb band contains V_2, V_1, and J_4-C_4 all in the germline configuration (also see Fig. 1). *Asterisks* indicate gene segments thought to be in germline configuration. Faintly hybridizing bands are cross-hybridizing bands; $C_{\gamma 1}$ (13.5 kb), C57BL/6-$C_{\gamma 3}$ (7.5 kb), and AKR-$C_{\gamma 3}$ (6.4 kb). *Hin*dIII-cut λ-size markers are indicated at the left in kb. For X and Y see the text.

used without further purification. The BW5147-specific $V_{\gamma 4}$-$J_{\gamma 1}$-junction probe, STP.069, had the following sequence: 5′-CTGAGC-TCCTCTCTCCTTTAGCCGTAGG-3′. Hybridization of this probe with DNA in phage plaques was carried out according to Miyada and Wallace (32) at 52°C. The probe for $V_{\gamma 7}$, STP.056, had the following sequence: 5′-GGGAGGGCCCCAGAGCACCTTCTCTACTATAACTT-CGTCAGTTCCACAACTGTGGT -GGATTCCAGA-3′. The primers for DNA sequencing were STP.028 (5′TTGGGGGAAATGTCTGCAT-3′) for $C_{\gamma 1} \rightarrow J_{\gamma 1} \rightarrow V_{\gamma}$ sequencing, STP.066 (5′AAGAAAATGGAGGCAAG-T-3′)for$V_{\gamma 4} \rightarrow J_{\gamma 1}$ sequencing,STP.067(5′TTGTTTCAGCAGCAGAAG-G-3′) for $C_{\gamma 4} \rightarrow J_{\gamma 4}$ sequencing, STP.061 (5′-GTTCCATTTTTCATGA-TG-3′) for $C_{\delta} \rightarrow J_{\delta 1} \rightarrow V_{\delta}$ sequencing, and M13 (Universal) and M13 (Reverse) from United States Biochemical Corp. (Cleveland, OH).

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method of Sanger (33) using dGTP and dITP reaction of Sequenase Kit (United States Biochemical) (34). Sequencing reaction was done directly with cDNA inserted in λgt-10 or subcloned into pUC13 vector (35, 36).

RESULTS

Generation of γδ-bearing hybridomas from adult thymus. T cell hybridomas were prepared by fusing DN

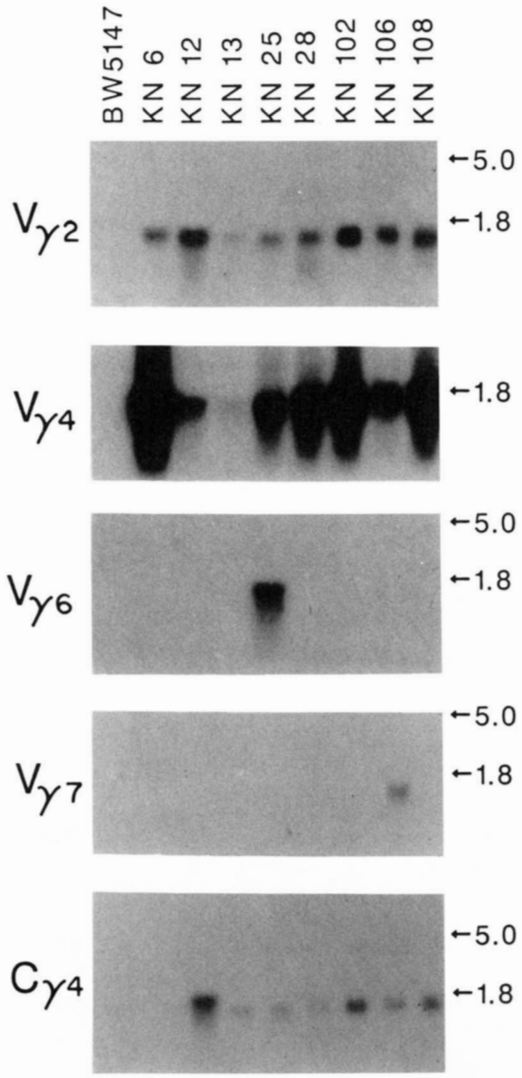

Figure 5. Northern blot analysis for γ-gene transcripts. Gamme gene probes used are indicated on the left. Molecular weight of the rRNA markers are indicated on the right. All the hybridomas gave low levels of RNA hybridizing with the $C_{\gamma 4}$-cDNA probe. We believe that these RNA bands are due to cross-hybridization of the $C_{\gamma 4}$-probe with the $V_2J_2C_2$ RNA. However, the relatively more intense $C_{\gamma 4}$-positive band displayed by KN12 RNA is likely to be genuine $V_{\gamma 1}C_{\gamma 4}$-RNA.

thymocytes isolated from 5-wk-old C57BL/6 mice with BW5147, an AKR thymoma. Forty independent $CD3^+$ hybridomas were identified by staining with anti-murine CD3 mAb (2C11). These hybridomas were analyzed by immunoprecipitation of ^{125}I-labeled cell surface proteins with the anti CD3 mAb as well as with anti-γ mAb, KN365. The latter mAb has been shown to immunoprecipitate C_1 and C_2 γ-gene products (10). Eight γδ-bearing hybridomas were obtained which exhibited sufficient but varying degrees of stability with respect to the surface expression of the γδ-CD3 complex.

