Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3814–3818, June 1981
Immunology

Expression of an antigen receptor on T cells does not require recombination at the immunoglobulin J_H-C_μ locus

(Southern blotting/immunoglobulin genes/cytotoxic T lymphocytes)

YVON CAYRE*†, MICHAEL A. PALLADINO*, KENNETH B. MARCU‡, AND JANET STAVNEZER*§

*Molecular Biology and Genetics Unit of the Graduate School, Sloan Kettering Institute, 1275 York Avenue, New York, New York 10021; and ‡Biochemistry Department, State University of New York at Stony Brook, Stony Brook, New York 11794

Communicated by J. Michael Bishop, March 13, 1981

ABSTRACT **Considerable evidence has accumulated suggesting that the antigen receptor(s) on T cells is coded for by genes for the variable (V) region of the immunoglobulin heavy (H) chains. In B cells, a complete gene for the immunoglobulin V_H region is formed by somatic recombination of V_H and joining region heavy chain (J_H) gene segments [through an intermediate diversity (D) region gene segment]. In an attempt to determine whether a complete immunoglobulin V_H region is expressed on T cells that bear an antigen receptor, we analyzed the restriction map of the J_H-C_μ locus in genomic DNA from two cloned murine cytotoxic T-lymphocyte (CTL) lines specific for the x-ray-induced leukemia RL♂1. We found no rearrangement of the J_H-C_μ locus in the CTL lines, indicating that the T-cell antigen receptor(s) in these CTLs is not coded for by a complete immunoglobulin V_H gene formed by joining of V_H, (D_H), and J_H genes. In addition, we determined that C_μ genes on both chromosomes were present and that there was no rearrangement of the C_α, C_κ, or λ chain genes in these CTL cells.**

Thymus derived lymphocytes (T cells), like antibody-producing cells (B cells), show an exquisite specificity in their capacity to recognize antigens. Although it is well documented that membrane immunoglobulins serve as the antigen receptor on B cells (1–3), the nature of the T-cell receptor(s) remains obscure. Serological and genetic data have suggested that the T-cell receptor(s) is coded for by heavy (H) chain variable region (V) genes (4–15), but whether the receptor(s) is also coded for by H chain constant region (C) genes or light (L) chain V or C region genes has been widely debated (16–20).

In B lymphocytes, a complete active immunoglobulin gene is created by joining of V and joining region (J) genes [in the case of H chain, usually through an intermediate diversity (D) region gene segment; refs. 21–26]. After joining, these two or three gene segments code for the V region of an immunoglobulin polypeptide chain. If the antigen receptor(s) expressed on T cells were coded for by a complete V_H gene, created by the joining of a V_H, (a D_H), and a J_H gene, the restriction enzyme sites around the J_H genes should be altered on T cells bearing an antigen receptor(s). It has recently been shown that, in two murine T-lymphoma cell lines (27, 28), and in two cloned cytotoxic T-lymphocyte (CTL) lines (29), restriction enzyme sites around the J_H and C_μ genes were altered relative to germ-line DNA. The location of the restriction sites that were altered suggested that DNA recombination events had occurred at the J_H locus, as would be expected if a V_H (D) gene had recombined with a J_H gene.

To investigate the putative recombination event at the J_H-C_μ locus in T cells that bear an antigen-specific receptor, we hybridized various immunoglobulin cDNA and genomic DNA probes with Southern blots (30) of genomic DNA to examine the restriction maps of the J_H-C_μ, C_α, C_κ, and λ (both V and C) gene loci in two cloned murine CTL cell lines. Our results indicate that no rearrangement at any of these loci has occurred. We also examined the immunoglobulin gene loci in a cloned T cell hybridoma line made from thymocytes from a nonimmunized mouse. Again, no rearrangement of the immunoglobulin gene loci was detected.

