Monoclonal Lym-1 Antibody-Dependent Lysis of B-Lymphoblastoid Tumor Targets by Human Complement and Cytokine-Exposed Mononuclear and Neutrophilic Polymorphonuclear Leukocytes

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Lym-1 is a murine IgG2a monoclonal antibody that recognizes a polymorphic variant of HLA-DR antigens on malignant B cells, with minimal cross-reactivity with normal tissues. Because it can be safely administered in vivo, a detailed knowledge of its ability to recruit and trigger the antitumor immune effector systems is required to optimize potential therapeutical approaches in B-lymphoma patients. By using Raji cells as a model of B-lymphoma targets, we found that Lym-1 activates complement-mediated lysis efficiently. Moreover, Lym-1 was capable of triggering the antibody-dependent cellular cytosis (ADCC) by peripheral blood mononuclear cells (MNCs). On the contrary, it failed to trigger neutrophilic polymorphonuclear leukocytes (PMNs). Owing to these properties, Lym-1 is presently considered an attractive candidate for developing MoAb-based therapies of B lymphomas.

A preliminary clinical trial with Lym-1 intravenous infusion, performed in 10 patients with refractory lymphoma, showed an evident reduction of lymph node size only in some cases. Although a number of factors can contribute to these partial responses, the inadequacy of host immune effector systems is likely to play a relevant role. To improve Lym-1 antibody-based therapeutic approaches, it is therefore critical to understand whether cell-mediated cytosis can be enhanced by biologic response modifiers. In this regard, two findings are promising, i.e., the capacity of interleukin-2 (IL-2) and γ-interferon (γ-IFN) to augment Lym-1 antibody-dependent cytosis by MNCs and neutrophilic polymorphonuclear leukocytes (PMNs), respectively.

In the present study, we first examined the capacity of Lym-1 to activate the lytic potential of the complement system, peripheral blood MNCs and PMNs. Then, in the attempt to increase the cell-mediated cytotic activity, we tested a panel of nine biologic response modifiers having specific receptors on leukocytes.

**MATERIALS AND METHODS**

**Culture medium and reagents.** The following culture medium was used: RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated (56°C for 45 minutes) fetal calf serum (FCS; HyClone Europe Ltd, Cramlington, NE) and 2 mmol/L glutamine (Irvine Scientific; RPMI-FCS). Hanks’ Balanced Salt Solution (HBSS) was from Irvine Scientific. Ficol-Hypaque was purchased from Seromed (Berlin, Germany). Sodium chromate Cr51 was from the Radiochemical Center (Amersham, UK). Triton X-100, ethidium bromide, and fluorescein diacetate were purchased from Sigma Chemical Co (St Louis, MO). Heparin was obtained from Roche (Milano, Italy). Gmisera was purchased from Merck (Darmstadt, Germany). Human AB serum, pooled from three normal donors, was stored in aliquots at −80°C immediately after preparation or immediately after heat-inactivation (56°C for 45 minutes). Human C5-deficient serum was purchased from Sigma Chemical Co and stored in aliquots at −80°C. Monoclonal Lym-1 antibody was a gift of Prof F. Indiveri (University of Genova, Genova, Italy). A nonsense mouse IgG2a MoAb (Dako S.p.A., Milano, Italy) was used as an isotype control.

**Cytokines and chemotaxins.** Human recombinant tumor necrosis factor α (TNFα), human recombinant insulin-like growth factor-1 (IGF-1), and human recombinant 72 amino-acids IL-8 were purchased from BioSource International (Camarillo, CA). Human recombinant γ-IFN was obtained from Genzyme srl (Milano, Italy). Human recombinant C5a and N-formyl-met-leu-phe (FMLP) were purchased from Sigma Chemical Co. Human recombinant IL-2 was a gift of Prof F. Indiveri (University of Genova, Genova, Italy). Human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and human recombinant granulocyte colony-stimulating factor (G-CSF) were kindly donated by Dr G. Guidi (Sandoz Italia, Milano, Italy). Each of these mediators was stored in aliquots.

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at -80°C and was diluted with culture medium immediately before using.

