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Enhancement of Anti-tumor Activity of Natural Killer Cells by BALL-1, a B Cell Lymphoma Line

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The anti-tumor activity of human peripheral blood mononuclear cells (PBMC) against various tumor cell line cells (K562, Daudi, KMG-2, and KATOIII) was enhanced by coculture with irradiated BALL-1, but not with other irradiated B cell line cells (NALM-1, Namalwa, and Daudi). PBMC cocultured with BALL-1, however, failed to exhibit evident cytotoxicity against autologous concanavalin A-induced lymphoblasts. The enhancement of the anti-tumor activity seemed not to be correlated with EBNA and HLA-DR expression on B cell line cells. Monoclonal antibodies (mAbs) against interleukin (IL)-2, interferon-γ, IL-12, IL-15, tumor necrosis factor-α and lymphotoxin showed little or no suppression of the anti-tumor activity of PBMC treated with irradiated BALL-1. Furthermore, the culture supernatants of BALL-1 failed to enhance the anti-tumor activity of PBMC, suggesting no involvement of soluble factors in the induction of the anti-tumor activity. The anti-tumor activity of PBMC treated with BALL-1 was synergistically enhanced by an additional IL-2 stimulation. Periodate-lysine-paraformaldehyde-fixed, but not ethanol- or acetone-fixed, BALL-1 could significantly enhance the anti-tumor activity. Furthermore, BALL-1-derived membrane fraction, but not that of Daudi, enhances the anti-tumor activity. It was thus suggested that some membrane glycoproteins on the cell surface of BALL-1 play a crucial role in the induction of the anti-tumor activity. By analysis using mAbs against human leukocytes, we found that depletion of CD11b, CD16, and CD56-positive cells resulted in decreased anti-tumor activity, suggesting that the main effector cells in the BALL-1-induced anti-tumor activity were natural killer (NK) cells. The present results thus raise the possibility that BALL-1, probably via membrane glycoproteins, modulates NK cell-mediated anti-tumor activity.

Key words: Anti-tumor — BALL-1 — Lymphoma — NK cells

Numerous avenues have been pursued in cancer immunotherapy. Several investigators have attempted *in vitro* generation of tumor-specific cytotoxic lymphocytes.[1, 2] Vose *et al*.[3] and Vanky *et al*.[4, 5] have succeeded in the induction of autologous tumor-specific cytotoxic lymphocytes by using mixed cultures of peripheral blood mononuclear cells (PBMC) with untreated autologous tumor cells. Grimm *et al*.[6] and Lotze *et al*.[7] have shown that lymphokine-activated killer (LAK) cells are induced by culturing PBMC in interleukin-2 (IL-2)-containing medium. Since IL-2 provides the second signal for the proliferation and differentiation of activated T lymphocytes,[8, 9] the factor enhances proliferation of the effector T lymphocytes *in vitro* in the presence of autologous tumor stimulator cells.[10–12] Much interest has been focused on the relationships among surface phenotype, major histocompatibility complex restriction for antigen recognition, and the effector function of tumor-specific cytotoxic T lymphocyte subsets.[3–5, 13–15]

The induction of nonspecific cytotoxicity is thought to be convenient and effective in cancer immunotherapy. Recent advances in genetic engineering have made it easy to obtain large amounts of pure recombinant cytokines. The world-wide use of these cytokines in cancer immunotherapy is expected to provide much data not only about their usefulness, but also about some hazardous side effects that limit their clinical applications.

In our previous paper, we have shown that some glycoproteins of BALL-1 (a B cell line) cells stimulate CD4-positive T cells to produce a unique eosinophil chemotactic factor and a fibroblast proliferation factor.[16–18] We report here that some glycoproteins of BALL-1 not only stimulate CD4-positive T cells, but also enhance natural killer (NK) cell-mediated non-specific and cytokine-independent anti-tumor activity.

MATERIALS AND METHODS

Generation of cytotoxic cells Heparinized venous blood was obtained from healthy volunteers. PBMC were

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enriched by a single centrifugation with Sepracell-MN (Sepratech, Oklahoma, OK). PBMC were collected, washed with 0.1% bovine serum albumin-phosphate-buffered saline (PBS), and suspended in RPMI 1640 solution supplemented with 10% heat-inactivated fetal calf serum, 300 μg/ml glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin (complete medium). The viable cell concentration was adjusted to 1×10^6/ml in complete medium. PBMC were cultured in plastic dishes at 37°C in a humidified 5% CO_2 atmosphere.