MATERIALS AND METHODS

Cell Lines. To produce tumor-specific CTL cell lines, (BALB/c × C57BL/6)F_1 mice were immunized by intradermal injection of 5×10^5 viable BALB/c x-ray-induced leukemia (RL♂1) cells as described (31). Spleen cells from immunized mice, 2 months after the tumor had regressed, were resensitized *in vitro* with irradiated RL♂1 cells for 6 days. These cells were grown in medium containing T-cell growth factor, prepared from CD rat spleen cells according to the method of Gillis and Smith (32). The specificity of T-cell killing activity was analyzed by 4-hr ^{51}Cr-release assays (33). In both direct tests and competitive inhibition assays with unlabeled tumor cells, reactivity was detected only against RL♂1 cells; other BALB/c tumors (seven leukemias and two sarcomas) and allogeneic tumors (three leukemias) failed either to be killed or to inhibit killing of RL♂1 cells. Lytic activity could be blocked by adding (without complement) anti-H-2D^d antiserum or monoclonal anti-Lyt-2.2 and anti-Lyt-3.2 antibodies. Blocking by anti-Lyt-2 and anti-Lyt-3 antibodies was at the level of the target cells (31). Cells were cloned twice by limiting dilution (<1 cell per well) in medium containing T-cell growth factor. The initial uncloned line and clone 1 (CTL.1) killed 50–60% of RL♂1 cells at an effector/target ratio of 40:1. One of the other 25 reactive clones (CTL.5) showed a greater reactivity (50–60% of RL♂1 cells were killed at an effector/target ratio of 30:1). As was found for the uncloned CTLs, the lytic activity of clones CTL.1 and CTL.5 against RL♂1 cells could also be blocked by anti-H-2D^d antiserum or by monoclonal anti-Lyt-2.2 and 3.2 antibodies (34). The cell surface phenotype of the cloned CTLs was determined by absorption analyses using monoclonal antibodies. Residual activity after absorption with cloned CTLs was tested against C57BL/6J thymocytes; the phenotype of the CTL lines was Lyt-1.2, 2.2, 3.2 and Thy-1.2 (34).

The hybridoma T-cell line, 23C, was produced by fusion of thymocytes from a 5-week-old C57BL/6 (H-2^b, Thy-1.2) mouse with the AKR (H-2^k, Thy-1.1) thymoma cell line BW5147 (de-

Abbreviations: V, C, J, and D, variable, constant, joining, and diversity (regions); H and L, heavy and light (chains); CTL, cytotoxic T lymphocytes; RL♂1, BALB/c x-ray-induced leukemia; kb, kilobase(s).

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† Present address: Département d'Hématologie, Hôpital Pitié-Salpêtrière, 91, Boulevard de l'Hôpital, 75634 Paris Cedex 13, France.
§ To whom reprint requests should be addressed.

rived by R. Hyman; ref. 35), which lacks the enzyme hypoxanthine-guanine-phosphoribosyltransferase. The method for cell fusion, using 41% polyethylene glycol 1500 as the fusing agent, was described by Galfre *et al.* (36). The hybridoma cells expressed the H-2 and Thy-1 markers of both parental strains (unpublished data). This hybridoma line might represent medullary thymocytes as it expresses the enzyme 20α-hydroxysteroid dehydrogenase which has been described as a marker of this thymocyte subpopulation (37) (BW5147 cells lack 20α-hydroxysteroid dehydrogenase).

Isolation of High Molecular Weight Cellular DNA. DNA was isolated by modification of the method of Hughes *et al.* (38). Cells were pelleted, and the pellets were washed with 100 mM NaCl/20 mM Tris·HCl, pH 7.4/1 mM Na_2EDTA and resuspended in the same buffer. Pronase (750 μg/ml, preincubated for 2 hr at 37°C), 1% $NaDodSO_4$ and RNase (50 μg/μl, previously heated at 100°C for 10 min) were added, and the lysates were incubated 2 hr at 37°C. DNA was isolated by three extractions with phenol/chloroform and dialyzed for 2 to 3 days against 10 mM Tris·HCl, pH 7.4/0.1 mM Na_2EDTA.

