Large Granular Lymphocyte Proliferation: An Analysis of T-Cell Receptor Gene Arrangement and Expression and the Effect of In Vitro Culture With Inducing Agents

By Wing C. Chan, Carol Dahl, Thomas Waldmann, Susan Link, Alison Mawle, Janet Nicholson, Fritz H. Bach, Kathleen Bongiovanni, Peter A. McCue, and Elliott F. Winton

The status of the T cell receptor β and γ genes in natural killer (NK) cells was investigated in two patients with a marked expansion of CD2+, CD3− NK cells. Both genes were found to be in the germline state. The Tα and complete Tγ gene transcripts were not detected, but a 1.0-kilobase Tγ gene transcript could be demonstrated at low levels in freshly isolated cells and at a much higher level in interleukin-2 (IL-2)-cultured cells. The transcript coding for the δ chain of the CD3 complex was also absent. These cells were cultured in IL-2 with or without the addition of the differentiation-inducing agents: retinoic acid, N,N-hexamethylene bisacetamide, and sodium butyrate. The cultured cells retained their NK activity except in culture with sodium butyrate at ≥1 mmol/L. Expression of CD3 or other T cell surface markers by the NK cells was not observed in these cultures. Either CD2+, CD3− NK cells are derived from a non-T lineage, or they have diverged from the T cell lineage earlier than the stage of Tγ gene rearrangement and CD3 δ chain expression; they are refractory to many induction signals in undergoing further T cell differentiation.

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MATERIALS AND METHODS

Immunophenotyping: Peripheral blood mononuclear cells (PBMC) were obtained from Ficol-Hypaque density gradient centrifugation. Immunophenotyping was performed on fresh, cryopreserved, or cultured cells. Lymphocytes bearing the sheep erythrocyte rosette receptor were evaluated by using the monoclonal antibody T11. Other monoclonal antibodies used included anti-CD3, (mature T lymphocytes), anti-CD4 (inducer/helper T cells), anti-CD8 (suppressor/cytotoxic T cells), anti-CD16, anti-Leu7, NKH-1 (NK/K cells), anti-CD11 (monocytes/granulocytes), anti-CD25 (IL-2 receptor), and anti–HLA-DR. Surface markers were determined by using an indirect immunofluorescence technique and analyzed by using a fluorescence-activated cell sorter.

Cytotoxicity assays: The erythroleukemia cell line K562 was used as target cells in the NK assays. The mouse mastocytoma line P815 was sensitized with rabbit antimesoprone lymphocyte serum and used as ADCC target cells. The cell lines were labeled with 125I during their exponential growth phase, mixed with various concentrations of effector cells, and incubated in 5% CO2 at 37°C for four hours. The amount of radioactivity in the supernatant was determined and percent cytotoxicity calculated as follows: 100 × [cpm (test well) − cpm (spontaneous release)]/[cpm (maximum release) − cpm (spontaneous release)].

Culturing LGL in IL-2-containing medium. The PBMC from the patients were put in RPMI 1640 medium containing 20% fetal calf serum and 10% IL-2-containing conditioned medium (Cellular Products, Buffalo) without any prior lectin stimulation. The initial concentration was adjusted to between 5 × 105 to 1 × 106 cells/mL. In actively growing cultures, the cell concentration was kept below 2 × 105/mL. After three days of culture, retinoic acid, Kodak Co., Rochester, MN, (10−3, 10−4, 10−5 mol/L), sodium butyrate, Sigma Co., Milwaukee, (0.5 mmol/L, 1.0 mmol/L, 2 mmol/L) and N,N-hexamethylene bis-acetamide (HMBA), Aldrich Co., Milwaukee, (0.3 mmol/L, 3 mmol/L, 6 mmol/L) were added to the cultures. Surface marker and NK assays were performed on the third and sixth day after the addition of these differentiation-inducing agents. Cytotoxicity results were analyzed by using Student's t test to
determine whether the inducing agents caused any significant change from IL-2 cultures alone.

**Determination of the T cell receptor β and γ gene rearrangement.** Assays for T cell receptor gene rearrangements were performed as described previously. High-molecular weight DNA was extracted from about 10⁸ PBMC and digested with the restriction endonuclease Bam HI and Eco RI (International Biotechnologies, Inc, New Haven, CT and New England BioLabs, Beverly, MA). The DNA digest was size fractionated on 0.5% to 0.9% agarose gels (10 µg/lane) and then electrotransferred to nitrocellulose paper. A 32P-labeled probe that would hybridize to both constant regions of the Tγ gene and another 32P-labeled probe (kindly provided by Dr J. Seidman) that would hybridize to both Jγ region of the Tγ gene were used in the hybridization experiments. Autoradiography was performed after washing at the appropriate stringency.