Leukocyte preparation. Heparinized venous blood (10 U/mL, heparin) was obtained from healthy volunteers (20 to 37 years old) after informed consent had been obtained. No donor had an infectious disease or was under medication at the time of and for 2 weeks before sampling. MNCs were isolated by centrifugation (400g for 30 minutes) on a Ficoll-Hypaque density gradient, washed with HBSS, and resuspended in RPMI-FCS, as previously described. The resulting MNC preparations contained 70% to 87% lymphocytes, 13% to 29% monocytes, and less than 1% neutrophils or eosinophils, as determined by morphologic analysis of Giemsa-stained cytopreps. Cell viability was determined by ethidium bromide-fluorescein diacetate test and was greater than 98%. Neutrophilic PMNs were prepared by dextran sedimentation, followed by centrifugation (400g for 30 minutes) on a Ficoll-Hypaque density gradient, as previously described. PMNs resuspended in RPMI-FCS were greater than 97% pure and greater than 98% viable, as determined by the assays described above.

Target cells. Lymphoblastoid Raji cells were used as targets in the cytolytic assays. The Raji cell line was grown in RPMI-FCS and subcultured every 3 days. The capacity of these cells to bind Lym-1 antibody was measured by indirect immunofluorescence with flow cytometry using a rabbit antimouse IgG (F(ab')2) polyclonal antibody conjugated with fluorescein isothiocyanate (Dako). The Raji cell population contained 93.9% ± 0.8% positive cells (mean ± 1 SD, n = 5). The mean fluorescence intensity of the cells was 23.2 ± 1.1 (mean ± 1 SD, n = 5), with a standard deviation ranging from 30.0 to 35.1. For cytolytic assays, 4 × 10^4 Raji cells were labeled with 100 to 200 μCi sodium chromate Cr 51 by incubating for 1 hour at 37°C (final volume, 0.5 mL; medium, RPMI 1640 plus 5% FCS). After washing, labeled cells were resuspended in RPMI-FCS.

Cytolytic assays. Cytolytic activity of MNCs and PMNs was measured as described elsewhere in detail. Briefly, target cells (2 × 10^4) were mixed with MNCs or PMNs as an effector:target ratio of 20:1, with and without Lym-1 MoAb and cytokines/chemokines appropriately diluted in RPMI-FCS. The effectortarget ratio of 20:1 was chosen on the basis of preliminary experiments. To test the cytolytic activity of human complement, 2 × 10^6 cells were mixed with appropriate dilutions of human AB serum or heat-inactivated human AB serum or human C5-deficient serum in the presence or absence of Lym-1 or the control IgG2a MoAb. The assays were performed in triplicate and in a final volume of 150 μL, using round-bottom microplates (Falcon, Becton-Dickinson Italia, Milano, Italy). After 14 hours of incubation in a humidified atmosphere of 95% air and 5% CO₂, the "Cr-release was determined in the cell-free supernatant. A series of experiments was also performed by preincubating (1 hour) either effector cells or target cells with biologic response modifiers. Some experiments were also performed using 20% of the percentage of cytolytic was calculated according to the formula:

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\text{Percent cytolysis} = \frac{\text{cpm released in the presence of effector cells (or complement)}}{\text{cpm released after lysing target cells with 5% Triton X-100}} \times 100
\]

Statistical analysis. Results were expressed as the mean ± 1 SD and/or as median with the 95% confidence interval. Statistical differences were analyzed by the Mann-Whitney test. Significance was accepted when \( P < 0.05 \).