Plasmid Purification and Preparation of Probes for Hybridization. Plasmids were isolated from cleared lysates (modified from ref. 39) by phenol/chloroform extraction at pH 4.0 (40) and gel filtration on Sepharose 4B. Hybridization probes were prepared by isolation of restriction fragments from the plasmids. DNA probes were nick translated (41) to a specific activity of $\approx 2 \times 10^8$ cpm/μg, using [α-^{32}P]TTP at 3000 Ci/mmol (1 Ci = 3.7×10^{10} becquerels). All bacteria containing recombinant DNA were contained at the P1/EK1 level as specified by the National Institutes of Health guidelines for recombinant DNA research.

Hybridization Probes. The hybridization probe for C_μ was a *Bam*HI/*Hin*dIII fragment isolated from the plasmid pμ (3741)[9], which contained cDNA coding for C_μ (42). Fig. 1 shows the sequences coding for the μ probe on the restriction map of the BALB/c genomic μ gene. Probes for J_H genes were from the plasmid clones pj_0 and pj_{11}, which contain BALB/c genomic DNA sequences coding for the J_{H1}-J_{H2}, and J_{H3}-J_{H4} segments, respectively (Fig. 1; ref. 42). The probe for C_α was a *Msp* I fragment containing nearly the entire C_α sequence (isolated from the cDNA plasmid pα(J558)[13] (42). The probe for Igλ chain genes was a *Hha* I fragment containing cDNA coding for the entire λ chain of the myeloma H2020 [from the plasmid A12, which was similar to the plasmid B1 described by Brack *et al.* (21)]. A12 was a gift from S. Tonegawa. The probe for immunoglobulin C_κ sequences was a *Hin*dIII fragment containing cDNA sequences coding for C_κ and a small portion of the V region of the MOPC384 κ chain [from the plasmid pM384κ (43)].

Restriction Enzyme Digestion, Gel Electrophoresis, and DNA Blotting. Samples (21 μg) of high molecular weight DNA were digested with the indicated restriction enzymes (purchased from New England BioLabs, Beverly, MA, or Bethesda Research Laboratories, Rockville, MD). To monitor the extent of digestion, 5% of the reaction mixture was added to 0.3 μg of pBR322 or λ DNA and incubated along with the main reaction (38). The test samples were analyzed by electrophoresis in a 0.7% agarose gel in 40 mM Tris·HCl/20 mM NaOAc/18 mM NaCl/2 mM Na_2EDTA, pH 8.1. When the digestion was complete, the DNA of the main reaction was precipitated with ethanol, and 10-μg portions were subjected to electrophoresis on 6-mm-thick 0.7% agarose gels in the same buffer.

DNA was transferred to nitrocellulose filters (30). The blots were baked, prehybridized for 6–20 hr, and hybridized for 3 days at 41°C in a solution containing 50% deionized formamide/0.45 M NaCl/45 mM sodium citrate, pH 7/0.3 mM EDTA/0.02% each bovine serum albumin, Ficoll, and polyvinyl pyrrolidine/1 mg of yeast RNA per ml/100 μg of *E. coli* DNA/ml. The blots were exposed at −80°C to x-ray film (Kodak XR), using a Du Pont Lightning Plus intensifying screen. If the blot was to be hybridized with a second probe, the first probe was