B cell line cells NALM-1, Namalwa, Daudi, and BALL-1 were used. All were generous gifts from Dr. Sagawa of Kurume University Medical School. Namalwa was EBNA-positive. HLA-DR expression was assessed by FITC-labeled monoclonal anti-HLA-DR antibody (Tago, Burlingame, CA).

***In vitro* sensitization** Primary *in vitro* sensitization was performed in plastic dishes. PBMC were cocultured with irradiated (120 Gray) or fixed B cell line cells at various responder-to-stimulator ratios (R/S ratio) for 7 days unless otherwise specified. As a control, LAK activity of PBMC was induced by treatment with recombinant IL-2 (TGP-3, a gift from Takeda Pharmaceutical Corp., Osaka).

Fixation of B cell line cells In some experiments, BALL-1 cells fixed with fixatives were used instead of irradiated BALL-1. Ethanol (95%), acetone and periodate-lysine-paraformaldehyde (PLP) solution were used as fixatives. BALL-1 cells were harvested, washed twice with PBS and put into each fixative. After 10-min fixation in an ice bath, the fixed cells were washed, and suspended in complete medium.

Cytotoxicity assays KMG-2 (glioma),[19] KATOIII (gastric carcinoma),[20] K562 (NK-sensitive erythroleukemic cell line), Daudi (LAK-sensitive Burkitt lymphoma) and autologous concanavalin A-induced lymphoblasts (ConA-blasts, non-tumor cells) were used as target cells. These cell line cells were maintained in complete medium and subcultured every 3 days. ConA-blasts were prepared by 3-day culture of autologous PBMC with 5 μg/ml of ConA. These target cells were labeled with 50 μCi/10^6 cells of $Na_2{}^{51}CrO_4$ (Daiichi Radioisotope Laboratories, Tokyo; specific activity, 1 mCi/ml) for 60 min at 37°C. The cells were washed twice, incubated at 37°C for another 30 min, washed, and resuspended at 1×10^5/ml. The cells were added at a concentration of 1×10^4 cells/well in triplicate and incubated with effector cells at effector:target (E:T) ratios of 10 to 80 (target: KMG-2, KATOIII, Daudi, ConA-blast) or 4 to 32 (K562) in 96-well round-bottomed microtiter plates. Target cells that had been incubated in medium alone or with 1 *N* HCl were used to determine spontaneous and maximum Cr-release, respectively. The plates were incubated for 4 h at 37°C, then centrifuged at 350*g* for 6 min, and 100 μl aliquots of the supernatants were harvested. The radioactivity of the supernatants was counted with a gamma counter (Aloka, Tokyo). The percentage of lysis was calculated as follows:

(experimental release−spontaneous release)×100/(maximum release−spontaneous release)

Cytotoxicity was expressed as the mean±SE and differences were assessed for statistical significance by using Student's *t* test. Spontaneous release was always less than 15% of maximum release.

In some experiments, monoclonal antibodies (mAbs) against cytokines, such as anti-IL2 (Otsuka Pharmaceutical Corp., Tokyo), anti-tumor necrosis factor-α (TNFα; Endogen, Boston, MA), anti-lymphotoxin (LT; Endogen), anti-IL-12 (Wako Life Sci., Osaka), anti-IL-15 (Wako Life Sci.), and anti-interferon-γ (IFNγ; Otsuka Pharmaceutical Corp.) mAbs were used to clarify whether such cytokines were involved in the enhancement of anti-tumor activity in this system.

Identification of PBMC subsets by using mAbs Depletion of CD3, CD4, CD5, CD8, CD11b, CD16, CD19, CD20, and CD56 populations of PBMC treated with BALL-1 for 7 days was achieved by complement-mediated lysis. PBMC treated with BALL-1 were incubated in an ice bath with appropriate concentrations of CD3, CD5, CD20 (Nichirei, Tokyo), CD4, CD8, CD11b (Ortho Pharmaceutical Corp., Raritan, NJ), CD16 (Becton-Dickinson, Mountain View, CA), CD19 (Cosmo Bio, Tokyo), and CD56 (Dako A.S., Glostrup, Denmark) antibodies for 60 min, centrifuged, and resuspended in rabbit complement (C) (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) for 60 min at 37°C. Viable cells were washed twice, resuspended to make 4×10^6 cells/ml and used for cytotoxicity assay. An aliquot of the cells was reincubated with appropriate FITC-conjugated antibodies, and analyzed for residual antibody-positive cells.