RESULTS

When added to ^3HCr-labeled Raji cells in the presence of 10 μg/mL Lym-1, human serum induced a dose-dependent lysis of Raji target cells as measured by a 14-hour ^3Cr release assay (Fig 1). The magnitude of human serum-mediated lysis was also dependent on the Lym-1 concentration (Fig 2). These data suggest that (1) 10 μg/mL Lym-1 and about 20% human serum are sufficient to induce the lysis of the majority of Raji target cells; and (2) relatively low concentrations of Lym-1 (0.1 to 1 μg/mL), combined with 5% to 10% of human serum, are still capable of inducing substantial levels of cytosis. Time-course experiments showed that the lysis reaches the plateau after 1 hour, ie, the serum-mediated Lym-1 antibody-dependent cytolytic reaction is over at the end of 1 hour of incubation (data not shown). Heat-inactivated (56°C for 45 minutes) human serum, added to Raji cells in the presence of 10 μg/mL Lym-1, was ineffective (percentage of cytosis by 5% heat-inactivated serum, 0.7 ± 0.6 [mean ± 1 SD]; n = 3). Similarly, C5-deficient human serum did not promote Lym-1 antibody-
MODULATION OF Lym-1 ADCC BY CYTOKINES

Fig 1. Effect of Lym-1 concentration on the cytolysis induced by human serum. Human serum concentrations were 10% (●), 5% (■), and 1% (▲). 3H-labeled Raji cells were at 2 x 10⁶. The incubation time was 14 hours. The results shown are representative of two experiments.

Fig 2. Effect of Lym-1 concentration on the cytolysis induced by human serum. Human serum concentrations were 10% (●), 5% (■), and 1% (▲). 3H-labeled Raji cells were at 2 x 10⁶. The incubation time was 14 hours. The results shown are representative of two experiments.

consistent with the resistance of Raji cells to natural killer (NK) cells, MNCs caused negligible lysis (Fig 3). The addition of 10 μg/mL Lym-1 resulted in significant cytolysis, i.e., MNCs appear to mediate Lym-1 ADCC (Fig 3 and its legend). On the other hand, PMNs were incapable of mediating significant spontaneous and Lym-1 antibody-dependent cytolysis (Fig 3 and its legend). As shown in Fig 3, there was intersubject variability in MNC-mediated ADCC, apparently unrelated to the number of monocytes in the MNC populations used (data not shown). Moreover, PMNs from relatively few individuals were found to exert ADCC (Fig 3). Nevertheless, repetitive testing of same donors at various times indicated that the majority had consistent profiles of both MNC- and PMN-mediated ADCC, i.e., high responders usually displayed high activity, whereas low responders generally had low activity (data not shown). As summarized in Fig 4, the addition of 200 U IL-2 at the beginning of the assay significantly (legend to Fig 4) augmented MNC-mediated Lym-1 ADCC. On the other hand, when added at the beginning of the assay in the absence of Lym-1, IL-2 did not significantly (P = .420) affect the negligible activity of MNCs (percentage of cytolysis by MNCs, 0.9 ± 1.1 [mean ± 1 SD]; n = 5; median, 0.6; confidence interval 95%, −0.5 to 2.4; percentage of cytolysis by MNCs plus 200 U IL-2, 2.7 ± 2.7 [mean ± 1 SD]; n = 5; median, 2.9; confidence interval 95%, −0.7 to 6.1). Therefore, under the present conditions, IL-2 stimulates MNC-mediated Lym-1 ADCC without affecting the antibody-independent activity, i.e., without inducing lymphokine-activated killer (LAK) cell activity. Stimulation of MNC-mediated Lym-1 ADCC could also be detected using Lym-1 at the concentration of 1 μg/mL in-

Fig 3. MNC- and PMN-mediated cytolysis in the absence or presence of 10 μg/mL Lym-1. 3H-labeled Raji cells were at 2 x 10⁶. The MNC:Raji cell ratio and PMN:Raji cell ratio were 20:1. The incubation time was 14 hours. MNC-mediated cytolysis was 1.5 ± 1.9 (mean ± 1 SD, n = 10), with a median of 0.85 (confidence interval 95%, 0.1 to 2.9), in the absence of Lym-1 and 10.7 ± 10.5 (mean ± 1 SD, n = 35), with a median of 19.9 (confidence interval 95%, 17.1 to 24.3), in the presence of Lym-1. MNC-mediated cytolysis in the absence of Lym-1 versus that observed in the presence of Lym-1 was P < .0001. PMN-mediated cytolysis was 0.9 ± 1.3 (mean ± 1 SD, n = 13), with a median of 0.0 (confidence interval 95%, 0.1 to 1.7), in the absence of Lym-1 and 3.0 ± 5.6 (mean ± 1 SD, n = 42), with a median of 0.7 (confidence interval 95%, 1.3 to 4.8), in the presence of Lym-1. PMN-mediated cytolysis in the absence of Lym-1 versus that observed in the presence of Lym-1 was P = .1309.
the absence versus that in the presence of IL-2 was 24.7 ± 2.6 (mean ± 1 SD, n = 19), with a median of 19.3 (confidence interval 95%, 16.1 to 27.7), in the absence of IL-2 and 31.5 ± 14.0 (mean ± 1 SD, n = 19), with a median of 32.4 (confidence interval 95%, 24.7 to 38.3), in the presence of IL-2. Cytolysis in the absence versus that in the presence of IL-2 was \( P = .0343 \).