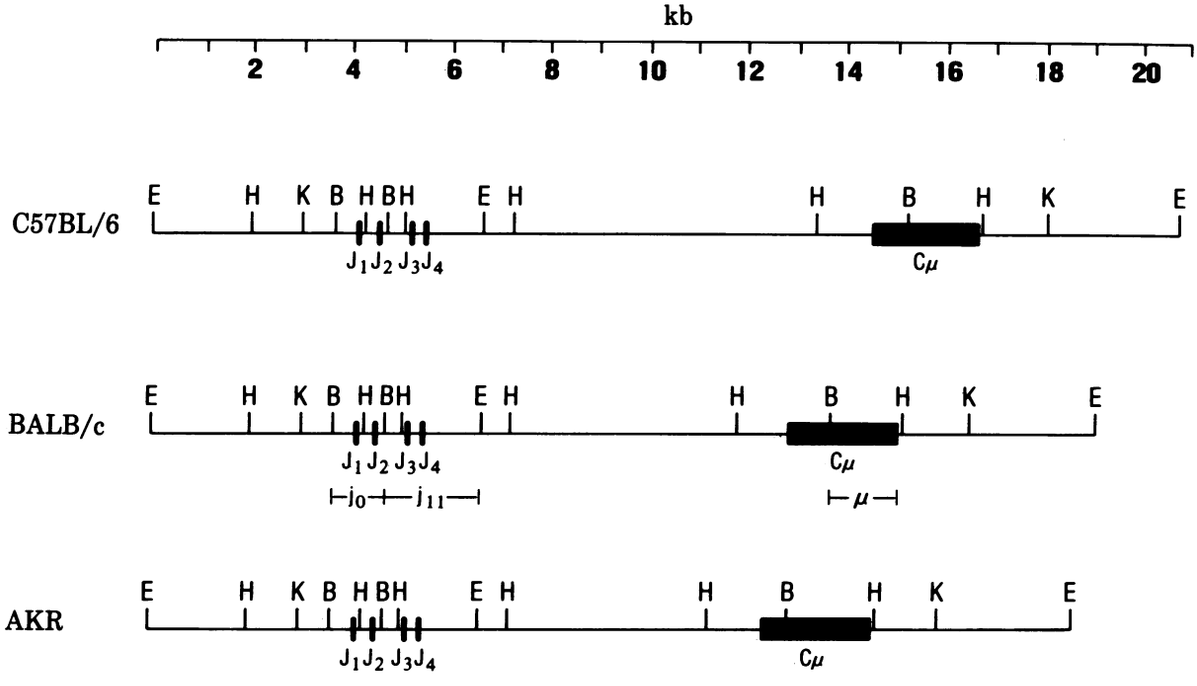

FIG. 1. Restriction enzyme maps of germ-line J_H-C_μ loci in three strains of mice. Maps were derived from data reported here and also from ref. 42. Coding sequences for J_H and C_μ genes are indicated by black boxes. BALB/c genomic DNA sequences used as probes for J_{H1} and J_{H2} and for J_{H3} and J_{H4} genes are indicated as j_0 and j_{11} below the BALB/c map. These sequences were subcloned into pBR322 (42). Genomic DNA sequences coding for the C_μ cDNA probe, labeled μ, are also indicated below the BALB/c map. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I.

removed by incubation of the blot at 68°C for 15–30 min in hybridization mix (44).

RESULTS

Genomic DNAs from two cloned CTL lines specific for RLδ1 leukemia cells (CTL.1 and CTL.5), a thymocyte hybridoma (23C), and livers of the appropriate strains of mice were digested with restriction enzymes, subjected to electrophoresis on agarose gels, blotted to nitrocellulose filters, and hybridized with ^{32}P-labeled DNAs coding for various immunoglobulins, or portions thereof. Liver cells contain immunoglobulin genes in the germ-line context, as in sperm or embryo cells (42, 45–47). Below the map of the BALB/c germ line J_H-C_μ locus (Fig. 1) are indicated the DNA sequences that we used as probes for this locus. Fig. 2 shows the *Eco*RI fragments detected by the μ cDNA probe. Due to differences among different strains of mice in the size of the *Eco*RI fragments containing the germ-line μ gene (Fig. 1; ref. 42), it can be seen that the CTL.1 cells, which were derived from a F_1 mouse (BALB/c × C57BL/6), contain μ genes on *Eco*RI fragments of the same size as in liver DNA on both of the homologous chromosomes. The hybridoma, 23C, also contains μ genes on *Eco*RI fragments of the same size as in liver DNA from its parental strains AKR/J and C57BL/6 (Fig. 2).