Analysis of γδ-proteins. The apparent m.w. of the γ-chains, as estimated by the mobility in SDS-PAGE, was somewhat variable depending on the hybridoma sources and fell between 37.5 and 34 kDa (Fig. 2). After N-glycanase treatment the M_r of all the γ-chains was reduced by approximately 3 kDa as exemplified in Figure 2 for KN6, KN12, KN25, and KN28. This indicates that at least one N-glycosylated chain is attached to these γ-chains, and thus these are not the $V_2J_2C_2$ γ-gene product which is known to lack N-glycosylation site (2). All the δ-chains were about 47 kDa except for that of KN25 which was slightly lighter. After N-glycanase treatment the M_r of all δ-chains was reduced by approximately 6 kDa, indicating that the δ-chains are glycosylated about twice more than the γ-chains.

γ- and δ-genes used for surface expression. To determine which γ- and δ-genes code for the surface-expressed γδ-heterodimers on the various hybridomas we analyzed their DNA and RNA by Southern and Northern blotting methods. These experiments help to narrow down the candidates for the genes responsible for the surface γδ expression but do not usually allow the unequivocal identification of these genes because of the multiple rearrangements in the thymocyte genome and the contribution by BW5147 genome. The final answer can therefore be obtained only by the combination of these results with those of DNA cloning and sequencing. Below we will first described γ-genes and then δ-genes.

γ-genes. The results of the Southern and Northern blot analysis of γ-genes are shown in Figures 3, 4, and 5, whereas the nucleotide sequences of the V_γ-J_γ-junctions are shown in Figure 6. Table I summarizes the state of rearrangement and expression at the $J_{\gamma 1}$ and $J_{\gamma 4}$ sites in the hybridomas and BW5147.

In Figure 3, the various genomic DNA digested with *Hin*dIII was analyzed with the $J_{\gamma 1}$ probe. As reported previously this combination of an enzyme and a probe allows the identification of all conventional rearrangements involving not only the $J_{\gamma 1}$-but also $J_{\gamma 2}$- and $J_{\gamma 3}$-gene segments (13). In figure 4, the same set of DNA digested with *Eco*RI is analyzed with a $C_{\gamma 4}$-probe to detect rearrangements at the $J_{\gamma 4}$-gene segment. It was previously reported that BW5147 carries a rearranged $V_4J_1C_1$ γ-genes and an out-of-frame $V_2J_2C_2$ γ-genes on one copy of chromosome 13 (on which all known γ-gene segments reside) (13, 37, 38). The other copy of chromosome 13 carries an unusual out-of-frame $V_7J_4C_4$ γ-gene (13). Because of the arrangement of the various γ-gene segments in the germ-line genome (see Fig. 1) this latter rearrangement causes the deletion of all other γ-gene segments from this chromosome. These conclusions are supported by the Southern blot patterns of BW5147 DNA shown in Figures 3 and 4.

The bands containing these three γ rearrangements observed in BW5147 are also detected in the DNA of all eight hybridomas analyzed (Figs. 3 and 4). In fact in three of them (KN6, KN28, and KN102) these seem to be the only bands containing γ rearrangements. The thymocyte-derived γ-genes coding for the surface-expressed γ-chains must therefore be included in one of these bands in each of these hybridomas. Among them $V_2J_2C_2$ can be excluded because it lacks N-glycosylation sites. $V_7J_4C_4$ can also be excluded because no V_7 probe-positive transcripts can be detected in these hybridomas (Fig. 5). Thus the $V_4J_1C_1$ γ gene for which transcripts are abundantly detected (Fig. 5) is the sole candidate for the surface-expressed γ-gene in KN6, KN28, and KN102. Indeed in the cDNA libraries prepared from each of the three hybridomas we could identify DNA clones that contain a unique, in-frame joined $V_4J_1C_1$ γ sequence (Fig. 6). We therefore conclude that these $V_4J_1C_1$ γ-genes code for the γ-chains expressed on the surface of these hybridomas.

In each of the other five hybridomas one or more γ rearrangement(s) was(were) detected in addition to the

potent agents capable of inducing a neutrophil influx, and although it is not directly chemotactic in vitro (34, 35) (our observations), it is thought to induce the generation of chemotaxins, the principal component possibly being IL-1, within an inflammatory lesion (36) (reviewed in Ref. 30).

There are relatively few reports concerning the skin test reactivities of purified or recombinant cytokines. Partially purified ETAF (identical with IL-1), IL-1 or recombinant IL-1 (36, 37) cause PMN infiltration and induration when injected intradermally into mice or rabbits. Although IL-1 is a potent lymphocyte chemotaxin in vitro (38) this property has not been confirmed in vivo, as recombinant IL-1 does not induce mononuclear cell infiltration at skin test sites, although it is occasionally evident with partially purified ETAF (37).