In some experiments, PBMC that had been stimulated by irradiated BALL-1 for 7 days were stained with FITC-conjugated mouse mAbs (CD16 and CD56; Dako). The cells were analyzed for fluorescence by using a fluorescence-activated cell sorter (FACStar; Becton-Dickinson). Ten thousand cells were collected for each sample, gated on forward light scatter and right-angle scatter to exclude cell debris, clumps and nonviable cells. To minimize nonspecific binding of the reagents, the cells were exposed to heat-inactivated human AB serum prior to incubation with the antibodies. The cells (1×10^6 cells) were placed in a tube and 50 μl of human AB serum was added. After 15 min, the cells were washed with PBS, and incubated with the appropriate amounts of antibody for 30 min in the dark at 4°C. They were then washed twice and submitted to flow cytometric analysis.

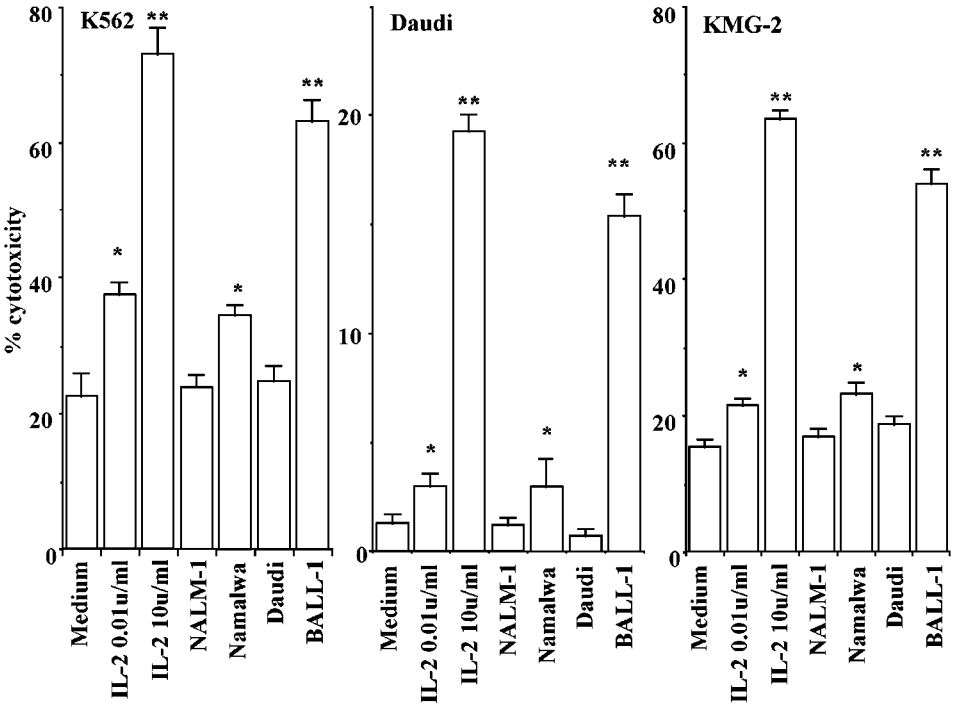

Fig. 1. Enhancement of anti-tumor activity of PBMC by B cell line cells. PBMC were treated with irradiated B cell line cells at an R/S ratio of 10. After 7-day incubation, the anti-tumor activity of treated PBMC was measured by Cr-release assay. Data at an effector/target ratio of 40 (target: KMG-2, Daudi) or 8 (K562) are presented. Data represent the mean±SE of four experiments in triplicate. Statistical significance of differences: * $P<0.05$; ** $P<0.005$.