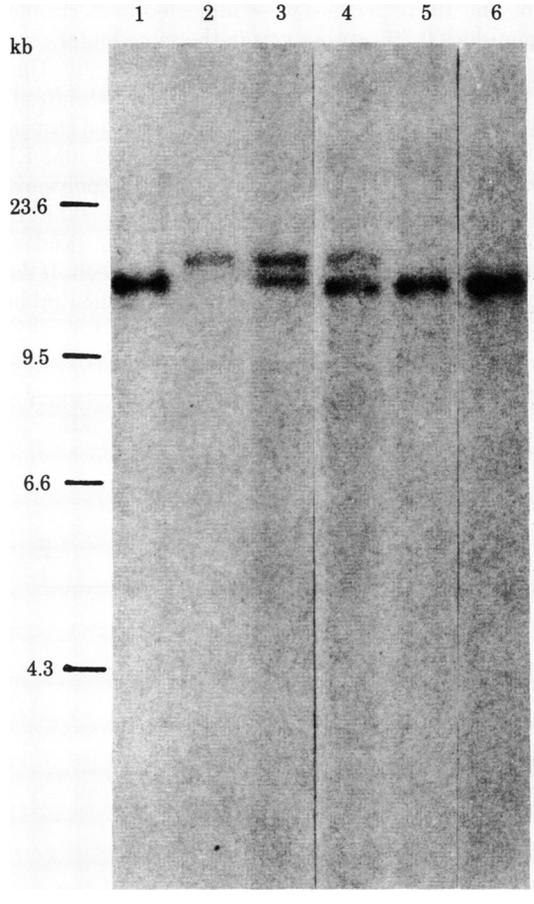

FIG. 2. Hybridization of μ cDNA with *Eco*RI DNA fragments. Genomic DNAs from T cells and liver were digested with *Eco*RI, subjected to electrophoresis, and blotted as described in *Materials and Methods*. The blots were hybridized with 8×10^6 cpm of μ cDNA, washed, and exposed to x-ray film for 4 days. CTL.1 was derived from a F_1 mouse (C57BL/6 × BALB/c) and 23C was from the fusion of C57BL/6 thymocytes and BW5147 cells (AKR strain). Positions and sizes (in kb) of *Hin*dIII fragments of λ DNA, which were subjected to electrophoresis in a parallel lane, are indicated on the left. Lanes: 1, BALB/c liver; 2, C57BL/6 liver; 3, CTL.1; 4, 23C; 5, BW5147; 6, AKR liver.

To investigate whether J_H genes in the CTL cells and in the thymocyte hybridoma were rearranged, as would be expected if a complete V_H gene were expressed in these cells, the blot of *Eco*RI fragments was hybridized sequentially with both J_H probes. Fig. 3*A* illustrates results with the j_{11} probe. No recombination at the J_H locus was detected; the *Eco*RI fragments containing the J_H genes in the CTL.1 and thymocyte hybridoma cells were identical in size to those in liver cells.

To eliminate the possibility that a rearrangement had occurred at the J_H locus without altering the size of the *Eco*RI fragments, DNAs from CTL.1 and the thymocyte hybridoma were digested with *Hin*dIII and hybridized with the j_0 probe (Fig. 3*B*). No rearrangement at the J_H locus was detected. DNA from CTL.1 and CTL.5 cells and from the thymocyte hybridoma were digested with *Kpn* I and hybridized with the μ, j_0, and j_{11} probes. Again, no rearrangement was detected (data not shown).

As α mRNA has been detected in thymocytes (49), we searched for rearrangement of α genes in the CTL.1 and 23C cells by digestion of DNA with *Hin*dIII and hybridizing with an α cDNA probe. As in the case of the μ genes, there is heterogeneity in the size of the *Hin*dIII fragments containing α genes among liver DNAs from different strains of mice (42), so we were able to determine that α genes were present on both chromosomes on *Hin*dIII fragments of the same size as in liver DNA (data not shown).