**Demonstration of transcripts of the Tγ and Tc genes and the δ chain of the CD3 complex.** Total cytoplasmic RNA was extracted from PBMC before or after culture in IL-2-containing medium for eight days. Cell membranes were disrupted with Triton X-100 in the presence of the RNase inhibitor vanadyl ribonucleoside complexes. Nuclei were removed by a low-speed centrifugation, and proteins were removed by sequential extractions with phenol and phenol-chloroform. The RNA was precipitated with ethanol and quantitated.

Samples containing 8 µg of RNA were electrophoresed on a 6% formaldehyde, 1.5% agarose gel and then transferred to GeneScreen Plus membranes (New England Nuclear, Boston). The blots were prehybridized and then hybridized with 32P-labeled nick-translated cDNA clones for the CD3e (clone pGBC-9), Tα (clone pY1-1), and Tγ (clone Juré 2) molecules. After hybridization, the blots were washed twice with 2x SSC (1 x SSC contained 150 mmol/L sodium chloride and 15 mmol/L sodium citrate at pH 7.0) at room temperature, followed by two washes with 2x SSC and 1% sodium dodecyl sulphate at 60°C and finally by two more washes in 0.1 x SSC at 50°C. Blots were autoradiographed for four to 72 hours.

Controls consisted of RNA extracts from a cytotoxic T cell clone generated in an alloreactive response, PBMC after 28 hours of culture in phytohemagglutinin (PHA), and the erythroleukemia cell line K562.

### RESULTS

Immunophenotyping showed that the two patients had a proliferation of LGL having a CD2+, CD3−, Leu7−, CD11+ phenotype. NKH1 was detected on a high proportion of the lymphocytes whereas CD16 was absent (Table 1). Cells from these patients exhibited strong NK and ADCC activity (Table 1).

DNA from PBMC of patients 1 and 2 was digested with Bam HI and Eco RI. On Southern blot analysis using a 32P-labeled Cγ probe no extra band was detected on both Bam HI and Eco RI digests. The 11-kilobase (kb) and 4-kb bands...
on Eco RI digests of LGL from the patients were almost of equal intensity. These results indicated the absence of monoclonal or polyclonal T cell receptor β chain gene rearrangement in the LGL (Fig 1). Furthermore, using the J region probe for the Tγ gene, only germline bands were observed (Fig 2). Clonal rearrangements as seen in T cell leukemia and oligoclonal bands as seen in polyclonal T cells were not detected. The cells from patient 1 were grown in IL-2–containing supernatants and their cytoplasmic mRNA extracted and analyzed by the Northern blot technique. No mRNA corresponding to the T cell receptor α chain could be detected (Fig 3). A 1.0-kb transcript of the β gene could, however, be demonstrated (Fig 4). This transcript was also present in unstimulated PBMC from patient 1 but in lower amounts compared with stimulated cells (Fig 4). Further experiments demonstrated the lack of a transcript coding for the δ chain of the CD3 complex in IL-2–cultured cells from patient 1 (Fig 5).

The PBMC from patient 1 were also grown in IL-2, with or without the addition of sodium butyrate, HMBA, and retinoic acid. The immunophenotype of the cells in IL-2–containing supernatants was very similar to the unstimulated LGL except for a decrease in the number of cells bearing CDII and an increase in the percentage of HLA-DR+ cells (Table 2). NK activity in IL-2 culture was generally higher than that in unstimulated cells.6 Sodium butyrate, HMBA, and retinoic acid were added to the IL-2 cultures at concentrations that were capable of inducing differentiation of embryonal carcinoma cells and HL-60, an acute myelogenous leukemia cell line.21-22 Both retinoic acid and HMBA failed to induce any significant additional alteration of surface markers. Compared with cultures in IL-2 alone, significant decrease in NK activity was observed at low effector:target cell ratio (Tables 2 and 3). Sodium butyrate was clearly toxic to the cultured cells at concentrations of ≥1 mmol/L. Even in this toxic range, the immunophenotype of the cells was similar to plain IL-2 culture. However, cellular proliferation, the expression of the IL-2 receptor, and cytolytic activity were markedly diminished (Table 4). After seven days, all the cells were dead in the cultures containing 2 mmol/L sodium butyrate.

