Activation via the CD3 and CD16 Pathway Mediates Interleukin-2–Dependent Autocrine Proliferation of Granular Lymphocytes in Patients With Granular Lymphocyte Proliferative Disorders

By Shigeru Hoshino, Kazuo Oshimi, Masano Teramura, and Hideaki Mizoguchi

Granular lymphocytes (GLs) in patients with GL-proliferative disorders (GLPDs) are known to express the interleukin-2 receptor (IL-2R) β chain (p70-75) constitutively and to proliferate in response to stimulation with IL-2 via the β chain. In this report, we found that the anti-CD3 monoclonal antibody (MoAb) OKT3 could induce the proliferation of GLs from patients with T-cell lineage GLPDs (T-cell receptor-αβ /CD3/CD16), but not that of natural killer (NK) cell lineage GLs (T-cell receptor-αβ /CD3/CD16). In contrast, the anti-CD16 MoAb 3G8 that reacts with NK-lineage GLs could induce the proliferation of these GLs but not that of GLs with a T-cell phenotype. Furthermore, the anti-CD16 MoAbs CLB FcR gran1 (VD2) and OK-NK, which react with both T- and NK-lineage GLs, induced the proliferation of GLs with both T- and NK-cell phenotypes. The proliferative response induced via the CD3 or IgG Fc receptor III (FcRIII: CD16) pathway was shown to be associated with the IL-2–dependent autocrine pathway by various findings, including the induction of endogenous IL-2 production, the coexpression of the IL-2Rα chain (p55) and the IL-2Rβ chain, and the inhibition of GL proliferation by anti-IL-2 or anti-IL-2R MoAb. These results suggest that GL proliferation is mediated at least partly through the IL-2–dependent autocrine pathway, and that the TCR/CD3 complex in T-cell phenotype GLs and the FcRIII in both T- and NK-cell phenotype GLs play a role in their activation in GLPDs.

**MATERIALS AND METHODS**

**Patients and cells.** Ten patients with GLPDs were studied. The diagnosis was made on the basis of persistent granular lymphocytosis of 2,000/μL or more for more than 6 months. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Conray gradient centrifugation, and these PBMCs contained 77% to 98% GLs as assessed by morphologic analysis. Purification of GLs from PBMCs was performed by negative selection with goat antihuman IgG-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) after coating accessory cells with saturating amounts of the appropriate MoAbs. In brief, selected MoAbs at designated concentrations were added to PBMC suspension (1 × 10^6 cells/mL) in RPMI 1640 medium (Flow Laboratories, Irvine, CA), and the suspensions were incubated for 30 minutes at 4°C. After incubation, the PBMCs were washed three times to remove unbound MoAbs, and then incubated again with goat antihuman IgG-coated magnetic beads for 30 minutes at 4°C. The bead-coated cells were then removed by magnetic separation. MoAbs used for the purification of T-lineage GLs included OKT4 (anti-CD4; Ortho Pharmaceutical Corp, Raritan, NJ), B4 (anti-CD19; Coulter Immunology, Hialeah, FL), and MY4 (anti-CD14; Coulter Immunology) for the removal of CD4+ T cells, B cells, and...
monocytes, respectively. MoAbs used for the purification of NK-lineage GLs included OKT3 (anti-CD3; Ortho), B4, and MY4 for the removal of T cells, B cells, and monocytes, respectively. Thus, the separated cells obtained contained greater than 99.5% GLs on morphologic analysis, and less than 0.5% CD4+ cells as assessed by flow cytometry. Contamination by monocytes was less than 0.2% as determined by staining for nonspecific esterase.

These studies were approved by the Institutional Review Board. Patients were informed that blood samples were obtained for research purposes and that their privacy would be protected.

