Analysis of the Methylation State of the T Cell Receptor β Chain Gene in T Cells and Large Granular Lymphocytes*

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We have evaluated the methylation state of the T cell receptor β chain gene (TcR β) in freshly isolated human large granular lymphocyte (LGL) and T cell DNA in order to investigate the relationship between LGL and T cells with regard to methylation of this region of genomic DNA. In addition, we wished to determine whether hypomethylation of specific regions of the β chain gene DNA might account for the production of only a nonfunctional 1.0-kilobase (kb) TcR β mRNA transcript in LGL. Our analysis indicates that the heterogeneous pattern of methylation seen in LGL DNA lies predominantly in the Jβ1 region of the TcR β DNA structure. The CCGG sequences located at the beginning of the HFR island (CG-rich region) in the Jβ2 region are nonmethylated in both LGL and at least half of the T cell DNA, suggesting that the HFR island is nonmethylated in both LGL and T cell DNA. In addition, specific methylation differences between T cell and LGL DNA can be detected 0.7 kb 3' to the last exon of Cβ1, just 5' to the first exon and within the second exon of the Cβ2 region. The heterogeneous methylation state of the TcR β Jβ1 region in LGL DNA may be due to and a result of the differential use of the Jβ1 segment for generation of the nonfunctional 1.0-kb mRNA in LGL. These results and our previous studies (Sakamoto, S., Ortaldo, J. R., and Young, H. A. (1988) J. Immunol. 140, 654-660 and Sakamoto, S., and Young, H. A. (1988) Nucleic Acid Res. 16, 2149-2163) indicate that DNA methylation may be one method by which functional gene expression is controlled in specific lymphoid cell populations.

In 1948, Hotchkiss (3) reported that bases in DNA are methylated. A few years later, Wyatt (4) reported that methyl C is the only modified base in higher eukaryotic cells. In 1962, J. Dosoccoli and F. Strom (5) demonstrated that most of the methyl C nucleotides in higher eukaryotic DNA reside in CpG sequences. After the discovery of a restriction enzyme which recognizes methylated CpG (6), there appeared many reports which suggested a possible biological role for this specific modification of DNA. A gene which is actively transcribed in a tissue is often hypomethylated in that tissue, but the same gene is highly methylated in tissue where the gene is not transcribed (7-16). Treatment of cells with inhibitors of DNA methylation, such as 5-azacytidine, often results in expression of genes which are not usually expressed in those cells (17-22). More recent studies of the DNA structure have shown that there exists in genomic DNA CpG rich islands, which are nonmethylated in all cells (23). The occurrence of such islands appears to be associated with genes required for basic cell functions (i.e. housekeeping genes) (23) and may be responsible for constitutive expression of these genes. Methylation of DNA also appears to play a role in development as the maternal and paternal genetic contributions to a mammalian zygote nucleus do not function equivalently during subsequent development (24-28), and differences in methylation of the genome during male and female gametogenesis may be responsible for the different parental genetic contributions to the zygote nucleus (i.e. allele-specific methylation) (29, 30). Furthermore, methylation of DNA is reported to play an important role in X chromosome dosage compensation (31). Additional unique features of gene methylation is that the pattern of methylation of a gene is maintained from cell cycle to cell cycle except when cell differentiation is associated with an expression of the gene (somatic heritability) (32-34).

LGL are a population of lymphoid cells that mediate natural killer activity (35-39). There have been, however, conflicting reports about the cell classification of LGL. Although LGL have unique functional and phenotypic characteristics (36, 37, 40), some reports have shown that they also have many of the phenotypic and genotypic properties of T cells. For example, LGL can be grown in culture with interleukin 2 (41), produce interleukin 2, and interferon-γ (42, 43), and express CD3, T10, and CD8 antigens (36, 38, 40). Analysis of T cell receptor gene rearrangement in cloned cells or leukemic cells having LGL-like properties indicated that most of these cell lines and leukemic populations have rearranged T cell receptor genes (i.e. a marker of T cell lineage) (44-48). However, Lanier reported that CD16-positive, low density lymphocyte of human peripheral blood (enriched for natural killer cells) did not contain rearranged TcR β genes (49), and there have been no reports of TcR β chain protein expression in LGL. Furthermore, this laboratory reported previously that both human and rat LGL do not express a full length 1.3-kb TcR β mRNA but do express a nonfunctional 1.0-kb TcR β mRNA (50).