RESULTS

Enhancement of PBMC-mediated anti-tumor activity by B cell line cells When PBMC were cultured with irradiated BALL-1 (R/S ratio, 10) for 7 days, PBMC-mediated anti-tumor activity was enhanced by the coculture (Fig. 1). Irradiated NALM-1, Namalwa and Daudi had little or no enhancing effect on PBMC-mediated anti-tumor activity at any E/T ratio tested. The anti-tumor activity was observed not only against leukemic cell line cells (K562 and Daudi), but also against glioma cell line cells (KMG-2) and gastric cancer cell line cells (KATOIII; non-treated, 13.7±0.64%; treated, 25.0±0.94%*; * $P<0.0001$). PBMC treated with irradiated BALL-1 failed to show significant cytotoxicity against autologous ConA-blasts at any E/T ratio tested (non-treated, 3.06±1.21%; treated, 3.35±1.44%*; * not significant). As a control, we used *rh*IL-2 at 2 different concentrations (0.01 u/ml and 10 u/ml) for induction of anti-tumor activity. As also shown in Fig. 1, a high dose of IL-2 enhanced anti-tumor activity against 3 tumor cell line cells, whereas a low dose of IL-2 failed to do so.

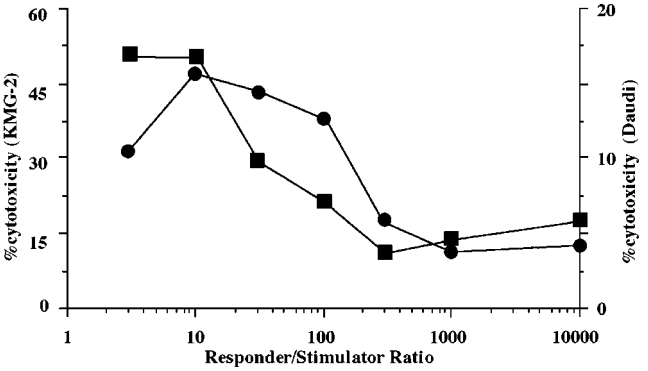

Fig. 2. Effect of R/S ratio on anti-tumor activity of BALL-1. PBMC were treated with irradiated BALL-1 cells at various R/S ratios. After 7-day culture, the anti-tumor activity of treated PBMC against (●) Daudi and (■) KMG-2 was tested by Cr-release assay at an E/T ratio of 40. Data represent the mean±SE of two experiments in triplicate. Statistical significance of differences: R/S ratio=100 (Daudi) and 30, $P<0.05$; R/S ratio=10 and 3, $P<0.005$; R/S ratio=100 (KMG-2) or more, not significant.

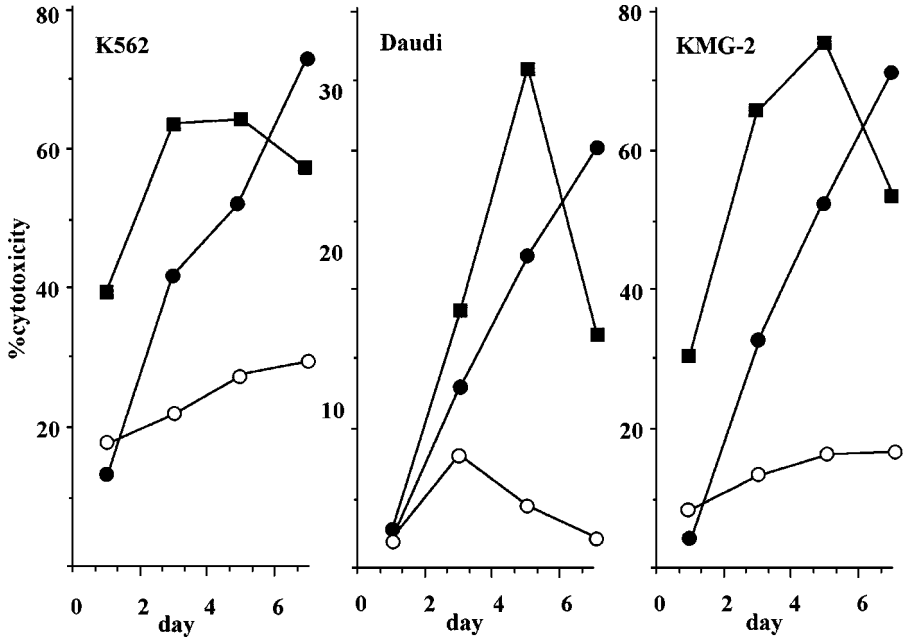

Fig. 3. Kinetics of enhancement of anti-tumor activity. PBMC were treated with PBS (○), BALL-1 (●) or 10 u/ml of IL-2 (■). One, 3, 5 and 7 days later, the anti-tumor activity of treated PBMC was measured using Cr-release assay. Data at an E/T ratio of 40 (target: KMG-2, Daudi) or 8 (target: K562) are presented. Data represent the mean±SE of three experiments in triplicate.