**Phenotypic analysis.** Phenotypic analysis was performed by indirect immunofluorescence with the following MoAbs as described previously:46 OKT1 (anti-CD2), OKT3, OKT4, OKT8 (anti-CD8), and OK-NK (anti-CD16) from Ortho; WT31 (anti-TCR-αβ) from Sanbio (Uden, The Netherlands); CLB FcR gran1 (VD2) (anti-CD16) kindly provided by Dr Pedro A.T. Tetteroo (The Netherlands Red Cross, Amsterdam); and 3G8 (anti-CD16) kindly provided by Dr Hideo Yagita (Juntendo University, Tokyo, Japan). VD2 was used as ascites form, and 3G8 was purified from ascites using a protein A column.

**Flow cytometric analysis of IL-2R chain expression.** IL-2R chain expression was evaluated by flow cytometric analysis as described previously.15 The MoAb anti-Tac (CD25), which recognizes the IL-2Rα chain (kindly provided by Drs Takashi Uchiyama and Masanori Kamio, Kyoto University, Kyoto, Japan),26 and the MoAb Mik-pl, which recognizes the IL-2Rβ chain (kindly provided by Dr Misuzu Tsudo, Yunitsuka Chuo Hospital, Kyoto, Japan, and Dr Masayuki Miyasaka, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan),24 were used for flow cytometric analysis.

**Preparation of MoAb-coated microtiter plates.** For the proliferation assay, 75 μL of 0.1 N Tris Buffer (pH 9.6) containing 10 mL of MOPC 31C and UPC 10; Sigma) and anti-CD8 MoAb (OKT8) were used instead of the anti-CD3 or anti-CD16 MoAb. For inhibition studies, anti–IL-2Rα chain MoAb (anti-Tac) and/or anti–IL-2Rβ chain MoAb (Mik-b1), or else anti–IL-2 MoAb (Shionogi Pharmaceutical Co Ltd), was added at the beginning of the culture period at the indicated concentrations.

**IL-2 synthesis assay.** Cells were incubated for 24 hours under conditions similar to those described for the proliferation assay. At the end of the culture period, the cell-free supernatant was collected from each well and assayed for IL-2 activity. IL-2 activity was measured using the IL-2–dependent murine CTLL cell line, as previously described.25 Calculation of the IL-2 activity was based on a standard source of recombinant human IL-2 that contained a known amount of activity determined in international standard units.

**RESULTS**

**Surface phenotypes and IL-2R chain expression of GLs from 10 GLPD patients.** As shown in Table 1, GLs from patients 1 through 7 exhibited the T-cell phenotype (TCR-αβ+/CD3+16+), while patients 8 through 10 showed the proliferation of TCR-αβ−/CD3+16− GLs, indicating the NK-cell phenotype. In all seven patients with T-lineage GLs, the presence of CD16 antigen (Ag) was detected by the CLB FcR gran1 (VD2) and OK-NK MoAbs, but in six

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### Table 1. Surface Phenotypes and IL-2R Chain Expression of GLs From 10 GLPD Patients

<table>
<thead>
<tr>
<th>Case No.</th>
<th>CD2 (OKT1)</th>
<th>CD3 (OKT4)</th>
<th>TCR-αβ (WT31)</th>
<th>CD4 (OKT3)</th>
<th>CD8 (OKT8)</th>
<th>CD16 (3G8)</th>
<th>VD2 (OK-NK)</th>
<th>IL-2R Chain</th>
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</tr>
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</table>

Surface phenotypes and IL-2R chain expression were evaluated by flow cytometric analysis.

Abbreviations: α, the IL-2Rα chain; β, the IL-2Rβ chain.

*Weakly positive by flow cytometric analysis.*
of these seven patients it was not detected by the 3G8 MoAb. In contrast, in all three patients with NK-lineage GLs, CD16 Ag was detected by the VD2, OK-NK, and 3G8 MoAbs. On both T- and NK-lineage GLs, only the IL-2RG chain and not the IL-2Rα chain was expressed constitutively, as has been reported previously.35