As shown in Figs 5 and 6, two cytokines (GM-CSF and TNFα) and two chemotaxins (FMLP and C5a) induced or enhanced PMN-mediated Lym-1 ADCC significantly (legends to Figs 5 and 6). When added to PMNs plus Raji cells, none of the agents had an effect on the \(^{51}Cr\) release from target cells. Stimulation of PMN-mediated Lym-1 ADCC by GM-CSF and FMLP and C5a was also detected using Lym-1 concentrations less than 10 \( \mu \)g/mL (Fig 7). The same phenomenon could be observed using TNFα and C5a (data not shown). As for MNC-mediated Lym-1 ADCC, other agents were tested for their ability to increase the target lysis by PMNs (Table 2). Among them, γ-IFN and IL-8 significantly augmented PMN-mediated Lym-1 ADCC (Table 2), but the magnitude of the target lysis was relatively low as compared with that observed using GM-CSF, TNFα, FMLP, and C5a. Other cytokines were completely ineffective, with low intersubject variability (Table 2), and were unable to stimulate PMN-mediated Lym-1 ADCC even after preincubation with PMNs or Raji cells (data not shown). As summarized in Fig 8, GM-CSF and TNFα were found to significantly increase PMN binding to Raji cells in the presence of Lym-1, whereas other biologic response modifiers were ineffective.

**DISCUSSION**

The present study, performed using Raji cells as a model of B-lymphoma targets, shows that (1) Lym-1 antibody activates the cytolytic sequence of human complement system efficiently; (2) Lym-1 triggers the ADCC activity of MNCs through a process susceptible of significant enhancement by

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**Table 1. Effect of Various Mediators on Lym-1 Antibody-Dependent Cytolysis by MNCs**

| Mediator | Type     | Dose       | Mean ± 1 SD | Median | Confidence Interval 95% | Mean ± 1 SD | Median | Confidence Interval 95% | n  | P Value |
|----------|----------|------------|-------------|--------|--------------------------|-------------|--------|--------------------------|----|---------|
| GM-CSF   | 1 ng/mL  | 19.3 ± 7.2 | 21.1        | 14.4-24.1| 17.8 ± 5.3                | 18.6        | 14.2-21.4|                        | 11 | .519    |
| G-CSF    | 1 ng/mL  | 24.5 ± 12.3| 27.7        | 13.0-35.9| 20.9 ± 11.1               | 20.4        | 10.5-51.2|                        | 7  | .535    |
| γ-IFN    | 100 U/mL | 24.1 ± 8.3 | 20.8        | 15.3-32.9| 24.2 ± 5.4                | 23.4        | 19.5-39.9|                        | 6  | .618    |
| TNFα     | 1 ng/mL  | 24.5 ± 14.2| 23.4        | 13.6-35.5| 22.6 ± 10.5               | 23.9        | 14.5-30.7|                        | 9  | .730    |
| IGF-1    | 100 ng/mL| 27.5 ± 10.4| 29.4        | 19.4-35.5| 22.6 ± 10.5               | 23.9        | 14.5-30.7|                        | 9  | .340    |
| IL-8     | 1 μmol/L | 20.2 ± 10.9| 21.2        | 11.1-29.4| 19.8 ± 10.6               | 20.2        | 10.6-28.7|                        | 8  | .720    |
| FMLP     | 1 μmol/L | 23.7 ± 11.5| 24.0        | 14.9-32.5| 22.6 ± 10.5               | 23.9        | 14.5-30.7|                        | 9  | .663    |
| C5a      | 1 μmol/L | 26.2 ± 16.5| 18.5        | 13.4-38.9| 22.6 ± 10.0               | 23.9        | 14.5-30.7|                        | 9  | 1.000   |