Responses to C5a and IL-1/ETAF differ from those induced by MPIF in several respects. It is distinct from the potent chemotactic peptide C5a in chemotaxis assays by virtue of its sensitivity to heat and lack of reactivity with anti-C5a antibody. It is also unlikely that the chemotactic activity of MPIF results from the activation of a "co-chemotaxin" present in heated FCS (39) as chemotaxis was somewhat enhanced when BSA was substituted for FCS in the assay. In vivo experiments indicate that MPIF causes a sustained cellular infiltrate, whereas PMN accumulation and induration induced by IL-1/ETAF peaks at 3 to 4 h and returns to background levels after 6 h (35) and reactions with C5a diminish after 1 h (see above). In addition, a single injection of 3 to 29 U IL-1 induces hemorrhage and erythema (40), whereas MPIF did not elicit these effects.

Our results show that MPIF is chemotactic for both PMN and macrophages, that the response is dose-dependent (Table II) and not due to random migration because a positive gradient of MPIF was required. IL-1/ETAF is chemotactic for phagocytic cells (41). The 20% solution of MPIF (the optimal concentration in chemotaxis assays, Table II) contained 0.5 U IL-1/ml and lower concentrations (5 and 10%) of MPIF had correspondingly lower levels of IL-1 but were still strongly chemotactic. Higher concentrations (30 to 60 U/ml IL-1/ETAF) were required by Luger et al. (41) to enhance migration of human mononuclear cells but 15 U/ml IL-1/ETAF had insignificant activity; PMN chemotaxis was maximal with 60 U/ml IL-1/ETAF with low activity at 15 U/ml. Subsequent studies have shown that IL-1/ETAF is a more potent lymphocyte chemoattractant (38). In addition, TNF-α is chemotactic for phagocytic cells (42) but TNF has properties distinct from MPIF, and does not induce MPCA (4, 5). The chemotactic activity of MPIF for cell types other than PMN and macrophages remains to be investigated.

Murine LDCF are poorly defined (for review see Ref. 2), making comparison with MPIF difficult. Leonard and Meltzer (43) have described a LDCF produced by stimulated mouse spleen cells which is chemotactic for macrophages. In contrast to MPIF-α and -β, this factor bound DEAE-Sephacel and had M_r 38 kDa, although some minor activity with M_r 13 kDa was found. Because LDCF was produced in the presence of serum which could result in the formation of complexes of LDCF, its relation to MPIF is unknown. Our studies have shown that the chemotactic activity co-eluted with MPIF and that the low mwt fractions (20 and 14 kDa) had the major activities in both assays. MPIF-$\alpha\beta$ differs from the macrophage chemotactic factor characterised by Harita et al. (44) which has M_r 43 kDa, pI = 6.6 to 6.8, and is stable to heating at 80°C for 15 min. MPIF is somewhat similar to human LDCF which has M_r 12.5 and 25 kDa and basic pI (45, 46).

Studies reported here have been carried out on highly enriched fractions of MPIF-$\alpha\beta$ which have chemical characteristics distinct from other factors which are chemotactic for PMN and macrophages (4, 5). The instability of MPIF has, to date, made further purification difficult and greater quantities would be necessary for further studies. Nevertheless, these experiments suggest that MPIF plays a key role in the histopathological features—fibrin deposition and cellular infiltration—characteristic of inflammatory reactions observed as a result of cell-mediated immunity response.

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p22 (Fig. 8) that differed in the J regions. In fact p23 was joined in-frame whereas p22 was out-of-frame. The V_δ-sequence of these clones differs from the previously described $V_{\delta5}$-sequence only by seven nucleotides which may very well be attributed to strain polymorphism (Fig. 7) (16). All three cDNA clones from KN12 were identical (represented by clone D1 in Figs. 7 and 8). They are derived from $V_{\delta4}$-gene segment joined in-frame with $J_{\delta1}$. The δ-cDNA clone D4 was isolated from KN25. The V region of this clone is encoded by a previously unknown V_δ-gene segment (Fig. 7; we refer to its as $V_{\delta7}$) joined in-frame with $J_{\delta1}$. The $V_{\delta7}$-sequence differs from the V_α-sequence of a Th cell clone FN1-18 ($V_{\alpha10}$) only by 10 of 210 nucleotides (39). We therefore believe that the $V_{\delta7}$- and $V_{\alpha10}$-gene segments belong to the same V subfamily.

