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 serotherapeutic 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). In an attempt to enhance Lym-1 ADCC by MNCs and PMNs, nine biologic response modifiers were tested. MNC-mediated Lym-1 ADCC was significantly stimulated by interleukin-2 (IL-2) and unaffected by other mediators, including γ-interferon (γ-IFN), tumor necrosis factor (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF). On the other hand, PMN-mediated Lym-1 ADCC was induced or significantly augmented by various cytokines, such as GM-CSF, TNFα, and γ-IFN, and chemotaxins, such as formyl peptides (FMLP), complement fragment C5a, and IL-8. Both MNC- and PMN-mediated ADCC was unaffected by granulocyte colony-stimulating factor (G-CSF) and insulin-like growth factor-1 (IGF-1). Finally, only GM-CSF and TNFα augmented the number of PMNs actually engaged in the binding of Raji target cells. The findings presented here, in particular those showing stimulatory activity of biologic response modifiers, may inspire new attempts for developing Lym-1 antibody-based approaches to the therapy of B lymphomas.

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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.13 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 test14 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.13 Contaminating erythrocytes were removed by hypotonic lysis.13 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 antiserum IgG (Fab'), polyvalent 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 × 10⁴ Raji cells were labeled with 100 to 200 µCi sodium chromate Cr51 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.14 Briefly, target cells (2 × 10⁴) 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 effector:target ratio of 20:1 was chosen on the basis of preliminary experiments. To test the cytolytic activity of human complement, 2 × 10⁴ 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 ⁵¹Cr-release was determined in the cell-free supernatants. 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 hours of incubation. The percentage of cytolysis was calculated according to the formula 100 × (E−S)/(T−S), in which E is the cpm released in the presence of effector cells (or complement), T is the cpm released after lysing target cells with 5% Triton X-100, and S is the cpm spontaneously released by target cells incubated with medium alone (<1% purity with 4 exceptions: 20.4%, 21.4%, 22.1%, and 25.7%).

**Target cell binding assay.** The assay was performed under conditions similar to those used for cytolytic assays. Briefly, 10⁵ effector cells were mixed with 2 × 10⁴ Raji cells in the presence of Lym-1 MoAb, with or without cytokines or chemokines. This effector:target ratio was chosen on the basis of preliminary experiments (higher effector:target ratios lead to cell overcrowding, therefore hampering the reading). Tests were performed in polypropylene 1.5 mL capped Eppendorf-type tubes (Greiner GmbH, Frickenhausen, Germany) in a final volume of 150 µL. The tubes were centrifuged (50g for 5 minutes) and incubated for 20 minutes at 37°C in a humified atmosphere of 5% CO₂. After resuspension, the number of effector cells adherent to 100 target cells was determined by counting at least 200 Raji cells on Giemsa-stained cytopreps.

**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 ⁵¹Cr-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 ⁵¹Cr 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 cytolysis. 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 cytolysis 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-
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Fig 2. Effect of Lym-1 concentration on the cytolysis induced by human serum. Human serum concentrations were 10% (■), 5% (□), and 1% (▲). 15Cr-labeled Raji cells were at 2 x 10⁴. 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 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 PMNs 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. 15Cr-labeled Raji cells were at 2 x 10⁴. 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 20.7 ± 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. PMN-mediated cytolysis in the absence of Lym-1 versus that observed in the presence of Lym-1 was P < .0001. PMN-mediated cytolysis was 6.9 ± 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

dence interval

each of these agents was found to enhance ADCC by MNCs
crease MNC-mediated Lym-1 ADCC (Table 1). Although
of 200 U (data not shown). Finally, a panel of other cytokines
been previously claimed to enhance Lym-1-dependent
cytolysis was 21.9 ± 12.6 (mean ± 1 SD, n = 19), with a median of
19.9 (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 (confi-
dence 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.

instead of 10 µg/mL and 100 U but not 50 U/mL IL-2 instead
of 200 U (data not shown). Finally, a panel of other cytokines or
biologic mediators were tested for their potential to in-
crease MNC-mediated Lym-1 ADCC (Table 1). Although
each of these agents was found to enhance ADCC by MNCs
from one or more donor, none of them also had significant
activity (Table 1) after preincubations with MNCs or target
cells (data not shown). Because γ-IFN and GM-CSF have
been previously claimed to enhance Lym-1-dependent
MNC cytolysis in a 20-hour assay,6,16 parallel experiments
were performed using 14-and 20-hour incubation periods.
Both 100 U/mL γ-IFN and 1 ng/mL GM-CSF also did not
significantly stimulate MNC-mediated Lym-1 ADCC in the
20-hour assay (percentage of stimulation of MNC ADCC by
γ-IFN, 13.2 ± 2.6 [P = .222] and 14.9 ± 7.9 [P = .420]
during 14- and 20-hour incubation periods, respectively; per-
centage of stimulation of MNC ADCC by GM-CSF, 13.0 ±
6.1 [P = .222] and 9.5 ± 9 [P = .547] during 14- and
20-hour incubation periods, respectively [mean ± 1 SD; n = 5].