**IL-2-induced proliferation of GLs.** GLs were incubated with various concentrations of recombinant human IL-2, and proliferation was measured by ³H-TdR incorporation. Both T- and NK-lineage GLs showed a marked proliferative response when cultured in the presence of 10 U/mL or more of IL-2 (Fig 1). To evaluate the inhibitory effects of anti-IL-2R MoAbs or anti-IL-2 MoAb, we examined the changes of the IL-2-dependent proliferation of GLs. GLs were cultured with IL-2 in the presence or absence of anti-IL-2Rα chain and/or anti-IL-2Rβ chain MoAb. As shown in Fig 2A, the IL-2-dependent proliferation of T-lineage GLs was partially inhibited by anti-IL-2Rα chain MoAb or anti-IL-2Rβ chain MoAb alone, but maximal inhibition was observed when both MoAbs were added to the cultures. An inhibitory effect of these anti-IL-2R MoAbs was similarly observed with NK-lineage GLs (data not shown). As shown in Fig 2B, the IL-2-dependent proliferation of T-lineage GLs was inhibited by anti-IL-2 MoAb in a dose-dependent manner, and a similar result was observed for NK-lineage GLs (data not shown).

**Effects of anti-CD3 MoAb or anti-CD16 MoAb on GL proliferation.** To examine whether GLs proliferated in response to stimulation other than the addition of IL-2, we studied the effects of various MoAbs. We used the OKT3 MoAb (anti-CD3) that reacts with T-lineage GLs and not with NK-lineage GLs, the 3G8 MoAb (anti-CD16) that reacts with NK-lineage GLs and has little or no effects on T-lineage GLs, and the VD2 and OK-NK MoAbs (anti-CD16) that react with GLs of both lineages. Because it was suggested by preliminary experiments that soluble MoAbs alone had no effect on GL proliferation (data not shown), PBMCs or GLs from the GLPD patients were cultured with solid-phase immobilized MoAbs, or with soluble MoAbs in the presence of PMA.

When PBMCs from patients with T- and NK-lineage GLPDs were cultured with solid-phase immobilized OKT3 MoAb (Fig 3A) or with soluble OKT3 MoAb in the presence of PMA (Fig 3B), a significant increase in ³H-TdR incorporation was observed. However, when GLs were purified by immunomagnetic methods, the proliferative effect of the OKT3 MoAb was partially decreased for the T-lineage GLs and was lost for the NK-lineage GLs. These results suggested that purified T-lineage GLs themselves could respond to the OKT3 MoAb but that purified NK-lineage GLs did not respond, and that contaminating CD3⁺ cells also affected the OKT3 MoAb-induced cell proliferation of GLs in both lineages. Accordingly, GLs were purified from PBMCs and cultured with solid-phase...
immobilized OKT3 MoAb or with soluble OKT3 MoAb in the presence of PMA. As shown in Fig 3, C and D, a significant increase in \(^3\)H-TdR incorporation was observed for T-lineage GLs purified from the PBMCs of the seven patients with T-lineage GLPDs, but not for NK-lineage GLs purified from the PBMCs of the three patients with NK-lineage GLPDs.

Regarding the anti-CD16 MoAbs, the 3G8 MoAb, which reacts with NK-lineage GLs but not with T-lineage GLs, induced the proliferation of purified NK-lineage GLs to the same degree as that of unpurified PBMCs (Fig 4, A and B). As shown in Fig 4, C and D, a significant increase in \(^3\)H-TdR incorporation was observed for the NK-lineage GLs obtained from all three patients, but not for T-lineage GLs. These results suggested that the 3G8 MoAb could stimulate NK-lineage GLs directly, but had no effect on T-lineage GLs or contaminating cells.

In contrast, the VD2 and OK-NK (anti-CD16) MoAbs, which react with both T- and NK-lineage GLs, induced the proliferation of GLs of both lineages in a similar manner (Fig 5). However, the proliferation of T-lineage GLs induced by anti-CD16 MoAbs was lower than that by anti-CD3 MoAb, ie, mean stimulation indices of immobilized OKT3, soluble OKT3 with PMA, immobilized VD2, soluble VD2 with PMA, immobilized OK-NK, and soluble OK-NK with PMA were 8.23, 7.36, 3.76, 3.17, 2.80, and 3.18, respectively.