We prepared hybridization probes from each of the three types of V_δ-segments, $V_{\delta4}$, $V_{\delta5}$, and $V_{\delta7}$ and used them in Northern blot analysis of RNA from five other hybridomas, which gave an apparently full-length transcript detected by one of the three hybridization probes (Fig. 9). All of these transcripts seem to hybridize also with a $J_{\delta1}$-probe. We therefore screened cDNA libraries prepared from these hybridomas using the appropriate V_δ-probes and determined the nucleotide sequences of the V-J junctional regions. In agreement with the Northern blot data, the library of KN13 contained cDNA clones in which $V_{\delta4}$ is rearranged to $J_{\delta1}$ in-frame (Fig. 8). Similarly $V_{\delta7}$- and $J_{\delta1}$-gene segments are joined in-frame in KN28 whereas $V_{\delta5}$- and $J_{\delta1}$-gene segments are joined in-frame in KN102, KN106, and KN108 (Fig. 8). The Southern blot data shown in Figure 10 are entirely consistent with the Northern and cDNA sequence data. Thus KN6, KN102, KN106, and KN108 all have rearranged $V_{\delta5}$-$J_{\delta1}$-bands, KN12 and KN13 rearranged $V_{\delta4}$-$J_{\delta1}$-bands, and KN25 and KN28 rearranged $V_{\delta7}$-$J_{\delta1}$-bands. KN25, KN28, KN102, KN106, and KN108 have additional $J_{\delta1}$-rearrangements that are not detected by the $V_{\delta4}$-, $V_{\delta5}$-, or $V_{\delta7}$-probe. Presumably these rearrangements involve either D and J gene segments only or other V_δ-gene segments. Inasmuch as the cDNA sequencing identified a δ-gene joined in-frame in each of these hybridomas (Fig. 8), the second $J_{\delta1}$-bands presumably reflect out-of-frame rearrangements. We therefore did not characterize these bands further.

V_δ-J_δ-junctional sequences shown in Figure 8 show that all but KN102 use both $D_{\delta1}$- and $D_{\delta2}$-gene segments. KN102 uses only $D_{\delta2}$. All three reading frames are used both in $D_{\delta1}$ and $D_{\delta2}$. All have extra nucleotides (N regions) between the different gene elements except for KN25. These findings are in good agreement with the previous report (16) and are strikingly different from the junctional sequences of fetal thymocytes, which use only $D_{\delta2}$ without N regions (15).

DISCUSSION

Figure 11 summarizes the γ- and δ-gene segments used for expression in the hybridomas and deduced amino acid sequences around the V-J junctions of the expressed γ- and δ-chains. Among eight hybridomas analyzed, five use $V_{\gamma4}$ with three in combination with $V_{\delta5}$ (KN6, KN102,

```
                                                                M   V   R   P   F   F   L   W   V   L   F   L   S
KN12-D1                              CTGATCTGAAGCCGGCTTCTAGC ATG GTA CGG CCG TTC TTC CTG TGG GTG CTC TTC CTT TCC

                                                                M   K   R   L   L   C   S   L   L   G   L   L   C
KN25-D4   CCACAGCCCAGGGACTGGTTACTTGCTTCTGTCTCCAGCAGCCACACAAGCACC ATG AAG AGG CTG CTG TGC TCT CTG CTG GGG CTT CTG TGC
