Analysis of the T Cell Receptor in the Lymphoproliferative Disease of Granular Lymphocytes: Superantigen Activation of Clonal CD3⁺ Granular Lymphocytes¹

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ABSTRACT

To investigate whether cell populations in CD3⁺ lymphoproliferative disease of granular lymphocytes (LDGLs) were skewed toward the use of specific Vβ regions, we studied the repertoire of T-cell receptor (TCR) Vβ gene products in 18 patients, as well as their relationship to the clonal bands in the Southern blot and the activation mediated by superantigens. Using a panel of monoclonal antibodies (mAbs) for conserved Vβ segments and PCR, a dominant population expressing a specific Vβ region was demonstrated in all patients. In five (27%) cases, granular lymphocytes (GLs) were found to express the Vβ 13.1, while Vβ 38 and Vβ 36 were each expressed in three (17%) cases. The remaining cases were characterized by the proliferation of TCR Vβ2, Vβ3, Vβ4, Vβ9, Vβ11, Vβ16, and Vβ20. This finding indicates a biased usage of a limited TCR Vβ in LDGLs, since nearly 60% of the cases utilized only three families of the TCR Vβ genes. In all of the cases studied, we proved that the subset recognized by mAb and PCR was identical to that accounting for the extra band(s) of the Southern blot. This finding confirms the clonal nature of the population identified according to TCR Vβ expression both by phenotype and PCR. On functional grounds, we evaluated whether GLs can be activated through the specific TCR using the superantigens recognizing discrete Vβ families, such as staphylococcal proteins, including SEA, SEB, SEC1, SEC2, SED, and SEE. We demonstrated that the TCR-α/β of clonal GLs in LDGL patients is functionally active in delivering cytotoxic and proliferative signals upon superantigen activation.

INTRODUCTION

LDGL³ is characterized by a chronic proliferation of GLs which could belong either to CD3⁺ T or CD3⁻ natural killer cell lineage (1–3). CD3⁺ LDGL account for the major part of these disorders and are characterized by the clonal proliferation of TCR-α/β GLs expressing CD8 and CD16 antigens. Although functional properties of CD3⁺ GLs indicate that they can be efficiently activated through CD3 antigen stimulation (4, 5), the putative antigen(s) recognized by these discrete cell populations and whether these cells are equipped with a TCR able to transduce the activation signal still remains elusive. These concepts have an obvious relevance to understanding the pathogenesis of the disease. In particular, the presence of associated disorders, such as RA or chronic viral infections (the most common are EBV, hepatitis C virus, hepatitis B virus, human T-cell lymphotropic virus type I/II) (2, 3, 6–8), prompted us to investigate the mechanisms by which these putative inciting agents are likely to act (directly or indirectly) on the CD3⁺CD16⁺ lymphocyte population.

SAGs are molecules which include endogenous retroviral gene products and microbial toxins. They can stimulate proliferation and in turn cause clonal anergy or deletion of T cells bearing a particular TCR Vβ sequence (9, 10). Recently, Kasten-Sportes et al. (11), taking advantage of the description of a patient with a monoclonal proliferation of CD3⁺ GLs, suggested that an immune-mediated selection process, occurring after the transformation event that established the clone, could have affected the TCR-α chain expression. Such a mechanism indicated that TCR was under antigenic pressure and was reminiscent of a SAG-driven selection of GLs. Interestingly, a SAG has been recently claimed to be involved in the pathogenesis of RA (12) which represents a disease frequently associated with LDGL, although the pathogenesis of the two disorders is obviously different.

This study was aimed at exploring the possibility that a bias of TCR Vβ region usage could take place in GLs of LDGL patients and putative causal factors. To this end, using the available mAbs recognizing different Vβ regions of TCR and PCR, we investigated the TCR Vβ repertoire in a large series of CD3⁺ LDGL. To definitively prove the clonality of the population under study, we evaluated its relationship to the extra bands detected by the Southern blot. We also evaluated the cytotoxic and proliferative response of purified GLs to SAGs of recognized specificities such as staphylococcal enterotoxins, including SEA, SEB, SEC1, SEC2, SED, and SEE. Our data definitively demonstrate that the cells recognized by specific mAbs included the clonal population identified by Southern blot analysis. The ability of these cells to respond to the relevant SAG indicates that their TCR is functional.