To confirm whether other MoAbs that react with GLs can induce the proliferation of GLs, we examined the effects of anti-CD8 MoAb (OKT8). However, solid-phase immobilized OKT8 MoAb or soluble OKT8 MoAb with PMA did not induce the proliferation of CD8+ GLs (data not shown), suggesting that the proliferative effects of anti-CD3 and anti-CD16 MoAbs for GLs are specific.

**Inhibitory effects of anti-IL-2R MoAbs or anti-IL-2 MoAb on GL proliferation induced by anti-CD3 and anti-CD16 MoAbs.** To evaluate the relationship between MoAb-induced GL proliferation and IL-2, we examined the effects of anti-IL-2R MoAbs or anti-IL-2 MoAb. As shown in Fig 6, anti-CD3 and anti-CD16 MoAb-induced GL proliferation was inhibited by anti-IL-2R MoAbs or anti-IL-2 MoAb.

**IL-2 synthesis.** To confirm whether anti-CD3 or anti-CD16 MoAb-induced GL proliferation was mediated through the IL-2 autocrine pathway, we next investigated IL-2 synthesis by cells from seven patients with GLPDs (Table 2). When PBMCs and purified GLs from the T-lineage GLPD patients were cultured with the OKT3 MoAb in the presence of PMA, the culture supernatants found to contain 16.7 to 18.3 U/mL and 14.0 to 17.0 U/mL...
of IL-2 activity, respectively. These results suggested that purified T-lineage GLs could produce IL-2 and that contaminating cells may also produce small amounts of IL-2, because a decrease in IL-2 activity was observed after the immunomagnetic removal of contaminating cells from the PBMCs. Purified NK-lineage GLs produced 0.4 to 0.5 U/mL of IL-2 activity when stimulated with the OKT3 MoAb in the presence of PMA, and this was similar to the...
IL-2-DEPENDENT AUTOCRINE GROWTH IN GLPD

Fig 6. Inhibitory effects of anti-IL-2R MoAbs or anti-IL-2 MoAb on GL proliferation induced by anti-CD3 and anti-CD16 MoAbs. GLs were cultured with the indicated MoAbs (OKT3, □; 3G8, □; VD2, □; OK-NK, □; and subclass-matched control MoAb, ■) as described in Figs 3 through 5. At the beginning of culture, anti-IL-2R MoAbs (combining both 100 μg/mL of anti-Tac and 40 μg/mL of Mik-p1, □), or 50 μg/mL of anti-IL-2 MoAb (□) were added. Proliferation was evaluated as described in Figs 3 through 5. Representative results for T-lineage (case 1) and NK-lineage (case 8) GLs are shown.

In contrast, the VD2 and OK-NK MoAbs induced the IL-2 production by GLs of both lineages.

Induction of IL-2Ra chain expression on GLs by anti-CD3 and anti-CD16 MoAbs. As described previously, both T- and NK-lineage GLs usually expressed the IL-2Rβ chain alone and not the IL-2Ra chain. However, GLs began to express the IL-2Rα chain after treatment with anti-CD3 or anti-CD16 MoAb (Fig 7). The OKT3 MoAb induced the IL-2Rα chain expression on T-lineage GLs but not on NK-lineage GLs, and the 3G8 MoAb induced the α chain expression on NK-lineage GLs but not T-lineage GLs. In contrast, the VD2 and OK-NK MoAbs induced the α chain on both T- and NK-lineage GLs. However, as shown in Fig 7, anti-CD16 MoAb-induced expression of IL-2Rα chain on T-lineage GLs was lower than that on NK-lineage GLs or