_________

           F   A   Y   K   D   V   L   C   I   T   L   T   Q   S   S   T   D   Q   T   V   A   S   G   T   E   V   T
KN6-p23   TTT GCC TAC AAG GAT GTG CTG TGC ATC ACG CTG ACC CAG AGC TCC ACT GAC CAG ACA GTG GCA AGC GGC ACT GAA GTA ACA
           T   S   L   E   A   S   M   A   Q   T   V   S   Q   P   Q   K   K   K   S   V   Q   V   A   E   S   A   T
KN12-D1   ACT TCT CTT GAA GCC AGC ATG GCT CAG ACA GTG TCT CAG CCT CAG AAG AAA AAG TCT GTG CAG GTG GCA GAA TCA GCA ACC
           T   Q   V   C   W   V   K   G   Q   Q   V   Q   Q   S   P   A   S   L   V   L   Q   E   G   E   N   A   E
KN25-D4   ACC CAG GTT TGC TGG GTG AAA GGA CAG CAA GTG CAG CAG AGT CCT GCA TCC TTG GTT CTG CAG GAG GGG GAG AAC GCA GAG
                                      1                                  10                                      20
_________

           L   L  (C)  T   Y   N   A   D   S   P   N   P   D   L   F   W   Y   R   K   R   P   D   R   S   F   Q   F
KN6-p23   CTG CTC TGC ACG TAC AAT GCG GAT TCT CCA AAC CCA GAT TTA TTT TGG TAT CGC AAA AGG CCA GAC AGA TCC TTC CAG TTC
           L   D  (C)  T   Y   D   T   S   D   T   N   Y   L   L   F   W   Y   K   Q   Q   G   G   Q   V   T   L   V
KN12-D1   CTG GAC TGC ACC TAT GAC ACA AGT GAT ACT AAT TAC CTC TTG TTC TGG TAC AAA CAG CAA GGA GGG CAG GTG ACT CTC GTC
           L   Q  (C)  N   F   S   S   T   A   T           R   L   Q   W   F   Y   Q   R   P   G   G   S       L   V
KN25-D4   CTG CAG TGT AAC TTT TCC TCC ACA GCA ACC --- --- CGG CTG CAG TGG TTT TAC CAA CGT CCT GGG GGA AGC --- CTC GTC
                                                  30                                      40
_________

           I   L   Y   R   T   S   T   S   S   H   D   A   D   G   V   Q   G   R   F   S   V   K   H   S   K   A   N
KN6-p23   ATC CTT TAT AGG ACG AGC ACT AGT TCC CAT GAT GCA GAT TTT GTT CAA GGT CGA TTT TCT GTG AAG CAC AGC AAG GCC AAC
           I   L   Q   E   A   Y   K   Q   Y   N   A   T   L               N   R   F   S   V   N   F   Q   K   A   A
KN12-D1   ATT CTC CAA GAA GCA TAC AAG CAG TAT AAT GCA ACG TTA --- --- --- AAC CGC TTC TCT GTG AAC TTC CAG AAA GCA GCT
           S   L   L   S   N   P   S   G   T   K   H   T                   G   R   L   T   S   T   T   V   T   K   E
KN25-D4   AGC CTG TTG TCC AAT CCT TCT GGG ACA AAG CAC ACT --- --- --- --- GGA AGA CTG ACA TCC ACC ACA GTC ACT AAA GAA
                   50                                      60                                      70
_________

           R   T   F   H   L   V   I   S   P   V   S   L   E   D   S   A   T   Y   Y  (C)  A   S
KN6-p23   AGA ACC TTC CAT CTG GTG ATC TCT CCA GTG AGC CTT GAA GAC AGC GCT ACT TAT TAC TGT GCC TCG G
           K   S   F   S   L   E   I   S   D   S   Q   L   G   D   A   A   T   Y   F  (C)  A   L   M   E
KN12-D1   AAG TCC TTC AGC CTG GAG ATC TCC GAC TCG CAG CTG GGG GAT GCT GCG ACG TAT TTC TGT GCT CTC ATC GAG C
           R   R   G   S   L   H   I   S   S   S   Q   I   T   D   S   G   T   Y   L  (C)  A   M   G
KN25-D4   CGT CGC GGC TCT TTG CAC ATT TCC TCC TCC CAG ATC ACA GAC TCA GGC ACT TAT CTC TGT GCT ATC GG
                                  80                                      90
```