* Percentage of cytosis at 20:1 MNC:Raji cell ratio. Lym-1 concentration was 10 μg/mL. Incubation time was 14 hours.

† When added to MNCs plus Raji cells in absence of Lym-1 or mixed with Raji cells plus Lym-1, none of the agents was found to induce Raji cell lysis.
IL-2; and (3) although per se ineffective, Lym-1 induces ADCC by PMNs in the presence of GM-CSF, TNFα, FMLP, and C5a. Therefore, Lym-1 alone or in combination with biologic response modifiers is capable of directing and activating some of the major immune cytolytic systems towards B-lymphoblastoid tumor cells.

Previous experiments showed that complement lyases Raji cells presensitized with high doses of Lym-1. In agreement with previous data, Lym-1 was also found to trigger MNC-mediated lysis, an event enhanced by IL-2. This finding, coupled with the incapacity of IL-2 to induce LAK activity in the present setting, suggests that the signals delivered by Lym-1 and IL-2 converge and synergize to amplify the ADCC activity of MNCs. None of the other cytokines herein tested, including γ-IFN and GM-CSF, was target lysis can be induced by 5% to 20% human serum in presence of Lym-1 concentrations achievable in vivo. In the absence of GM-CSF and 19.2 ± 10.1 (mean ± 1 SD, n = 23), with a median of 20.0 (confidence interval 95%, 14.8 to 23.6), in the presence of GM-CSF. Cytolysis in the absence of GM-CSF versus that in the presence of GM-CSF was P < .0001. (B) The cytolysis was 5.1 ± 7.6 (mean ± 1 SD, n = 19), with a median of 1.1 (confidence interval 95%, 1.1 to 7.1), in the absence of TNFα and 17.1 ± 10.8 (mean ± 1 SD, n = 19), with a median of 17.2 (confidence interval 95%, 12.5 to 21.5), in the presence of FMLP. Cytolysis in the absence of FMLP versus that in the presence of FMLP was P < .0001. When added to PMNs and Raji cells in the absence of Lym-1 or mixed with Raji cells plus Lym-1, both GM-CSF and TNFα had no effect.

**Fig 5.** Effect of GM-CSF and TNFα on PMN-mediated Lym-1 antibody-dependent cytolysis. 11Cr-labeled Raji cells were at 2 x 10⁶. The PMN:Raji cell ratio was 20:1. Lym-1 was at 10 μg/mL. The incubation time was 14 hours. (A) The cytolysis was 1.8 ± 2.6 (mean ± 1 SD, n = 23), with a median of 0.5 (confidence interval 95%, 0.6 to 2.9), in the absence of GM-CSF and 19.2 ± 10.1 (mean ± 1 SD, n = 23), with a median of 20.0 (confidence interval 95%, 14.8 to 23.6), in the presence of GM-CSF. Cytolysis in the absence of TNFα versus that in the presence of TNFα was P < .0001. (B) The cytolysis was 5.1 ± 7.6 (mean ± 1 SD, n = 19), with a median of 1.1 (confidence interval 95%, 1.1 to 7.1), in the absence of TNFα and 17.1 ± 10.8 (mean ± 1 SD, n = 19), with a median of 17.2 (confidence interval 95%, 12.5 to 21.5), in the presence of TNFα. Cytolysis in the absence of TNFα versus that in the presence of TNFα was P < .0001. When added to PMNs and Raji cells in the absence of Lym-1 or mixed with Raji cells plus Lym-1, both GM-CSF and TNFα had no effect.
found to enhance Lym-1 ADCC by MNCs, even during a 20-hour assay and after 1 hour of preincubation with either effector or target cells. Therefore, our data do not confirm the findings of other investigators showing that γ-IFN6 and GM-CSF16 stimulate Lym-1 ADCC by MNCs in most individuals.