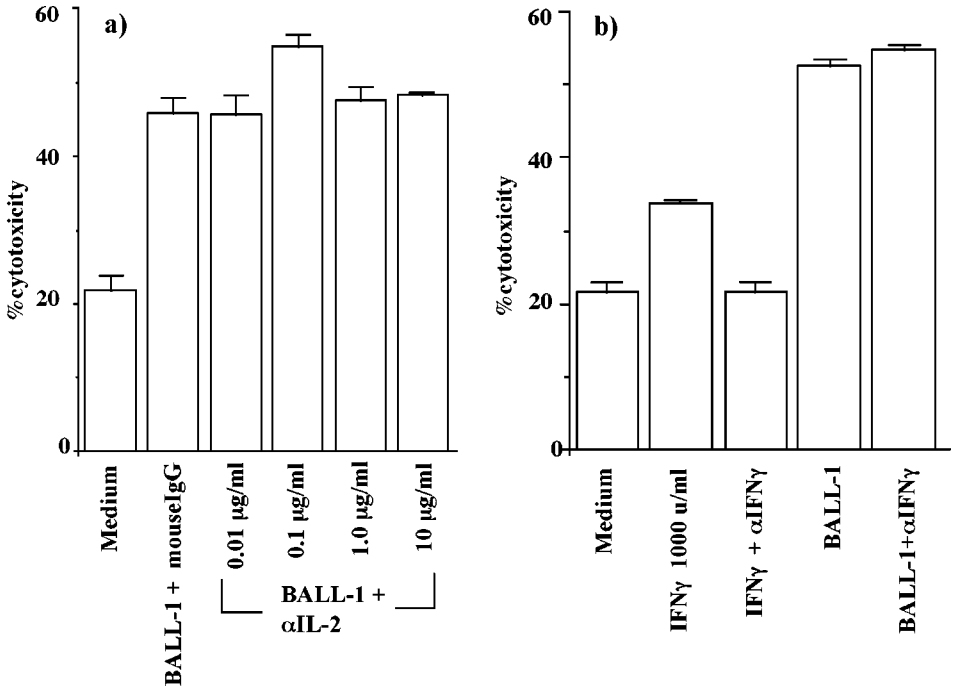

Fig. 4. Effect of anti-IL2 and anti-IFNγ monoclonal antibody on anti-tumor activity against KMG-2. PBMC and irradiated BALL-1 were cocultured for 7 days with or without (a) anti-IL2 and (b) anti-IFNγ mAb, and the anti-tumor activity of PBMC was assessed. Data at an E/T ratio of 40 are presented.