Figure 7. V region sequences of the δ-genes used in the hybridomas. KN6-p23 = $V_{\delta5}$, KN12-D1 = $V_{\delta4}$ (16), and KN25-D4 = $V_{\delta7}$. The first amino acid after the cleavage site of the putative signal peptide is referred to as 1. Cysteine residues within the Ig-like domain are circled.

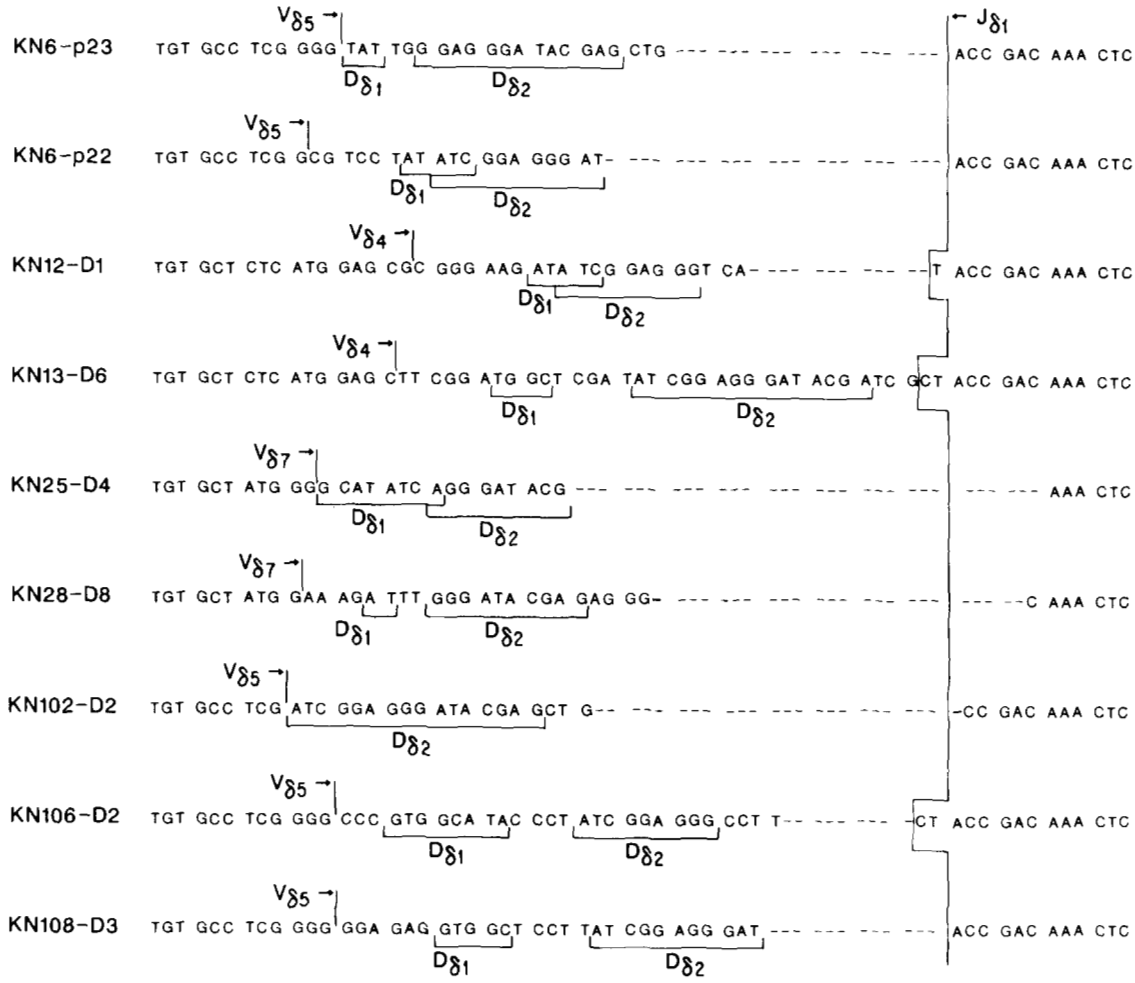

Figure 8. Nucleotide sequences of the V-J junctions of the δ-genes expressed in the hybridomas. Boundaries of $D_{\delta 1}$-, $D_{\delta 2}$-, and $J_{\delta 1}$-coded regions were deduced from the published genomic DNA sequences (15). Boundaries of $V_{\delta 4}$- and $V_{\delta 5}$-coded regions are estimated from the published cDNA sequences (14, 16).

and KN108), and the rest with $V_{\delta 4}$ (KN12) or $V_{\delta 7}$ (KN28). Two hybridomas use $V_{\gamma 7}$ and $V_{\delta 4}$ (KN13) or $V_{\delta 5}$ (KN106). The last hybridoma, KN25, uses $V_{\gamma 6}$ in combination with $V_{\delta 7}$. Thus, the adult DN thymocytes seem to use $V_{\gamma 4}$ predominantly (i.e, five of eight cases). $V_{\gamma 7}$ and $V_{\gamma 6}$ are also used but less frequently. Similarly $V_{\delta 5}$ is used in half of the cases whereas $V_{\delta 4}$ and $V_{\delta 7}$ are used less frequently. Although the total number of cases analyzed is relatively small, V_γ and V_δ do not appear to have any preferential pairing. Rather, the pairing appears to be random only restricted by the frequency of the use of individual V_γ- and V_δ-gene segments.

The frequency of the usage of V_γ- and V_δ-segments found in this study was only partially borne out by the previous studies carried out using populations of adult DN thymocytes. In the case of γ, Garman et al. (11) detected abundant RNA expression of $V_{\gamma 2}$ and $V_{\gamma 4}$, and no $V_{\gamma 5}$ and $V_{\gamma 6}$ RNA in 4-wk-old BALB/c DN thymocytes. We also observed predominant use of $V_{\gamma 4}$, but, so far no hybridomas bearing $V_2J_2C_2$ γ-chains. All of the eight hybridomas harbor one or two copies of thymocyte-derived $V_2J_2C_2$ γ-genes which seem to be transcribed along with the out-of-frame rearranged BW5147 $V_2J_2C_2$ γ-gene. Although we did not attempt to characterize these thymocytes-derived $V_2J_2C_2$ γ-genes with respect to in-frame or out-of-frame joining, it is unlikely that all of these are out-of-frame joined. The reason why $V_2J_2C_2$ γ-chain is not expressed at the surface is unclear. It may be due to the lack of an appropriate δ-chain partner for pairing or other regulatory mechanisms.

In the case of δ-genes, Elliott et al. (16) found that the frequency of $V_{\delta 4}$ and $V_{\delta 5}$ usage in the total adult DN thymocyte population is similar to the one reported here. However, $V_{\delta 7}$ described here was not detected in their study whereas $V_{\delta 6}$ reported was not seen in the present study. It is possible that the apparent discrepancy is due to the difference in the cell populations used: we studied only those thymocytes expressing the γδ-CD3 complex on the cell surface, whereas Elliott et al. (16) analyzed a pool of adult DN thymocytes in which only 5% express the γδ-CD3 complex on the cell surface (6, 17). We should, however, also keep in mind that the two studies used different mouse strains.