MATERIALS AND METHODS

Patients. Eighteen patients (10 males and 8 females, mean age 59 ± 15 years) were studied. In all cases, a chronic lymphocytosis (lasting >6 months) sustained by at least 2000 GLs/mm³ was present in the peripheral blood (1). At the time of this study, none of the patients had received treatment. Relevant immunological data of cases under study are reported in Table 1.

Isolation of Granular Lymphocytes. The cell suspension was initially depleted of adherent cells by incubation for 45 min in plastic Petri dishes at 37°C in an atmosphere of 95% air and 5% CO₂. GLs were further purified by magnetic separations over columns (Mini MACS System; Miltenyi Biotec, GMBH, Bergisch Gladbach, Germany). Following this multistep selection procedure, >97% of the cells were viable when tested by the trypan blue exclusion test. The cells were then resuspended in complete culture media and cultured for 7 days (5 days for patient 8). The cells were then used for the experiments described in this study.

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³The abbreviations used are: LDGL, lymphoproliferative disease of granular lymphocytes; TCR, T-cell receptor; RA, rheumatoid arthritis; SAG, superantigen; mAb, monoclonal antibody; GL, granular lymphocyte.

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Science (Boston, MA), and anti-Vβ3, anti-Vβ5, anti-Vβ8.1/8.2, anti-Vβ13.1, anti-Vβ13.6, anti-Vβ14, anti-Vβ16, anti-Vβ17, anti-Vβ18, anti-Vβ20, anti-Vβ21.3, and anti-Vβ22 were obtained from Immunotech (Marseille, France); anti-Vβ1 and anti-Vβ9 were obtained from Pharmingen (San Diego, CA). For flow cytometric analysis, cells were scored using a FACScan analyzer (Becton Dickinson), and data were processed by using Consort 30 Software program, as previously reported (14).

Proliferative Activity. Freshly separated GLs from patients at the concentration of 1 × 10⁶ cells/ml were cultured in round-bottomed 96-well plates for 72 h at 37°C in a 5% CO₂ atmosphere with staphylococcal toxins (SEA, SEB, and toxic against the HLA-DRF natural killer-resistant Raji target cells was evaluated in the presence of staphylococcus toxins at the concentrations of 10, 100, and 1000 pg/ml. For the last 18 h of culture, plates were pulsed with 1 μCi/well [3H]thymidine (26 mCi/mM; CEA Ire Sorin, Saluggia, Italy); cells were then harvested, and the radioactivity was measured with a beta counter. Results are expressed as cpm ± SEM.

Cytotoxic Activity. Cytotoxic activity was assessed by the lysis of 51Cr-labeled Raji target cells in a 4-h assay, as already reported (15). The panel of the available mAbs recognizing specific Vβ families is less represented than PCR primers to different Vβ regions; however, when the results of the two methodologies were compared (Table 1), our data demonstrated a strict correlation between the clonal populations identified by PCR and those recognized using anti-TCR Vβ mAbs.

RESULTS

A survey of the TCR Vβ repertoire in LDGL using mAbs and PCR analysis is reported in Table 1. The data related to PCR analysis in four representative LDGL patients are given in Fig. 1. The panels show that a population expressing a discrete Vβ gene product can be easily identified for each patient. Although considering all of the patients together the pattern was quite heterogenous, a bias toward a preferential use of a discrete TCR Vβ results from our data. In fact, 5 (27%) of 18 cases expressed the Vβ13.1 product, while 3 (17%) cases expressed Vβ8.1/8.2 and 3 (17%) cases expressed Vβ6.7 product, the remaining cases being characterized by cell proliferations using different TCR Vβ regions (one case each Vβ2, Vβ3, Vβ4, Vβ9, Vβ12, Vβ16, and Vβ20). In other words, nearly 60% of the cases preferentially used only TCR Vβ13.1, Vβ6, or Vβ8 gene products.