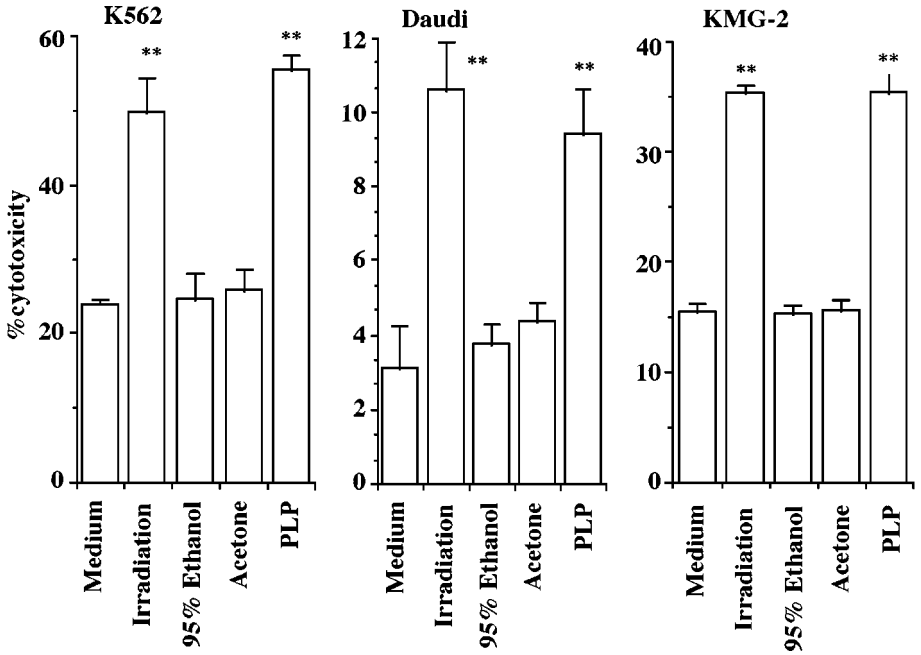

Fig. 5. Effects of fixatives on BALL-1-induced anti-tumor activity. Cultured BALL-1 cells were harvested, washed and fixed with 95% ethanol, acetone or periodate-lysine-paraformaldehyde. PBMC were treated with fixed BALL-1 cells at the R/S ratio of 10. After 7-day culture, the anti-tumor activity of treated PBMC was assessed. Data represent the mean±SE of three experiments in triplicate. Statistical significance of differences: * $P<0.05$; ** $P<0.005$.

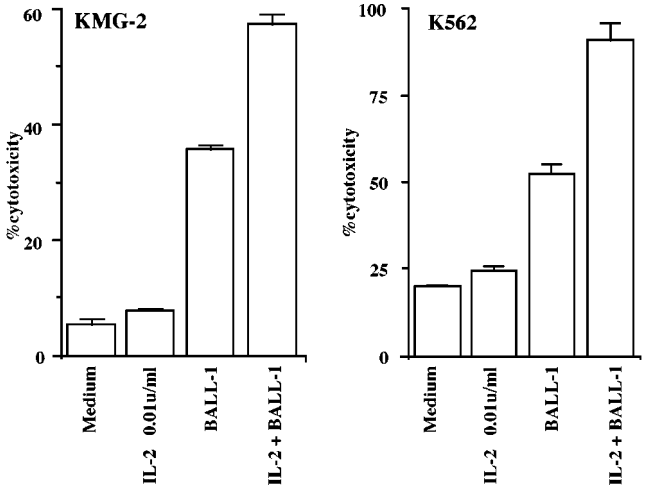

Fig. 6. Synergistic effect of IL-2 on BALL-1 induced anti-tumor activity. PBMC were cultured with IL-2 and/or BALL-1 for 7 days. Then the cells were collected and the anti-tumor activity was assessed. Data at an E/T ratio of 40 (KMG-2) or 8 (K562) are presented. Data represent the mean ECA±SEM of four experiments in triplicate. Statistical significance of differences: treated with IL-2 and BALL-1 alone vs. in combination, $P<0.005$.

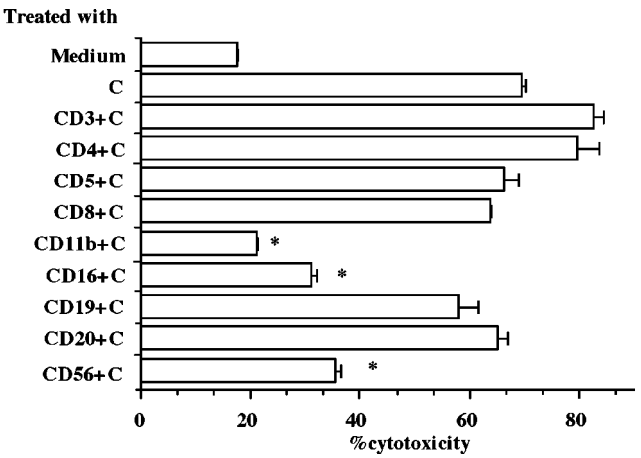

Fig. 7. Analysis of effector cells activated by irradiated BALL-1. PBMC were treated with irradiated BALL-1 for 7 days, incubated with the respective monoclonal antibodies (mAbs), and suspended in complement (C). The PBMC were then washed and their anti-tumor activity was assessed. Data represent the mean±SE of three experiments in triplicate. Statistical significance of differences: treated with complement alone vs. treated with mAb and C. * $P<0.001$.

Irradiated BALL-1 that enhanced anti-tumor activity was EBNA-negative. Though HLA-DR expression on Namalwa (36.9%) was more prominent than on BALL-1 (24.8%), the enhancement of anti-tumor activity against KMG-2 induced by irradiated BALL-1 (38.6±2.9%) was greater than that induced by irradiated Namalwa (12.8±0.4%). It is unlikely that the enhancement of the anti-tumor activity was correlated with EBNA and HLA-DR expression on BALL-1. We used PBMC from more than 10 donors, who were expected to have different HLA typings, and a similar tendency of enhancement was observed in every PBMC donor, supporting the idea that BALL-1-induced anti-tumor activity was not correlated with EBNA and HLA-DR expression on BALL-1.