Although the present manuscript was in the process of being reviewed Korman et al. (40) published data which describes V_δ usage different from that obtained in the present work. Of five adult DN thymocyte hybridomas they analyzed, two expressed $V_6J_1C_1$ γ-chains and V_1J_2C δ-chains, which were previously shown by us (18) and others (15) to be expressed predominantly in late fetal thymocytes. In contrast they obtained only one hybridoma expressing $V_5J_1C_1S$ chain which we have shown

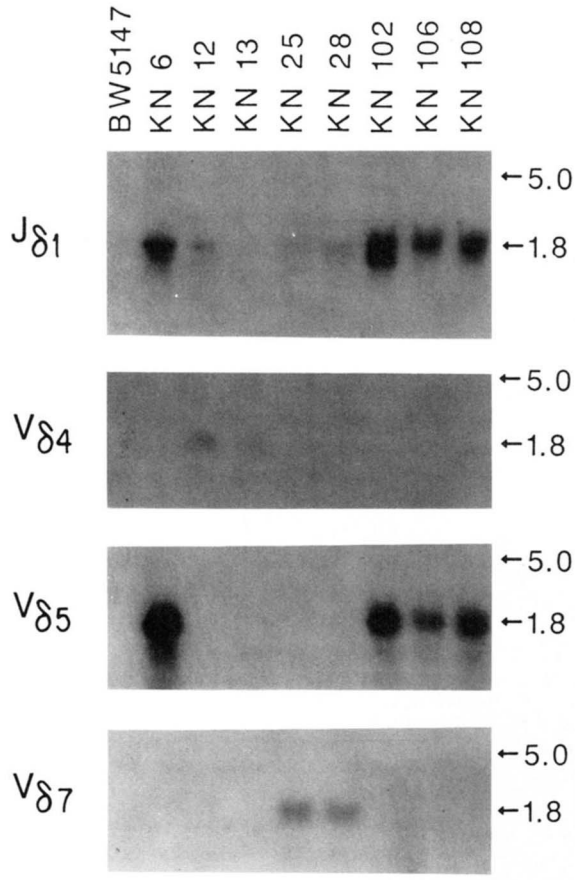

Figure 9. Northern blot analysis for δ-gene transcripts. Delta gene probes used are indicated on the left. Molecular weight of rRNA markers are indicated on the right in kb.

here to be the major δ-chain expressed on the surface of adult thymocytes. Despite these findings Korman et al. (40) concluded $V_4J_1C_1$ γ-chains and V_5J_1C δ-chains to be the predominant types used in C57BL/6 adult DN thymocytes on the basis of their population study of DNA isolated from γδ-enriched DN thymocytes. Thus our present hybridoma results are at variance with their hybridoma study but in line with their DNA study (40) as well as the population study of RNA by Elliott et al. (16).

The results obtained in the present study together with those of our earlier study on the hybridomas prepared with fetal thymocytes (18) revealed a dramatic switch of the types of γδ-receptors expressed on thymocytes as the animal develops from a fetus to a new born and to an adult. In the early fetus, the heterodimers composed of $V_5J_1C_{1\gamma}$ and V_1DJ_2C δ are the predominant types whereas a majority of late fetal thymocytes bear $V_6J_1C_1$ γ-chains paired with the same V_1DJ_2C δ-chains (18). Thymocytes bearing the γ-chain encoded by a third type of γ-gene, $V_4J_1C_1$ start appearing about day 17 of gestation but remain relatively infrequent during the embryonic life. However, they become the major type in adulthood. Another γ-chain encoded by $V_7J_1C_1$ γ-gene has been observed only on adult thymocytes. The δ-chains expressed on adult thymocytes are also distinct from those present on fetal thymocytes and are encoded by genes containing several "adult type" V_δ-gene segments rearranged via D segments to the $J_{\delta1}$-gene segment instead of the $J_{\delta2}$-gene segment which is highly preferred by the fetal thymocytes (18).

One feature of the fetal vs adult switch of the receptor is that its expressed repertoire in thymus is substantially increased during the development. The early and late fetal thymus seem to use primarily only one combination of V_γ and V_δ gene segments: $V_{\gamma5}$ and $V_{\delta1}$ for early and $V_{\gamma6}$ and $V_{\delta1}$ for late. By contrast adult thymocytes use several different V_γ- and V_δ-gene segments in random combinations. Thus the CDR1 and CDR2 diversity which is minimal in fetal thymocytes is substantially increased in adult thymocytes. As to the CDR3 diversity, this age-dependent increase in the size of γδ-repertoire seems to be even greater because of the fetal vs adult difference in the way D_δ-gene segments and N nucleotides are used.

Three possibilities could be raised for the fetal vs adult transition of γδ-receptors on thymocytes: first, a developmental stage-dependent elimination of the thymocytes bearing certain types of γδ-receptors (11); second, sequential rearrangements of genes from fetal to adult type (11, 13); and third, a developmental stage-dependent generation and emigration of various types of γδ-thymocytes (41). The second possibility was postulated on the basis of the presence of an extra, heptameric recombinational signal within the $V_{\gamma5}$- and $V_{\gamma6}$-gene segments (11, 13). However, the sequence data shown in Figure 6 suggest that none of the γ-genes studied is generated by this type of secondary rearrangement. However, the recent findings of $V_{\gamma5}$-expressing dendritic epidermal cells (42, 43) seem to support the third possibility.