As shown in Figs 5 and 6, two cytokines (GM-CSF and
TNFa) and two chemotaxins (FMLP and C5a) induced or
enhanced PMN-mediated Lym-1 ADCC significantly (leg-
eads to Figs 5 and 6). When added to PMNs plus Raji cells
in the absence of Lym-1 or to Raji cells incubated with Lym-
1 in absence of PMNs, they had no effect on the 51Cr release
from target cells. Stimulation of PMN-mediated Lym-1
ADCC by GM-CSF and FMLP was also detected using Lym-
1 concentrations less than 10 µg/mL (Fig 7). The same
phenomenon could be observed using TNFa 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 (Ta-
ble 2), but the magnitude of the target lysis was relatively
low as compared with that observed using GM-CSF, TNFa,
FMLP, and C5a. Other cytokines were completely ineffec-
tive, 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 TNFa 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 acti-
vates 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**

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Dose</th>
<th>Mean ± 1 SD</th>
<th>Median</th>
<th>Confidence Interval 95%</th>
<th>P Value</th>
</tr>
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<tr>
<td>GM-CSF</td>
<td>1 ng/mL</td>
<td>19.3 ± 7.2</td>
<td>21.1</td>
<td>14.4-24.1</td>
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<tr>
<td>G-CSF</td>
<td>1 ng/mL</td>
<td>24.5 ± 12.3</td>
<td>27.7</td>
<td>13.0-35.9</td>
<td></td>
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<tr>
<td>γ-IFN</td>
<td>100 U/mL</td>
<td>24.1 ± 8.3</td>
<td>20.8</td>
<td>15.3-32.9</td>
<td></td>
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<tr>
<td>TNFα</td>
<td>1 ng/mL</td>
<td>24.5 ± 14.2</td>
<td>23.4</td>
<td>13.6-35.5</td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>100 ng/mL</td>
<td>27.5 ± 10.4</td>
<td>29.4</td>
<td>19.4-35.5</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>1 μmol/L</td>
<td>20.2 ± 10.9</td>
<td>21.2</td>
<td>11.1-29.4</td>
<td></td>
</tr>
<tr>
<td>FMLP</td>
<td>1 μmol/L</td>
<td>23.7 ± 11.5</td>
<td>24.0</td>
<td>14.9-32.5</td>
<td></td>
</tr>
<tr>
<td>C5a</td>
<td>1 μmol/L</td>
<td>26.2 ± 16.5</td>
<td>18.5</td>
<td>13.4-38.9</td>
<td></td>
</tr>
</tbody>
</table>

* 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 cell in absence of Lym-1 or mixed with Raji cells plus Lym-1, none of the agents was found to induce Raji
cell lysis.
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Fig 5. Effect of GM-CSF and TNFα on PM-mediated Lym-1 antibody-dependent cytolysis. ¹⁴Cr-labeled Raji cells were at 2 × 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 ± 13.1 (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 14.5 (confidence interval 95%, 11.8 to 22.3), 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.

Previously experiments showed that complement lyzes Raji cells presensitized with high doses of Lym-1.³ The present findings extend this observation, showing that high levels of target lysis can be induced by 5% to 20% human serum in presence of Lym-1 concentrations achievable in vivo.¹ In agreement with previous data,¹² 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

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.

Fig 6. Effect of FMLP and C5a on PMN-mediated Lym-1 antibody-dependent cytolysis. ¹⁴Cr-labeled Raji cells were at 2 × 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 4.1 ± 7.0 (mean ± 1 SD, n = 24), with a median of 1.1 (confidence interval 95%, 1.1 to 7.1), in the absence of FMLP and 17.5 ± 9.4 (mean ± 1 SD, n = 24), 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. (B) The cytolysis was 5.3 ± 8.0 (mean ± 1 SD, n = 17), with a median of 2.6 (confidence interval 95%, 1.1 to 9.4), in the absence of C5a and 11.6 ± 9.0 (mean ± 1 SD, n = 17), with a median of 11.4 (confidence interval 95%, 7.0 to 16.3) in the presence of C5a. Cytolysis in the absence of C5a versus that in the presence of C5a was P = .0103. When added to PMNs and Raji cells in the absence of Lym-1 or mixed with Raji cells plus Lym-1, both FMLP and C5a 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-IFN and GM-CSF 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. Despite this ability and consistent with the results obtained by other investigators, PMNs failed to exert Lym-1 ADCC. Nevertheless, two cytokines (GM-CSF and TNFα) and two chemotaxins (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. 18,21

The ability of Lym-1 shown here to mediate complement-dependent cytolyis efficiently, coupled with its particular reactivity for lymphoma cells, 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 inefficient 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. Therefore, although radiolabeled or toxin-conjugated Lym-1 may also be effective, 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,25 and the expected unwanted effects of FMLP and C5a 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,22 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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