As regards PMN-mediated ADCC, it is well-known that these phagocytes lyse lymphoid cells, including Raji cells, in the presence of rabbit antitarget antibodies.17 Despite this ability and consistent with the results obtained by other investigators,15 PMNs failed to exert Lym-1 ADCC. Nevertheless, two cytokines (GM-CSF and TNFα) and two chemokins (FMLP and C5a), per se ineffective in absence of Lym-1, were found to induce Lym-1 ADCC by PMNs.
Moreover, γ-IFN and IL-8 displayed significant but very low PMN stimulatory activity. As for the IL-2 enhancement of Lym-1 ADCC by MNCs, these results suggest the intervention of a synergistic interaction between Lym-1 and each of the aforementioned biologic mediators. However, at present, we have no hint as to the detailed biochemical processing whereby these distinct mediators exert the same stimulatory action. Nevertheless, the enhancement of PMN ADCC by GM-CSF and TNFα is associated with an augmentation of the number of target cell-bound effectors, whereas other mediators (FMLP, C5a, γ-IFN, and IL-8) stimulate ADCC of bound PMNs without affecting the effector-target conjugate formation. Finally, it is of note that, whereas the results obtained with γ-IFN are confirmatory, those observed with GM-CSF, TNFα, FMLP, and C5a are novel. In fact, to our knowledge, only GM-CSF was previously found to stimulate the MoAb-dependent tumoricidal activity of PMNs, using melanoma, neuroblastoma, or certain colorectal carcinoma target cells.10–21

The ability of Lym-1 shown here to mediate complement-dependent cytolyis efficiently, coupled with its particular reactivity for lymphoma cells,1 suggests its possible use for the purging of harvested bone marrow before reinfusion. On the other hand, preliminary clinical studies with Lym-1 intravenous infusion in patients with refractory lymphomas have shown low response rates, suggesting that the in vivo activation of complement- and MNC-mediated cytosis by Lym-1 is relatively insufficient as compared with in vitro findings. Nevertheless, the administration of Lym-1 might be effective in patients with a low tumor burden. As shown in biopsy specimens, Lym-1 can trigger a sort of inflammatory or immune response at tumor sites, at least in a subset of lymphoma patients. Similarly, certain antitumor and complement-activating MoAbs have been found to induce tumor-destructing inflammatory reactions in melanoma patients.22 Therefore, although radiolabeled or toxin-conjugated Lym-1 may also be effective,23 the possibility of augmenting the activity of immune cells by using biologic response modifiers appears to be a reasonable option to improve Lym-1 antitumor effects. Taking into account the intersubject variation of lymphoma cell ability to bind Lym-1, the combination of the antibody with IL-2 to increase MNC-ADCC might specifically target and potentially eradicate small numbers of residual tumor cells in selected patients. Moreover, the capacity of certain cytokines (GM-CSF and TNFα) and chemotaxins (FMLP and C5a) to trigger PMN-mediated Lym-1 ADCC raises potentially attractive possibilities to develop new approaches to the serotherapy of lymphomas. Although the infusion of cytokines or other mediators has its own set of problems, the known toxicity of intravenous administration of TNFα,11–24 the ability of IL-2 to promote the production of TNFα,11,25 and the expected unwanted effects of FMLP and C5a16,17,26 might be reduced by conjugation of these molecules with the antibody. As far as GM-CSF is concerned, it has been recently shown that this cytokine potentiates Lym-1 ADCC by macrophages.27 This finding and the present data, coupled with the ability of GM-CSF to increase monocyte-macrophage production28 and PMN production and survival,29,25 render the cytokine the best candidate for inducing phagocytes to express their Lym-1–dependent antitumor potential. Consistent with this possibility, the administration of an antitumor MoAb together with GM-CSF has yielded encouraging clinical responses in patients with colorectal carcinoma.30

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