In a previous study, we reported that the TcR β gene is more methylated in LGL than in T cells and LGL are heterogeneous in the methylation state of TcR β, suggesting that LGL are a heterogeneous lymphoid population distinct from T cells (1). However, we could not precisely localize which region of the TcR β gene in LGL DNA differed in its methylation state from T cell, B cell, and monocyte DNA. We could also not determine which portion of the LGL TcR β gene was associated with the heterogeneous methylation pattern, due to the limitation of the methods which we used in

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1 The abbreviations used are: LGL, large granular lymphocytes; TcR β, T cell receptor beta chain gene; kb, kilobase.
the previous study (i.e., comparison of the restriction fragment pattern after only HpaII orMspI cleavage). In this study, we utilized the combination of HpaII and other restriction enzymes to obtain more precise information concerning the methylation pattern of TcR β in LGL and T cell DNA. The predicted restriction enzyme digestion patterns of TcR β, based on the location of the cleavage sites for the restriction enzymes utilized in this study, were derived from published DNA sequence data (51) and our own restriction enzyme analysis and have enabled us to localize methylated and nonmethylated regions of DNA within the genomic DNA structure. Based on these results, we will offer a hypothesis on the origin and relationship of CD3-LGL to other lymphocyte populations.

**MATERIALS AND METHODS**

Cell Preparation—LGL and T cells were obtained from the buffy coats after leukopheresis from eight normal healthy volunteers as described previously (52). Mononuclear cells were separated by centrifugation on a Ficoll-Hypaque gradient and depleted of monocytes by adherence on a plastic surface. Nonadherent cells were applied onto a nylon wool column, and eluted cells were then fractionated on a seven-step discontinuous gradient of Percoll (Pharmacia LKB Biotechnology Inc.) at concentrations ranging from 40 to 60% (52). LGL were collected from the low density fraction (2 and 3 counting density bottom fraction). Contaminating T lymphocytes were further determined by morphological analysis of Giemsa-stained cytopreps of the prepared LGL and T cell DNA when the T cell DNA contains a nonmethylated M8 site. Since the intensity of the 0.7-kb fragment is much greater than that of the 1.0-kb fragment, most of the M30 sites are nonmethylated in T cell DNA. Further digestion of BglII-digested LGL DNA with HpaII revealed a 5.0-kb band and a 1.0-kb band (lanes 8, 9, 11, and 12), indicating that the M8 site is methylated and a MspI site or multiple MspI sites near M11 are nonmethylated (Fig. 1B). As extensive HpaII digestion (3X HpaII) does not change the hybridization pattern in any of the experiments reported here, incomplete digestion of the DNA sample by HpaII can be ruled out in all of these restriction enzyme studies.

Analysis ofDNA Digested with HindIII and HpaII—The results of restriction enzyme digestion with either HindIII alone or HindIII followed by HpaII of T cell DNAs and LGL DNAs are shown in Fig. 2A. HindIII digestion of T cell DNA (lanes 1 and 4) and LGL DNA (lanes 7 and 10) revealed 7.5- and 3.8-kb bands. As shown in Fig. 2B, the 7.5-kb band is the H5-H6 fragment, and the 3.8-kb band is the H4-H5 fragment. The H6-H7 fragment cannot be detected by our present experimental conditions. After further digestion of HindIII-digested T cell DNA with HpaII, the 7.5-kb band disappeared, the intensity of the 3.8-kb band decreased, and new 3.2-, 2.1-, and 0.6-kb bands appeared (lanes 2, 3, 5, and 6). The 3.8-kb band is the H4-H5 fragment, the 3.2-kb band is the H4-M5 fragment, the 2.1-kb band is the M28-H6 fragment, and the 0.6-kb fragment is the M29-H6 fragment. As the M28 or M29 sites are almost completely nonmethylated in T cell DNA (as shown in the Xbal + HpaII digestion below), very little of the 3.5-kb M27-H6 fragment can be detected. As the intensity of the 3.8-kb band is identical to or slightly weaker than the intensity of 3.2-kb band, about half of the M8 sites are nonmethylated in T cell DNA. In addition, since the intensity of the 0.6-kb band is much stronger than that of the 2.1-kb band, the M29 site is nonmethylated in more than half of T cell DNA. Much of the T cell DNA which contains a methylated M29 site has a nonmethylated M28 site as the intensity of the 2.1-kb band is relatively strong.