DISCUSSION

NK cells have been defined as cells that can kill tumor cells, cell lines, and some normal cells spontaneously without

![Fig 2](image2.png)

**Fig 2.** $T_\gamma$ chain gene arrangements of the PBMC from the two patients (Pt1 and Pt2) with LGL proliferation compared with the arrangement in a control EBV B cell line (C). The $T_\gamma$ J region probe identified 3.4 and 1.5-kb germline bands in Eco RI DNA digests of both the control and patient cell populations corresponding to the $J_{\gamma2}$ and $J_{\gamma1}$ loci. Thus the $T_\gamma$ genes are not rearranged but are retained in the germline configuration in LGL from these two patients with LGL proliferation.

![Fig 4](image4.png)

**Fig 4.** Northern blot analysis of $T_\delta$ chain gene expression in a cytotoxic T cell clone (lane A), the erythroleukemia line K562 (lane B), PHA-stimulated normal peripheral blood lymphocytes (lane C), IL-2–stimulated PBMC from patient 1 (lane D), and unstimulated PBMC from patient 1 express a large amount of the 1.0-kb transcript of the $T_\delta$ chain gene but not the 1.3-kb complete transcript. Patient's unstimulated PBMC also contain a small amount of the 1.0-kb message.
previous sensitization. This form of cytotoxicity is not restricted by the major histocompatibility complex (MHC) antigens. There is still much confusion concerning the cell lineage of NK cells and the target recognition structure used. Recently, it has been proposed that NK activity be renamed non-MHC-restricted cytotoxicity. Cells with classical T lymphocytic surface and functional characteristics that can mediate this type of cytotoxicity will be termed non-MHC-restricted cytotoxic T lymphocytes. Other effectors of non-MHC-restricted cytotoxicity will be termed NK cells. The CD3– LGL from our two patients belong to the latter category. The ontologic relationship between these two categories of non-MHC-restricted cytotoxic cells has not been entirely clarified. This study used multiple parameters to look for evidence of T lineage derivation in highly enriched populations of CD3– NK cells, both before and after culture with differentiation-inducing agents.

The NK cells studied were derived from patients with proliferations of CD2+, CD3– LGL. These cells were CD16– and probably represented proliferation of a small subset of normal peripheral blood LGL with the corresponding phenotype (CD3–, CD16–, and NKH1+). We analyzed the T cell receptor β and γ gene status in these CD3– LGL. The Tγ gene appeared to be in the germline state similar to the findings recently reported by Rambaldi et al in CD3– LGL populations of similar but not identical phenotypes. The Tγ gene was also in the germline configuration. There were no clonal bands as observed in clonal T cell populations or oligoclinal bands as seen in normal peripheral T lymphocytes. Although T cell receptor β and γ gene rearrangement cannot be taken as definitive evidence of T lineage derivation, the lack of such rearrangement indicates that the LGL have not even undergone the early steps of T cell differentiation.

The LGL from patient 1 was also cultured in IL-2 and a number of differentiation-inducing agents to gain further insight on their cell lineage and differentiation potential. We could not induce the expression of CD3 or other T cell markers in cells cultured in IL-2 for up to nine days. The addition of retinoic acid, HMBA, and sodium butyrate did not induce any further significant changes for up to six days. IL-2 has been found to enhance the NK activity of CD3– LGL, especially for relatively resistant target cells. When retinoic acid and HMBA were added to IL-2 cultures, NK activity was slightly depressed at the low effector-to-target cell ratios. The mechanism involved was not clear. Sodium butyrate at ≥1 mmol/L concentration appeared to be toxic to the cultured cells. NK activity was significantly diminished even at the concentration of 0.5 mmol/L. We also studied the expression of the mRNA of the δ chain of the CD3 molecule in IL-2–cultured LGL from patient 1. No transcripts could be detected, which indicated that there was no induction of CD3 expression at the mRNA level. Furley et al have recently shown the CD3– associated transcripts to be one of the earliest events in T cell differentiation and production of the T cell receptor. The lack of expression of CD3δ chain transcript, even after growth in IL-2–containing supernatant, would again strongly suggest that these cells do not belong even to a very early stage of T cell differentiation.
respectively. After the addition of HMBA, the control culture contained IL-2 but no added inducing agents, and the same control was used for experiment employing butyrate instead of HMBA (Table 4).