Table 2. IL-2 Synthesis by GLs

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Cells</th>
<th>PMA Alone</th>
<th>PMA With Control MoAb</th>
<th>Anti-CD3 OKT3</th>
<th>Anti-CD16 3G8</th>
<th>Anti-CD16 OK-NK</th>
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</tr>
<tr>
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</tr>
<tr>
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<td>6.3</td>
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PBMCs (2 × 10⁵) or GLs purified from 2 × 10⁵ PBMCs were cultured in 96-well microtiter plates in 150 μL of RPMI 1640 medium containing 10% fetal calf serum with OKT3 (1 μg/mL), 3G8 (10% vol/vol), VD2 (0.01% vol/vol), or OK-NK (1 μg/mL) in the presence of 5 ng/mL of PMA for 24 hours. For the control, cells were cultured with PMA alone or with PMA and subclass-matched control MoAb. At the end of culture, the cell-free supernatant was collected and assayed for IL-2 activity using an IL-2-dependent CTLL cell line. Results show the IL-2 activity in units per milliliter.

Abbreviation: ND, not done.
DISCUSSION

In this study, we showed that anti-CD3 and anti-CD16 MoAbs could induce the proliferation of GLs from patients with GLPDs. The proliferative response induced via CD3 or CD16 pathway is associated with a pathway involving IL-2R expression, endogenous IL-2 production, and subsequent binding of IL-2 to IL-2Rs. These results suggest that the proliferation of patient GLs is mediated at least partly through the MoAb-induced IL-2-dependent autocrine pathway. The OKT3 (anti-CD3) MoAb could induce IL-2-dependent autocrine proliferation in T-lineage GLs but not in NK-lineage GLs, whereas the 3G8 (anti-CD16) MoAb could induce the proliferation of NK-lineage GLs but not T-lineage GLs. In contrast, the VD2 and OK-NK (anti-CD16) MoAbs modulated the IL-2-dependent autocrine proliferation of both T- and NK-lineage GLs.

As described in Table 1 and a previous report, the presence of CD16 Ag on T-lineage GLs was detected by VD2 and OK-NK MoAbs, but not or little, if any, by 3G8, anti-Leu-11a and anti-Leu-11c MoAbs. Furthermore, even after the activation with OKT3 MoAb, T-lineage GLs did not express the 3G8 epitope of CD16 (data not shown). These results indicate that the structure of FcyRIII of T-lineage GLs is different from those of normal NK cells and patient GLs with NK lineage, because normal NK cells and patients GLs with NK lineage (Table 1) have FcyRIII epitopes recognized by both VD2/OK-NK MoAbs and 3G8/anti-Leu-11 MoAbs.

It has been reported that unstimulated patient GLs of both lineages constitutively express the IL-2Rβ chain and show a proliferative response to exogenous IL-2 mediated via the β chain. We also observed the constitutive expression of the β chain on GLs of both lineages (Table 1) and found that IL-2 induced the proliferation of these cells (Fig 1). This proliferative response to exogenous IL-2 mediated via the β chain was significant in the presence of 10 U/mL or more of IL-2, and was inhibited by anti-IL-2R MoAbs (Fig 2A) or by anti-IL-2 MoAb (Fig 2B).

Incubation of T-lineage GLs with OKT3, NK-lineage GLs with 3G8, and GLs of both lineages with VD2 and OK-NK resulted in expression of the IL-2Rα chain on the GLs (Fig 7). Because GLs of both lineages constitutively express the IL-2Rβ chain, the MoAb-induced expression of the IL-2Rα chain led to the formation of the high-affinity IL-2R, which is a membrane complex composed of the IL-2Rα and β chains. Thus, when patient GLs, which usually express the intermediate-affinity IL-2R, were stimulated with the appropriate MoAbs, they expressed the high-affinity IL-2R, suggesting that there was an increase in the affinity of patient GLs for IL-2. Furthermore, it is suggested that the increase in affinity is followed by a cellular response to lower levels of IL-2 in an autocrine fashion. However, because the expression of IL-2Rα chain on GLs after stimulation with IL-2 was reported, it is possible that IL-2 secreted by GLs themselves may also affect partly the expression of IL-2Rα chain on these cells.