According to this view these lymphocytes are generated

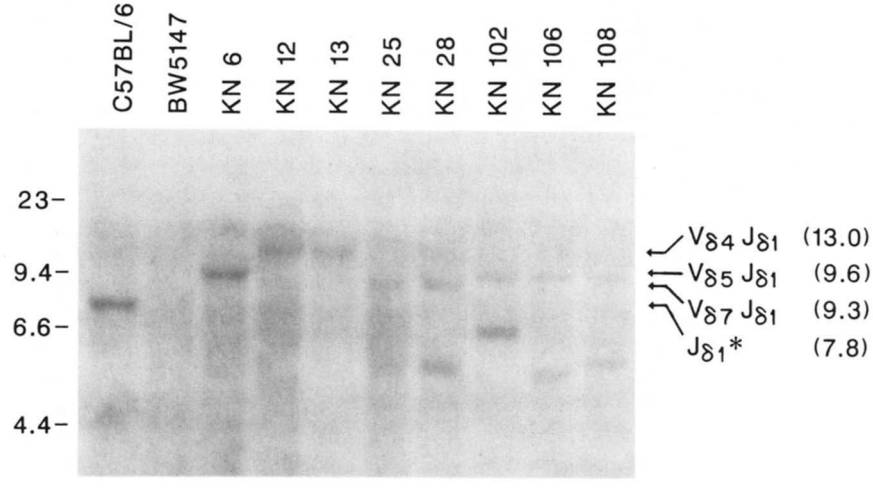

Figure 10. Southern blot analysis of δ-genes with $J_{\delta1}$-probe. $V_{\delta4}(D)J_{\delta1}$, $V_{\delta5}(D)J_{\delta1}$, and $V_{\delta7}(D)J_{\delta1}$ are 13.0, 9.6, and 9.3 kb, respectively. Germ-line $J_{\delta1}$, $V_{\delta1}DJ_{\delta1}$, germ-line $V_{\delta4}$ and germ-line $V_{\delta5}$ are 7.8, 8.8, 8.4, and 10.7 kb, respectively (15).

	γ	δ
KN 6	$V_{\gamma 4}$-CSYGQG——G $J_{\gamma 1}$	$V_{\delta 5}$-CASGYWEGYEL———TDK-$J_{\delta 1}$
KN 12	$V_{\gamma 4}$-CSYGQREYSSG-$J_{\gamma 1}$	$V_{\delta 4}$-CALMERGKISEGH——TDK-$J_{\delta 1}$
KN 13	$V_{\gamma 7}$———(N.D.)———$J_{\gamma 1}$	$V_{\delta 4}$-CALMELRMARYRRDTIATDK-$J_{\delta 1}$
KN 25	$V_{\gamma 6}$-CACWDWGDSSG-$J_{\gamma 1}$	$V_{\delta 7}$-CAMGHIRDT————K-$J_{\delta 1}$
KN 28	$V_{\gamma 4}$-CSYGD——SSG-$J_{\gamma 1}$	$V_{\delta 7}$-CAMERFGIREG————K-$J_{\delta 1}$
KN 102	$V_{\gamma 4}$-CSYGY——SSG-$J_{\gamma 1}$	$V_{\delta 5}$-CASIGGIRAA————DK-$J_{\delta 1}$
KN 106	$V_{\gamma 7}$-CASWAGRG- SG-$J_{\gamma 1}$	$V_{\delta 5}$-CASGPVAYPIGGPS—TDK-$J_{\delta 1}$
KN 108	$V_{\gamma 4}$-CSYGY——SSG-$J_{\gamma 1}$	$V_{\delta 5}$-CASGGEVAPYRRD——TDK-$J_{\delta 1}$

Figure 11. The summary of the γ- and δ-genes used by the hybridomas for the surface expression. The deduced amino acid sequences in the V-J junctions are indicated by the single letter codes except for the KN13 γ-chain for which no information is available.

primarily by the emigration of thymocytes produced in early ontogeny, whereas the γδ-cells present in the more conventional peripheral lymphatic organs such as spleen, lymph nodes, and peripheral blood are mostly derived from the $V_{\gamma 4}$- and $V_{\gamma 7}$-bearing thymocytes (41). The latter thymocytes begin to appear in embryonic thymus (18) but the primary site of their generation is adult thymus.

Finally our present study suggests that transcription of rearranged V_4 and V_7 γ-genes in αβ-cells are regulated by *trans*-acting factors. We have previously shown that many $\alpha\beta^+$ T cells harbor transcriptionally silent $V_4J_1C_1$ γ-genes (44). BW5147 also carries a transcriptionally silent $V_4J_1C_1$ γ-gene. (This AKR thymoma belongs to αβ-type T cells because it carries transcriptionally active α- and β-genes although αβ-heterodimers are not on the cell surface because of a defect in the expression of CD3 (39, 45).) This gene remains silent when BW5147 is fused to fetal thymocytes expressing $V_{\gamma 5}$ or $V_{\gamma 6}$ (18). Transcription of this γ-gene, however, is activated when BW5147 is fused to γδ-thymocytes of the adult type. Whether αβ-cells and fetal thymocytes lack $V_{\gamma 4}/V_{\gamma 7}$-specific positive transcriptional factor or carry $V_{\gamma 4}/V_{\gamma 7}$-specific repressor remain to be seen.

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