The panel of the available mAbs recognizing specific Vβ families was used. In brief, PCR was performed in 100-μl reactions for 30 cycles with 30-s melting at 94°C, 60-s annealing at 55°C, and 90-s extension at 72°C in a Perkin Elmer/Cetus thermal cycler using 1 μl cDNA and 2.5 units Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT) in the buffer recommended by the manufacturer. Twenty-two Vβ primers specific for 20 TCR Vβ families were used (Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ6, Vβ7, Vβ8, Vβ9, Vβ10, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ14, Vβ15, Vβ16, Vβ17, Vβ18, Vβ19, and Vβ20), as well as one Cβ primer for Vβ amplification and 3’ and 5’ Ca primers for use as standards for quantitation. For the PCR reaction, a solution consisting of 1.3 mM MgSO₄, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM concentrations of each deoxynucleotide triphosphate, 0.4 μM concentrations of 3’Ca and 3’Cβ 3’-end labeled primers, approximately 10⁶ cpm/tube, cDNA from 2 μg of total RNA, and 2.5 units ampliTaq polymerase (Perkin Elmer/Cetus) was added into each of the 22 tubes containing 20 pmol of one of the Vβ primers. To avoid contamination, reagent controls were performed. Ten μl of PCR products were electrophoresed on 2% TBE agarose gels, transferred on nylon filters (Hybond-N+; Amersham, Buckinghamshire, United Kingdom), and fixed by UV light. Filters were then exposed for 3 h at -80°C.

Statistical Analysis. Data are expressed as mean ± SE, and comparisons between values were made using the Cochran-Cox analysis. A P value of < 0.05 was accepted as significant.

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In all cases, more than 50% of purified GLs expressed HLA-DR antigen.

| nd, not done. |
largest human Vβ families with many functional Vβ genes in each family, to rule out the possibility that the preferential use of a Vβ region by GLs of patients might have happened by chance alone as a consequence of the fact that common Vβ was also more frequently used by the normal T-cell counterpart, we evaluated the respective frequencies of the various Vβ used in the CD3−CD16+ “residual” normal T cells of each patient by fluorescence-activated cell sorting analysis. As a matter of fact, the limit of resolution of the Southern blot does not allow us to rule out the presence of a small (1–5%) population of normal T cells with the same Vβ region. In all patients tested the expression of each Vβ never exceeded 4% of the total population, thus indicating that a preferential use of the common Vβ region in the clonal population was not detectable in the normal counterpart.

The possibility that a SAG triggers the GL activation was assessed by the ability of staphylococcal proteins to stimulate functional prop-
Fig. 3. Rearrangements of TCR β genes using Southern blot analysis on peripheral blood lymphocytes (PBL) and enriched GLs in a representative patient (case 17) with a JαCα probe. The two bands of germ line configuration shown after EcoRI digestion (Mr, 11,000 and Mr, 4,000) on control DNA were absent on peripheral blood lymphocytes and Vβ2* cells of the patient, whereas the Mr, 11,000 was not evident on purified Vβ2* cells (*), probably due to the lack of contaminating monocytes in this cell population. After HindIII digestion an extra band (C) was present in peripheral blood lymphocytes and Vβ2* cells. This latter population lacks the Mr, 3,500 germ line band (*). Vβ2* cells showed the expected 8.0, 6.5, and 3.5 germ line bands.

Fig. 4. Cytotoxic activity of purified GLs against the HLA-DR<sup>+</sup> Raji target cells in three LDGL patients. Staphylococcus toxins were added at the beginning of the test at the concentration of 10 pg/ml. In all instances, target cells were used at a concentration of 10 × 10⁶/ml, and the results referred to the 20:1 E:T ratio.

DISCUSSION

In this study, we demonstrated a trend toward a preferential usage of specific TCR Vβ13, Vβ8, and Vβ6 families in LDGL patients. The population bearing a definite Vβ specificity is within the clonal population identified by Southern blot. We also showed that triggering the TCR Vβ chains by SAGs induces purified GLs expressing discrete TCR Vβ regions to provide cytotoxic and proliferative in vitro activities.