It is accepted that anti-CD3 MoAb can induce IL-2-
dependent autocrine T-cell proliferation in normal T cells.20 Our results indicated that abnormally expanded T-lineage GLs responded similarly to anti-CD3 MoAb, and that IL-2-dependent autocrine proliferation was induced by this MoAb. These results suggest that patient T-lineage GL proliferation can be mediated via the TCR/CD3 complex. In addition, our results showed that anti-CD16 MoAbs (VD2 and OK-NK) could induce the proliferation of T-lineage GLs. Because CD4+ inducer T cells and CD8+ cytotoxic T cells usually do not possess the CD16 Ag, anti-CD16 MoAb-induced T-cell proliferation has not been previously reported in these populations. Thus, it is interesting that the proliferation of CD3+16+ GLs from T-lineage GLPD patients was mediated via the FcyRIII. However, the proliferation, the IL-2Ra chain expression, and the IL-2 production induced by activation via the CD16 pathway in T-lineage GLs is low as compared with that via the TCR/CD3 complex (Table 2 and Figs 3, 5, and 7).

NK-lineage GLs reacted with all three anti-CD16 MoAbs tested, and these anti-CD16 MoAbs could induce IL-2-dependent autocrine proliferation of the NK-lineage GLs. It has been reported that the FcyRIII of normal NK cells acts as a signaling pathway through which IL-2-dependent activation is enhanced,21 and cross-linked anti-CD16 MoAb acts synergistically with IL-2 to elicit the expression of the IL-2Ra chain on normal NK cells.23,24 Thus, it is suggested that the FcyRIII on normal NK cells is a signal-transducing molecule which induces the expression of the IL-2Ra chain. Our finding of anti-CD16 MoAb-induced IL-2Ra chain expression on abnormally expanded NK-lineage GLs confirms these previous observations in normal NK cells. In addition, it has been reported that the treatment of normal NK cells with anti-CD16 MoAb induces the production of various cytokines, such as interferon-γ, tumor necrosis factor, macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage CSF (GM-CSF).25 However, the ability of normal NK cells to produce IL-2 is controversial.26 In a previous study using T- and NK-cell clones that showed non-MHC-requiring cytotoxicity, the majority of the T-cell clones produced high levels of IL-2 after stimulation with phytohemagglutinin, whereas only 2 of 11 NK-cell clones produced small levels of IL-2 following stimulation with the calcium ionophore A23187 and PMA.27 Our study suggested that treatment of patient NK-lineage GLs with anti-CD16 MoAb induced IL-2 production.

We used highly purified GLs obtained by negative selection using immunomagnetic beads in this study. However, because complete elimination of nonleukemic normal cells was not possible, ie, CD8+ normal cells in T-lineage GLPDs or CD16+ normal cells in NK-lineage GLPDs, we could not exclude the possibility that these contaminating cells also respond to anti-CD3 or anti-CD16 MoAb and affect the cell proliferation and/or IL-2 production. Furthermore, because GLs of both lineages constitutively express the IL-2RB chain and proliferate when cultured with exogenous IL-2, it is suggested that the IL-2 secreted by accessory cells may also play a critical role in GL proliferation in vivo, where it could act in a paracrine fashion. Indeed, our results suggested that the accessory cells in the PBMC population affected OKT3-induced IL-2 production (Table 2). Thus, it appears that an MoAb-induced IL-2-dependent paracrine mechanism also has an effect on GL proliferation in patients with GLPDs.

ACKNOWLEDGMENT

We thank Drs M. Tsudo, M. Miyasaka, T. Uchiyama, K. Kamio, H. Yagita, and P.T.A. Tettero for kindly providing the MoAbs Mik-Bl, anti-Tac, 3G8, and CLB FcR gran1.
patients with abnormally expanded large granular lymphocytes. J Immunol 140:4199, 1988


Activation via the CD3 and CD16 pathway mediates interleukin-2-dependent autocrine proliferation of granular lymphocytes in patients with granular lymphocyte proliferative disorders

S Hoshino, K Oshimi, M Teramura and H Mizoguchi