Concentrations of Ta mRNA and the complete 1.3-kb Tα transcript were not detected in the IL-2–cultured cells from patient 1, but a 1.0-kb truncated Tα transcript could be demonstrated. Ritz et al.39 studied the Tα and Tβ gene expression in cloned NK cell lines with the CD2+, CD3– phenotype and also found the expression of a 1.0-kb Tα transcript. Both the 1.0-kb and the 1.3-kb complete transcripts of the Tα gene are expressed in many T cells, and the 1.0-kb transcript is expressed at relatively high levels in immature T lymphocytes.29 The transcription of the 1.0-kb Tα message is, however, not limited to cells of the T lineage and has in fact been demonstrated in B lymphoblastoid cell lines.30 Its presence, therefore, cannot be taken as proof of T cell differentiation.

The PBMC of the patients, though consisting predominantly of CD2+, CD3– LGL, were contaminated with a small population of CD3+ cells. The experimental results, however, appeared to reflect the behavior of the LGL, and the contaminating cells were present in such a low concentration as not to interfere with the interpretation of the data.

The detection of the 1.0-kb mRNA of the Tα gene in cloned NK cells and in IL-2 cultures of LGL from our patients may indicate that its expression is dependent on IL-2 stimulation. This mRNA could, however, also be detected in PBMC freshly isolated from patient 1, although at a lower level, thus indicating that such a transcript is present in resting LGL from this patient.

Reynolds et al.31 studied four cytotoxic LGL leukemia cell lines from rats and failed to detect T cell receptor β chain gene rearrangement and the expression of the functional 1.3-kb mRNA. Similar findings were reported for human LGL isolated from peripheral blood lymphocytes (90% CD16+, NKH1+ population).32 Recently, Lanier et al.33

### Table 3. Immunophenotype and NK Activity of Patients’ PBMC Cultured in IL-2–Containing Medium With and Without the Addition of HMBA

<table>
<thead>
<tr>
<th>HMBA (mmol/L)</th>
<th>CD2</th>
<th>CD3</th>
<th>CD8</th>
<th>NKHI</th>
<th>CD25</th>
<th>NK Activity (Percent Cytotoxicity ± 1 SD): Effector: Target Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A</td>
<td>99</td>
<td>8</td>
<td>4</td>
<td>87</td>
<td>31</td>
<td>96 ± 4.6  99 ± 0.8  101 ± 1.8  80 ± 1.8</td>
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<tr>
<td>B</td>
<td>94</td>
<td>3</td>
<td>3</td>
<td>91</td>
<td>45</td>
<td>83 ± 9.0  85 ± 1.2  88 ± 8.9  66 ± 8.4</td>
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<tr>
<td>0.3</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>A</td>
<td>98</td>
<td>7</td>
<td>5</td>
<td>81</td>
<td>17</td>
<td>86 ± 14.2 95 ± 8.5  96 ± 2.7  81 ± 5.9</td>
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<td>B</td>
<td>91</td>
<td>2</td>
<td>1</td>
<td>61</td>
<td>26</td>
<td>75 ± 3.0  84 ± 1.7  85 ± 3.4  56 ± 2.8</td>
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<tr>
<td>A</td>
<td>95</td>
<td>7</td>
<td>4</td>
<td>80</td>
<td>22</td>
<td>96 ± 7.7  104 ± 0.7† 107 ± 4.2  87 ± 4.8</td>
</tr>
<tr>
<td>B</td>
<td>83</td>
<td>2</td>
<td>2</td>
<td>81</td>
<td>34</td>
<td>89 ± 0.1  92 ± 1.8†  87 ± 3.5  39 ± 1.6*</td>
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<tr>
<td>6.0</td>
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<td>A</td>
<td>98</td>
<td>7</td>
<td>4</td>
<td>78</td>
<td>15</td>
<td>89 ± 0.4  93 ± 5.6  90 ± 4.0  62 ± 1.8*</td>
</tr>
<tr>
<td>B</td>
<td>97</td>
<td>3</td>
<td>3</td>
<td>80</td>
<td>35</td>
<td>80 ± 3.8  87 ± 9.0  85 ± 11.0 51 ± 2.8</td>
</tr>
</tbody>
</table>

The experimental design and data expression are the same as described for Table 2. A and B indicate cultures that were tested three and seven days, respectively, after the addition of HMBA. The control culture contained IL-2 but no added inducing agents, and the same control was used for the experiment employing butyrate instead of HMBA (Table 4).