Finally, to determine whether a recombination had occurred at the J_κ-C_κ or the V_λ and J_λ-C_λ loci, we examined the size of the *Eco*RI fragments detected with a C_κ cDNA probe and a λ cDNA probe. No rearrangement of the J_κ-C_κ nor λ loci was detected (Fig. 4). As *Eco*RI recognizes sites located 5′ to J_κ and J_λ gene segments (21–23), recombination at the J_κ or J_λ genes would have been detected. Other authors have also reported no rearrangement of C_κ genes in T cells (27, 29).

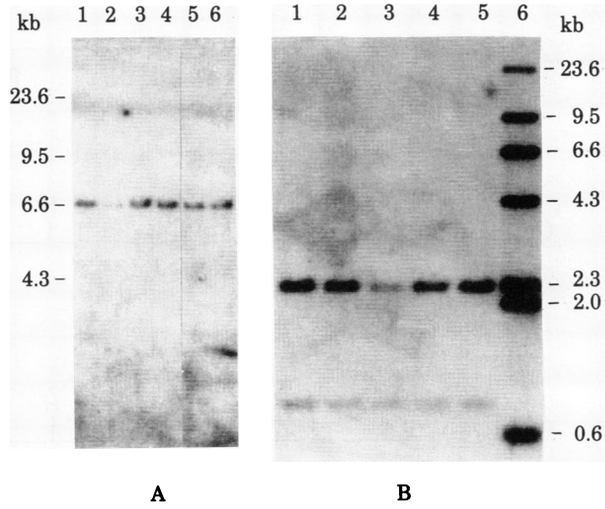

FIG. 3. Hybridization of J_H probes with T-cell DNAs. (*A*) The various DNAs were digested with *Eco*RI (same blot as in Fig. 2), and the fragments were hybridized with the j_{11} probe (10×10^6 cpm), which contains DNA sequences coding for J_{H3} and J_{H4} genes: (Identical results were obtained with the J_{H1} and J_{H2} probe, j_0.) The blots were exposed for 3 days. Lanes: 1, CTL.1; 2, C57BL/6 liver; 3, BALB/c liver; 4, AKR liver; 5, BW5147; 6, 23C. (*B*) DNAs were digested with *Hin*dIII, and the fragments were hybridized with the j_0 probe (11.5×10^6 cpm). The blots were exposed for 10 days. The *Hin*dIII fragments of λ DNA were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase, by modification of the method of Maxam and Gilbert (48). Lanes: 1, BALB/c liver; 2, CTL.1; 3, C57BL/6 liver; 4, 23C; 5, AKR liver; 6, λ *Hin*dIII.

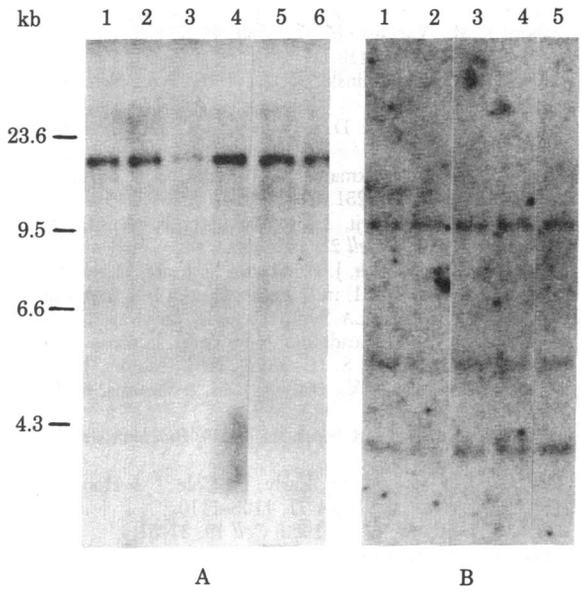

FIG. 4. Hybridization of cDNAs for immunoglobulin L chains with *Eco*RI DNA fragments from T cells. (*A*) The DNA blot used in Figs. 2 and 3*A* was hybridized with 4×10^6 cpm of C_κ cDNA from pM384κ, and the blot was exposed for 3 days. Lanes: 1, AKR liver; 2, BALB/c liver; 3, C57BL/6 liver; 4, CTL.1; 5, 23C; 6, BW5147. (*B*) The blot used in Fig. 4*A* was hybridized with 11.6×10^6 cpm of cDNA coding for the λ chain of myeloma H 2020 (from the plasmid clone A12), and the blot was exposed for 10 days. Lanes: 1, 23C; 2, BW5147; 3, AKR liver; 4, BALB/c liver; 5, CTL.1.