After the further digestion of HindIII-digested LGL DNA with HpaII, the 7.5-kb band disappeared, the 3.8-kb band remained relatively constant, and a new 2.1-kb and a very faint 0.6-kb band (not visible in Fig. 2A) appeared (lanes 8, 9, 11, and 12). As shown in Fig. 2B, the region of hybridization at approximately 3.8 kb represents both the 3.8-kb H4-H5 and the 3.5-kb M27-H6 fragment, whereas the 2.1-kb band is the M28-H6 fragment, and the very faint 0.6-kb band is the M29-H6 fragment. These results indicate that the M8 site is methylated, and most of the LGL DNA contains a methylated M29 site since only a very faint 0.6-kb band appeared. As the
Fig. 1. Digestion of T cell and LGL DNA with BglII and HpaII. A, T cell or LGL DNA was either digested with BglII (B) alone (lanes 1, 4, 7, and 10) or first digested with BglII and then twice (2 X) (lanes 2, 5, 8, and 11) or three times (3 X) (lanes 3, 6, 9, and 11) with HpaII (Hp). 10 µg of digested DNA was subjected to electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled T cell receptor @ chain cDNA probe. Roman numerals represent different donors, and numbers on the side of the figure refer to size of the DNA in kilobases.

B, schematic representation of the BglII + HpaII restriction fragment pattern in T cell and LGL DNA. The cDNA probe utilized hybridizes with the first exon of both Cγl and Cγ2 genomic DNA.

intensity of the 2.1-kb band is relatively intense, the M28 site in most of the DNA is nonmethylated as it was cleaved by HpaII. However, since the M27-H6 fragment overlaps with the H4-H5 fragment, the methylation state of M27 cannot be analyzed by double digestion with HpaII and HindIII as the two bands cannot be resolved under these electrophoresis conditions.

Analysis of DNA Digested with XbaI and HpaII—The results of restriction enzyme digestion with either XbaI alone or XbaI followed by HpaII of T cell DNA and LGL DNA is shown in Fig. 3A. XbaI digestion of T cell DNA (lanes 1 and 4) and LGL DNA (lanes 7 and 10) revealed 10- and 2.5-kb DNA fragments. The 10.0-kb band of T cell DNA is less distinct and broader than that observed in LGL DNA due to the polyclonal rearrangement of TcR @ in T cells which includes the δ2 and β2 segments. As seen in Fig. 3B, the 2.5-kb band is the X2-X3 fragment and the 10-kb band is the X4-X5 fragment. After the further digestion of XbaI-digested T cell DNA with HpaII, the 10-kb band disappeared, the intensity and width of the 2.5-kb band increased, and a new 1.0-kb band and a very faint 5.0-kb band appeared (lanes 2, 3, 5, and 6). The 2.5-kb band is a summation of fragments, X2-M8, X2-X3, M28-M30, the 1.0-kb band is the M29-M30 fragment, and the 5.0-kb band is the M28-X5 fragment. These results indicate the majority of T cell DNA contains M28, M29, and M30 sites which are not methylated.