The anti-tumor activity of PBMC was assessed at various responder-to-stimulator ratios (R/S ratio) (Fig. 2). Anti-tumor activity against KMG-2 that was enhanced by irradiated BALL-1 appeared at an R/S ratio of 100 and reached its maximum at an R/S ratio of 3 to 10. In the case of Daudi, the strongest enhancing effects were observed at an R/S ratio of 10 to 100. Irradiated BALL-1 cells were thus cocultured with PBMC at an R/S ratio of 10 in the following experiments.

Kinetics of BALL-1-induced anti-tumor activity The kinetics of the enhancement of anti-tumor activity against K562, Daudi, and KMG-2 induced by irradiated BALL-1 were assessed. The effect of IL-2 (10 u/ml) was evident even on the 1st culture day, reached its maximum on the 5th culture day, and then declined (Fig. 3). In contrast, enhancement of the anti-tumor activity by irradiated BALL-1 cells was not remarkable on the 1st culture day. However, it gradually increased to surpass the enhancement by high-dose IL-2 on the 7th culture day.

Effects of mAbs against cytokines Experiments were done to clarify whether the culture supernatant of irradiated BALL-1 has enhancing activity on the anti-tumor activity. PBMC were cultured with the 7-day culture supernatants from BALL-1 for 7 days, and the anti-tumor activity of PBMC was assessed. We found that the cultured supernatants of BALL-1 failed to enhance the anti-tumor activity of PBMC (data not shown), suggesting that no soluble factor is involved in the induction of the anti-tumor activity.

In order to confirm that no soluble factor is involved in the anti-tumor activity, PBMC and irradiated BALL-1 were cocultured for 7 days with or without mAbs against cytokines, and anti-tumor activity was assessed. PBMC cocultured with irradiated BALL-1 cells exhibited significant anti-tumor activity. Treatment with both anti-IL-2 (Fig. 4a) and anti-IFNγ (Fig. 4b) mAbs failed to suppress the anti-tumor activity induced by irradiated BALL-1. Furthermore, we found that treatment with anti-IL-12, anti-IL-15, anti-TNFα and anti-LT mAbs also had no significant effect on the anti-tumor activity of PBMC, whereas these mAbs could inhibit the activity of the respective cytokine almost completely (data not shown).

Effects of fixation of BALL-1 on the enhancement of anti-tumor activity Experiments were done to clarify whether the anti-tumor activity of PBMC was enhanced by coculture with BALL-1 cells even when the BALL-1 cells were fixed with fixatives. As shown in Fig. 5, PBMC cocultured with BALL-1 that had been fixed with PLP solution exhibited evident anti-tumor activity against 3 tumor cell line cells, the effect being equivalent to that obtained with irradiated BALL-1 cells. On the other hand, BALL-1 cells that had been fixed with ethanol and acetone failed to induce significant anti-tumor activity of PBMC (Fig. 5).

Synergistic effects of BALL-1 and IL-2 When PBMC were treated with a low dose of IL-2 (0.01 u/ml), PBMC failed to exhibit evident anti-tumor activity towards KMG-2 and K-562. However, when PBMC were simultaneously exposed to low-dose IL-2 and irradiated BALL-1, the anti-tumor activity of PBMC against both KMG-2 and K-562 was synergistically enhanced by an additional IL-2 stimulation (Fig. 6).

Identification of effector cells Experiments were done to clarify the effector cells in the anti-tumor activity induced by BALL-1. PBMC cocultured with irradiated BALL-1 were collected after 7-day culture, and anti-tumor activity was assessed after the elimination of a specific population of treated PBMC. The elimination of CD11b, CD16, and CD56-positive cells resulted in a significant decrease in the anti-tumor activity (Fig. 7). Depletion of CD11b resulted in almost complete suppression of anti-tumor activity, and suppression of anti-tumor activity by depletion of CD16 and CD56 was significant, but less than that by CD11b. In contrast, the elimination of CD3, CD4, CD5, CD8, CD19, and CD20-positive cells failed to decrease BALL-1-induced anti-tumor activity.