DISCUSSION

The cloned CTL lines (CTL.1 and CTL.5) studied in these experiments bear antigen receptors as they specifically kill RLδ1 leukemia cells. Their cytotoxic activity is H-2 restricted (34). The fact that no recombination has occurred at the immunoglobulin J_H or J_L loci of these cells suggests that the antigen receptor(s) on CTLs does not contain a complete immunoglobulin V_H or V_L region of the type found on antibody-producing cells. However, the fact that antigen receptors on T cells have been found to bear serological determinants specific for V_H regions (4–9, 13–15) and to be coded for by genes linked to the immunoglobulin H chain locus (10–12, 14) strongly suggests that at least a portion of the T-cell receptor(s) is coded for by V_H genes. Therefore, it appears likely that in some CTLs, V_H genes may have been recombined with genes other than an immunoglobulin gene(s) to form a complete gene(s) coding for the antigen receptor(s). The sizes of the polypeptides tentatively identified as T-cell receptors (70,000–90,000 daltons; refs. 8, 9, 15, 50) are too large for the receptor(s) to consist solely of an immunoglobulin V_H region; hence the receptor(s) is probably coded for by an additional gene(s) joined to the V_H gene. These putative T-cell-specific genes may be located 5′ to the J_H-C_μ locus; the J_H-C_μ genes are not deleted from the chromosomes in these two CTL lines, and there is some evidence that the DNA sequence intervening between genes that are joined to form active immunoglobulin genes in B cells is deleted from the chromosome during the recombination process (23, 46, 51). Other less-likely possibilities for the gene(s) coding for the T-cell antigen receptor(s) are that (*i*) immunoglobulin V_H genes and sequences located immediately 3′ to them code for the T-cell receptor(s) without prior recombination with any other gene; (*ii*) immunoglobulin V_H genes recombine with genes located on a different chromosome; or (*iii*) there exists another set of V_H genes, similar to immunoglobulin V_H genes, on a different chromosome.

Our data disagree with those of other investigators (27–29), who found that rearrangements of the J_H-C_μ genes occur in T cells. The T-lymphoma lines discussed in refs. 27 and 28 have not been shown to bear an antigen-specific receptor and are transformed cells. However, the cloned CTL cells studied in ref. 29 are antigen-specific, H-2 restricted, and not transformed. As we found, by using different cloned CTL cells from those studied in ref. 29, that rearrangement of J_H genes is not necessary to form a functional T-cell receptor, it is possible that there is heterogeneity among CTLs, so that only some of them use immunoglobulin J_H genes in forming the gene for the antigen receptor(s), or that the rearrangements of the J_H genes in these other T cells do not produce a gene coding for a T-cell receptor. Perhaps these latter rearrangements are nonfunctional, but occur because DNA sequences used for joining V_H genes to the T-cell receptor C_T genes may be similar to those used for joining V_H and J_H genes and because the enzymes that perform the recombination may be similar in antibody-producing cells and in T cells. Or perhaps the enzymes that perform recombination of immunoglobulin genes are sometimes activated prematurely in precursor cells common to both B and T lymphocytes.

After this paper was submitted for review, similar results were reported by Kronenberg *et al.* (52).

We wish to thank Susumu Tonegawa for the A12 clone, Alex Barkas for his helpful discussions, Kathy Slavin and Sonia Sirlin for excellent technical assistance, and Briann Packard for photography. The research was supported by Grants AI GM 14617, GM 26939, and CA 09149 from the National Institutes of Health; a Basil O'Connor Starter Research Grant from the March of Dimes-Birth Defects Foundation, and a grant from the Kleberg Foundation. Y.C. was supported by a grant from the French Foreign Affairs Ministry.

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