* Cytotoxicity significantly decreased compared with IL-2 culture alone (P < .05).
† Cytotoxicity significantly increased compared with IL-2 culture alone (P ≤ .05).

### Table 4. Immunophenotype and NK Activity of Patients’ PBMC Cultured in IL-2–Containing Medium With and Without the Addition of Butyrate

<table>
<thead>
<tr>
<th>Butyrate (mmol/L)</th>
<th>CD2</th>
<th>CD3</th>
<th>CD8</th>
<th>NKHI</th>
<th>CD25</th>
<th>NK Activity (Percent Cytotoxicity ± 1 SD): Effector: Target Ratio</th>
</tr>
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<tbody>
<tr>
<td>Zero</td>
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<tr>
<td>A</td>
<td>99</td>
<td>8</td>
<td>4</td>
<td>87</td>
<td>31</td>
<td>96 ± 4.6  99 ± 0.8  101 ± 1.8  80 ± 1.8</td>
</tr>
<tr>
<td>B</td>
<td>94</td>
<td>3</td>
<td>3</td>
<td>91</td>
<td>45</td>
<td>83 ± 9.0  85 ± 1.2  88 ± 8.9  66 ± 8.4</td>
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<td></td>
</tr>
<tr>
<td>A</td>
<td>98</td>
<td>9</td>
<td>4</td>
<td>81</td>
<td>14</td>
<td>92 ± 2.5  88 ± 3.3* 91 ± 0.4* 53 ± 1.8*</td>
</tr>
<tr>
<td>B</td>
<td>90</td>
<td>7</td>
<td>4</td>
<td>51</td>
<td>19</td>
<td>67 ± 4.3  66 ± 4.5* 63 ± 1.6* 32 ± 1.8*</td>
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<tr>
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<tr>
<td>A</td>
<td>98</td>
<td>9</td>
<td>4</td>
<td>77</td>
<td>14</td>
<td>71 ± 3.7* 70 ± 11.5 58 ± 7.0* 22 ± 0.8*</td>
</tr>
<tr>
<td>B</td>
<td>91</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>45 ± 4.1* 47 ± 3.9* 45 ± 4.0* 25 ± 4.6*</td>
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<td>A</td>
<td>99</td>
<td>6</td>
<td>3</td>
<td>49</td>
<td>2</td>
<td>1 ± 0.2* 0 ± 0.1* 1 ± 0.9* 1 ± 1.1*</td>
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<tr>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND       ND       ND       ND</td>
</tr>
</tbody>
</table>

The experimental design and data expression are the same as described for Table 3. Many cells died in cultures containing sodium butyrate at concentrations of 1.0 mmol/L or above, and all the cells were dead after seven days of culture at 2.0 mmol/L.

Abbreviation: ND, not determined.

* Cytotoxicity significantly lower than that in IL-2 culture alone (P < .05).
demonstrated the lack of T cell receptor γ gene rearrangement in human peripheral blood CD3−, CD16+ cells. These findings are very similar to what we found in our patients’ LGL with the CD3−, CD16− phenotype. Interpretations of cell lineage based on single parameters are quite unreliable because few markers are absolutely lineage specific. Multiple parameters of T cell differentiation were scrutinized in this study, and they failed to support the hypothesis that CD3− NK cells are derived from the T lineage. Even stimulation of these cells with IL-2 and a number of differentiation-inducing agents failed to induce the appearance of further evidence of T cell differentiation.

The expression of CD2 on CD3− NK cells deserves some comment. There is now mounting evidence that NK cell and T cell function and state of activation can be altered through the CD2 ligand.34 CD2 also appears to bind to LFA-3,35 a widely distributed cell surface molecule. It is possible that CD2 is a primitive recognition molecule important in cell-cell interaction. It may precede the appearance of specific T cell receptors on lymphoid effector cells of cell-mediated immunity. If this hypothesis is correct, its expression on CD3− NK cells indicates that these cells are one component of the primitive lymphoid effector system and does not imply T lineage differentiation.

REFERENCES


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WC Chan, C Dahl, T Waldmann, S Link, A Mawle, J Nicholson, FH Bach, K Bongiovanni, PA McCue and EF Winton