The recent availability of a series of mAbs for conserved portions of TCR Vα or Vβ regions has offered new pieces of information to the understanding of the biology of expanding cells in LDGL. In this regard, the demonstration that CD3*CD16<sup>+</sup> GLs express a preferential TCR has permitted the identification of putatively clonal populations. The evidence we provided in this study, namely, that the cell population identified by mAbs is consistent with that identified using Southern blot has a clinical relevance, supporting the concept that these reagents are useful for the screening of suspected cases and/or monitoring of LDGL patients, particularly in those with low numbers...
of circulating clonal GLs. Of course, this type of analysis must be viewed in a general context taking into account clinical and laboratory findings. In fact, the demonstration that in healthy elderly individuals monoclonal populations of cells expressing a specific TCR V@ region can easily be demonstrated (21, 22) implies that the presence of clonal populations should be interpreted with caution and the diagnosis of LDGL should not be based exclusively on the presence of clonal T cells.

The V@ pattern of the TCR we detected should be compared with recent reports describing the TCR V@ repertoire in patients with LDGL (23–25). Davey et al. (23) demonstrated in 12 cases that the process of clonality randomly affects CD3+ GLs, although from their data a correlation exists between GL proliferations expressing V@6 and the presence of RA as an associated disease. A report claiming the prevalence of V@5.3 in patients with LDGL using mAbs was biased by the reactivity of the reagent to V@5.3 with a CD8+ cell-related antigen (24, 25). A large series of patients should be evaluated to establish a definite response, possibly on the basis of the different setting in which the GL proliferation has been developed.

The demonstration that the CD3+ proliferating population responds to staphylococcal SAGs confirms and expands previous data, indicating that clonal GLs are equipped with a fully functional CD3 apparatus (4, 5, 26). In this regard, this is the first study in which the functional ability of TCR V@ regions of clonal GLs in patients with LDGL was explored. It can be speculated that the initial event leading to proliferation of GLs could interact with TCR by selecting a population expressing a preferential TCR V@ region in a SAG-driven like mechanism. The demonstration we provided that clonal CD3+ GLs are able to respond to SAGs is consistent with the above quoted mechanism. However, since the activation of T cells induced by SAGs is polyclonal (9, 10), one must conclude that a second event could ultimately account for the lymphocyte proliferation. As a matter of fact, our data indicate that the population expanded in our cases which expressed a specific TCR V@ region is the clonal population identified by Southern blot. Although the nature of the putative second event is still unknown, a series of data suggests a role for chromosomal abnormalities or viral agents (3, 27, 28).

The association between LDGL and RA has been reported in nearly one third of LDGL patients (8, 29). This finding is of interest since RA has been associated with SAG-driven T-cell proliferation (12). As a matter of fact, the presence of oligoclonal populations has been reported in lymphocytes recovered from peripheral blood and more frequently in cells obtained from affected joints of patients (30). The more frequently expressed TCR V@ regions were V@83, V@84, and V@17 (12, 30). Although a positive RA test was reported in two patients of our series, none of them has been shown to suffer from RA. Collectively, our data are not consistent with the suggestion that the same pathogenetic noxa could account for RA and LDGLs.

Since V@13-positive cells represent the population more commonly expanded in LDGL, we investigated whether the increase in this cell population could be to some extent associated with some specific disorders. To the best of our knowledge, V@13+ cell populations have been demonstrated to be overrepresented only in the lips of patients with Sjogren’s disease (31). However, Sjogren’s disease was not associated with any of the LDGL patients belonging to our study nor could the presence of characteristic autoantibodies be demonstrated (data not shown).

Our data support the concept that proliferating cells in patients with LDGL are characterized by the preferential use of discrete TCR V@ families. Although SAGs display a broad potential for stimulating T cells, the demonstration herein determined that discrete monoclonal GLs responding to SAGs represents an intriguing feature. In this regard, a SAG-driven activation could be involved in establishing the setting for a clonal transformation of the GL proliferation, possibly mediated by additional and still unknown factors.

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