Further experiments were done to clarify whether the treatment of PBMC with BALL-1 results in the expression of CD11b, CD16 and CD56. We failed to detect any significant increment of mean fluorescence intensity or percentage of positive cells (data not shown).

DISCUSSION

We present evidence that BALL-1 can enhance the anti-tumor activity of PBMC against various tumor cell line cells. Irradiated BALL-1 stimulated PBMC to produce anti-tumor activity against leukemic (K562 and Daudi), glioma (KMG-2), and gastric cancer (KATOIII) cell line cells (Fig. 1). B cell line cells have been shown to produce various cytokines including IFNγ by which the cytotoxic activity of PBMC is enhanced.[21–28] However, it is unlikely that irradiated BALL-1 produce sufficient amounts of soluble factors to enhance the anti-tumor

activity, because the culture supernatants of irradiated BALL-1 fail to enhance the anti-tumor activity.

PBMC treated with Namalwa produce IL-2 activity in excess of 0.01 u/ml (data not shown), an amount capable of inducing weak but significant LAK activity. On the other hand, irradiated BALL-1 induce significant anti-tumor activity without production of a significant amount of IL-2 from PBMC. These results suggest that the anti-tumor activity induced by BALL-1 is independent of IL-2 production. This was confirmed by the evidence that anti-IL-2 mAb has no significant effect on the enhancement of anti-tumor activity induced by BALL-1. IFN, TNFα, IL-12, IL-15 and LT are cytokines capable of augmenting NK activity[29–33] or activating cells to become LAK cells that have a wider range of specificity than conventional NK cells.[34–36] These cytokines could also widen the spectrum of cytotoxic T cell specificity.[37, 38] However, we have found by enzyme-linked immunosorbent assay that the amounts of these cytokines are negligible in the supernatants of PBMC cocultured with BALL-1 (data not shown). Furthermore, we have found that treatment of PBMC with anti-IFNγ, anti-TNFα, anti-IL-12, anti-IL-15, and anti-LT mAbs failed to suppress the enhancement of anti-tumor activity, suggesting that these cytokines are not involved in BALL-1-induced enhancement of anti-tumor activity.

It has become evident that IL-2-activated human killer cells are derived from phenotypically heterogeneous precursors.[39] Even B lymphocytes exhibit natural cytotoxic activity.[40] The anti-tumor activity of PBMC treated with irradiated BALL-1 decreases significantly upon treatment with CD11b, CD16 or CD56 and complement (Fig. 7). Depletion of CD3, CD4, CD5, CD8, CD19, and CD20-positive lymphocytes fails to eliminate enhancement of anti-tumor activity by irradiated BALL-1. Analysis of effector cells of anti-tumor activity by BALL-1 indicates that the main effector cells are NK cells. We can not exclude the possibility that macrophages are also effector cells, since depletion of CD11b-positive cells is more effective for suppression of the anti-tumor activity by treated PBMC than that of CD16 and CD56 (Fig. 7). Furthermore, the percentage of NK cells, unexpectedly, seems not to be associated with the enhancement of NK activity in this system, though cytokine-induced enhancement of NK activity is thought to be mediated by an increase of the number and/or percentage of NK cells.[41] This suggests that BALL-1-induced enhancement of anti-tumor activity is mediated by a different pathway from cytokine-induced enhancement, though further study is required on this point.

The degree of HLA expression on the stimulator cells is well correlated with the enhancement of the anti-tumor activity.[42, 43] However, the degree of EBNA and HLA-DR expression in the B cell line cells does not parallel the observed enhancement of the anti-tumor activity, suggesting that the surface antigen capable of inducing enhancement of the anti-tumor activity is not EBNA or HLA-DR antigen, and that BALL-1-induced anti-tumor activity is not the result of mixed lymphocyte reaction. We found that enhancement of the anti-tumor activity is induced by PLP-fixed cells, but not by ethanol- and acetone-fixed cells (Fig. 6). Fixation with PLP is frequently used in the immunocytochemical analysis for glycoproteins on cell surface because PLP may preserve the antigenicity of glycoprotein.[44] Furthermore, we have found that the enhancement of the anti-tumor activity is induced by a crude membrane fraction of BALL-1 (data not shown). We posit here that some membrane glycoproteins of BALL-1 have cytokine-independent immunopotentiating activity on NK cell-mediated anti-tumor activity.

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