After the further digestion of XbaI-digested LGL DNA with HpaII, the 10-kb fragment disappeared and new 6.2- and 5.0-kb bands appeared, whereas the 2.5-kb band showed no change (lanes 8, 9, 11, and 12). The 6.2-kb band is the M27-X5 fragment, and the 5.0-kb band is the M28-X5 fragment. As the intensities of the 6.2- and 5.0-kb bands are almost identical, the majority of LGL DNA contains methylated M29 and M30 sites. These data are in agreement with the results seen with double digestion with HpaII and HindIII or HpaII and EcoRI. In addition, about half of the M27 and M28 sites are nonmethylated in LGL DNA. As the intensity of the 2.5-kb band did not change following HpaII digestion, there is no M28-M30 fragment in the 2.5-kb band.

Analysis of DNA Digested with EcoRI and HpaII—The
results of restriction enzyme digestion with either EcoRI alone or EcoRI followed by HpaII of T cell DNA and LGL DNA are shown in Fig. 4A. EcoRI digestion of T cell DNA revealed a 4.0-kb band and a faint broad band of hybridization from 7 to 12 kb (lanes 1 and 4). As shown in Fig. 4B, the 4.0-kb band is the E3-E4 fragment, and the faint, broad hybridization is due to a polyclonal rearrangement which includes the Dβ1 and Jβ1 region. EcoRI digestion of LGL DNA revealed a 12- and 4.0-kb band (lanes 7 and 10). As shown in Fig. 4B, the 12-kb band is the E1-E2 fragment, and the 4.0-kb band is the E3-E4 fragment. After the double digestion of T cell DNA with EcoRI and HpaII, the broad band of hybridization disappeared, the intensity of the 4.0-kb band decreased and new 1.5- and 1.0-kb bands appeared (lanes 2, 3, 5, and 6). Most of the 4.0-kb band consisted largely of the M6 or M7-E2 fragment, since the broad hybridization disappeared following HpaII digestion. The 1.5-kb band is the E3-M30 fragment and the 1.0-kb band is the M29-M30 fragment. This result indicates that most T cell DNA contain a nonmethylated M30 site. However, the intensity of the 1.0-kb fragment is two or three times stronger than that of the 1.5-kb band, indicating that T cell DNA contains a low level of nonmethylated M29 sites.

As shown in Fig. 4A, after double digestion of LGL DNA with EcoRI followed by HpaII, the intensity of the 12-kb band decreased considerably, the 4.0-kb band showed almost no change, and the new 7.5-, 5.5-, and 1.5-kb bands appeared (lanes 8, 9, 11, and 12). Another faint 1.0-kb band appeared
in LGL DNA from one donor (lanes 11 and 12). The 7.5-kb band is fragment M1 or M2-E2, the 5.5-kb fragment is M4 or M5-E2, and the 4.0-kb band consists largely of the E3-E4 and M6 or M7-E2 fragments. This indicates that LGL DNA contains a heterogeneous methylation pattern in the JB1 region. The 1.5-kb band is the E3-M30 fragment, and the 1.0-kb band is the M29-M30 fragment. These results indicate that a small fraction of LGL DNA contains a nonmethylated M30 site and that in one donor at least some of the M29 sites are nonmethylated. Thus, the methylation state of the M29 site can differ among different donors.

**DISCUSSION**

Based on the results of these studies, a comparison of the methylation pattern of the TcR β chain gene among the different leukocyte populations is shown in Fig. 5 and can be summarized as follows.

**Dβ1, Jβ1, and Cβ1 Regions**—1) LGL DNA contains a heterogeneous methylation pattern in the Jβ1 region; 2) T cell DNA which still contains the Cβ1 region has almost all nonmethylated M6 or M7 sites in the Jβ1 region; 3) B cell and monocyte DNA have a highly methylated 5β2 region (2); 4) LGL, B cell (2), and monocyte DNA (2) contain a methylated MS site, but about half of T cell DNA in which TcR β has not been deleted contains a nonmethylated MS site.

**Dβ2, Jβ2, and Cβ2 Regions**—1) specific MspI sites of the Jβ2 HTF island are nonmethylated in T cell, LGL, B cell (2), and monocyte DNA (2), and it is hypothesized that the entire HTF island in Jβ2 is nonmethylated; 2) about half of LGL DNA, half of monocyte DNA, and all of B cell DNA have a

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**FIG. 3. Digestion of T cell and LGL DNA with XbaI and HpaII.** A, T cell or LGL DNA was either digested with XbaI (X) alone (lanes 1, 4, 7, and 10) or first digested with XbaI and then twice (2 X) (lanes 2, 5, 8, and 11) or three times (3 X) (lanes 3, 6, 9, and 12) with HpaII (Hp). 10 µg of digested DNA was subjected to electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled T cell receptor β chain cDNA probe. Roman numerals represent different donors, and numbers on the side of the figure refer to size of the DNA in kilobases. B, schematic representation of the XbaI + HpaII restriction fragment pattern in T cell and LGL DNA. The cDNA probe utilized hybridizes with the first exon of both Cβ1 and Cβ2 genomic DNA.
FIG. 4. Digestion of T cell and LGL DNA with EcoRI and HpaII. A, T cell or LGL DNA was either digested with EcoRI (E) alone (lanes 1, 4, 7, and 10) or first digested with EcoRI and then twice (2 ×) (lanes 2, 5, 8, and 11) or three times (3 ×) (lanes 3, 6, 9, and 12) with HpaII (Hp). 10 μg of digested DNA was subjected to electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled T cell receptor β chain cDNA probe. Roman numerals represent different donors, and numbers on the side of the figure refer to size of the DNA in kilobases. B, schematic representation of the EcoRI + HpaII restriction fragment pattern in T cell and LGL DNA. The cDNA probe utilized hybridizes with the first exon of both Cβ1 and Cβ2 genomic DNA.

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- Approximately half of LGL DNA and all of B cell and monocyte DNA has a methylated M28 site (3′ to the Jβ2 HTF island) (2).
- A small fraction of LGL DNA has a nonmethylated M28 site (3′ to the Jβ2 HTF island) (2), whereas at least half of T cell DNA has a nonmethylated M28 site; 4) a small fraction of LGL DNA has a nonmethylated M29 site (immediately 5′ to the Cβ2 region), this site is completely methylated in B cell and monocyte DNA (2), and most of T cell DNA has a nonmethylated M29 site. 5) A small fraction of LGL DNA has a nonmethylated M30 site (in the Cβ2 region), B cell and monocyte DNA band contain a methylated M30 site (2), and most (but not all) T cell DNA contains a nonmethylated M30 site.

The results of this study indicate that LGL are a distinct cell population from other leukocyte cell types. However, the observed pattern of methylation places LGL as being more closely related to T cells than to B cells and monocytes with regard to cell differentiation pathways. Our preliminary data (not shown) that T cell and LGL but not B cells or monocytes have an equally hypomethylated T cell receptor γ chain gene also supports this hypothesis. The main cause of the heterogeneity in the methylation state of the Jβ1 region of LGL DNA is not precisely known, but it could possibly be due to the fact that LGL may be composed of distinct subpopulations which contain more homogeneous methylation patterns or as discussed below, this heterogeneity could arise due to the selection of various Jβ1 segments for production of a 1.0-kb mRNA of TcR β in LGL. We hypothesize that T cells and LGL share an initial stem cell and differentiation pathway to
be considered to be closely related to T cells, the elucidation of TcR promoters. The heterogeneity of the Jpl region might transcribe a 1.0-kb mRNA of TcR gene. We have demonstrated here that both the Jpl and Jp2 regions are transcribed in B cells, as Jpl but not Jp2 is nonmethylated in B cell DNA, although there is as yet no evidence to rule out transcription from the Jpl promoter in B cells. LGL also synthesize a 1.0-kb mRNA, and the methylation state of the Jpl and Jp2 regions and the transcriptional status in leukocyte DNA of the TcR gene are involved in rearrangement of TcR gene. Therefore, the elucidation of the biochemical mechanisms which control methylation and the effect of methylation on site specific recombination in these cell populations, appear to be important areas of further investigation.

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